**Role of kinases MAP2K1, PDHK and NEK1 on Hedgehog Signaling Activation**

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**Fall 2019 Rotation Report**

This report was written by the student, has a distinct and reachable goal, and represents a reasonable amount of work for one rotation project earning 4 units of academic credit. The student will learn new techniques and approaches consistent with the goals of the rotation program.

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**Introduction**

Basal Cell Carcinoma (BCC) is the most abundant skin cancer in the US with 4 million diagnoses a year 1. Evidence supports that this cancer depends on uncontrolled activation of the Hedgehog (Hh) Signaling Pathway. The Hedgehog Signaling Pathway becomes active when a Hedgehog Signaling ligand binds to the Patched1 (PTCH1) receptor on the primary cilia of a cell, thus activating the signal transducer Smoothened (SMO). SMO then moves to the cilium where it binds and inhibits Suppressor of Fused (SUFU), resulting in transcription factor activation of Glioma-Associated Oncogene (GLI) 2. Mutations that occur in regulators of the Hh Signaling Pathway such as PTCH1, SMO and GLI cause uncontrolled activation of the Hh pathway leading to the development of cancers such as BCC 3. Small molecule drugs that inhibit SMO, such as Vismodegib, have been used to modulate the Hedgehog Signaling pathway by preventing release of GLI transcription factors to treat BCC. It has been found that BCC tumors can acquire resistance to SMO inhibitors, thus causing a loss of SMO inhibitor Sufu or the increase of transcription factors in the system 4,5. Additionally, mutations in SMO have caused the drug to be ineffective in suppressing SMO and thus allowing the pathway to proceed as normal 5. BCC resistance and mutations of SMO have directed attention to GLI transcription factors downstream of SMO as targets for BCC treatment. It has been previously found that multi-site phosphorylation regulates the conversion of GLI proteins into transcriptional activators and kinases activate the response in Hedgehog Signaling activity and tumor growth 6. The purpose of this project is to assess Hedgehog Signaling Pathway activation by inhibiting kinases predicted to be active at recurrent GLI mutation sites.

Mitogen-Activated Protein Kinase 2 (MAP2K1) is active at re-current GLI mutations sites determined experimentally to activate Hh Signaling. Mutations in MAP2K1 are correlated with melanoma and inhibiting this kinase reduces metastasis in pancreatic cancer 7–9. Additionally, MAP2K1 stabilizes GLI and activates Hedgehog Signaling 9. Cobimetinib is effective as a MAP2K1 inhibitor used to treat melanoma with an IC50 of 4.2 nM 10. Pyruvate Dehydrogenase Kinase (PDHK) regulates glycolysis which is more highly activated in cancer cells than normal cells 11. A PDHK inhibitor, Dichloroacetic acid (IC50, 81.03 mM), upregulates apoptosis of ovarian cancer cells 12. The prediction of PDHK activity on re-current GLI mutation sites, makes it of interest in assessing its inhibition in Basal Cell Carcinoma. NIMA Related Kinase (NEK1) affects Hh signaling through its role in primary cilia formation and has demonstrated activity in cancer 13,14. Zinc05007751 is a novel selective NEK1 inhibitor with an IC50 of 3.4 μM. Evidence suggests that inhibition of NEK1 would reduce GLI activation and Hedgehog Signaling. As MAP2K1, PDHK and NEK1 kinases are predicted to be active at recurrent mutation sites in GLI, their inhibition is predicted to influence transcriptional activity and activate Hedgehog Signaling.

**Hypothesis**

We hypothesize that MAP2K1, PDHK and NEK1 kinases increase Hedgehog Signaling activity due to their predicted activity at recurrent GLI mutation sites and insight to their role in Hedgehog Signaling or cancer.

**Materials and Methods**

**Kinase Prediction**

A compilation of GLI1 and GLI2 mutations that occur three or more times in cancer cells in the Catalogue of Somatic Mutations in Cancer (COSMIC) database have been generated and previously screened for activation of the Hedgehog Signaling Pathway in the Atwood Lab. Using Group-Based Prediction Software (GPS) and PhosphoNet Kinase Predictor, kinases which are predicted to be active at phosphosites four positions up or downstream from a recurring mutation side are compiled. Kinase candidates having previous evidence of mediating phosphorylation and activating Hedgehog Signaling at a mutation site were eliminated from the compilation. Previous insight into their activity in Hedgehog Signaling or cancer allowed for the selection of MAP2K1, PDHK and NEK1 to evaluate Hedgehog Signaling effects due to kinase activity experimentally.

**Cell Dosing, RNA Extraction, RT-qPCR**

Cobimetinib, Dichloroacetic Acid, and Zinc05007751 are selected as inhibitors for MAP2K1, PDHK and NEK1 due to their availability, specificity and IC50 range. To promote the generation of primary cilia and Hedgehog Pathway activation, Wild Type (3T3) cells are serum starved and serum starved with Hedgehog conditioned media immediately before treatment with these inhibitors. Inhibitors are added at their IC50s and serially increased on a logarithmic scale to three higher concentrations. Similarly, Basal Cell Carcinoma Cells are serum starved and treated with inhibitors as in Wild Type cells. These cell types and serum conditions are all treated with diluent only (DMSO) as a control. After 24 hours of incubation, RNA is extracted and quantified from both diluent and drug treated cells using the Zymo Research Direct-Zol RNA Miniprep Plus kit. Extracted RNA is amplified using RT-qPCR with primers specific for the GLI1 gene as a means of quantifying Hedgehog Signaling activation, as well as a GAPDH experimental control to quantify GLI expression. Fold change was normalized to the diluent only (DMSO) control in Serum Starved media (SS) for each cell line (3T3 and BCC). Standard error bars denote technical replicates between drug treated samples.

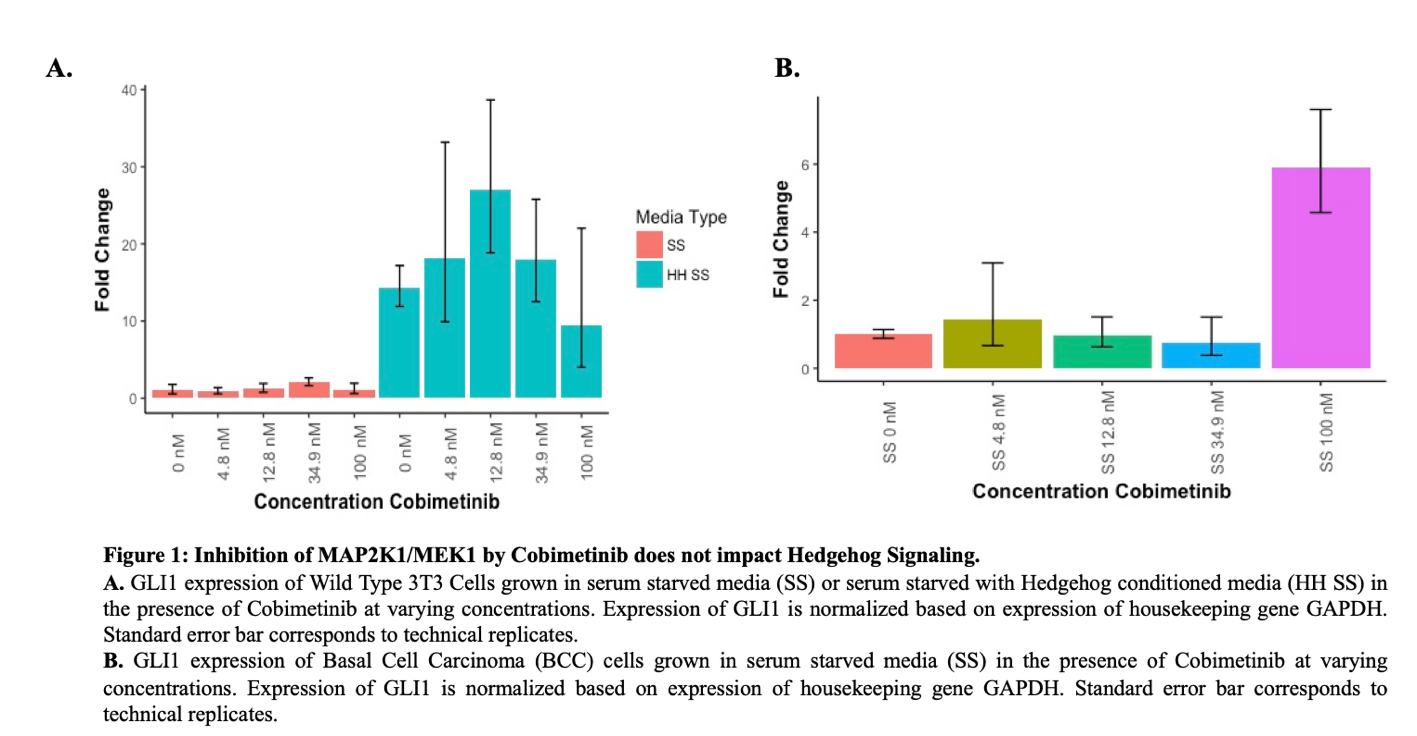
**Metabolic Activity (MTT) Assay**

To confirm that kinase inhibitors would not cause cell death *in vivo*, an MTT Assay is conducted on diluent (DMSO) and drug treated BCC cells. Cells are seeded at 2,000 cells/well and grown to 50% confluence. Once desired confluence is achieved, kinase inhibitors (Cobimetinib, Dichloroacetic Acid, and Zinc05007751) are added at their IC50 and three logarithmically increased concentrations to the cells in replicates of six to allow for variability. When performing the assay, MTT reagent in PBS is added to the each well and allowed to incubate at 36°C for two hours protected from light. After incubation, the MTT reagent is removed and MTT solvent (DMSO) is added to the wells and allowed to shake. Cell proliferation is quantified by measuring the absorbance at 570 nm for BCC cells at 0, 2, 4 and 6 days post drug addition.

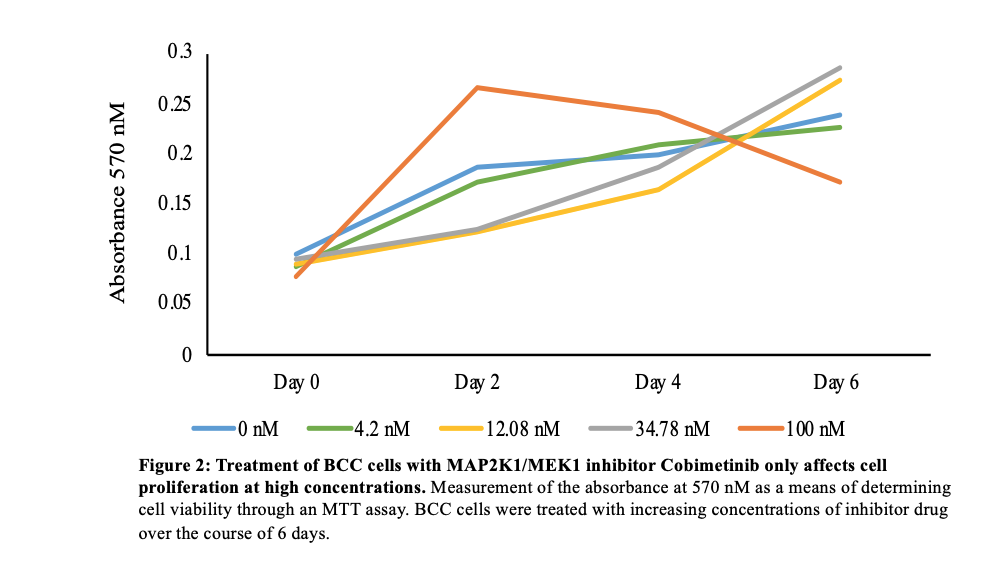
**Results**

**Inhibition of MAP2K1/MEK1 does not impact Activation of Hedgehog Signaling**

Previous evidence of the role of MAP2K1 in cancer and its ability to activate Hedgehog Signaling brought the hypothesis that inhibiting this kinase with Cobimetinib would decrease Hedgehog Signaling activity. GLI1 expression, indicative of Hedgehog Signaling, is not affected in the presence of MAP2K1/MEK1 Cobimetinib kinase inhibitor at the concentrations tested (**Figure 1**). Increased expression of GLI1 is observed for Wild Type (3T3) cells under serum starved with Hedgehog conditioned media (HH SS) in comparison to serum starved media as expected. However, fold change does not vary between Cobimetinib concentrations of SS or HH SS treated 3T3 cells (**Figure 1A**). This observation is consistent in the Basal Cell Carcinoma (BCC) cell line with the exception of the 100 nM concentration of Cobimetinib (**Figure 1B**).

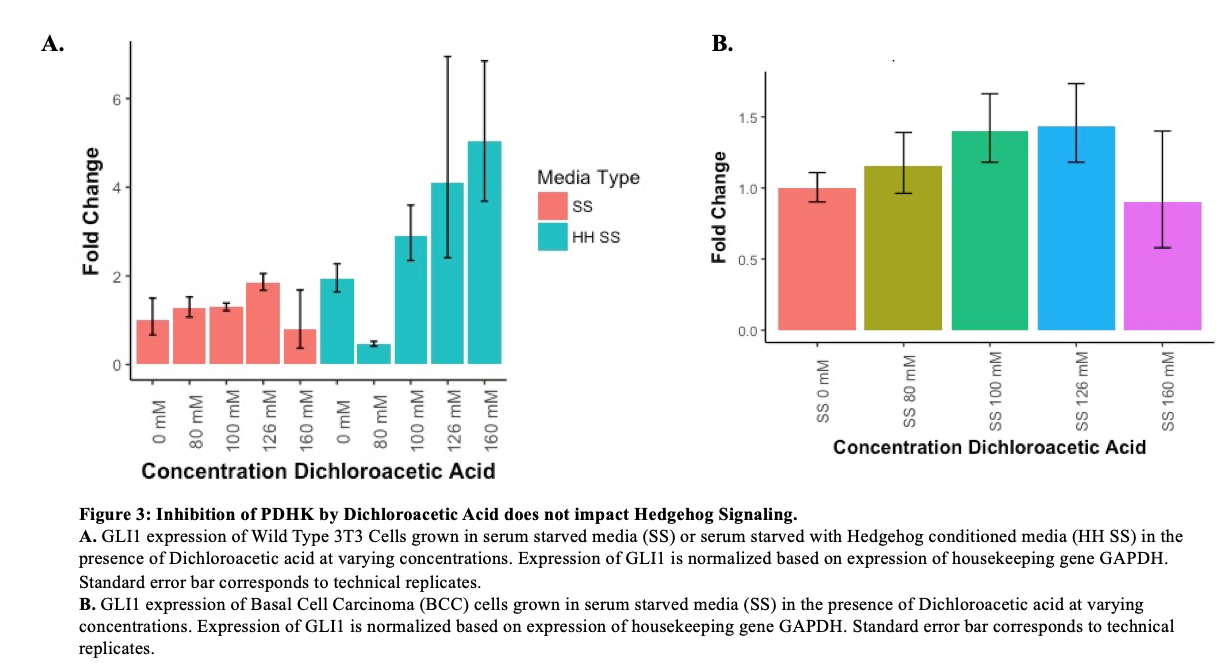


In assessing the cell viability in the BCC cell line, as the concentration of Cobimetinib increases, the cell death with Cobimetinib drug treatment is consistent over a period of 6 days with the exception of the highest concentration 100 nM (**Figure 2**).

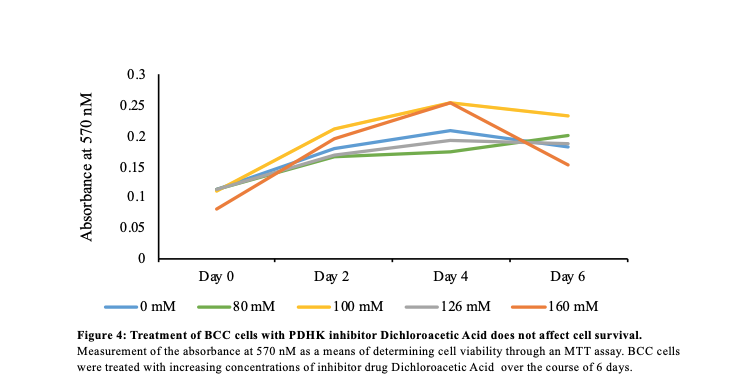


**Hedgehog Signaling activity is not impacted by PDHK Inhibition with Dichloroacetic Acid**

Increased activity of PDHK in cancer cells suggests that inhibition with Dichloroacetic acid could reduce Hedgehog Signaling. Hedgehog signaling activity measured through GLI1 expression is not affected in the presence of Dichloroacetic acid at the concentrations tested (**Figure 3**). Overall, fold change of GLI in the HH SS Wild Type cells is higher than in the SS Wild Type cells as expected. Between concentrations of Dichloroacetic Acid, GLI expression levels are within the standard deviation in the SS condition. In the case of the HH SS treated Wild Type cells, GLI expression is only lower at the IC50 of 80 nM Dichloroacetic Acid (**Figure 3A**). In BCC cells treated with Serum Starved (SS) Media, GLI1 expression is within the standard error bars for all concentrations of inhibitor (**Figure 3B**).

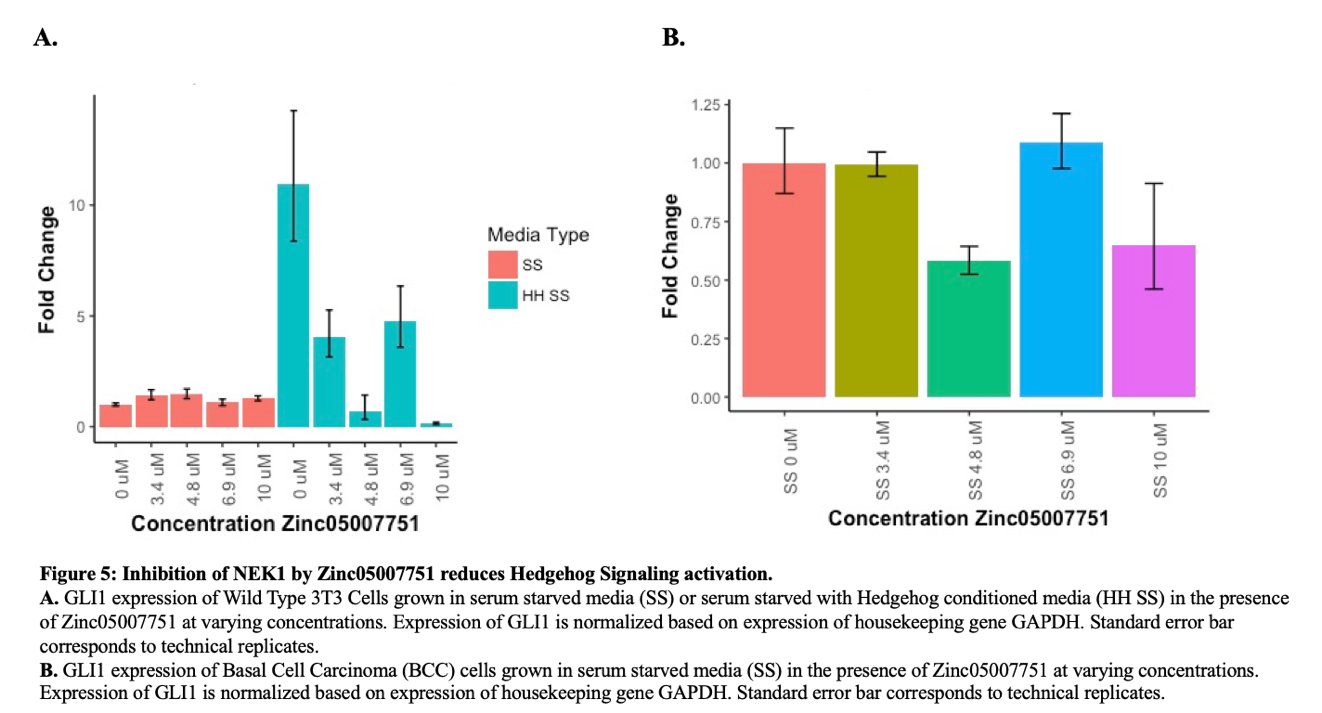


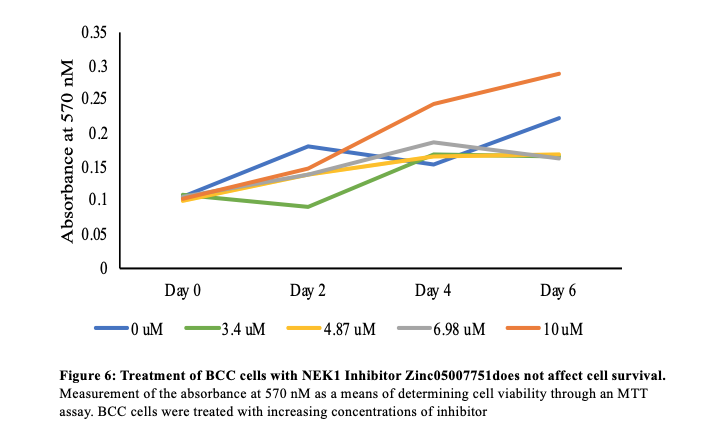
To assess *in vivo* toxicity of the drug, an MTT assay was conducted where absorbance is consistent for all concentrations of PDHK inhibitor over a period of 6 days in the (**Figure 4**).



**NEK1 Inhibition Demonstrates Activation of Hedgehog Signaling**

The effect of NEK1 on cilia regulation and its correlation in cancer suggests inhibition of NEK1 with Zinc05007751 likely affects activation of Hedgehog Signaling. The fold change of GLI in diluent only Wild Type samples increased in the HH SS media condition versus the SS media as expected (**Figure 5**). However, in the HH SS samples, GLI expression decreased beyond the error bars with drug treatment as low as at the IC50 of 3.4 μM (**Figure 5A**). Additionally, there is a decrease in fold change with drug treatment in the BCC cell line (**Figure 5B**). The addition of this drug does not influence cell death in an *in vivo* setting which confirms that the decrease in GLI expression with drug addition is not due to cell viability (**Figure 6**).





**Discussion**

Due to their activity in Hedgehog Signaling or cancer and predicted presence at recurrent GLI mutation sites, MAP2K1/MEK1, PDHK and NEK1 increase Hedgehog Signaling measured through GLI expression in the presence of kinase inhibitors. Although both inhibitor drugs do not contribute to general cell death, MAP2K1/MEK1 and PDHK do not demonstrate significant activation of Hedgehog Signaling in Wild Type and BCC cells treated with inhibitor drug as compared to diluent only treated cells. Using the Cobimetinib inhibitor of MAP2K1/MEK1 which is currently used as treatment for melanoma, there is no activation of Hedgehog Signaling by MAP2K1/MEK1 (**Figures 1, 2**). Due to the direct application of the inhibitor drug to skin cancer, it does not seem to be applicable to continue to pursue the MAP2K1/MEK1 kinase for Hedgehog Signaling Activation. Dichloroacetic Acid, the PDHK inhibitor selected for this project due to its accessibility, did not have an effect on Hedgehog Signaling (**Figure 3**). However, with an increase in the range of drug dosage there is potential this kinase is worthwhile to investigate. As this inhibitor is a general acid, it might be interacting with multiple pathways within the cell; therefore, it would be critical to knock down the PDHK kinase with a short hairpin RNA or CRISPR to determine that the acid is acting on the anticipated target kinase. Overall, the data collected with MAP2K1/MEK1 and PDHK indicates that these kinases, although predicted at mutation sites with GLI activation are directly related to activation of the Hedgehog Signaling Pathway, do not impact the pathway as expected. The final kinase tested, NEK1 which is responsible for regulating ciliogenesis activates Hedgehog Signaling through the assay tested without impacting cell death (**Figures 5, 6**). Expression of GLI in Wild Type serum starved cells with Hedgehog conditioned media treated with diluent only is much higher than the Wild Type cells that are only serum starved. Additionally, when treated with concentrations of inhibitor Zinc05007751, GLI expression decreases, demonstrating the role of NEK1 in activating Hedgehog Signaling. GLI expression additionally decreases when Zinc05007751 is added to BCC cells. As NEK1 demonstrated expected activation of Hedgehog Signaling, it would be of interest to expand the treatment concentration range to determine how robust the treatment is. Additionally, it is critical to validate the target of Zinc05007751 by knocking out NEK1 kinase with short hairpin RNA or CRISPR. It would also be of interest to validate the cell cytotoxicity of Zinc05007751 using a control mouse keratinocyte line, rather than our BCC line which has developed resistance over time. After completion of these experiments to solidify our original result, it would be of interest to look further into the mechanism by which GLI is being activated as regulation of GLI transcription factors is poorly understood. We can do this by…..If this project was to be continued it would also be in need of a larger kinase screen using the methodology utilized in this project to expand the search of Hedgehog Activating kinases. Overall, evidence leads us to believe that NEK1 kinase plays a role in activating the Hedgehog Signaling pathway.

**Works Cited**