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UNIVERSITY OF CALIFORNIA,
IRVINE

Phosphorylation dependent regulation of GLI transcription factors and Hedgehog signaling

DISSERTATION

Submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

In Biological Sciences

By

Eric Tarapore

Dissertation Committee:
Associate Professor Scott Atwood, Chair
Professor Lee Bardwell
Associate Professor Rahul Warrior
Professor Maxsim Plikus
Professor Xing Dai

2022

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DEDICATION

To
My family and friends who have always been there to support me

My Mother
Who's constant encouragement,
Allowed me to persevere through many tough times

My Father
Even though he is no longer with us,
I know that he still offers guidance to me

My Brothers
Who have always been there,
Able to make me smile and laugh at any moment

My Friends
Who made graduate school a blast,
Who were always willing to go to Disneyland with me.

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To my thesis advisor, Scott Atwood, Ph.D., thank you for giving me an opportunity to learn and develop as a scientist. I've been able to develop a lot of life skills and research skills that I will now be able to carry into my future career as a scientist.

I would like to thank all members of my thesis committee. Lee Bardwell, Ph.D., Xing Dai, Ph.D., Maxsim Plikus, Ph.D., and Rahul Warrior, Ph.D., thank you for all your time and effort. And, for always offering constructive criticism on where to take the next steps experimentally.

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To each one of my many undergraduate students that I had the pleasure to work with; Thank You. Shaun Cruz, Manjot Dhaliwal, Ivan He, Michelle Lee, Jerald Lim, Kristeen Shamas, Bao Ton That, Yvonne Tran, Ariana Valencia, and David Young. Thank you all for being there and working with me. The days when you guys were in the lab were always my favorite (especially the days where we would sneak off early and go to Disneyland) and I am truly thankful for all the work and effort you all put towards these projects. Each of you has a bright future ahead of you and I look forward to hearing about all your accomplishments.

Lastly, I would like to thank my family. My parents and my brothers have been the best support system I could ask for during this long journey and I wouldn't have been able to do it without you. Unfortunately, my father was not able to see me through to the end, but I know he is still proud of this accomplishment. Once again Thank You all for being there for me!

VITA

ERIC TARAPORE

EDUCATION

University of California – Irvine June 2022
Doctor of Philosophy in the Biological Sciences

University of California - Irvine June 2014
Bachelor of Science in Developmental & Cell Biology
GPA: 3.624
Biological Sciences GPA: 3.782

HONORS & AWARDS

Recipient of American Society of Cell Biology Travel Grant (2019)
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Recipient of the Undergraduate Research Opportunities Program Grant (January 2014)
Recipient of the Robert Ernst Prize for Excellence in Research in the Biological Sciences (June 2014)
Recipient of the Excellence in Research Award (June 2014)
Ten-time Dean's Honors List recipient (September 2010-June 2014)

RESEARCH EXPERIENCE AND TRAINING

Activating GLI mutants in Cancer 2016 to 2022

For over four years, I have been working closely with Dr. Scott Atwood conducting a large variety of molecular and cellular biology experiments to interrogate the effect of clinical GLI mutations on the Hedgehog pathway. During my time in the lab, I have generated a 100+ stable cell lines each of which overexpress a different clinical mutation of either GLI1 or GLI2. I have also been responsible for testing these cell lines through qRT-PCR, immunostaining, and Western Blotting. Along with my responsibilities for generating over expression cell lines, I have also used lentivirus in order to generate knock down cell lines to assist experiments conducted by other students in the lab. During my work with Dr. Atwood, I have **learnt a variety of skills** some of which include tissue culture technique, generation of stable cell lines, shRNA knockdown, utilization of CRISPER/Cas9, DNA and RNA isolation, qRT-PCR, and Western blotting.

Memory Th17 cell response January 2016 to March 2016

Working under Dr. Manuela Raffatellu, I was able to gain experience working with mice and knowledge on conducting flow cytometry experiments. The range of experiments conducted during my brief time with Dr. Raffatellu included handling mice and collecting fecal samples in order to test for bacterial load to dissection of mice and collection of organs needed for cell isolation and flow cytometry. The emphasis of my work was focused on the latter where I isolated a variety of organs from mice and was responsible for isolating cells needed for flow cytometry and staining with antibodies associated with B Cells, T Cells, T Helper Cells, and Memory T Helper Cells. During my time working under Dr. Raffatellu I have **acquired a variety of skills** such as mouse husbandry, tissue dissection, cell isolation, DNA and RNA isolation, qRT-PCR, and cell marker staining for flow cytometry.

Bio 199 Undergraduate Researcher September 2012 to June 2014
For close to two years, I worked under Dr. Kavita Arora, as an undergraduate researcher. I was able to gain firsthand laboratory experience and learn several laboratory techniques. I have performed various types of experiments that support the research of the lab. These experiments include performing genetic crosses to create strains of flies, tissue dissection, fixation and staining, sample collection, and processing for immunohistochemistry, colorimetric assays, and selecting animals in order to run various viability and mating assays. During my time as an undergraduate researcher, I was able to write a grant and receive funds from the Undergraduate Research Opportunities Program at UCI.

TEACHING AND MENTORING EXPERIENCE
Supervised Students:
Kristeen Shamas (Undergraduate Student, UCI) 2016-2017
Shaun Cruz (Undergraduate Student, UCI) 2016-2017
- *Chapman University MMS Physician Assistant Studies Program Student*

Ariana Valencia (Undergraduate Student, UCI) 2017-2018
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Yvonne Tran (Undergraduate Student, UCI) 2017-2019
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Bao That (Undergraduate Student, UCI) 2018-2020
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Jerald Lim (Undergraduate Student, UCI) 2019-2021

Head Teaching Assistant January 2020 to March 2020
Attended weekly lecture sessions. Facilitated group activities with my assigned sections during the lecture. Under the supervision of the instructor, taught one assigned section for an upper-division laboratory section consisting of ~20 students per section. Lead discussion within the lab section. Gave in class demonstrations during laboratory sections. Graded weekly lab notebooks. Along with these responsibilities as Head Teaching Assistant it was my responsibility to write and grade weekly quizzes. I was also responsible for writing and grading the final examination.

COSMOS Teaching Assistant July 2019 to August 2019
Teaching assistant for the California State Summer School for Mathematics and Science (COSMOS). Designed a four-week experiment testing the effectiveness of peptides to inhibit the activity of Atypical Protein Kinase C (aPKC). Worked directly with 20 High School students from across California to design their own peptide inhibitor and subsequently clone, express, purify, and test the effectiveness of this peptide to block aPKC function. Attended daily lectures and led group discussions on the topics that were covered. Led discussions analyzing scientific literature related to experiments that the students would work on during the week. Worked directly with the students in our daily laboratory sessions, where I would supervise the students set up and run their experiments as well as walk them through how to analyze their results or troubleshoot the experiment.

Head Teaching Assistant April 2019 to June 2019
Attended weekly lecture sessions. Facilitated group activities with my assigned sections during the lecture. Under the supervision of the instructor, taught one assigned section for an upper-

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Teaching Assistant April 2018 to June 2018
Attended weekly lecture sessions. Facilitated group activities with my assigned sections during the lecture. Under the supervision of the instructor, taught two assigned sections for an upper-division laboratory section consisting of ~20 students in each section. Lead discussion within the lab section. Gave in class demonstrations during laboratory sections. Graded weekly lab notebooks.

Head Teaching Assistant April 2017 to June 2017
Attended weekly lecture sessions. Facilitated group activities with my assigned sections during the lecture. Under the supervision of the instructor, taught one assigned section for an upper-division laboratory section consisting of ~20 students per section. Lead discussion within the lab section. Gave in class demonstrations during laboratory sections. Graded weekly lab notebooks. Along with these responsibilities as Head Teaching Assistant it was my responsibility to write and grade weekly quizzes. I was also responsible for writing and grading the final examination.

PROFESSIONAL EXPERIENCE

Laboratory Assistant June 2014 to July 2015
Under the supervision of the Principal Investigator, I have performed various procedures in support of ongoing research activities in a laboratory setting. Duties include organizing and maintaining chemical inventory, making stock solutions, routine molecular and biochemistry assays, managing and maintaining fly stocks, routine experimental procedures such as tissue sample collection, fixation and processing for immunohistochemistry, qRT-PCR, and colorimetric assays.

PUBLICATIONS (** Denotes equal contribution, # Denotes co-corresponding author)

Chen A., **Tarapore E.,** To A.G., Catolico D.M., Nguyen K.C., Coleman M.J., Spence R.D. 2022. Introducing Immunohistochemistry to a Molecular Biology Laboratory. Biochemistry and Molecular Biology Education. https://doi.org/10.1002/bmb.21611

Tarapore, E.,** Shaffer, J.F.**, Atwood, S.X. 2022. Online engagement in an undergraduate cell biology course. Journal of College Science Teaching. 51(4):27-34.

Beasley, S., Vandewalle, A., Singha, M., Nguyen, K., England, W., **Tarapore, E.,** Dai, N., Correa Jr., I., Atwood, S.X., Spitale, R.C. 2022. Exploiting endogenous enzymes for cancer-cell selective metabolic labeling of RNA *in vivo*. Journal of the American Chemical Society. 144(16):1085-7088.

Wang S.**, Drummond M.L.**, Guerrero-Juarez C.F., **Tarapore E.**, MacLean A.L., Stabell A.R., Wu S.C., Gutierrez G., That B.T., Benavente C.A., Nie Q.#, Atwood S.X.#. 2020. Single cell transcriptomics of human epidermis reveals basal stem cell transition states. Nature Communications. 11(4239) 10.1038/s41467-020-18075-7

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Tarapore E, Atwood SX. 2019. Defining the genetics of basosquamous carcinoma. Journal of Investigative Dermatology. 139(11):2258-60. 10.1016/j.jid.2019.04.011

Brown A., Meera P., Altindag B., Chopra R., Perkins E., Paul S., Scoles D.R., **Tarapore E.**, Magri J., Jackson M., Shakkottai V.G., Otis T.S., Pulst S.M., Atwood S.X., Oro A.E. 2018. MTSS1/Src family kinase dysregulation contributes to multiple neurodegenerative disorders. Proceedings of the National Academy of Sciences. 115(52):E12407-E12416 10.1073/pnas.1816177115

Drummond ML, Li M, **Tarapore E**, Nguyen TTL, Barouni BJ, Cruz S, Tan KC, Oro AE, Atwood SX. 2018. Actin polymerization controls cilia-mediated signaling. Journal of Cell Biology. 217(9):3255-66 10.1083/jcb.201703196

Tarapore E, Arora K. 2014. Analysis of the role of TGF-β/Activin signaling in regulation of energy homeostasis. Journal of Undergraduate Research.

PRESENTATIONS & POSTERS

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Tarapore E., Stabell A., Mauzy-Melitz D. (2019, June). Dazed & Diffused: A hands-on approach to understanding diffusion in biology. Workshop presented at annual meeting of the Association for Biology Laboratory Education, Ottawa, Canada.

Tarapore E., Tran Y., Valencia A.M., Atwood S.X. (2018, December). Phosphorylation dependent regulation of C2H2 Zinc Finger transcription factors. Poster presented at annual meeting of the American Society for Cell Biology, San Diego, CA.

Brown A., Meera P., Altindag B., Chopra R., Perkins E., Paul S., Scoles D.R., **Tarapore E.**, Magri J., Jackson M., Shakkottai V.G., Otis T.S., Pulst S.M., Atwood S.X., Oro A.E. (2018, December). MTSS1/Src family kinase dysregulation contributes to multiple neurodegenerative disorders. Poster presented at annual meeting of the American Society for Cell Biology, San Diego, CA.

Tarapore E., Li L., Chapman D., Mauzy-Melitz D. (2018, June). Barking up a storm: what dog DNA testing can tell us about statistical errors. Workshop presented at annual meeting of the Association for Biology Laboratory Education, Columbus, Ohio.

Drummond M.L., Wang S., MacLean A.L., **Tarapore E.**, Benavente C., Nie Q., Atwood S.X. (2018, March). Single cell transcriptome profiling of human interfollicular epidermis reveals robust heterogeneity and divergent differentiation lineages. Poster presented at Center for Complex Biological Systems retreat, Los Angeles, CA.

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Tarapore E., Arora K. 2014. Analysis of the role of TGF-β/Activin signaling in regulation of energy homeostasis. University of California Irvine Excellence in Research Symposium. Irvine, CA, USA. 2014.

Tarapore E., Arora K. 2014. Analysis of the role of TGF-β/Activin signaling in regulation of energy homeostasis. University of California Irvine Undergraduate Research Opportunities Symposium. Irvine, CA, USA. 2014.

ABSTRACT OF DISSERTATION

Phosphorylation dependent regulation of GLI transcription factors and Hedgehog signaling

By

Eric Tarapore

Doctor of Philosophy in the Biological Sciences

University of California, Irvine, 2022

Associate Professor Scott Atwood, Chair

Basal cell carcinoma (BCC) is one of the most prevalent cancers and is mainly driven by the overactivation of the Hedgehog (HH) signaling pathway. Although easily treatable via Smoothened inhibitor vismodegib, drug resistance is common and as such alternative avenues to treat BCCs must be identified. One way to target pathway activity is through targeting the transcription factors responsible for relaying the signals. Here we use a combination of molecular biology techniques to identify additional ways to regulate the HH signaling pathway. We identify phosphorylation as being a key regulator of GLI activity and binding. We identify that AURKA plays a key role in regulating BCC growth *in vitro* and *in vivo*. We found that pharmacological inhibition of AURKA in the *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mouse BCC tumor model resulted in significantly reduced levels of BCC cell growth. Inhibition of AURKA in BCC cells *in vitro* resulted in reduced levels of HH signaling and cell growth. This goes with work that was conducted showing that phospho-mimetic mutation of the zinc finger domain of GLI results in differential levels of DNA binding and activity. We identify a specific region of the zinc finger domain that is permissive to phospho-mimetic mutation whereas other regions of the zinc finger completely

abolish DNA binding and activity. We have termed these as the permissive regulatory region along with the more restrictive DNA binding region and linker region. Further studies into additional zinc finger transcription factors seem to show similar results although not to the same extent as GLI. Together these finding identify alternate means of GLI regulation, either through phosphorylation of GLI via AURKA or phosphorylation of the zinc finger binding domain of GLI.

CHAPTER 1

INTRODUCTION

BASAL CELL CARCINOMA

Adapted from Nguyen, Tarapore, and Atwood 2019

BCCs are locally invasive epithelial cancers driven by activating mutations in the Hedgehog (HH) pathway (Atwood et al., 2012). They are the most common skin cancers and make up approximately 60–80% of all skin cancer cases (Cameron et al., 2019), affecting approximately 4.88 million patients in the United States (Wu et al., 2013), 1.28 million in Europe (Reinau et al., 2014), less than 1 million in Asia (Sng et al., 2009), and approximately 450,000 in Australia (Richmond-Sinclair et al., 2009) based on current population counts. Accurate numbers are difficult to pin down due to a lack of uniform reporting guidelines for non-melanoma skin cancer and a lack of universal application of a staging system by dermatologists. Although BCCs are typically treated by surgical excision, five-year recurrence rates of 3.3% indicate that a large number of patients are not cured by this method (Chren et al., 2013). While alternative therapies such as SMO inhibitors (SMOinh: i.e., vismodegib and sonidegib) are FDA-approved to treat advanced BCCs by targeting and suppressing the HH pathway (Atwood et al., 2012), nearly 60% of advanced tumors display inherent SMOinh resistance and 20% of tumors that initially respond acquire resistance every year (Chang et al., 2012). This is a highly relevant issue as advanced BCC cases, which are defined as surgically nonresectable, are estimated at 1–10% of total BCC cases (Mohan et al., 2014). Inappropriate HH pathway activation also drives growth of a variety of cancers including blood, bone, brain, breast, lung, pancreas, prostate, and stomach cancer, accounting for up to 25% of all human cancer deaths (Epstein, 2008). These statistics point to a need to understand how oncogenic HH signaling is controlled and how we can target this pathway to suppress HH-driven cancers.

Risk Factors

The likelihood of developing BCC derives from a combination of genetic and environmental factors. On the far end of the genetic spectrum, basal cell nevus syndrome (BCNS) patients harbor germline alterations in the PTCH1 gene and can develop hundreds of BCCs throughout their lifetimes (Huq et al., 2017), presumably driven by persistently high activation levels of the HH pathway. On the other end of the environmental spectrum, ultraviolet (UV) radiation from indoor tanning significantly increases the risk of BCC by approximately 4% alone as determined by a systematic review and meta-analysis of appropriate research articles (Wehner et al., 2012). A combination of these two risk factors significantly increases the number of tumors developed by BCNS patients (Chiang et al., 2018). Interestingly, genetics seems to play a role in preventing too many more tumors from forming in BCNS patient population as these patients display a lower mutational load, lower proportion of UV mutagenesis, and increased genomic stability in comparison to patients with sporadic BCC tumors that often develop after a lifetime of UV exposure. Light-skinned individuals have far higher incidence than darker-skinned individuals, as seen in the statistics at the beginning of the chapter and reviewed by Porcia Bradford (Bradford, 2009). Photosensitizing drugs that induce either a phototoxic or photoallergic reaction upon UV exposure have been shown to enhance the risk of developing BCC in a population-based case-control study (Robinson et al., 2013). Other types of radiation, such as those exposed to the atomic bomb, have also been shown to have higher rates of BCC formation, especially when the exposure occurred earlier in life (Sugiyama et al., 2014). Of final note on the risk factors for BCC, immunosuppression is a frequently reported significant risk factor that increases over time, especially in organ transplant recipients (Adami et al., 2003). The risk of subsequent BCC tumors after the first tumor appearance is high in transplant recipients, where patients who develop BCCs typically continue to develop BCCs (Wisgerhof et al., 2010). Frequent BCC formation is also a clinical marker for inherited cancer risk of other malignancies, presumably due in part to an increase in pathogenic mutations in DNA repair genes (Cho et al., 2018).

Cell of Origin

Multiple stem cell populations are found in the basal layer of the epidermis and hair follicles in both mice and humans. At least in mice, BCC can arise from nearly all of the stem cell populations in the basal layer depending on the strength of the oncogenic driver. Loss of *PTCH1* induces tumor formation in ~70% of human BCCs, whereas overexpression of constitutively active SMO is found in ~20% of tumors and constitutively active GLI2 in ~8% of tumors (Bonilla et al., 2016). In mice, loss of *Ptch1* can induce BCCs in the hair follicle bulge, secondary hair germ, and epidermis (Kasper et al., 2011; Wang et al., 2011). Interestingly, when *Ptch1* is lost using specific drivers within the skin and hair follicle compartments, the isthmus and infundibulum also show tumor induction, but the epidermis is largely resistant to tumor growth except at the touch dome epithelia where nerve innervation promotes HH pathway activity (Peterson et al., 2015). Overexpression of constitutively active SMO, a relatively weak oncogenic driver, can induce BCCs from the upper infundibulum and epidermis (Wong et al., 2011; Youssef et al., 2010). Using a strong oncogenic driver like constitutively active GLI2 can drive nodular BCC formation when expressed in the lower bulge, secondary hair germ, infundibulum, and sebaceous gland compartments of the hair follicle, whereas such expression in the epidermis resulted in superficial BCCs (Grachtchouk et al., 2011). In humans, BCCs are observed to arise from the epidermis and hair follicle, but the exact cells of origin are not known. If one were to assume superficial BCCs arise from the epidermis and nodular BCCs arise from the hair follicle, ~16% of human BCCs are superficial and 57–78% of human BCCs are nodular (Bastiaens et al., 1998; Scrivener et al., 2002). One intriguing observation is that Merkel cells, which are associated with the mouse touch dome, are seen in more superficial human tumors, suggesting at least one common origin at mechanosensory niches (Peterson et al., 2015).

Common Treatments

Surgical excision is the most common therapy to treat BCC, although management is often guided by anatomical location and histological features (Rubin et al., 2005). Common surgical methods include cryosurgery, curettage, electro dissection, Mohs surgery, and standard surgical excision. These methods are typically used for superficial and nodular BCC but are inappropriate for tumors in cosmetically sensitive areas or tumors that are overly large or metastatic. Mohs surgery does appear to be the most effective way to prevent recurrence in primary BCCs and recurrent BCCs compared with standard surgical excision (Mosterd et al., 2008).

Nonsurgical methods to treat BCC include radiotherapy, photodynamic therapy, and chemotherapy. Radiotherapy is used for patients who are not good candidates for surgery because of the tumor location or metastasis and is typically avoided in patients younger than 60 years of age. Photodynamic therapy is an alternative way to treat BCC, which uses visible light to activate a photosensitizing drug to generate reactive oxygen species and kill tumor cells (Cohen et al., 2016). This type of therapy is more effective for superficial BCC than the nodular subtype, and for smaller tumors, where response rate increases with more sessions (Christensen et al., 2012). For larger tumors, photodynamic therapy leads to higher recurrence rates compared with surgical excision (Rhodes et al., 2007), so a trade-off between a better cosmetic outcome with photodynamic therapy compared with surgical excision must be weighed when using this therapy.

The major types of topical chemotherapy treatments include imiquimod, 5-fluorouracil (5-FU), and SMOinh (i.e., vismodegib and sonidegib). Imiquimod is a Toll-like receptor 7 agonist that induces local skin inflammation through interferon-α, TNF-α, and other cytokines and can clear up to 75% of superficial BCCs (Geisse et al., 2004). Serendipitously, imiquimod also serves to suppress HH pathway activation by reducing the active form of GLI (Wolff et al., 2013). 5-FU is a well-tolerated treatment for BCC with little to no pain or scarring and works by interfering with DNA synthesis by blocking methylation of deoxyuridylic acid and inhibiting thymidylate synthetase (Gross et al., 2007). In a five-year randomized controlled trial comparing the effectiveness of

photodynamic therapy of imiquimod and 5-FU in 601 patients with superficial BCC, imiquimod resulted in an 80.5% tumor-free survival compared with 70% for 5-FU and 62.7% for photodynamic therapy (Jansen et al., 2018).

Vismodegib was the first SMOinh to be FDA-approved to treat locally advanced and metastatic BCC and is quite effective at suppressing both late-stage tumor growth and Gorlin's syndrome patients (Sekulic et al., 2012; Tang et al., 2016; Tang et al., 2012). However, many tumors regrow after cessation of drug therapy with nearly 60% of advanced tumors displaying innate resistance and 20% of tumors that do respond to drug acquire resistance every year (Chang et al., 2012), suggesting use of SMOinh are more appropriate for neoadjuvant therapy in combination with a surgical technique. Side effects of this therapy are more pronounced than the other topical chemotherapy treatments and include alopecia, decreased appetite, diarrhea, fatigue, muscle spasms, nausea, and taste and weight loss. Acquired resistance to SMOinh are mainly SMO mutations that prevent drug interaction or promote constitutive activity even in the presence of drug (Atwood et al., 2015). As such, alternative therapies to treat SMOinh-resistant BCC should target genes that promote HH pathway activity downstream of SMO, such as the SRF-MKL1 (Whitson et al., 2018), aPKC (Atwood et al., 2013), or HDAC1 (Mirza et al., 2017). Interestingly, recent data point to hair follicle-derived BCCs as more sensitive to SMOinh than ones with an epidermal-derived origin (Biehs et al., 2018; Sanchez-Danes et al., 2018). BCCs are plastic and can switch from a hair-follicle signature to an epidermal signature to evade SMO inhibition and persist in the presence of drug. However, targeting with a WNT inhibitor in combination with SMOinh leads to the eradication of resistant tumor cells and prevents tumor relapse.

HH SIGNALING AND BCC

Adapted from Nguyen, Tarapore, and Atwood 2019

Inappropriate activation of the HH pathway and its target genes are thought to be the sole drivers of BCC with enrichment of GLI protein or RNA signal used as a clinical marker that is present in all BCCs so far observed (Atwood et al., 2015). HH signaling is essential for the development of all vertebrates and drives proliferation, migration, and differentiation of progenitor cells to pattern organs (Varjosalo et al., 2008). Vertebrate HH signals through a microtubule-based sensory organelle called the primary cilium, which largely resides in progenitor cells of the skin and hair follicle (Croyle et al., 2011). HH signaling components are recruited to this membrane-enclosed structure to sense and respond to extracellular cues. HH ligand binds and inactivates the receptor PTCH1, removing it from the primary cilium and allowing the G protein-coupled receptor SMO to traffic into the cilium to activate the GLI transcription factors. Loss of the primary cilium suppresses tumor growth when the driving mutation is at the receptor level; however, tumor growth can be accelerated in the absence of cilia when the driving mutation is at the level of GLI (Wong et al., 2009).

Stem cell compartments that are competent to receive HH signal and activate GLI and its target genes drive tumor growth (Nilsson et al., 2000; Oro et al., 1997; Oro et al., 2003). Additionally, pathways that enhance HH signaling are often intimately entwined with tumor growth. For instance, atypical protein kinase C iota/lambda (aPKC), a HH target gene and GLI1 kinase, phosphorylates and activates GLI1 to maintain high HH pathway activation levels in both normal and tumor contexts (Atwood et al., 2013). Inappropriate aPKC activity correlates with poor patient clinical outcome and mortality (Murray et al., 2011) and loss of aPKC results in severe reduction in GLI activity and tumor growth (Atwood et al., 2013). aPKC-specific phosphorylation of GLI1 recruits HDAC1, promoting GLI1 deacetylation and activity, and that pharmacological suppression of HDAC1 also suppresses tumor growth (Mirza et al., 2017). Actin cytoskeletal dynamics driven by RHO can activate SRF and MLK1, which serve as GLI1 coactivators on target

genes to drive tumor growth (Whitson et al., 2018). These are but a few examples of pathways that also activate HH signaling to drive tumor growth.

GLI PROTEINS AS THE EFFECTORS OF HH SIGNALING

GLI proteins are transcription factors involved in the intracellular signal transduction controlled by the HH family of ligands. In mammalian cells glioma-associated oncogene family members 1, 2, and 3 (GLI1, GLI2, GLI3) are dedicated transcription factors for the HH pathway (Jiang et al., 2008). These proteins are essential to activate downstream transcriptional target genes of the HH pathway that drives proliferation, migration, and differentiation (Varjosalo et al., 2008). The GLI transcription factors were among the first to have their DNA binding site determined (Kinzler et al., 1990) and the 3D structure of their DNA binding domain resolved (Pavletich et al., 1993).

The GLI family of transcription factors falls under the larger Kruppel family of transcription factors, each containing five C2H2 zinc fingers that serve as the DNA binding domain. Zinc finger 4 and zinc finger 5 bind specifically to the consensus binding sequence of 5' – GACCACCCA – 3' whereas zinc finger 1-3 stabilize this binding by interacting with the phosphate backbone (Kinzler et al., 1990; Pavletich et al., 1993). A nuclear export sequence (NES) and a nuclear localization sequence (NLS) ensure proper shuttling of the GLI transcription factors (Han et al., 2017). Zinc finger transcription factors make up the largest proportion of transcription factors in mammals accounting for roughly 80% (Vaquerizas et al., 2009). C2H2 zinc finger transcription factors (C2H2 ZF-TFs) consist of two cysteine and two histidine residues that fold into a two-stranded antiparallel β-sheet and an α-helix after interacting with zinc ions (McCarty et al., 2003). Two to three zinc finger motifs are suitable to serve as a DNA binding domain.

The GLI family of C2H2 ZF-TFs consist of five zinc finger domains, and although these factors are highly conserved each play a distinct functional role as part of the HH signaling

pathway. All GLI proteins contain a SUFU binding sequence which is required for cytoplasmic retention of GLI1/2/3 (Han et al., 2015). In addition, all GLI proteins also contain a C-terminal trans-activation domain (TAD), but only GLI2 and GLI3 contain a N-terminal repressor domain that allows these two to act as both activator and repressor of HH signaling (Tsanev et al., 2009). GLI1 is not required for early development and appears to function mostly as part of a positive feedback loop (Bai et al., 2002). It is believed that GLI1 plays the role of a strong transcriptional activator of the HH pathway. Studies conducted in mice have shown that *Gli1*, but not *Gli2* or *Gli3* can mimic HH pathway function by activating HH target genes in the CNS (Park et al., 2000). As previously described GLI1 is also reported as being overexpressed in a variety of different cancers. Two isoforms of GLI1 have been previously described GLI1ΔN and tGLI1. GLI1ΔN is generated through alternative splicing creating a protein with a 128 amino acid deletion, which prevents SUFU binding but leaves intact the zinc finger domain. GLI1ΔN acts on similar target genes as full-length GLI1 and does not seem to be preferentially expressed in cancer (Shimokawa et al., 2008). tGLI1 generated through splicing losing 41 amino acids but maintains the same domains as full length. Studies have shown that tGLI1 does have tumor specific expression and is involved in regulating genes associated with epithelial to mesenchymal transition (Lo et al., 2009).

Similar to GLI1, GLI2 plays the role of transcriptional activator albeit to a lesser extent due to the N-terminal repressor domain found in GLI2. Due to this repressor domain GLI2 can act as a bifunctional transcription factor. This bifunctional activity applies to GLI3 as well however in most cases GLI3 is reported to be a strong repressor of HH signaling (Tsanev et al., 2009). Whereas GLI1 is dispensable for development mutant studies on GLI2 and GLI3 have shown that they are essential for early development. Loss of mouse *Gli2* results in embryonic lethality with defects in early brain development (Mo et al., 1997). In addition, mutation in murine *Gli3* results in embryonic lethality with defects in multiple organs such as the brain (Hui et al., 1993). Due to

the nature of the GLI transcription factors having bifunctional roles as both activators and repressors of the HH pathway, a fine balance between activator and repressor needs to be achieved in order to output signal. The combinatorial and cooperative function of the three GLI transcription factors is known as the GLI code (Ruiz et al., 2007). In vertebrae tissues this is achieved through gradients of activator and repressor functions across distinct spaces during specific times of development (Ruiz et al., 1998). Due to this the GLI transcription factors need to be highly regulated in order to achieve a balance of activator and repressor function. This regulation can be achieved in several ways such as post translational modification.

POST TRANSLATION MODIFICATION AS A MEANS TO REGULATE TRANSCRIPTION FACTOR ACTIVITY

Post translational modification (PTM) refers to any modification of proteins following synthesis. PTMs could be isolated single events that regulate an aspect of a transcription factors function likewise, PTMs can be sequentially linked. One PTM may promote or inhibit the second or third PTM on the same protein (Filtz et al., 2014). Most common forms of PTMs include phosphorylation, acetylation, glycosylation, methylation, SUMOylation and ubiquitination (Khoury et al., 2011). These modifications may alter a transcription factors localization, stability, DNA binding affinity, or association with co-factors (Filtz et al., 2014).

GLYCOSYLATION

Glycosylation, specifically O-GlcNAcylation occurs mostly on nuclear proteins but can also occur on intracellular compartments including mitochondria. The modification to a serine or threonine residue of nuclear or cytoplasmic proteins was originally discovered in the 1980s (Torres et al., 1984). This differs from other forms of glycosylation due to the small size and dynamic nature (Filtz et al., 2014). As O-GlcNAcylation targets the same motifs as phosphorylation, there is an extensive cross talk between these two PTMs on proteins (Wang et

al., 2008). Inhibition of GSK3β has been shown to increase glycosylation on cytoskeletal proteins and decreases glycosylation of transcription factors (Wang et al., 2007). Studies done on the overexpression of O-GlcNAc transferase (OGT) has shown that it results in polyploidy of cells and reduces overall phosphorylation of proteins (Slawson et al., 2005). Competition between O-GlcNAcylation and phosphorylation occurs through distinct mechanisms. Transcription factor c-Myc gets reciprocally modified at the same site by either O-GlcNAcylation or phosphorylation (Chou et al., 1995). Other proteins that get competed for O-GlcNAcylation or phosphorylation include factors such as p53 (Yang et al., 2006), vimentin (Slawson et al., 2008), and RNA polymerase II (Kelly et al., 1993) to name a few.

SUMOYLATION

SUMOylation is a reversible modification by a small ubiquitin like modifier that can dramatically affect the activities, localization, or protein-protein interactions of transcription factors (Geiss-Friedlander et al., 2007). SUMOylation ends up being a far larger PTM then others such as phosphorylation, acetylation, and even larger than ubiquitination. This addition in the size of the protein ends up playing a role in many transcription factors that undergo SUMOylation. SUMOylation of transcription factors is most often associated transcriptional repressive activity (Cubenas-Potts et al., 2013) and several mechanisms have been proposed as to why this occurs. The presence of the large SUMO moiety may serve as an interacting platform for other proteins containing SUMO interacting motifs (SIM). For example, the assembly of the transcriptionally repressive complex of LSD1/CoREST1/HDAC requires the interaction the SIM of CoREST1 with SUMO2/3 (Ouyang et al., 2009). Additionally, SUMOylation plays a key role in the assembly and function of polycomb repressive complex (Luis et al., 2011). Although mostly associated with repression of transcriptional activity, SUMOylation has been associated with transcriptional

activation for a variety of factors such as Ikaros (Gomez-del Arco et al., 2005) and PAX6 (Yan et al., 2010).

UBIQUITINATION

Ubiquitination and SUMOylation share many similarities between the two modifications. Although the consensus sequences for these processes may differ, both modifications result in the addition of a moiety to a lysine residue (Jadhav et al., 2009). These modifications alter the function of transcription factors in a variety of ways. Ubiquitination has been well studied in regard to protein degradation but, ubiquitination can also regulate activity of transcription factors in other ways. Mono-ubiquitination of transcription factor FOXO4 results in increased nuclear localization and transcriptional activity (van der Horst et al., 2006)). Poly-ubiquitination of transcription factors often results in proteolytic processing which may be necessary for regulating transcription factor activity. Ubiquitination and processing of NF-κB is required for the activation of the transcription factor (Geng et al., 2012). In other cases, poly-ubiquitination results in the degradation of the transcription factor preventing signal transduction, as in the case with WNT ligands (Latres et al., 1999).

ACETYLATION

Acetylation is a dynamic modification that can alter the functions of several proteins. Targeting lysine residues of many histones as well as transcription factors this modification serves as a key regulatory mechanism for proteins. Initially studied in the context of histone acetylation, eventually non-histone protein acetylation was found as the research continued, and now several transcription factors have been found to be regulated through acetylation. Acetylation of p53 results in the activation of additional DNA binding sites (Glozak et al., 2005). Transcription factor STAT3 acetylation enhanced transcriptional activity, a process which also involves the reduction

of HDAC1 (Chen et al., 2015). Zinc finger transcription factor SNAI1, a transcription factor involved in epithelial to mesenchymal transition has been shown that increased levels of acetylation results in an increased metastatic potential of cancer cells (Hsu et al., 2014). Acetylation of Myc, a transcription factor involved in the induction of apoptosis, has been shown to increase Myc stability and activity (Kenneth et al., 2007).

PHOSPHORYLATION

Phosphorylation is the most common form of PTM a protein can undergo and is often the first PTM to be studied when looking at protein activity. Rapidly reversible, this PTM is utilized as a mechanism to transduce extracellular signals into the nucleus, and affects transcription factor stability, localization, structure, and protein-protein interactions (Filtz et al., 2014). Phosphorylation of proteins is executed via kinases and reversed through phosphatases. Phosphorylation is a dynamic PTM as it is easily reversible and can serve as a 'switch' to alter the activity of transcription factors. Phosphorylation may also promote or prevent other PTMs or lead to protein degradation. Phosphorylation occurs at serine, threonine, or tyrosine residues and as such transcription factors may harbor multiple sites of phosphorylation to occur. Multisite phosphorylation creates another layer of regulation where multiple phosphorylation may result in increasing or decreasing levels of transcription factor activity. For example, transcription factor TORC2 requires dephosphorylation at multiple sites to allow for translocation (Screaton et al., 2004). Phosphorylation not only directly affects a transcription factor, but also affects other PTMs that occur on the same protein.

Phosphorylation has been linked with both increasing and decreasing levels of SUMOylation of different transcription factors. MEF2 transcription factors which are associated with myogenesis (Black et al., 1998), have been shown to undergo phosphorylation, acetylation, and SUMOylation all of which are associated with regulation of the transcription factors.

Phosphorylation of amino acid S408 of MEF2A has been shown to promote the SUMOylation of MEF2A. On the other hand, dephosphorylation of S408 promotes acetylation of the same residue which restores the ability of MEF2A to activate downstream target genes (Shalizi et al., 2006). Multisite phosphorylation via ERK1/2 and p38 of the transcriptional regulator BCL11B results in the deSUMOylation of the protein (Zhang et al., 2012). Phosphorylation has also been linked to the acetylation and ubiquitination of transcription factors. Studies conducted on FOXO1 have shown that phosphorylation reduces nuclear localization and DNA binding (Zhao et al., 2004) which ultimately leads to its ubiquitination and proteasomal degradation. Methylation has also been shown to have a connection with protein phosphorylation. Phosphorylation results in the conformational changes that destabilize the CARM1-C/EPBβ complex. This change allows for C/EPBβ to undergo demethylation allowing interaction with SWI/SNF and Mediator complexes to induce target gene expression (Kowenz-Leutz et al., 2010). Phosphorylation plays a critical part during the lifespan of a transcription factor and often converges multiple signaling networks or PTMs on a single transcription factor, allowing for phosphorylation to regulate a wide variety of pathways and mechanisms.

These are just a few examples of the different types of PTMs and how they regulate the activity of transcription factors. Multiple PTMs expand the possibilities for transcriptional regulation, and sequential modifications allow for time dependent control of gene expression in response to an initial stimulus (Filtz et al., 2014). It is no surprise then that PTMs play a key role in regulating the HH signaling pathway through the modification of GLI.

REGULATION OF GLI PROTEINS THROUGH POST TRANSLATION MODIFICATION

GLI proteins are extensively modified post translationally and these PTMs play a critical role in determining localization of these proteins and output of the HH signaling pathway. GLI1/2/3

are modified by several types of PTMs some of which include phosphorylation, ubiquitination, and acetylation.

Phosphorylation is the best studied PTM of the GLI proteins and to date several kinases and phosphorylation sites have been identified. One of the most well studied phosphorylation events of GLI include the sequential multisite phosphorylation via protein kinase A (PKA), casein kinase 1 (CK1), and glycogen synthase kinase 3-β (GSK3β). There have been identified six sites of phosphorylation via PKA within GLI2/3 (Niewiadomski et al., 2014) and once phosphorylated primes these transcription factors for subsequent phosphorylation via CK1 and GSK3β (Tempe et al., 2006; Pan et al., 2006). This multisite phosphorylation triggers ubiquitination of the proteins and cleavage into the repressor construct of GLI2 and GLI3. This all occurs while the HH pathway is in an inactive state, in the presence of HH ligand, PKA sites are dephosphorylated resulting in the accumulation of active GLI and transcriptional activity. Outside of PKA several other kinases have been identified which regulate GLI activity. CK1 which plays a role in the creation of the GLI repressor form is also involved in stabilizing active GLI by protecting it from Cul3/Spop proteasomal degradation (Shi et al., 2014). AMP-activated protein kinase (AMPK) phosphorylates GLI1 at three residues resulting in protein destabilization and subsequent downregulation of HH signaling (Li et al., 2015). Mitogen activated kinase kinase kinase (MEKK1) has been shown to phosphorylate GLI1 at multiple sites two of which overlap with known PKA sites (Antonucci et al., 2019). Citron Rho-interacting kinase phosphorylates GLI2 and increases nuclear translocation and upregulation of target genes in breast cancer cells (Xing et al., 2014). As described previously in BCC phosphorylation of GLI1 via aPKC has been shown to increase GLI1 DNA binding (Atwood et al., 2013). Tyrosine kinases have also been studied as playing a part in GLI regulation, however not to the extent as serine/threonine kinases. Tyrosine kinase HCK has been shown to phosphorylate GLI1 and increase transcriptional activity (Shi et al., 2015).

Ubiquitination is another method by which GLI is regulated through PTM. After the sequential phosphorylation via PKA, CK1, and GSK3β GLI2/3 becomes able to interact with the Cul1/β-TrCP complex. This interaction causes GLI3 to be ubiquitinated and processed leading to the formation of a repressor form (Wang et al., 2006). Cul1/β-TrCP complex is also involved in the ubiquitination of GLI1 and GLI2 however, unlike GLI3 these two are not usually processed but rather completely degraded after ubiquitination (Bhatia et al., 2006). Whereas Cul1/β-TrCP complex is involved in the ubiquitination and degradation of GLI while they are in an inactive state, Cul3/Spop is involved in the degradation of the active forms of GLI. *In vitro* studies have shown that Cul3/Spop interacts with both GLI2 and GLI3 and increasing levels of Spop are correlated with decreases in full-length/active forms of GLI2 and GLI3 (Wang et al., 2010). However, this observation seems to be dependent on the cell line as other studies have no decreases in full length versions of GLI or even decreases in the repressor form of GLI (Cai et al., 2016). GLI1 on the other hand does not seem to be a target for Cul3/Spop, but instead its degradation is executed via Numb an adaptor for E3 ligase Itch (Di Marcotullio et al., 2006). Another ubiquitin ligase PCAF has also been shown to ubiquitinate GLI1 which subsequently leads to proteasomal degradation (Mazza et al., 2013). Proteasomal degradation of GLI can be prevented via deubiquitination. Deubiquitinase HAUSP has been shown to interact with all three GLI proteins and protects them from degradation (Zhou et al., 2015).

Acetylation is another means to regulate GLI transcriptional activity at a post translational level. GLI1 and GLI2 have both been shown to be acetylated and this modification serves as a way to alter activity of these transcription factors. Acetylation of GLI serves as a means to reduce GLI transcriptional regulation through sequestering GLI at the nuclear lamina preventing DNA binding and nuclear export (Mirza et al., 2019). Whereas HDAC1 deacetylation and aPKC phosphorylation has been shown to increase pathway activity (Mirza et al., 2019).

SUMOylation also plays a role in regulating the HH pathway. When the pathway is in the off-state GLI2 undergoes SUMOylation, which is then reduced upon pathway stimulation or in constitutively active GLI2 mutants suggesting negative regulation (Han et al., 2012). On the other hand, Pias1, a SUMO ligase, appears to increase activity of GLI proteins, directly interacting with the GLIs and reducing protein ubiquitination protecting them from proteasomal degradation (Cox et al., 2010). Other modification such as O-GlcNAcylation and methylation have both been shown to enhance GLI activity, increasing the nuclear accumulation and transcriptional activity of GLI (Das et al., 2019; Fu et al., 2016). These are just a handful of examples as to how PTMs are able to regulate GLI transcription factor activity.

Outside of PTMs, GLI is further regulated via its interactions with other proteins, such as those within the primary cilia. Intraflagellar transport (IFT) plays a key role in the processing of GLI into the active or repressor state (GLIA or GLIR respectively). IFT mutant mice show defects similar to defective GLI3R (Huangfu et al., 2003; Caspary et al., 2007). The conversion of GLI into GLIR, relies on interactions with two key components SUFU and PKA. SUFU sequesters GLI in the cytoplasm, preventing translocation into the nucleus during pathway inactivity (Humke et al., 2010). As previously described above, interaction of PKA with GLI results in phosphorylation and subsequent degradation into the GLIR form (Niewiadomski et al., 2013). Importin $\alpha/\beta1$ and exportin 1 both interact with GLI through their NLS/NES respectively to accommodate nuclear/cytoplasmic shuttling of GLI proteins (Barnfield et al., 2005). Mastermind-like 1 has been shown to directly interact with GLI serving as a transcriptional coactivator (Quaranta et al., 2017). C2H2 transcription factor ZIC interact with GLI through their zinc finger domains and regulate each other's transcriptional activity (Koyabu et al., 2001). Both GLI1 and GLI3 can bind to the pluripotency factor NANOG, resulting in lowered transcriptional activity (Li et al., 2016). Kinesin motor protein (KIF7) interacts with GLI resulting in positive regulation of HH signaling (Hsu et al.,

2011). The combination of protein-protein interactions and PTM allows for dynamic regulation of the HH pathway.

SUMMARY

The aim of this dissertation is to contribute to the understanding of how PTMs control GLI transcription factor activity, and whether targeting these PTMs can provide new avenues for treatment of BCCs. In Chapter 2, we conducted a large-scale screen on known GLI1/2 mutations in cancer in order to identify mutations which regulate HH signaling. We found that many mutation sites that show changes in HH signaling also reside near kinase sites. We then aimed to interrogate how different kinases affect BCC growth. We identify that inhibition of Aurora kinase A reduces HH signaling and BCC cell growth *in vitro* as well as reducing tumor size in a murine model of BCC. In Chapter 3, we targeted the zinc finger domain of the GLI transcription factors to elucidate how phosphorylation affected DNA binding and transcriptional activity. We found that the zinc finger domain allows for phosphorylation within a certain region, whereas phosphorylation outside this domain restricts DNA binding and activity. Together these findings demonstrate additional roles of regulation through PTM, specifically through the phosphorylation of the GLI transcription factors.

CHAPTER 2

AURORA A KINASE REGULATES HEDGEHOG SIGNALING THROUGH PHOSPHORYLATION OF GLI1/2

CONTRIBUTIONS

This project was conceived by Scott Atwood and Eric Tarapore. Scott Atwood supervised the research. Eric Tarapore performed the experiments. Mice injections and tissue collection were conducted by Rachel Chow and Kirsten Wong. RNA sequencing analysis was conducted by Adam Stabell. Yvonne Tran and Michelle Lee generated and maintained stable cell lines. Bao Ton That, Ivan He, Tejasvi Bhyravabhotla, Nikita Rao, Kendall Kearns, Peggy Chen, Stella Shamas and Tanin Zadeh assisted with qRT-PCR. Jerald Lim and David Young assisted in quantifying immunostained cell lines. Scott Atwood and Eric Tarapore wrote the manuscript. All authors analyzed and discussed the results and commented on the manuscript. The work in this chapter has not yet been published.

SUMMARY

Basal cell carcinomas (BCCs) are driven by uncontrollable activation of the Hedgehog (HH) signaling pathway. Many cases of BCCs possess resistance to common treatments such as the use of Smoothened (SMO) inhibitors. Identifying upstream activators of the HH pathway has potential to reveal ways to bypass SMO inhibitors and rather target these alternative activators of the HH pathway. Here, we performed a large-scale screen of known GLI1 and GLI2 mutations in cancer to identify mutations that regulate HH signaling. Through this screen we identified many of these cancerous mutations reside at or near kinase sites, thus we further screened kinase inhibitors effects on HH signaling. Inhibition of aurora a kinase (AURKA) activity in BCC cells resulted in reduced HH signaling and cell proliferation. Additionally, we have shown that AURKA is able to phosphorylate GLI1/GLI2. Similarly, treatment of the *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mouse BCC tumor model with alisertib, an AURKA inhibitor, reduces tumor growth. Together this data has shown that AURKA regulates HH pathway activity, potentially through phosphorylation of GLI.

INTRODUCTION

Basal cell carcinoma (BCC) is the most common of cancers, with nearly 5 million new cases in the United States every year (Nguyen et al., 2019). BCCs result from aberrant activation of the Hedgehog (HH) signaling pathway, an important pathway normally involved in embryonic development and adult tissue homeostasis (Hui et al., 2011). Although BCCs are common the cancer is also easily treatable through surgery or when surgery is not possible with Smoothened inhibitors. Smoothened (SMO) inhibitors such as vismodegib and sonidigib are commonly used to suppress tumor growth. Unfortunately, SMO inhibitor treatment is only effective in ~40% of advanced patients (Sekulic et al., 2012), with ~20% of patients who do respond eventually developing resistance each year (Chang et al., 2012). Developing therapies to bypass SMO inhibitor resistance is a critical need and an active area of investigation, especially as inappropriate HH pathway activation also drives other cancers such as rhabdomyosarcoma, medulloblastoma, and basal cell carcinoma (Zibat et al., 2010; Rutkowski et al., 2005; Epstein 2008).

HH ligand binds to receptor Patched1 (PTCH1), derepressing SMO allowing for the full length GLI transcription factors to translocate into the nucleus and activate target genes involved in proliferation, migration, and invasion (Varjosalo et al., 2008). In many cases of BCCs patients carry either inactivating mutations of PTCH1 (~70%) or activating mutations of SMO (~20%), either of these mutations leads to uncontrollable activation of the HH signaling pathway (Bonilla et al., 2016). Treatments of BCCs include SMO inhibitors however resistance to these treatments commonly occurs through additional mutations to SMO. Due to this fact research is now being pursued to identify ways to bypass SMO inhibition by directly targeting the GLI transcription factors.

The HH signaling pathway consists of three GLI transcription factors, although these proteins are highly related, they each play a distinct role in signaling. Of these three GLI1 and

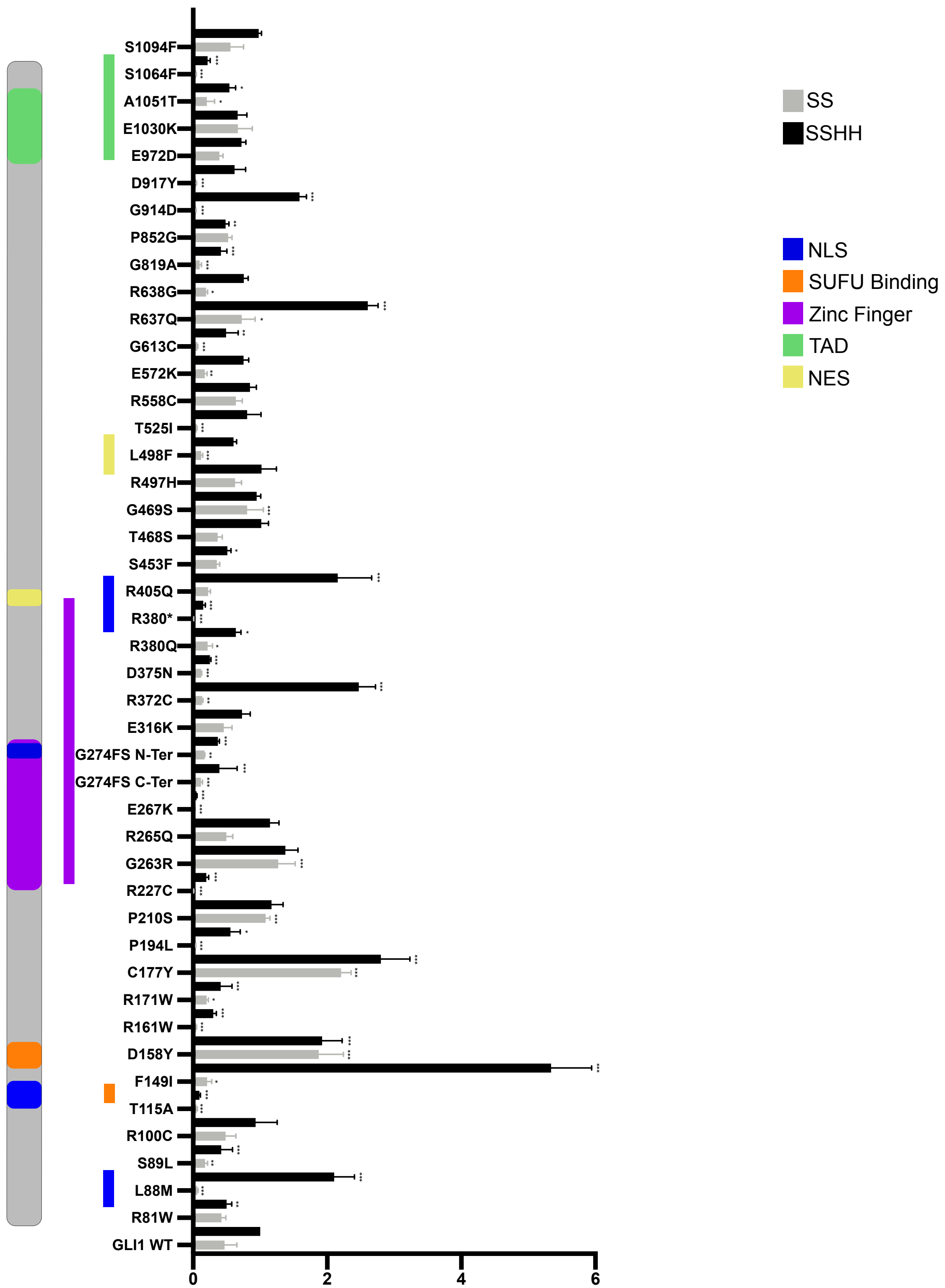
GLI2 play the role of activators of the HH gene transcription when the pathway is active (Hui et al., 2011). The GLI proteins can act as bifunctional transcription factors through regulated proteolytic processing of full-length active form to a cleaved repressor form. Several mutations within GLI have been discovered that are associated with disease states. Mutation found in the zinc finger domain of GLI1 has been associated with Ellis-van Creveld syndrome (Palencia-Campos et al., 2017). Mutations within the repressor domain of GLI2 has been shown to result in holoprosencephaly (Rosseler et al., 2003). Whereas mutations within the zinc finger domain of GLI3 have been associated with Greig cephalopolysyndactyly syndrome (Volodarsky et al., 2014). As such post-translational modification (PTM) can play a key role in the function of the GLI transcription factors. Transcription factors can undergo a variety of PTMs such as methylation, acetylation, and phosphorylation to name a few. Understanding how the GLI proteins are modified and how these modifications affect function have been researched previously. Phosphorylation is considered the most common form of PTM a protein can undergo (Ramazi et al., 2021). Previous work done on GLI has shown that a variety of kinases can phosphorylate GLI resulting in differing functions based on the site of modification. Phosphorylation via kinases PKA, GSK3Beta, and CK1 results in proteolytic cleavage of GLI and inhibition of the HH pathway (Tempe et al., 2006). Additionally, phosphorylation via aPKC has been shown to increase GLI1 DNA binding and transcriptional activity (Atwood et al., 2013). Aurora A kinase (AURKA) is a Serine/Threonine kinase that is commonly overexpressed in a variety of cancers. Originally identified as a mitotic factor controlling chromosome segregation, AURKA plays additional roles outside of the cell cycle such as cilia disassembly, neurite extension, and cell motility (Bertolin et al., 2020). AURKA has also been studied in the context of the HH signaling pathway via its control over ciliogenesis (Pejskova et al., 2020). Although AURKA has been shown to play a role in regulating the HH pathway via the cilia very little has been done to show whether AURKA directly acts upon the GLI transcription factors.

Here, we provide evidence that AURKA can directly phosphorylate GLI1 and GLI2 transcription factors. We demonstrate that *in vitro* and *in vivo* inhibition of AURKA results in significant reduction of BCC growth and HH signaling activity. Our results here show that AURKA may be a viable target to treat SMO inhibitor resistant BCCs.

RESULTS

Cancerous GLI1/2 mutations show a variety of HH signaling responsiveness

In order to identify sites of interest within GLI1 and GLI2, we performed a large-scale screen of known mutations within these transcription factors. Using the Catalogue of Somatic Mutations in Cancer (COSMIC) we were able to identify 44 mutations within GLI1 and 66 mutations within GLI2. The majority of these mutations were missense mutations as these were less likely to create a non-functioning protein. Along with the previous criteria we chose mutations that were recurrent in the COSMIC database, where there were >/= 3 cases. Stable cell lines carrying these mutations in GLI1 or GLI2 were generated and HH signaling was assayed through qRT-PCR (Figure 2.1A and Figure 2.2A). We saw that several mutations showed significant increases or decreases in both GLI1 and GLI2. The mutations for these stable cell lines are also tagged with the RFP variant mApple. As such we can immunostain these cells with anti-RFP to visualize the localization of each mutant (Figure 2.1B and Figure 2.2B). We saw that there was no correlation between HH signaling and localization of the protein, suggesting that mutations are not causing increased nuclear shuttling of GLI and that changes to the transcriptional activity of these mutations may be due to an alternative factor. As we tested mutations that reside all throughout the GLI protein these mutations may be altering function of the protein by disrupting one of more of the domains within GLI or altering the ability of GLI to interact with partner proteins.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S1064F
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E1030K
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G914D
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G613C
E572K
R558C
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L498F
R497H
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E267K
R265Q
G263R
R227C
P210S
P194L
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R171W
R161W
D158Y
F149I
T115A
R100C
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NLS
SUFU Binding
Zinc Finger
TAD
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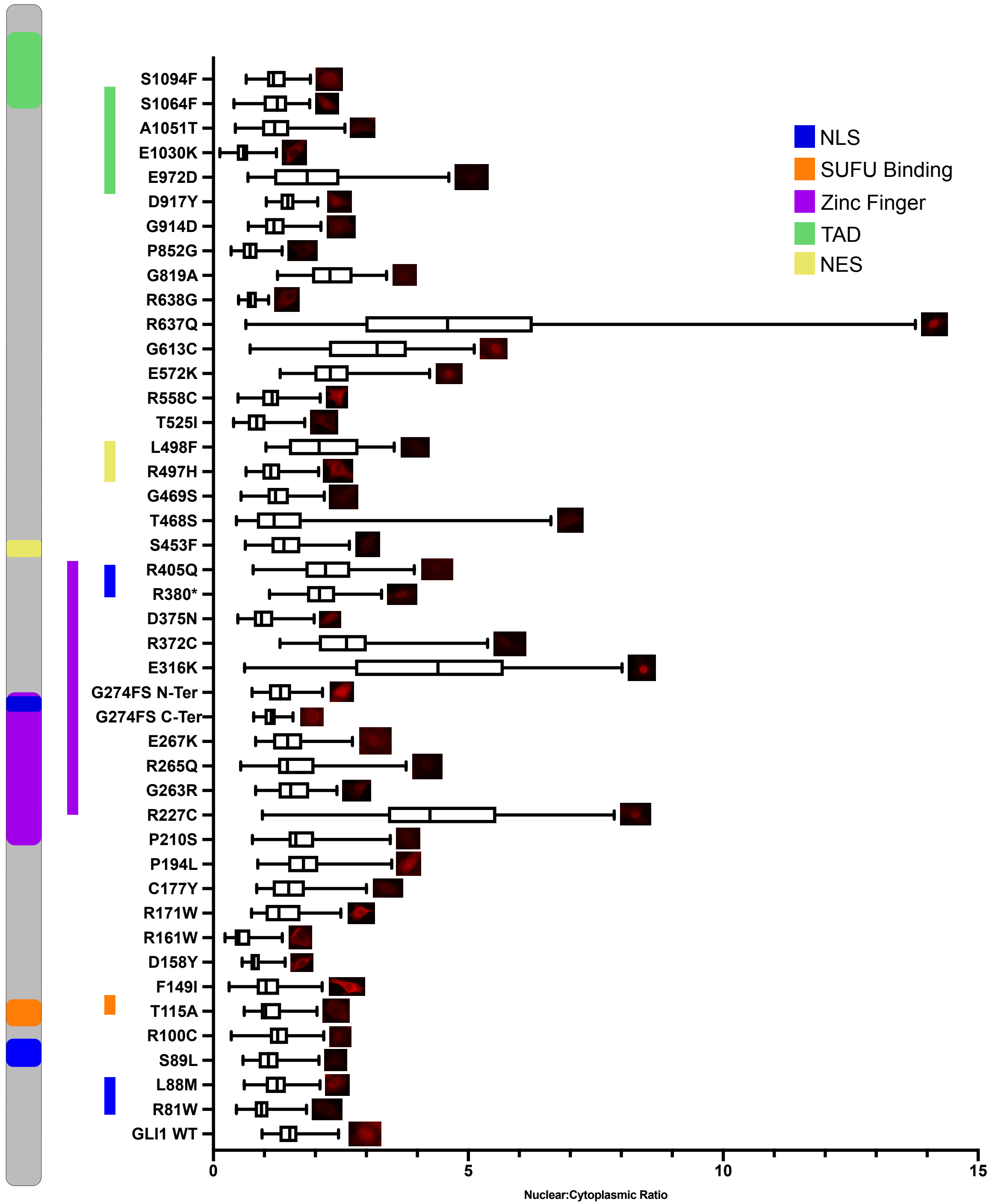

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E572K
R558C
T525I
L498F
R497H
G469S
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S453F
R405Q
R380*
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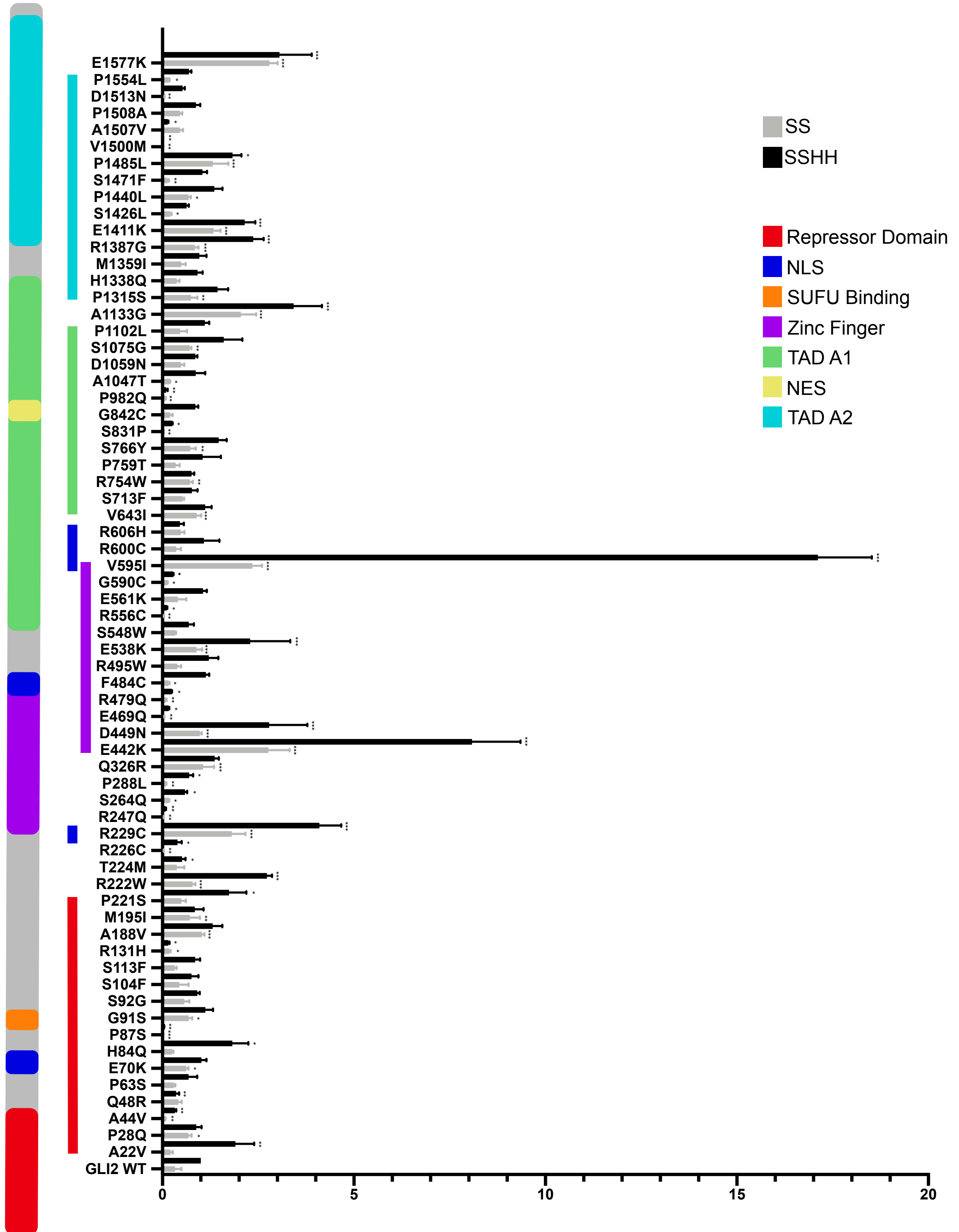
R372C
E316K
G274FS N-Ter
G274FS C-Ter
E267K
R265Q
G263R
R227C
P210S
P194L
C177Y
R171W
R161W
D158Y
F149I
T115A
R100C
S89L
L88M
R81W
GLI1 WT
0
5
10
15
Nuclear:Cytoplasmic Ratio
NLS
SUFU Binding
Zinc Finger
TAD
NES

Figure 2.1 GLI1 mutant HH signaling and localization. A) qRT-PCR analysis of GLI1 mutations in either serum starved, or serum starved with hedgehog ligand conditions along with schematic of GLI1 structure and known domains. n = 3. Error bars represent SD. Significance was determined by One-Way ANOVA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **B)** Nuclear to cytoplasmic ratios of GLI1 mutations in serum starved conditions along with schematic of GLI1 structure and known domains. n = 50 individual cells. Plotted in Box and Whisker.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D1513N
P1508A
A1507V
V1500M
P1485L
S1471F
P1440L
S1426L
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R1387G
M1359I
H1338Q
P1315S
A1133G
P1102L
S1075G
D1059N
A1047T
P982Q
G842C
S831P
S766Y
P759T
R754W
S713F
V643I
R606H
R600C
V595I
G590C
E561K
R556C
S548W
E538K
R495W
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R479Q
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S264Q
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R226C
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S113F
S104F
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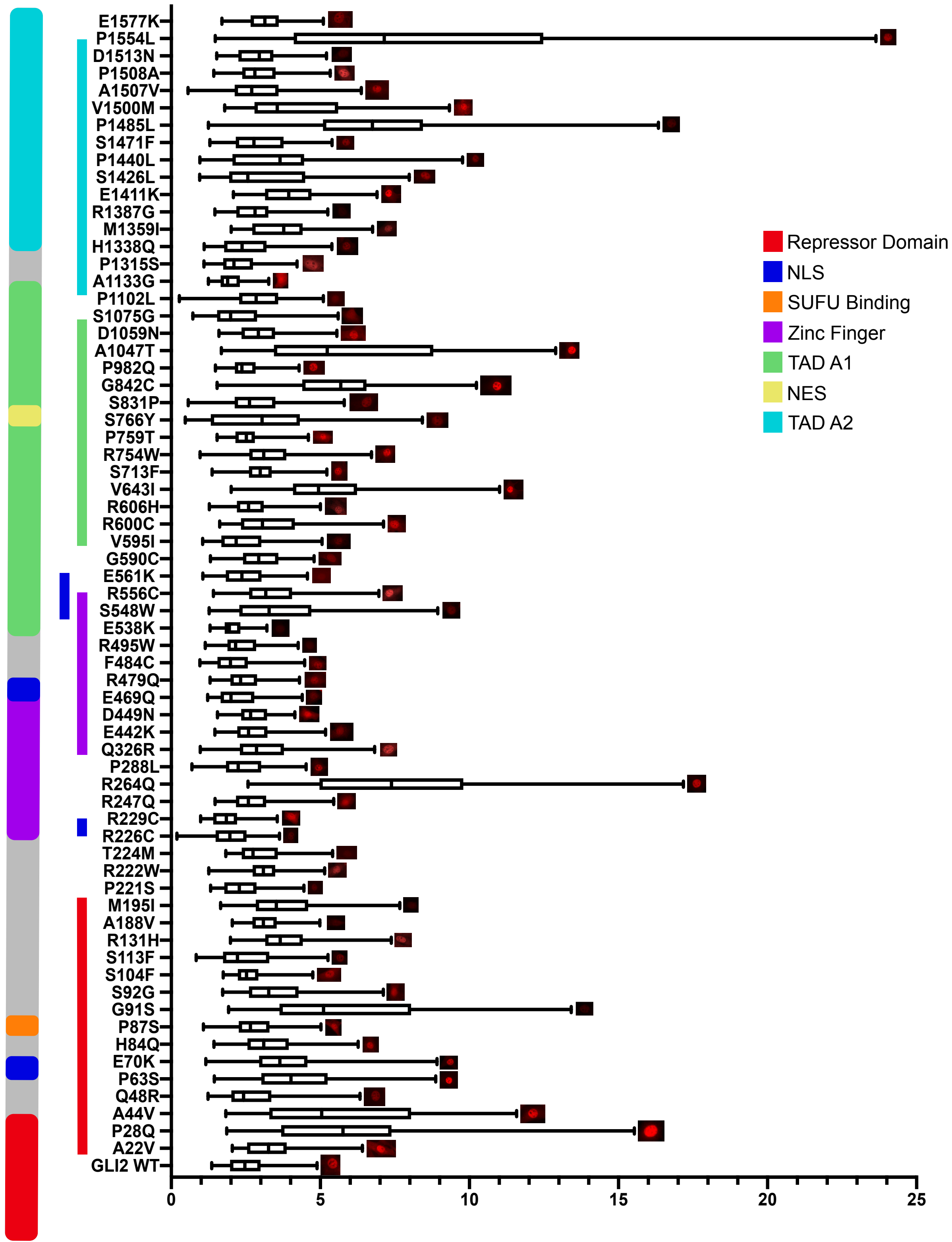
G91S
P87S
H84Q
E70K
P63S
Q48R
A44V
P28Q
A22V
GLI2 WT
0
5
10
15
20
SS
SSHH
Repressor Domain
NLS
SUFU Binding
Zinc Finger
TAD A1
NES
TAD A2



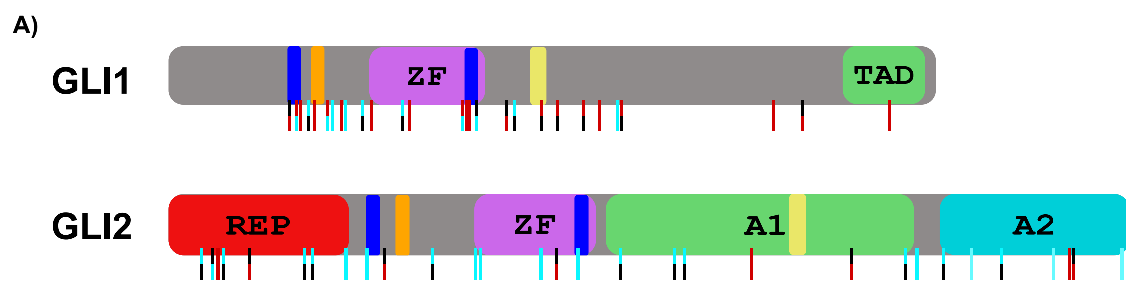
E1577K
P1554L
D1513N
P1508A
A1507V
V1500M
P1485L
S1471F
P1440L
S1426L
E1411K
R1387G
M1359I
H1338Q
P1315S
A1133G
P1102L
S1075G
D1059N
A1047T
P982Q
G842C
S831P
S766Y
P759T
R754W
S713F
V643I
R606H
R600C
V595I
G590C
E561K
R556C
S548W
E538K
R495W
F484C
R479Q
E469Q
D449N
E442K
Q326R
P288L
R264Q
R247Q
R229C
R226C
T224M
R222W
P221S
M195I
A188V
R131H
S113F
S104F
S92G
G91S
P87S
H84Q
E70K
P63S
Q48R
A44V
P28Q
A22V
GLI2 WT
0
5
10
15
20
25
Repressor Domain
NLS
SUFU Binding
Zinc Finger
TAD A1
NES
TAD A2

Figure 2.2 GLI2 mutant HH signaling and localization. A) qRT-PCR analysis of GLI2 mutations in either serum starved, or serum starved with hedgehog ligand conditions along with schematic of GLI2 structure and known domains. n = 3. Error bars represent SD. Significance was determined by One-Way ANOVA. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **B)** Nuclear to cytoplasmic ratios of GLI2 mutations in serum starved conditions along with schematic of GLI2 structure and known domains. n = 50 individual cells. Plotted in Box and Whisker.

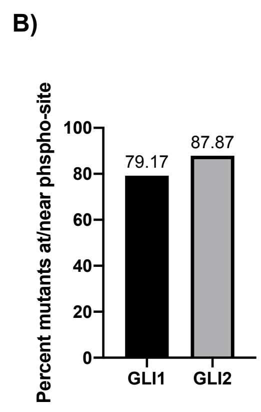
Majority of mutants in GLI1 and GLI2 reside at or near phosphorylation sites

After conducting our screen, we saw a variety of mutations have caused a significant change in HH activity (Figure 2.3A). As these mutations reside across the entirety of GLI, we hypothesized that these mutations maybe affecting function via disruption of known domains or sites of interaction. As PTMs can influence the function of the protein quite drastically we sought to investigate how these mutations would affect post-translational sites and since phosphorylation of GLI1 and GLI2 play a critical role in the resulting function of these proteins we decided to further investigate how these mutations affected phosphorylatable sites. We found that most of the mutations tested, ~79% in GLI1 and ~87% in GLI2, reside at or near phosphorylatable residues (Figure 2.3B) mainly at or near a Serine/Threonine residue (Figure 2.3C). Of this subset of mutations, we found that ~76% in GLI1 and ~51% in GLI2 caused a significant change in HH activity (Figure 2.3D). Due to this we decided to further investigate the role of kinases for GLI1 and GLI2 activity. Using a Group-based Prediction System (GPS 3.0) (Xue et al., 2008) we were able to identify potential phosphorylatable residues within GLI1 and GLI2. From there we were able to identify the kinases that were most predicted to phosphorylate GLI1/2 to test the effect of kinase inhibition on HH activity (Figure 2.3E).

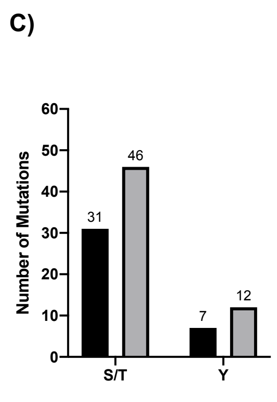
A)



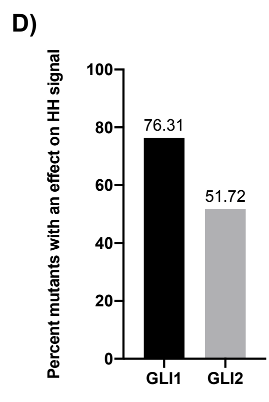
B)



C)



D)



E)

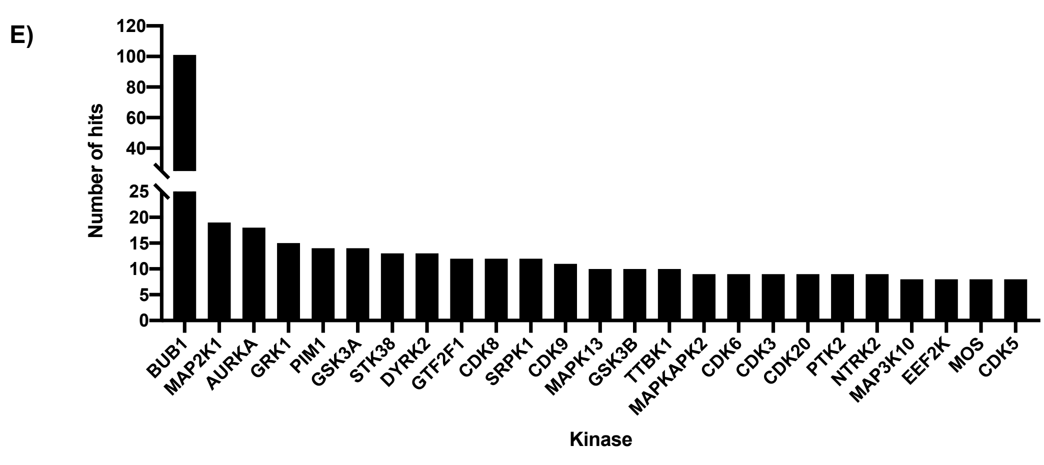
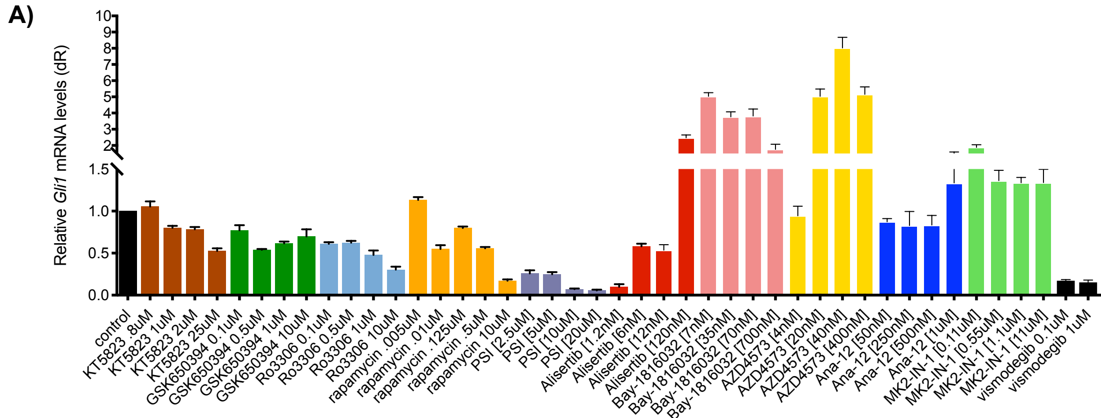
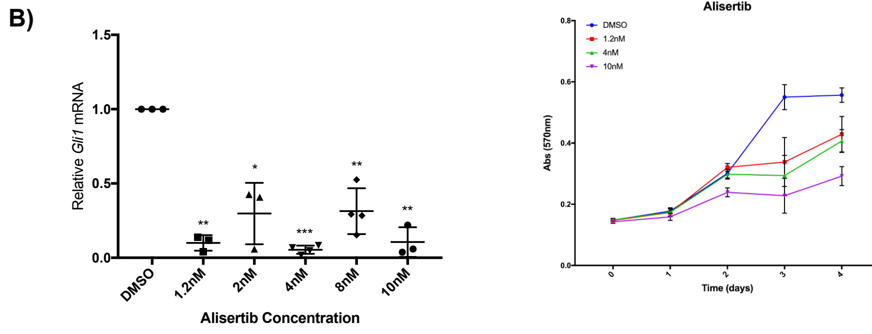


Figure 2.3 Majority of mutants tested reside at/near predicted phosphorylatable residues. A) Schematic of GLI1 and GLI2 with known domains illustrated. Mutations that reside +/- 5 amino acids indicated by tick mark. Color indicates effect on HH signaling. Blue showed an increased level of signaling, red showed a decreased level of signaling, black neutral. For tick marks that are multi-colored, top half represents activity in serum starvation conditions, bottom half represents activity in serum starvation with hedgehog ligand conditions. **B)** Percentage of mutants tested that were located +/- 5 amino acid residues away from a kinase site. **C)** Type of phosphorylatable residue within +/- 5 amino acid residues from a mutation. **D)** Percentage of mutations that are +/- 5 amino acid residues away from a phosphorylatable residue that showed a significant change in HH signaling. **E)** Histogram plotting the top 25 predicted kinases that phosphorylate residues +/- 5 amino acids from a mutation within GLI1/2.

Aurora A Kinase inhibition suppresses murine BCC cell growth and HH signaling

After generating a list of top predicted kinases for GLI1/2, we began testing how pharmacological inhibition of a select group of kinases affected HH signaling. We saw slight repression of HH activity with drugs such a rapamycin, an mTOR inhibitor, and ro3306, a CDK inhibitor (Figure 2.4A). Of interest though the Aurora A inhibitor Alisertib showed the greatest effect on HH signaling at low concentrations of drug (Figure 2.4A and Figure 2.4B). Along with the reduction in HH signaling we saw that treating mBCC cells with alisertib also reduced levels of proliferation (Figure 2.4B). However, when staining for proliferation marker Ki67 and apoptosis marker cleaved caspase 3, we saw no difference in positive stained cells between control mBCC cells and cells treated with Alisertib even though we saw reduced levels of cell growth via MTT (Figure 2.4C). This could be explained since the drug treatment for staining was only for 24 hours; and we didn’t see an effect growth until 72 hours of treatment. A longer treatment time of alisertib may show further changes with Ki67 and cleaved caspase 3. Form this data it suggests that AURKA plays a role in the HH signaling pathway of mBCC cells.


A)
Relative Gli1 mRNA levels (dR)
control
KT5823 .8uM
KT5823 1uM
KT5823 2uM
KT5823 25uM
GSK650394 0.1uM
GSK650394 0.5uM
GSK650394 1uM
GSK650394 10uM
Ro3306 0.1uM
Ro3306 0.5uM
Ro3306 1uM
Ro3306 10uM
rapamycin .005uM
rapamycin .01uM
rapamycin .125uM
rapamycin .5uM
rapamycin 10uM
PSI [2.5uM]
PSI [5uM]
PSI [10uM]
PSI [20uM]
Alisertib [1.2nM]
Alisertib [6nM]
Alisertib [12nM]
Alisertib [120nM]
Bay-1816032 [7nM]
Bay-1816032 [35nM]
Bay-1816032 [70nM]
Bay-1816032 [700nM]
AZD4573 [4nM]
AZD4573 [20nM]
AZD4573 [40nM]
AZD4573 [400nM]
Ana-12 [50nM]
Ana-12 [250nM]
Ana-12 [500nM]
Ana-12 [1uM]
MK2-IN-1 [0.11uM]
MK2-IN-1 [0.55uM]
MK2-IN-1 [1.1uM]
MK2-IN-1 [11uM]
vismodegib 0.1uM
vismodegib 1uM


B)
Relative Gli1 mRNA
DMSO
1.2nM
2nM
4nM
8nM
10nM
Alisertib Concentration
Alisertib
DMSO
1.2nM
4nM
10nM
Abs (570nm)
Time (days)

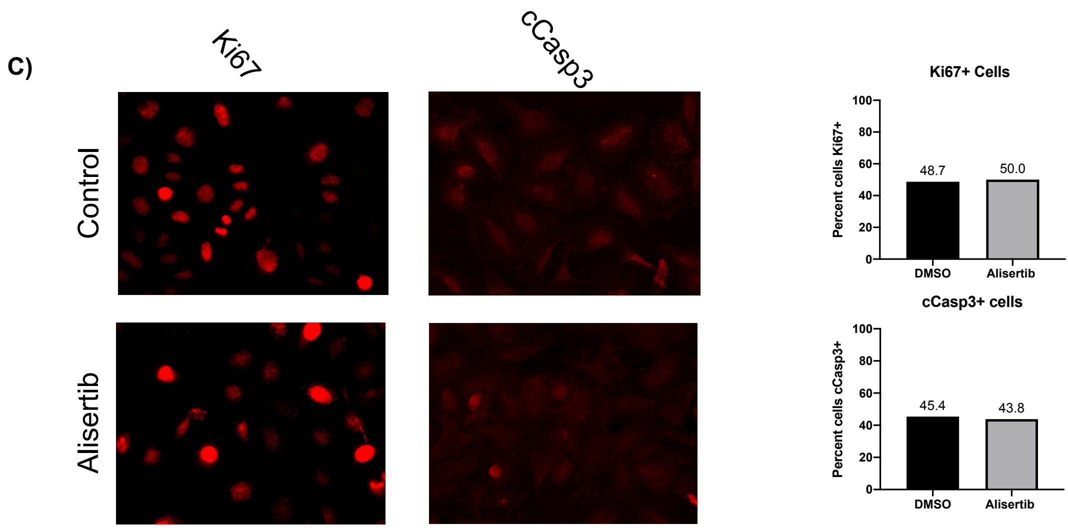
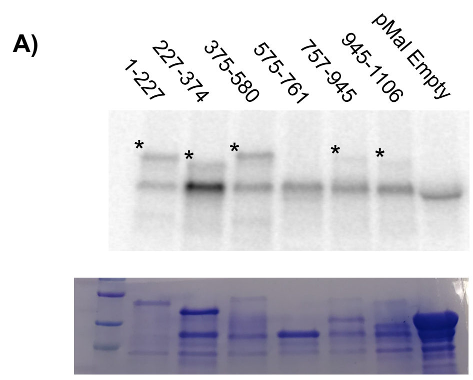

C)
Ki67
cCasp3
Control
Alisertib
Ki67+ Cells
Percent cells Ki67+
48.7
50.0
DMSO
Alisertib
cCasp3+ cells
Percent cells cCasp3+
45.4
43.8
DMSO
Alisertib

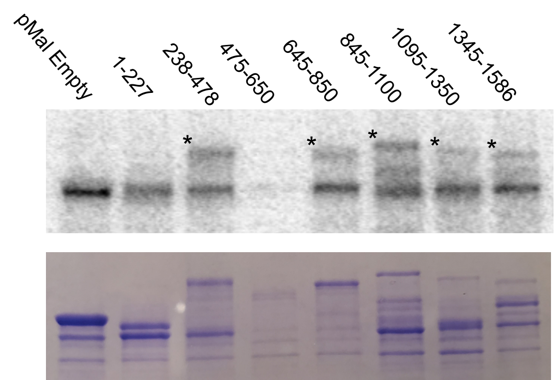
Figure 2.4 Aurora A inhibition shows reduced levels of BCC proliferation and HH signaling. A) qRT-PCR analysis of mBCC cells treated with a variety of different kinase inhibitors. Vismodegib serves as a control. n = 3. Error bars represent SD. **B)** qRT-PCR analysis and MTT assay of mBCC cells treated with alisertib for 24 hours. n = 3. Error bars represent SD. Significance was determined by Welch's t Test. *, $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **C)** Immunostaining of mBCC cells treated with DMSO control or alisertib for 24 hours and then stained for Ki67 or cCasp3 along with quantification of percent positive cells. n = >100 total cells counted.

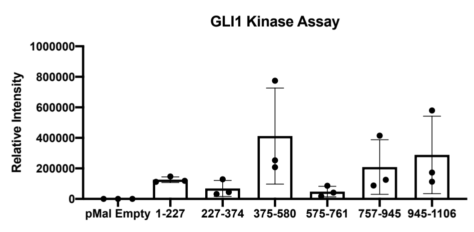
Aurora A kinase phosphorylates GLI1 and GLI2

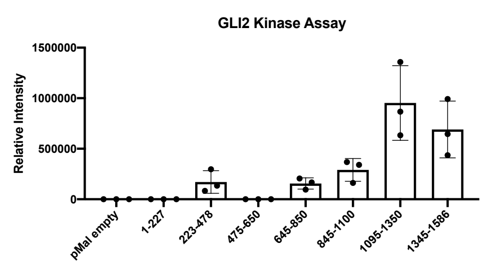
With AURKA inhibition having an impact on HH signaling and BCC proliferation we next wanted to test whether AURKA can directly phosphorylate GLI1/2. To test this, we created several recombinant fragments of GLI1/2 and tested them in a kinase assay with AURKA. We found that both GLI1 and GLI2 can be phosphorylated by AURKA *in vitro* (Figure 2.5A). We saw that several fragments of GLI1/2 showed levels of phosphorylation (Figure 2.5A). Using the kinase assay we were able to narrow down a list of potential AURKA sites located within GLI1. We then generated phospho-mimetic mutations at these sites of interest and tested for HH signaling in cells stably expressing these mutations. We saw that under serum starvation conditions that no site showed a significant increase over the WT control. However, under serum starvation plus HH ligand we saw that one site S116E showed a mild but significant increase over the WT control (Figure 2.5B). This suggests that S116 may be a target for phosphorylation in GLI1 when the HH pathway is in a more active state.

A)









B)

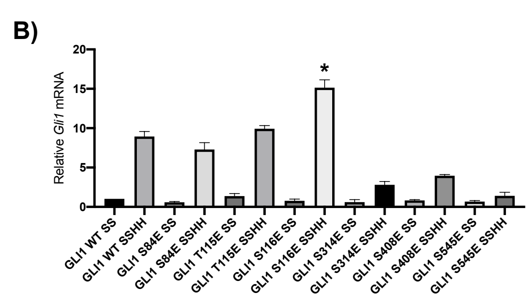
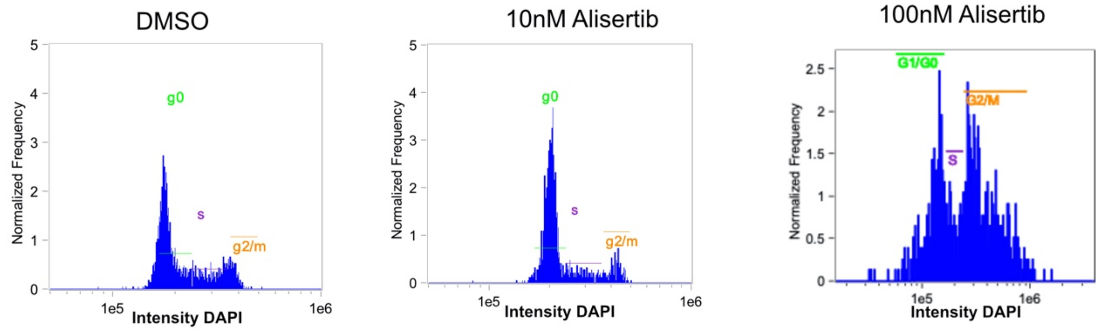


Figure 2.5 Aurora A kinase phosphorylates GLI1 and GLI2. A) Kinase assay of GLI1/2 along with commassie stained blot for protein load. Relative intensity normalized to empty pMal values. n = 3. **B)** qRT-PCR analysis of predicted AURKA phosphorylation sites within GLI1 in either serum starved, or serum starved with hedgehog ligand. Error bars represent SD. Significance determined by Welch's t Test. *, $p < 0.05$.

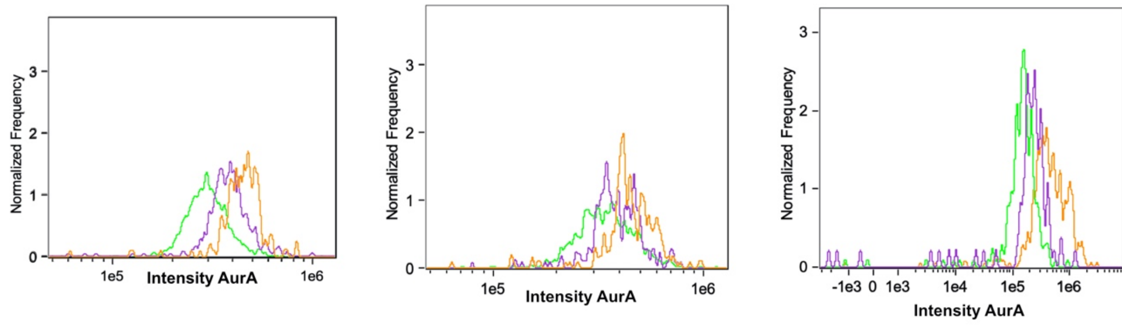
Low concentrations of Alisertib disrupt AURKA and GLI1 levels without disrupting the cell cycle

We next wanted to further test how AURKA inhibition would affect GLI1. To do this we chose a flow cytometry approach to assaying the effect of AURKA inhibition on mBCC cells. mBCC cells treated with a low concentration of alisertib showed little to no change in cell cycle profile compared to DMSO control. However, at a higher concentration of the drug we began to see a disruption in cell cycle profile (Figure 2.6A). We also see that unlike the DMSO control where we see increases in AURKA as the cell cycle progresses, when we treated with alisertib this gradual increase in intensity of signal is disrupted (Figure 2.6B). There does not appear to be any change in GLI1 intensity as the cell cycle progresses however, we do see that the frequency of GLI1 positive cells decreases during G2/M for alisertib treated cells (Figure 2.6C). We also noted a slight decrease in the overall intensity of active AURKA as the cell cycle progressed when treated with Alisertib (Figure 2.6D). Together this data suggests that lower concentrations of alisertib are able to disrupt the HH signaling pathway without disrupting normal cell cycle progression.

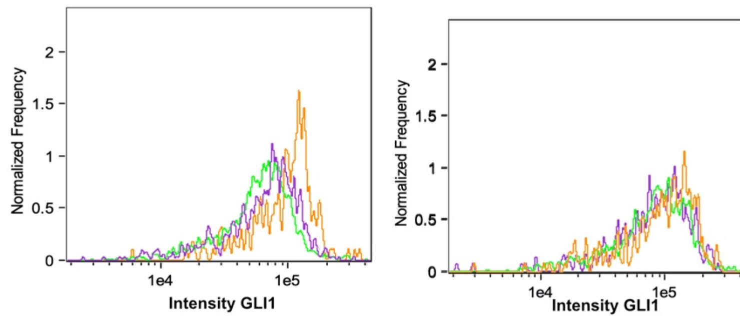
A)



B)



C)



D)

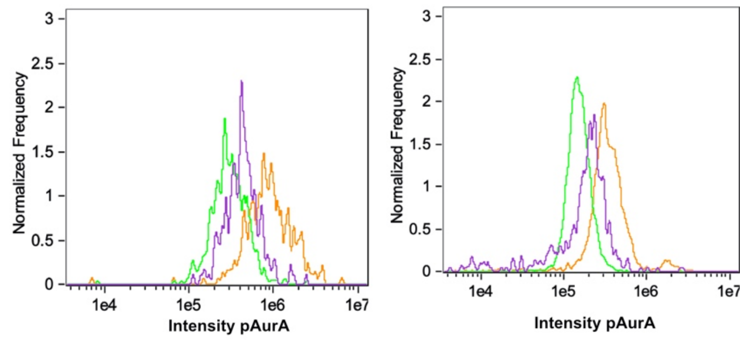
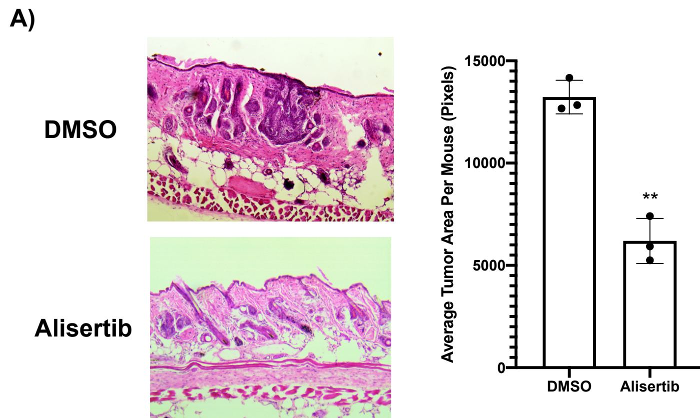
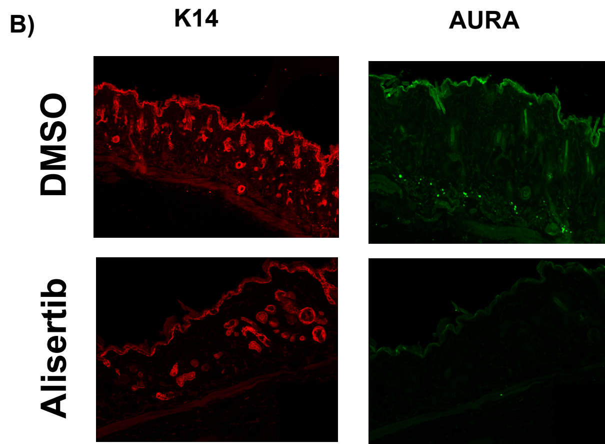


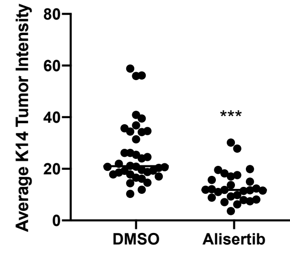
Figure 2.6 Aurora A inhibition disrupts GLI without disrupting the cell cycle. A) Flow cytometry analysis of cell cycle profile of mBCC cells treated for 24 hours with either DMSO control, 10nM alisertib, or 100nM alisertib. **B)** Flow cytometry analysis of AURKA stained mBCC cells over the course of the cell cycle. G0 = Green; S = Purple; G2/M = Orange. **C)** Flow cytometry analysis of GLI1 stained mBCC cells over the course of the cell cycle. G0 = Green; S = Purple; G2/M = Orange. Flow cytometry analysis of pAURKA stained mBCC cells over the course of the cell cycle. G0 = Green; S = Purple; G2/M = Orange.

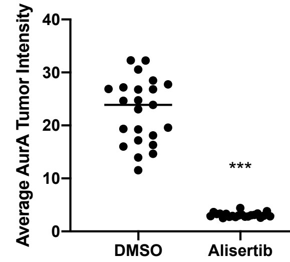
Alisertib inhibition of AURKA inhibits tumor growth in a murine model of BCC

Lastly, we wanted to examine how alisertib inhibition of AURKA affects tumor growth *in vivo*. To do so we used a mouse BCC model *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2}. We grew BCC tumors in *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} for 6 weeks after tamoxifen injection and intraperitoneally injected either DMSO or alisertib once a day for seven days. Histological staining of the dorsal back skin of alisertib treated mice revealed a significant decrease in the average tumor area per mouse compared to the DMSO control (Figure 2.7A). GLI and AURKA immunostaining showed a downward trend in alisertib treated mice compared to that of the control (Figure 2.7B). Lastly, we conducted bulk RNA sequencing on the dorsal back skin of these mice. We saw several genes that showed significantly altered levels of RNA expression between DMSO control and alisertib treated mice (Figure 2.7C). When looking at HH genes specifically we saw that in Alisertib treated mice there were significantly higher levels of GLI1 and GLI3 in alisertib treated mice along with higher levels of PTCH1, SMO, and SUFU (data not shown). This is surprising as we see significantly lowered HH signaling *in vitro*. With this data we have been able to show that *in vivo* reducing tumor area in these mice is reduced when treated with alisertib. Suggesting that AURKA plays a role in upregulating HH signaling in this BCC model.


A)
DMSO
Alisertib
15000
10000
5000
0
Average Tumor Area Per Mouse (Pixels)
**
DMSO
Alisertib


B)
K14
AURA
DMSO
Alisertib


80
60
40
20
0
Average K14 Tumor Intensity
***
DMSO
Alisertib


40
30
20
10
0
Average AurA Tumor Intensity
***
DMSO
Alisertib

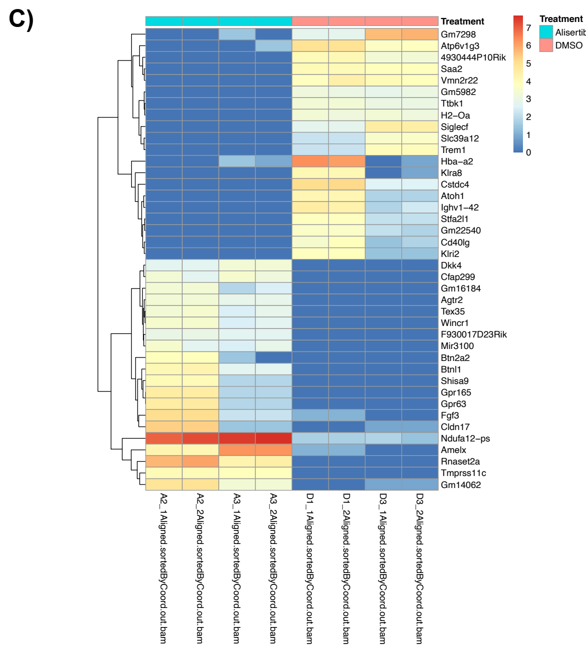

C)
Treatment
Alisertib
DMSO
7
6
5
4
3
2
1
0
Gm7298
Atp6v1g3
4930444P10Rik
Saa2
Vmn2r22
Gm5982
Ttbk1
H2−Oa
Siglecf
Slc39a12
Trem1
Hba−a2
Klra8
Cstdc4
Atoh1
Ighv1−42
Stfa2l1
Gm22540
Cd40lg
Klri2
Dkk4
Cfap299
Gm16184
Agtr2
Tex35
Wincr1
F930017D23Rik
Mir3100
Btn2a2
Btnl1
Shisa9
Gpr165
Gpr63
Fgf3
Cldn17
Ndufa12−ps
Amelx
Rnaset2a
Tmprss11c
Gm14062
A2_1Aligned.sortedByCoord.out.bam
A2_2Aligned.sortedByCoord.out.bam
A3_1Aligned.sortedByCoord.out.bam
A3_2Aligned.sortedByCoord.out.bam
D1_1Aligned.sortedByCoord.out.bam
D1_2Aligned.sortedByCoord.out.bam
D3_1Aligned.sortedByCoord.out.bam
D3_2Aligned.sortedByCoord.out.bam

Figure 2.7 Alisertib treatment decreases tumor area in murine BCC model. A) Hematoxylin and eosin staining of dorsal back skin collected from DMSO- or Alisertib-treated *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mice along with quantification of average tumor area per mouse. n = 3 mice tested in each condition. Significance determined by Welch's t Test. **, $p < 0.01$. **B)** Immunofluorescent staining of DMSO- or Alisertib-treated *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mice for the indicated markers. **C**) Heat map of top 40 differentially expressed genes in either DMSO-treated or Alisertib-treated mouse dorsal back skin.

DISCUSSION

The COSMIC database provided a plethora of mutations within GLI1/2 that are known to occur in various cancer types. Although cancer are heterogenous including BCC (Xie et al., 2022), these individual mutations were still enough to significantly alter HH signaling in cells. Thus, we were interested in identifying how these mutations are disrupting HH signaling. These mutations were spread throughout the entirety of the protein, as such could be affecting the function of the protein by disrupting protein domains. Mutations within the zinc finger domain of GLI1 such as D375N, reside near DNA binding residues. This mutation could disrupt GLI1 DNA binding resulting in loss of HH signal. Same could be applied to mutations within GLI2 zinc finger domain, many of which resulted in reduced HH signal. Mutations within the GLI2 transactivation domain (TAD) could result in changes to partner binding, such as binding of the androgen receptor which has been previously shown to bind to the TAD of GLI2 (Li et al., 2014). Disruption in GLI1 SUMOylation may play a role in the changes to activity that we saw. Mutations near K180 and K815 may play a role in enhancing or restricting SUMOylation of GLI1 (Cox et al., 2010). Mutations found in the repressor domain of GLI2 show both increasing and decreasing levels of activity. These mutations may play a role in altering the way that GLI2 is being processed into the repressor isoform resulting in the changes in HH activity. As phosphorylation plays a key role in several aspects of the cell, we chose to focus our efforts on this modification. Previous work by others have already shown that phosphorylation of the GLI transcription factors play critical roles in protein function and stability (Tempe et al., 2006; Pan et al., 2006). Through the efforts of our GLI mutation screen we were able to identify several kinases that could potentially regulate HH signaling.

The role of AURKA in the cell cycle has been well studied (Roy et al., 2018), slowly however additional roles for the kinase outside of the cell cycle have been found. Roles such as neurite elongation, cilia disassembly, and cell motility (Bertolin et al., 2020). A possible connection

between AURKA and the HH signaling pathway exists as ARUKA plays a role in the cilia and vertebrae HH signaling primarily takes place at the cilia. However, a direct link between AURKA and the HH signaling pathway remains unclear. Our data suggests that AURKA can directly act on GLI1/2 through phosphorylation and that this phosphorylation stimulates HH activity. Our data also suggests that the ability of AURKA to disrupt the HH pathway is outside of the kinase's role in the cell cycle. At low concentrations of alisertib, we were able to show significant changes to HH signaling and BCC proliferation, without disrupting AURKA other role with cell cycle progression. As AURKA is known to regulate the cilia, one aspect worth further investigation would be looking at how alisertib affects cilia in cells as a potential mechanism of how AURKA is regulating HH signaling.

Through kinase prediction we were able to identify six sites that could potentially be phosphorylated by AURKA which are conserved between GLI1 and GLI2. Of these sites we tested in GLI1 we found that S116E showed a significant increase in HH signaling compared to the WT. Interestingly, this site is already known to play a role in the HH signaling pathway. S116 has been previously shown to be a site where Suppressor of Fused (SUFU) interacts with GLI1 (Dunaeva et al., 2003). SUFU is a negative regulator of the HH signaling pathway and is absolutely required for early mouse development (Barnfield et al., 2005). Loss of SUFU in mice closely resembles the phenotype of PTCH1 null mice both of which are embryonic lethal at embryonic day 9.5. If AURKA does in fact phosphorylate S116 this may cause a disruption in the ability of SUFU to sequester GLI proteins and prevent them from activating transcriptional targets. This provides another potential means as to how AURKA can regulate HH signaling. As of now to true mechanism of how AURKA regulates HH signaling is still unknown. The phosphorylation of GLI1 by AURKA could improve GLI1 stability or shuttling into the nucleus furthermore the cilia could also play a role in this process if alisertib disrupts ciliogenesis. As we continue to test these additional avenues, we hope to tease apart the true mechanism of AURKA role in the HH pathway. Nonetheless the work here AURKA directly phosphorylates GLI1/2 *in vitro* and inhibition of

AURKA is enough to reduce murine BCC proliferation and HH signaling *in vitro*. We have also been able to show AURKA inhibition reduces tumor growth *in vivo*. Together this data suggests that targeting AURKA may be a viable option for drug resistant BCCs.

CHAPTER 3

REGULATION OF GLI TRANSCRIPTION FACTORS THROUGH ZINC FINGER PHOSPHO-MIMETIC MUTATION

CONTRIBUTIONS

This project was conceived by Scott Atwood and Eric Tarapore. Scott Atwood supervised the research. Eric Tarapore performed the experiments. Kristeen Shamas assisted in generating recombinant protein for kinase assays. Yvonne Tran and Arianna Valencia generated and maintained stable cell lines. Yvonne Tran and Arianna Valencia assisted with qRT-PCR. Scott Atwood and Eric Tarapore wrote the manuscript. All authors analyzed and discussed the results and commented on the manuscript. The work in this chapter has not yet been published.

SUMMARY

Transcription factors are key to cellular processes and are involved in the process of transcribing DNA into RNA. One such family of transcription factors are the C2H2 zinc finger transcription factors (ZF-TF) composed of a zinc finger binding domain. Historically, disruption of the zinc finger binding domain results in loss of transcription factor binding. Hedgehog signaling pathway (HH), a key pathway for early development and adult tissue homeostasis, is executed via three ZF-TFs; GLI1, GLI2, and GLI3. Here we show that phospho-mimetic mutations of GLI1 within the zinc finger domain is modular. Regions of the C2H2 zinc fingers of GLI1 are permissive to modification, such as phospho-mimetic mutation, whereas other regions of the zinc fingers are not. Furthermore, GLI2 and GLI3 share this feature of permissive/restrictive modification with GLI1 however not to the same degree. Lastly, we report that other C2H2 ZF-TFs that share a similar structure to that of the GLI family may be permissive to modifications in the same way.

INTRODUCTION

Gene expression is an essential process for any living organism. Transcription factors (TFs) are the major regulators of gene expression within cells. TFs can recognize specific DNA sequences, bind to these sequences, and regulate gene expression (Mitsis et al., 2020). Within eukaryotes there are several major families of TFs, such as C2H2-zinc finger (ZF), Homeodomain, basic helix-loop-helix (bHLH), basic leucine zipper (bZIP), and nuclear hormone receptor (NHR) (Lambert et al., 2018). Of these families the C2H2-zinc finger transcription factor (C2H2 ZF-TF) represents the dominant DNA binding motif in eukaryotes making up over 80% of transcription factors in primates (Vaquerizas et al., 2009). C2H2 ZF-TFs contain four amino acids on each zinc finger located at specific positions that are brought into direct contact with adjacent nucleotides of the DNA sequence as the binding domain wraps around the DNA (Stubbs et al., 2011). As ZF-TFs are one of the most abundant family of gene regulators in eukaryotes critical roles in many tissues such as the skin (Evans et al., 2008) and the adipose tissues (Perez-Mancera et al., 2007) along with being implicated in several cancers (Atwood et al., 2013; Lin et al., 2014; Lamouille et al., 2014).

As ZF-TFs play a major role in the regulation of gene expression, many of these factors need to be regulated themselves to have a proper balance of active and inactive transcription. One method of regulation is through post-translational modification (PTM) allowing a switch between an active and inactive state. Phosphorylation, being the most common form of PTM (Ramazi et al., 2021), has been previously looked at in the context of regulating C2H2 ZF-TF activity. Studies have shown that phosphorylation of the highly conserved linker peptide of C2H2 ZF-TFs results in loss of DNA binding (Dovat et al., 2002). During mitosis it has been seen that there is a global phosphorylation event of the linker region of C2H2 ZF-TFs removing them from DNA allowing access to the replication machinery (Rizkallah et al., 2011). Outside of this understanding how phosphorylation can regulate C2H2 ZF-TFs is not fully understood.

One signaling pathway that is regulated via C2H2 ZF-TFs is the hedgehog signaling pathway (HH). HH ligand binds to the receptor Patched1 (PTCH1), derepressing SMO allowing for the full length GLI transcription factors to translocate into the nucleus and activate target genes involved in proliferation, migration, and invasion (Varjosalo et al., 2008). HH signaling plays a critical role in early development and adult tissue homeostasis (Hui et al., 2011); and upon loss of regulation aberrant HH signaling drives the formation of basal cell carcinoma (BCC) (Bonilla et al., 2016). There are three C2H2 ZF-TFs that regulate the HH signaling pathway; these include GLI1, GLI2, and GLI3. GLI1 and GLI2 server as primary activators of the pathway upregulating gene transcription, whereas GLI3 servers as the major repressor of the pathway. All three of the HH pathway's C2H2 ZF-TFs share common features of their zinc-finger domain which include five C2H2 zinc fingers within close-proximity of one another.

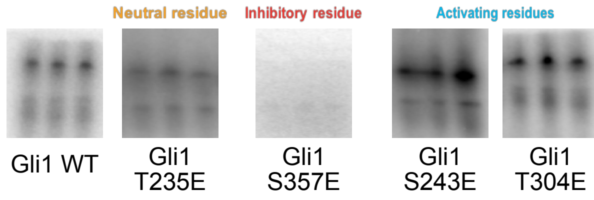
Previous work has shown that phosphorylation of the zinc fingers within GLI1 enhances DNA binding contrary to previously published works showing that phosphorylation is a negative regulator of DNA binding (Atwood et al., 2013). Along with this piece of evidence work on C2H2 ZF-TF Sp1 has also shown that phosphorylation enhances the ability of Sp1 to bind to DNA (Fojas de Borja et al., 2001). Furthermore, mutations found within the zinc finger domain negatively affect transcription factor binding. Mutations within the zinc finger have been found to be significantly more often than chance among three distinct cancer types (Munro et al., 2018). Zinc finger proteins such as ZNF750 and MDM2 have been shown to have mutations within the zinc finger domain that negatively regulate DNA binding and function of the transcription factor (Lin et al., 2014; Lindstorm et al., 2007). Here we provide evidence that phosphorylation of GLI1's zinc-fingers is modular where certain regions are permissive while others are restrictive to phosphorylation. Furthermore, GLI2 and GLI3 share this similar feature to GLI1 however, not to the same extent as GLI1. Lastly, we expand our search for additional C2H2 ZF-TFs that share similar features to that of the GLI family and ask whether they can also be regulated through phosphorylation in a similar manner.

RESULTS

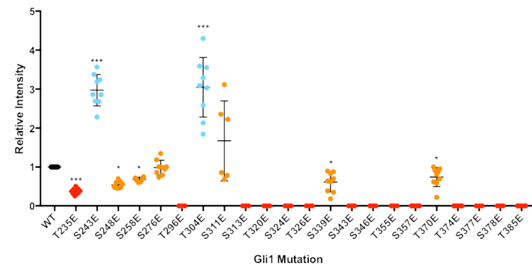
GLI1 DNA binding and transcriptional activity is regulated through phospho-mimetic mutation of the zinc finger binding domain

To begin testing how phosphorylation affects DNA binding and transcriptional activity we generated phospho-mimetic mutations at serine/threonine residues within the GLI1 zinc finger domain. Using *in vitro* translation, we generated GLI1 protein that we used for an electrophoretic mobility shift assay (EMSA). We saw that different glutamic acid mutations showed different levels of DNA binding capacity (Figure 3.1A) and quantified these levels of binding compared to the wild type (Figure 3.1B). Mutations showed either a neutral level of binding (similar to that of the wild type), enhanced levels of binding, or diminished levels of binding. We next wanted to test how these changes to DNA binding affected the activity of the HH pathway. To do so we stably transfected these mutations into NIH 3T3 cells and tested for HH signaling through qRT-PCR. For the most part DNA binding in the EMSA was a predictor for levels of HH signaling. We saw that enhanced DNA binding resulted in enhance HH signaling; whereas loss of DNA binding resulted in reduced levels of HH signaling (Figure 3.1C). Together, we were able to see that certain regions of the zinc finger domain were permissive to phospho-mimetic mutation (regulatory region); however, regions of the zinc finger such as DNA binding and the linker region are more restrictive.

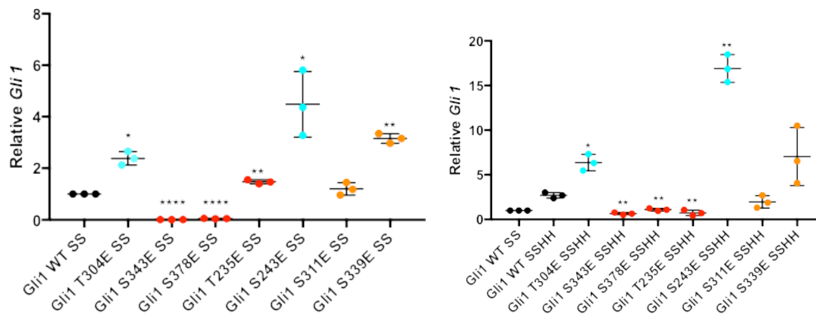
A)



B)



C)



D)

Regulatory region | DNA binding | Linker region

```
235      243    248
ETDCRWDGCSQE---FDSQEQLVHHINSEHIHGERK   ZF1
EFVCHWGGCSRELRPFKAQYMLVVHMRR-HTGEK     ZF2
    304    311   313     320     326
PHKCTFEGCRKS---YSRLENLKTHLRS-HTGEK     ZF3
         339   343  346       355
PYMCEHEGCSKA---FSNASDRAKHQNRTHSNEK     ZF4
         370        377 378
PYVCKLPGCTKR---YTDPSSLRKHVKTVHGPDA     ZF5
```

Neutral residues

Inhibitory residues

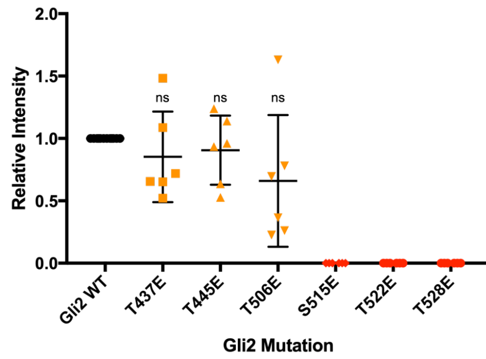
Activating residues

Figure 3.1 Phospho-mimetic mutation of GLI1 ZF alters DNA binding and transcriptional activity. A) Representative images of electrophoretic mobility shift assay of different GLI1 zinc finger mutants. Upper band shows bound GLI1. **B)** Relative intensity of bound GLI1 in electrophoretic mobility shift assay normalized to WT. n = >/= 3. Significance determined by Welch's t Test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **C)** qRT-PCR analysis of GLI1 zinc finger mutants in either serum starved, or serum starved with HH ligand. n = 3. Significance determined by Welch's t Test. * $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. **D)** Visual representation of GLI1 zinc finger domain with labeled phosphorylation sites. Determination of the activity of each residue was decided by the results of EMSA.

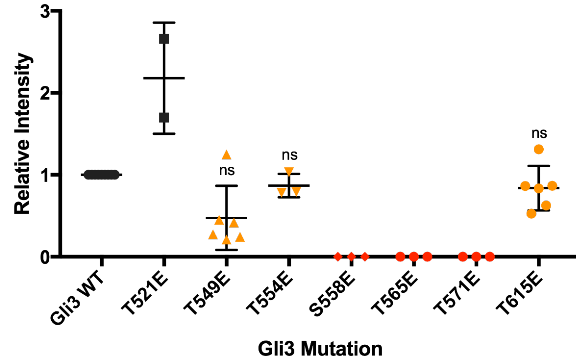
GLI2 and GLI3 share similar regulatory, DNA binding, and linker regions within zinc finger domain

Since GLI1 showed a modular response to phospho-mimetic mutation within the zinc finger domain we next wanted to test if other members of the HH signaling pathway shared similar features. GLI2 and GLI3 also have five C2H2 zinc fingers and so we mutated serine/threonine residues within the regulatory, DNA binding, and linker region of these zinc fingers and tested their ability to bind to DNA and regulate transcription. We first tested through EMSA, and we saw that mutations within the DNA binding and linker regions abolished the transcription factor's ability to bind DNA (Figure 3.2A and Figure 3.2B). We also saw that mutations within the regulatory region of GLI2/3 showed similar levels of binding to that of WT, however unlike GLI1 we never saw any mutation increase DNA binding (Figure 3.2A and Figure 3.2B). Furthermore, transcriptional activity for most of the GLI2/3 mutants show similar activity to that of the WT; even though some mutations completely abolished DNA binding (Figure 3.2C and Figure 3.2D). Together we were able to show that GLI2/3 DNA binding is regulated through phospho-mimetic mutation in a similar way to that of GLI1, but transcriptional activity does not correlate as well with DNA binding as it did for GLI1.

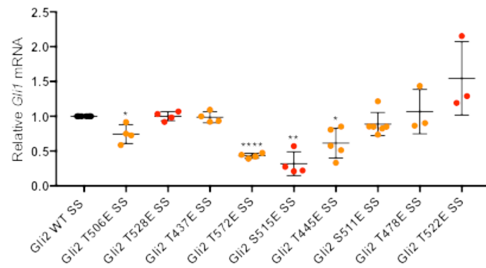
A)

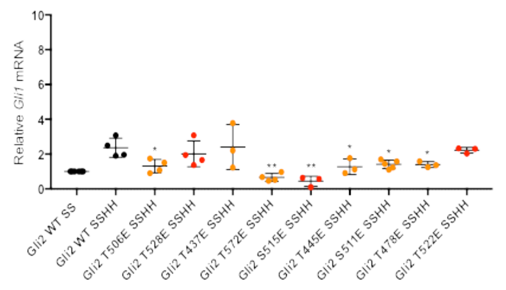


B)

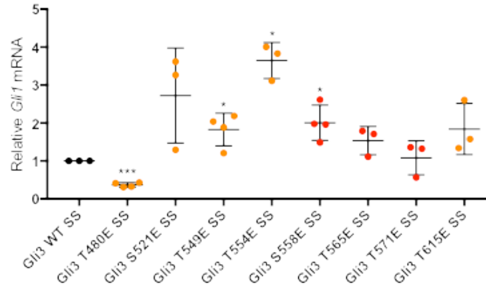


C)





D)



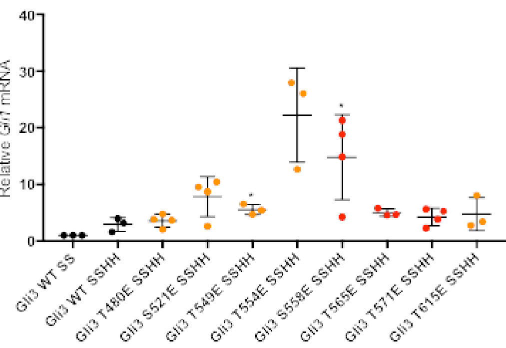


Figure 3.2 GLI2/3 zinc fingers DNA binding and activity regulated through phospho-mimetic mutation. A) Relative intensity of bound GLI2 in electrophoretic mobility shift assay normalized to WT. n = >/= 3. Significance determined by Welch's t Test. **B)** Relative intensity of bound GLI3 in electrophoretic mobility shift assay normalized to WT. n = >/= 2. Significance determined by Welch's t Test. **C)** qRT-PCR analysis of GLI2 zinc finger mutants in either serum starved, or serum starved with HH ligand. n = 3. Significance determined by Welch's t Test. * $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$. **D) C)** qRT-PCR analysis of GLI3 zinc finger mutants in either serum starved, or serum starved with HH ligand. n = 3. Significance determined by Welch's t Test. * $p < 0.05$; ***, $p < 0.001$.

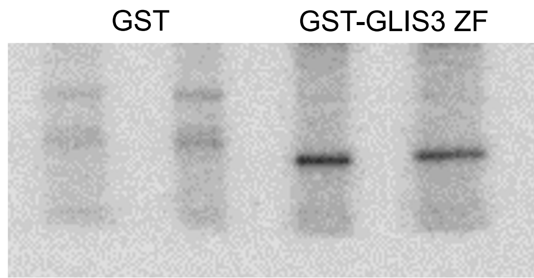
Additional C2H2 zinc finger transcription factors share similar features to GLI family

Lastly, we wanted to test if other C2H2 ZF-TFs shared similar features to that of the GLI family. One such transcription factor, GLI Similar 3 (GLIS3), which plays a critical role in the development and regulation of pancreatic beta cells (Scoville et al., 2019). GLIS3 is a five fingered C2H2 ZF-TF with all five fingers being near each other similar to the GLI family. Zinc finger three in particular shares high conservation with the GLI family zinc finger (Figure 3.3A). As such we first tested to see whether zinc finger three of GLIS3 could be phosphorylated. It has been previously shown that aPKC is the kinase that phosphorylates GLI1 at amino acid residue T304 (Atwood et al. 2013). We conducted an *in vitro* kinase assay using recombinantly expressed GLIS3 zinc finger domain with aPKC. We saw that indeed the GLIS3 zinc finger domain does get phosphorylated by aPKC (Figure 3.3B). To test whether the conserved residue T415 is being targeted by aPKC we mutated that residue to an alanine (phospho-dead mutant) and conducted an *in vitro* kinase assay. We saw reduced levels of phosphorylation compared to the WT for GLIS3 T415A (Figure 3.3C). We lastly wanted to test how phosphorylation affected GLIS3 DNA binding. To do so we generated a glutamic acid mutation at T415 and conducted an EMSA. We saw increased levels of DNA binding for GLIS3 T415E compared to the WT protein (Figure 3.3D). Together we see that GLIS3 is phosphorylated at the conserved region of the zinc finger similar to GLI1 and that this phosphorylation promotes DNA binding *in vitro*.

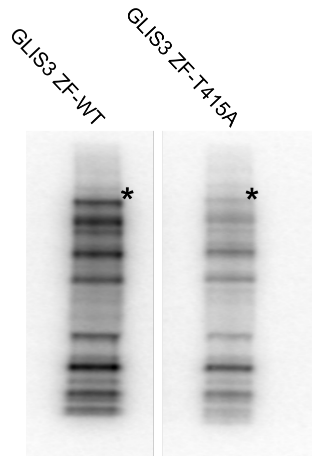
A)

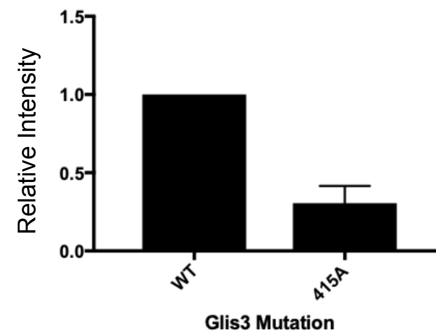
```
HKCTFEGCRKS---YSRLENLKTHLRS-HTGEKP   Gli1
HKCTGEGCSKA---YSRLENLKTHLRS-HTGEKP   Gli2
HKCTFEGCTKA---YSRLENLKTHLRS-HTGEKP   Gli3
NKCTFEGCEKA---FSRLENLKIHLRS-HTGEKP   Glis3
```

B)

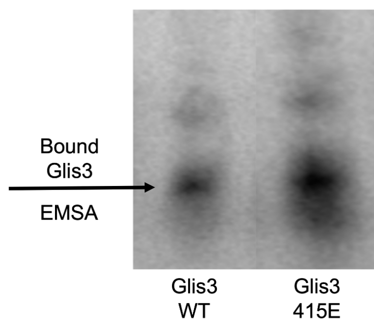


C)





D)



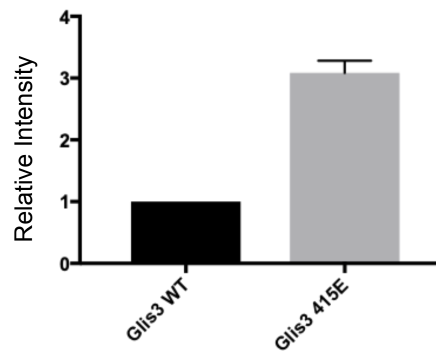


Figure 3.3 Conserved GLIS3 zinc finger shares similar properties to GLI family zinc fingers. A) Visual representation of GLI family and GLIS3 zinc finger three. Conserved phosphorylation site bolded. GLI1 serine/threonine residues highlighted based on results of EMSA analysis. **B)** Kinase assay of GST or GST-GLIS3 zinc fingers with aPKC. Relative intensity quantified compared to the WT normalized to protein load. **C)** Kinase assay analysis of recombinantly expressed GLIS3 zinc finger domain of WT or T415A mutant. Asterisk marks position of full-length product. Relative intensity quantified compared to WT. **D)** EMSA analysis of *in vitro* translated GLIS3 zinc finger domain of WT or T415E mutant. Lower band is bound GLIS3. Relative intensity of bound GLIS3 quantified compared to WT.

DISCUSSION

The role of post-translation modifications on protein function has been well studied (Karve et al., 2011). When it comes to ZF-TFs previous work has made it obvious that phosphorylation of the zinc fingers removes these transcription factors from DNA (Dovat et al., 2002). However, as more work has been done studying ZF-TFs, it has been revealed that this may not be the case in every context. Previous work done on C2H2 ZF-TFs Sp1 and GLI1 have shown that phosphorylation of the zinc finger domain doesn't always result in loss of DNA binding and activity (Atwood et al., 2013; Fojas de Borja et al., 2001). In this study we aimed to further elucidate the role of phosphorylation of the GLI family of C2H2 ZF-TFs.

Outside of the zinc finger domain the role of PTMs on the GLI transcription factors has been well characterized, whether it be non-canonical pathway activation through phosphorylation, inhibition through acetylation, or activation through SUMOylation (Liu et al., 2016; Canettieri et al., 2010; Cox et al., 2010). However, work on the zinc finger domain of the GLIs has been limited. Here, we were able to show that the zinc finger domain of the GLI transcription factors can be broken down into three major regions: the regulatory, DNA binding, and linker regions. The regulatory region of the zinc finger domain acts as permissive zone for phosphorylation. PTM within this zone allows for the modulation of DNA binding and activity either enhancing, repressing, or having a net neutral affect. These PTMs within the regulatory region could provide a means cofactor binding or overall protein structure. As the GLIs have many interacting partners, phosphorylation within the zinc finger domain could provide ways to alter partner binding or protein structure along with DNA binding affinity. This could explain as to why we saw neutral binding with GLI1 mutant S339E but slightly enhanced transcriptional activity. Looking to see how regulatory partners are being affected by these mutations could show how these mutants are affect GLI protein-protein interactions. Outside this regulatory region there lies a more restrictive zone, the DNA binding and linker regions. Both areas when phosphorylated abolish DNA binding

and transcriptional activity of GLI1. As the zinc finger domain of GLI1 has been crystalized bound to DNA, looking at that structure could provide insight as to why there are regions of permissive modification. From the crystal structure it has been seen that fingers four and five make extensive contact with DNA (Pavletich et al., 1993). In contrast the regions of zinc fingers one and three that carry the S243E and T304E mutations are seen to be located a distance away from DNA. This could explain why adding a negative charge in the form of a phosphate results in loss of DNA binding in certain regions, but no effect to DNA binding in other regions. As to why phosphorylation enhances DNA binding is still an open question, either through conformational changes to the protein or enhancing interactions with partners could result in the enhanced binding that we see. In the context of DNA binding, this held true for GLI2 and GLI3. We were not able to identify any sites within the regulatory regions that enhanced DNA binding, however we did identify sites that showed a neutral response. Outside of DNA binding the mutations did not correlate well with transcriptional activity like GLI1 did. These transcriptional activity studies were done by overexpressing these mutants in NIH 3T3 cells, as such the endogenous GLI transcription factors are still present in these cells. GLI1 being the main effector of the HH pathway may not have run across an issue, however GLI2 and GLI3 not being the central effectors of the pathway the endogenous proteins may have compensated for these mutants. Furthermore, GLI2 and GLI3 are well characterized to have both an activator and repressor forms (Li et al., 2017). As these studies are conducted the overexpressed versions of GLI2/3 could be processed differently resulting in differing responses. We believe that additional transcriptional activity studies utilizing a GLI2/GLI3 null cell line (Lipinski et al., 2008) may further elucidate an answer as to how these zinc finger mutants are affecting transcriptional activity. Along these lines we also hope to examine the zinc finger mutants of GLI2/3 in their activator and repressor isoforms. Investigating these mutations may also provide another avenue of regulation of the HH pathway when specific isoforms of GLI2/3 are present.

As C2H2 ZF-TFs make up roughly 80% of transcription factors in primates (Vaquerizas et al., 2009), we wanted to expand our search to beyond the HH signaling pathway. We identified several C2H2 ZF-TFs that met our criteria (five C2H2 zinc fingers within proximity of each other), and we hope in future work we further elucidate the role of phosphorylation of these transcription factors. From this list of factors that we generated one C2H2 ZF-TF caught our attention, that being GLIS3. This transcription factor shares high conservation with that of the GLIs especially within the zinc finger domain. As such we were interested in understanding whether a known kinase of GLI1's zinc finger, aPKC, could also phosphorylate GLIS3. Our data has shown that GLIS3 can be phosphorylated by aPKC, and most likely is being phosphorylated at T415. Similar to GLI1 we were also able to show that phosphorylation of GLIS3 enhances DNA binding *in vitro***.** This opens the possibilities that similar C2H2 ZF-TFs can be regulated in the same phosphorylation dependent manner that we have seen with GLI. Together, we have been able to show that phosphorylation of the zinc finger domain is as clear cut as previously defined. The zinc finger domain seems to be open to modification if those modifications happen at specific regions within the zinc finger. Additionally, these modifications have a functional effect on the transcription factor, altering DNA binding and transcriptional activity. Furthermore, similar C2H2 ZF-TFs may be regulated in this way, as seen with GLIS3. Additional work on GLI as well as similar C2H2 ZF-TFs is needed to fully elucidate the role of phosphorylation of the zinc finger domain and how this phosphorylation affects transcription factor function.

CHAPTER 4

CONCLUSIONS

Role of Aurora A in HH pathway regulation

In the context of the cell cycle AURKA has been well studied (Roy et al., 2018), however as time has progressed additional roles for the kinase outside of the cell cycle have been discovered. These include roles such as neurite elongation, cilia disassembly, and cell motility (Bertolin et al., 2020). AURKA also plays a critical role in cancer as it is often abnormally expressed in tumor cells and promotes cell proliferation by regulating mitotic substrates (Lin et al., 2020). Mitotic factors like p53, PLK1, and TPX2 are all phosphorylated and regulated by AURKA in cancers (Katayama et al., 2004; Macurek et al., 2008; Garrido et al., 2016). As HH signaling primarily takes place at the primary cilia, there is potential that AURKA may interact with components of the HH signaling pathway. But very little work has been done to elucidate whether there is a direct connection with AURKA and the HH pathway. Evidence has shown that AURKA does interact with the HH pathway however, these are often indirect interactions with an intermediary such as KIF14 (Pejskova et al., 2020) or mir-124 (Xu et al., 2017). Additionally, there is little work that has been done to elucidate the role of AURKA in BCC or other skin cancers. Work has been done showing that there is an overexpression of AURKA in squamous cell carcinoma and that this overexpression induces high levels of genomic instability (Torchia et al., 2009). However, if AURKA plays a role in BCC is still up in the air. There still is an open possibility that AURKA does play a more direct role on the HH pathway along with previous work that has demonstrated the effect that AURKA has on the pathway indirectly.

Our finding in Chapter 2, support the idea that AURKA plays a role in the HH pathway and furthermore we were able to demonstrate a potential direct connection between AURKA and the GLI transcription factors. In Chapter 2, we demonstrated that inhibition of AURKA via alisertib, a kinase inhibitor specific to AURKA, resulted in decreased levels of BCC cell growth both *in vitro* and *in vivo*. Additionally, we have been able to show that inhibition of AURKA reduces levels of

HH signaling *in vitro*. This initial data supports the previous finding of other studies showing that AURKA does influence HH signaling pathway and cell growth. To further elucidate whether this is a direct interaction on the HH pathway we wanted to show that AURKA phosphorylates the GLI transcription factors. And indeed, we saw that AURKA does phosphorylate both GLI1 and GLI2 *in vitro*. To further elucidate this, we mutated several sites within GLI1 and found that S116E showed a significant increase in HH signaling compared to WT. S116 has been previously shown to be a site where SUFU interacts with GLI1 (Dunaeva et al., 2003), suggesting that AURKA may play a role in the SUFU-GLI1 interaction. Further experimentation needs to be done to fully elucidate the role of AURKA with the HH pathway and GLI1, but these initial steps have shown the likely direct interaction of AURKA with GLI1. Phosphorylation of GLI1 S116 by AURKA may lead to disruption of SUFU interactions with GLI1 resulting in increased transcriptional activity. This may be the reason as to why we see that inhibition of AURKA reduces levels of HH signaling and cell growth in BCCs. This could provide a potential avenue for therapeutics of drug resistant BCCs, targeting AURKA as a means to reduce levels of HH signaling and cell growth.

Applications of GLI mutant screen and Kinase inhibitor screen

BCC is the most common of cancers, with nearly 5 million new cases in the United States every year (Nguyen et al., 2019). BCCs result from aberrant activation of HH signaling pathway, an important pathway normally involved in embryonic development and adult tissue homeostasis (Hui et al., 2011). The HH signaling pathway is regulated via a family of C2H2 ZF-TFs the GLI family. These transcription factors have been seen to be mutated in a variety of cancers such as rhabdomyosarcoma, medulloblastoma, as well as BCC (Zibat et al., 2010; Rutkowski et al., 2005; Epstein 2008). These transcription factors need to be under tight regulation to prevent aberrant activation. One way that this is achieved is through post-translational modification (PTM). Several works have shown that the GLI transcription factors are under tight regulation via PTM whether that be through phosphorylation, acetylation, or ubiquitination (Niewiadomski et al., 2014; Mirza

et al., 2019; Wang et al., 2006). These types of modifications can control GLI factors DNA binding ability, protein stability, or localization.

In Chapter 2, we conducted a screen of GLI1 and GLI2 mutations in order to assay how these individual mutations contribute to HH signaling. These mutations were collected from the COSMIC database, a database that collects known mutations in cancer. After we collected mutations that were recurrent, mutations that appeared >/= 3 times, we assayed how these mutations affected HH signaling. We identified that the majority of the mutations that we tested showed a significant change in HH signaling through qRT-PCR. Even though cancer is diverse and often carries multiple mutation to drive cancer, these single mutations with GLI1/2 were enough to significantly alter HH signaling. Furthermore, when looking at these mutations we identified that the majority of these mutants reside at or near a predicted/known kinase site. This led us to conduct a secondary screen on how different kinase inhibitors affect the HH pathway. We discovered in Chapter 2, that alisertib, an AURKA inhibitor, potently inhibits BCC cell growth *in vitro* and *in vivo*. We tested other kinase inhibitors and although some showed minor changes in HH signaling alisertib reduced HH levels at a greater extent. Although we focused on AURKA, other kinase inhibitors that we tested may also play roles in the HH signaling pathway. mTor, which is inhibited via rapamycin, has been shown to enhance GLI activation by preventing SUFU association (Wang et al., 2012). Additional kinases that we identified in our screen but did not test also have been shown to regulate HH signaling. For example, DYRK2 which induces GLI2 protein degradation (Varjosalo et al., 2008). Kinases from the CDK family have been shown to reduce cilia length when inhibited, thus reducing HH activity (Scheidt et al., 2020). Members of the MAP kinase family have been shown to inhibit GLI1 transcriptional activity (Antonucci et al., 2019). These are just a handful of kinases that appeared in our screen that are known to affect HH signaling pathway. However, many of the kinases we identified currently do not have any known association with the HH pathway. Future work can be done looking into these kinases and

identifying whether any of these play a role in regulating the HH pathway. This could potentially open up new avenues for therapeutics when it comes to treating HH pathway driven diseases such as BCC.

The data collected in Chapter 2 also provides a foundation for additional studies as to how these mutations regulate HH signaling. Although the focus was initially on mutation sites that reside near kinase sites, we tested several mutants that reside at or near other sites of PTM. Other PTM sites outside of phosphorylation may be disrupted by these mutations and offer additional avenues to further elucidate the role of PTM in regulating HH signaling. Along with this the mutations that we tested span the entire length of GLI1/2. As such these mutants reside in a variety of different known domains such as the repressor domain, SUFU binding domain, zinc finger domain, and trans-activation domain. The data collected in Chapter 2 through our GLI screen provides avenues to study the role of these domains and how these known cancer-causing mutants affect each domain of GLI1/2. Together the two screens conducted in Chapter 2, GLI screen and kinase screen, provide a strong foundation for future studies into understanding how these mutations regulate GLI through the distinct domains within the protein, or further elucidate how these different kinases regulate HH signaling either through direct or indirect pathway interaction.

How generalizable is zinc finger transcription factor regulation?

The GLI family of transcription factors falls under the larger umbrella of transcription factors, the C2H2 ZF-TFs. These types of transcription factors are one of the most abundant in eukaryotes, making up roughly 80% (Vaquerizas et al., 2009). These transcription factors play critical roles in many tissues such as the skin (Evans et al., 2008) and the adipose tissues (Perez-Mancera et al., 2007) along with being implicated in several cancers (Atwood et al., 2013; Lin et

al., 2014; Lamouille et al., 2014). These factors are tightly regulated by several means one of which includes PTM through phosphorylation. Several studies have shown how critical phosphorylation of the zinc finger domain is to the transcription factor's ability to bind to DNA. During mitosis it has been seen that there is a global phosphorylation event of the linker region of C2H2 ZF-TFs removing them from DNA allowing access to the replication machinery (Rizkallah et al., 2011). Few studies have shown how phosphorylation of the zinc finger domain can positively regulate DNA binding. Studies on C2H2 ZF-TF Sp1 have shown that phosphorylation of the zinc finger enhances Sp1 DNA binding (Fojas de Borja et al., 2001). Along with this phosphorylation of GLI1 zinc finger has been shown to increase levels of DNA binding (Atwood et al., 2013).

In Chapter 3, we wanted to further elucidate how phosphorylation of the zinc finger domain of GLI affects DNA binding and transcriptional activity. We demonstrated that phosphorylation of S243E and T304E showed increased levels of DNA binding *in vitro*, corroborating the previous work done by Atwood et al., 2013. Furthermore, we were able to show that both of these mutations increased levels of HH signaling when transfected into cells. These two sites within the zinc fingers of GLI1 enhanced both DNA binding and activity, however, we also were able to show that several sites within the zinc finger do not show any changes to binding or activity when phosphorylated and several others show dramatic reductions to binding and activity. This provided us with an idea that the zinc finger domain could be split into distinct zones: regulatory, DNA binding, and linker region. The regulatory region being an area where phosphorylation was permissive, allowing for phosphorylation to adjust the DNA binding and activity of the transcription factor without turning it off entirely, which is the case for the DNA binding and linker region of the zinc fingers. We wanted to further investigate this by mutating sites within GLI2/3. And although we saw similar results of DNA binding for the DNA binding and linker region, we did not see similar changes in transcriptional activity that we saw with GLI1. Along with this within the regulatory region of GLI2/3 we saw that phosphorylation was still permissive, but we did not see any

enhancements to DNA binding. This could be that the effect we were seeing is GLI1 specific, however it still does not rule out GLI2/3. Both GLI2/3 are known to exist in either an activator or repressor role within cells. Our transfected GLI2/3 may be being cleaved into a repressor form and as such we don't see much changed in transcriptional activity. Additionally endogenous GLI2/3 may also be playing a role in this case. As such we propose future studies to be conducted using GLI2/3 mutants in an activator or repressor form along with doing transcriptional studies in cells that no longer express GLI2/3.

In Chapter 3, we also identified additional C2H2 ZF-TFs that could be regulated in a similar fashion to GLI1. One of these transcription factors GLIS3 was of quite interest to us due to the high conservation of the zinc fingers between GLI and GLIS3. We initially tested the conserved site of GLIS3 to see if it could be phosphorylated. As aPKC is known to phosphorylate GLI1 we tested aPKC with GLIS3 and we did see that aPKC can phosphorylate GLIS3 zinc fingers. Furthermore, when the conserved site was mutated to an alanine, we saw reduced levels of GLIS3 phosphorylation by aPKC. Together this suggest that aPKC does in fact phosphorylate GLIS3. To further test GLIS3 we wanted to see how DNA binding was affected by phosphorylation. We generated a T415E mutant, and we did see that levels of DNA binding increased compared to the WT. This opens the possibility that GLIS3 is regulated in a similar fashion to GLI1 when phosphorylated. Although additional studies need to be conducted in order to confirm these initial findings. As we have seen with GLI1 and Sp1 along with GLI2/3 and GLIS3 to a certain extent, phosphorylation of the zinc finger domain does not always have to be a negative regulator. Phosphorylation of certain regions of the zinc fingers, such as with GLI1 zinc fingers that are not in direct contact with DNA, have been shown to enhance DNA binding or have a net neutral affect. Furthermore, the evidence that we provide strengthens the previous work done by others showing that phosphorylation of the linker region of C2H2 ZF-TFs causes loss of DNA binding (Dovat et al., 2002). Additional studies on the role of phosphorylation of C2H2 ZF-TFs, especially residues found within the proposed regulatory region, will provide further insight

into how generalizable this trend is. As of now the data provided in Chapter 3 provides further evidence about the role of phosphorylation of the linker region, as well as supporting previous work done on Sp1 and GLI1. This may provide an avenue to further regulate C2H2 ZF-TFs by modulated their DNA binding and activity through differential phosphorylation of the zinc fingers. As such we hope to expand our initial testing to additional C2H2 ZF-TFs in order to elucidate whether this is a generalizable form of regulation or if this is specific to certain ZF-TFs that contain a certain number of zinc fingers.

Future Perspectives

The body of work presented here focused on understanding how phosphorylation could potentially regulate GLI and hedgehog signaling. Additional experiments are still needed in order to fully flesh out the role of phosphorylation as well as other avenues that the data has provided. First and foremost, the validation of any of the phospho-mimetic mutations must be conducted. The putative AURKA site in GLI1 S116, needs to be validated through generation of a phospho-dead mutation (S116A) as well as through additional kinase assays that look to see how levels of GLI1 phosphorylation by AURKA change when this mutation is present. Additional phospho-dead mutations need to be tested when looking at the role of modifications within the zinc finger domain of GLI as well. Along with these validation experiments, in order to truly confirm that this site is indeed phosphorylated by AURKA, I propose conducting mass spectrometry experiments. We could perform *in vitro* kinase assays with AURKA and GLI1 WT along with GLI1 S116A. Once these kinase assays are performed these samples can be used for mass spectrometry to truly determine whether AURKA is phosphorylating GLI1 at S116. To further strengthen the data generated through our mutation studies we could also generate CRISPER mutations within the endogenous gene. As all the experiments were done through overexpression, the data may not be as accurate as we would want it to be, especially for the mutations within GLI2 and GLI3. Generating mutations within the endogenous gene would make sure that the mutation is the only

aspect driving the changes in signal and not due to the fact that endogenous WT GLI2/3 is present.

To further strengthen the data presented here, we would also propose doing additional functional studies on several of our mutation cell lines to show an effect both *in vitro* and *in vivo*. We plan on doing mouse injections with BCC cell lines containing the phospho-mimetic mutations within the zinc finger domain and characterizing how these mutations affect BCC growth in mice. We hypothesize that mutations such as S243E and T304E would show greater levels of BCC growth compared to WT as these two mutants showed significantly higher levels of DNA binding and transcriptional activity. Along with the zinc finger mutations additional mutations that we discovered during our GLI screen may be worth further investigation due to the dramatic response in HH signaling that we saw. For example, GLI2 V595I shows a much larger response in HH signaling compared to WT and even other mutations. I believe it would be worthwhile to further investigate this mutation in order to understand what is actually happening here. This mutation resides at the tail end of the zinc finger domain where a NLS is reported, there could be some disruption here resulting in enhanced levels of signaling. This mutation could also be responsible for disrupting/enhancing interacting partners. Lastly, how does this mutation affect BCCs? Generating a BCC cell line carrying this mutation in order to assay cell growth or tumor growth in mice in order to see if this enhanced level of HH signaling has a functional consequence on BCCs.

Summary

As BCC is a very common form of cancer, we sought to further understand the pathway responsible for driving activation of this cancer. As understanding how GLI is regulated, specifically, at a post-translational level may provide alternate ways to target GLI therapeutically. In Chapter 2 we demonstrated that several known cancerous mutations of GLI reside at or near kinase sites and these mutants significantly alter HH signaling. We were able to identify AURKA as a putative target for BCC treatment. As inhibition of AURKA reduced levels of BCC cell growth

in vitro and *in vivo* as well as reducing HH signaling *in vitro*. Additionally, in Chapter 3, we demonstrated that phosphorylation of the GLI zinc finger domain has differential effects on DNA binding and transcriptional activity based on the location of phosphorylation. We also were able to show that GLIS3 shares a similar type of phosphorylation dependent regulation as GLI1. Together this data shows several ways to target HH signaling by manipulating the PTM status of GLI.

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APPENDIX

MATERIALS AND METHODS

Cell Culture

ASZ001 cells (So et al., 2006) were grown in 154CF medium (Life Technologies) containing 2% Fetal Bovine Serum (FBS; Life Technologies) chelated overnight with ChelexÒ 100 Resin (Bio-Rad), 1% Penicillin-Streptomycin (P/S; Life Technologies), and 0.07 mM $CaCl_2$. NIH3T3 cells (ATCC, CRL-1658) were grown in DMEM medium (Life Technologies) containing 10% FBS and 1% Penicillin-Streptomycin.

Generation of Stable Cell Lines

Stable cells lines were generated using NIH3T3 cells and piggyBac™ transposons containing sequences for mApple, wild-type GLI1/2/3 or the listed GLI mutant. Cells were transfected with PEI (NC1038561, Fisher Scientific) per manufacturer's protocol and then selected for using 500µg/mL Geneticin G-418 (50841720, Fisher Scientific) until all non-transfected cells were no longer viable.

Hedgehog assay

ASZ001 cells were plated to confluence, serum-starved (SS), and treated with either DMSO or varying concentrations of KT5823, GSK650394, Ro3306, rapamycin, PSI, Alisertib, Bay-1816032, AZD4573, Ana-12, MK2-IN-1, or vismodegib for 24 hours. Additionally, NIH3T3 cell lines were also plated to confluency, SS, and treated with HH-conditioned media (1:100) for 24 hours. RNA was isolated using the Direct- zol RNA MiniPrep Plus (ZYMO Research). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the iTaq Universal SYBR Green 1-Step Kit (Bio-Rad) on a StepOnePlus Real-time PCR system (Applied BioSystem) using primers for *Gli1* (forward: 5'-GCAGGTG TGAGGCC AGGTAG TGACGA TG-3', reverse: 5'-CGCGGG CAGCAC TGAGGA CTTGTC-3') *Gapdh* (forward: 5'-AATGAA TACGGC TACAGC AACAGG GTG-3', reverse: 5'-AATTGT GAGGGA GATGCTCAGTGT TGGG-3'), and *mApple* (forward: 5'-ACCTAC AAGGCC AAGAAG CC -3', reverse: 5'- GCGTTC GTACTG TTCCAC GA -3'). Fold change in *Gli1* mRNA expression was measured using ΔΔCt analysis with *Gapdh* or *mApple* as

an internal control. Experiments were repeated three times and ran in triplicates.

***In Vitro* Kinase Assay**

GST/MBP-tagged GLI1/2/3 along with GLIS3 fragments wild-type or mutated sequences were cloned into protein expression vectors and harvested. GST-tagged GLI1 proteins were immunoprecipitated with anti-GST A/G plus beads for 1 h. Likewise with MBP-tagged proteins. The immunocomplexes were divided: one half was incubated with 0.4 ng of recombinant aPKC/Aurora A as per manufacturer's protocol (NEB) and 10 uCi of $[\gamma\text{-}^{32}P]$ ATP at 30 °C for 30 min and the other half was diluted in 2xSDS sample buffer then run on 10% SDS-PAGE gels with subsequent Coomassie staining. The kinase reactions were terminated by adding 2xSDS sample buffers, and the samples were separated by 10% SDS-PAGE and dried, followed by autoradiography. Relative kinase activity was calculated by dividing autoradiography signal by corresponding Coomassie signal on the same sample. Experiment was repeated n≥3.

Mouse Studies

All mice were housed under standard conditions and animal care was in compliance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at University of California, Irvine. *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2}mice were administered 100uL of 10mg/mL tamoxifen (Sigma) intraperitoneally for 3 consecutive days at 6 weeks of age. 6 weeks later, mice were treated with either DMSO or Alisertib (30mg/kg) intraperitoneally for 7 consecutive days. The final volume of all injections was 100 µL. At the end of treatment, mice were sacrificed, and their back skin collected. Half of the skin was used for RNA isolation which was sent for bulk RNA sequencing analysis and the other half was fixed in 4% paraformaldehyde (PFA, Electron Microscopy Sciences) for 30 minutes at room temperature, washed with DPBS (Life Technologies), immersed in 30% sucrose at 4°C overnight, and frozen in optimal cutting temperature (OCT) compound (Sakura Finetek). Samples were then cryo-sectioned (CryoStar NX50) at 14 µm for analyses. Three mice were used for each treatment.

Micro-tumor assessment

Mouse sections were H&E stained per standardized protocol and images were taken at 200x magnification on an AmScope microscope with an AmScope MU500B digital camera. Tumor size was measured using FIJI software. Micro-tumors were assessed in mouse back skin as average tumor per mouse.

Immunofluorescence staining

Skin sections were blocked using 10% BSA and 0.1% Triton X-100 in PBS for 1 hr at room temperature. The following antibodies were used: Krt14 (Chicken, Fisher Scientific 50-103-0174, 1:5000), Aurora A (rabbit, Fisher Scientific 10297-1-AP, 1:500) Sections were mounted with ProLong Diamond AntiFade Mountant with DAPI (Invitrogen). Images were acquired on a Zeiss LSM700 confocal microscope with a 63x oil immersion objective. FIJI was used to determine the average pixel intensity over five distinct tumors within a given skin section. Images were arranged with FIJI and Adobe Illustrator.

ASZ001 cells were seeded onto glass coverslips, serum-starved, and treated with DMSO or Alisertib (10 nM) for 24 hours. Cells were fixed with 4% PFA for 30 minutes, followed by incubation with antibodies against cCasp3 (R&D, 1:250) and Mki67 (ThermoFisher, 1:250), and then subsequent incubation with secondary antibodies donkey anti-mouse Cy3 or donkey anti-rabbit Cy3 (Jackson, 1:500). The coverslips were imaged using an EVOS fluorescence microscope.

NIH3T3 cells were seeded onto glass coverslips, serum-starved, and treated with DMSO or Alisertib (10 nM) for 24 hours. Cells were fixed with 4% PFA for 30 minutes, followed by incubation with antibodies against RFP (Rabbit, Rockland 1:1000), and then subsequent incubation with secondary antibodies donkey anti-mouse Cy3 or donkey anti-rabbit Cy3 (Jackson, 1:500). The coverslips were imaged using an EVOS fluorescence microscope.

ImageStream Analysis

ASZ001 cells were seeded and then treated with either DMSO or Alisertib (10nM/100nM) for 24

hours. Cells were then trypsinized, filtered, and collected for staining. Cells were fixed with 4% PFA for 15 minutes, washed 3X with PBS and 0.1% Triton X-100 and then shook at 4C for 20 minutes with PBS and 0.1% Triton X-100. Cells were then spun down and media was replaced with 3% BSA and 0.1% Triton X-100 in PBS for 30 minutes at 4C. The following antibodies were used to stain for 24 hours: Gli1 (Goat, R&D, 1:50), Aurora A (rabbit, Fisher Scientific, 10297-1-AP, 1:500), pAurora A (Rabbit, Fisher Scientific, 44-1210G 1:500). Following primary antibody, secondary antibody was added Alexa Fluor 488, 568 (Jackson, 1:500). Dapi was added after final wash for 5 minutes and then samples were washed again. Samples were analyzed on an ImageStream MKII and later analyzed using IDEAS 6.0.

Electrophoretic Mobility Shift Assay

Protein was generated via *in vitro* translation kit (Promega, PRL2080) as per the manufacturer's instructions. Once protein translation was finished half the sample was used for western blotting and the other half for DNA binding assay. 2uL of radio labeled GLI binding site was mixed with 8uL of protein and incubated at 37C for 30 minutes. Afterwards 2uL of DNA loading dye was added to solution and run on a gel under native conditions, and dried, followed by autoradiography. Relative levels of DNA binding was calculated by dividing autoradiography signal by corresponding protein signal on the same sample. Experiment was repeated $n \geq 3$.

Growth Assay

ASZ001 cells were seeded at 2000 cells/well into 96-well plates. After 48 hours, cells were treated with DMSO or varying concentrations of Alisertib for the indicated number of days. Growth assay was performed with MTT (Sigma-Aldrich) per manufacturer's protocol. Plates were analyzed with a Bio-Tek uQuant MQX200 plate reader. Experiments were repeated at least three times in 6 wells each.

Statistics

Statistical analyses were done using two-tailed *t* test or two-way ANOVA using GraphPad Prism.