UNIVERSITY OF CALIFORNIA,
IRVINE

Identification of Alternative HH Regulators in Basal Cell Carcinoma

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Rachel Y. Chow

Dissertation Committee:
Assistant Professor Scott Atwood, Chair
Professor Lee Bardwell
Assistant Professor Claudia Benavente
Assistant Professor Devon Lawson

2021

DEDICATION

to

My loved ones I've lost to cancer,
my late grandmother Oi New Tew,
my late Uncle Randy Wong,
and my late Auntie Patricia Shou,
you all are truly missed

My loved ones who have defeated cancer,
my Auntie Chaile Yip
and my father-in-law Romeo Gutierrez,
you give me hope in the work that I do

My babies, Bryce and Brynne,
know that there is no ceiling too high or mountain too tall for you to reach
Mommy loves you so much

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

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

To my committee members, Lee Bardwell, Ph.D., Claudia Benavente, Ph.D., and Devon Lawson, Ph.D., and former committee member, Irene Pedersen, Ph.D., thank you for all of your time, support, and meaningful insight on my projects.

To my labmates, Tuyen Nguyen, Eric Tarapore, Adam Stabell, Kirsten Wong, and Emmanuel Dollinger, thank you for all of your support through the years. You guys have made coming into the lab much more enjoyable for me. I especially want to thank Tuyen for being the best friend through all the highs and lows of grad school. I wouldn't have been able to survive without you.

To our former post-doc and my friend, Mike Drummond, Ph.D., thank you for all of your support and guidance while you were in the lab and for letting me bounce ideas off of you.

To my undergraduate student mentees, Ledia Tarabey, Taylor Levee, Daniel Cedeno, and Gurleen Kaur, thank you for all of your contributions in helping me to finish my projects.

To my graduate advisor, Christine Süetterlin, Ph.D., thank you for all of your support and guidance through some of the most stressful moments of grad school.

To my husband, Chris Gutierrez, thank you for your unwavering support through all our years together. The past several years have been rough with both of us being in grad school, but we're finally done and I can't wait to see what our future holds.

To my parents, thank you for being the best support system through all my years of schooling. I don't know how I would have been able to manage being a first time mom in grad school without your help.

CURRICULUM VITAE

Rachel Y. Chow

EDUCATION

University of California, Irvine — Irvine, CA
Ph.D. Developmental and Cell Biology — May 2021
Advisor: Scott X. Atwood, Ph.D.
Thesis: Identification of Alternative HH Regulators in Basal Cell Carcinoma

Albany Medical College — Albany, NY
M.S. Cell Biology and Cancer Research — May 2013
Advisor: Michelle R. Lennartz, Ph.D.
Thesis: The Role of PKC-ε in Membrane Mobilization in Response to Fc-γ Receptor Ligation

Dominican University of California — San Rafael, CA
B.S. Biological Sciences w/ Molecular Emphasis — May 2010
Minor: Chemistry
Summa cum laude
Advisor: Maggie C. Louie, Ph.D.
Thesis: The Effects of Vitamin D on Tamoxifen Resistant Breast Cancer Cell Growth

RESEARCH EXPERIENCE

Ph.D. Candidate, University of California, Irvine — 2014- 2021
Advisor: Scott X. Atwood
- *Identified alternative regulators of Hedgehog signaling in basal cell carcinoma to potentially uncover new therapies for this disease*

Graduate Student, Albany Medical College — 2010- 2012
Advisor: Dr. Michelle R. Lennartz
- *Established the role of PKC-ε in membrane mobilization and determined the signal transduction pathway by which PKC-ε affects phagocytosis*

Undergraduate Student/ Research Assistant, Dominican University of California — 2008- 2010
Advisor: Dr. Maggie C. Louie
- *Studied the role of vitamin D and the vitamin D receptor in tamoxifen resistant breast cancers*

Undergraduate Student, Dominican University of California 2007- 2008

Advisor: Dr. M. Graciela Carranza

- *Evaluated the potential anti-acetylcholinesterase activity of secondary metabolites isolated and purified from Umbellularia californica*

PROFESSIONAL EXPERIENCE

Research Associate, Natera (San Carlos, CA) 2013-2014

- *Processed fetal and parental DNA for paternity testing and disease screening*
- *Screened in-vitro fertilized blastomere samples for genetic diseases and chromosomal defects before implantation*

Staff Research Associate II, University of California, San Francisco- Clinical & Translational Science Institute- Sample Processing Lab (San Francisco, CA) 2012-2013

- *Processed human blood, urine, and cerebrospinal fluid samples for clinical research studies*

PUBLICATIONS

*equal contributions

Chow, R.Y., Jeon, U.S., Lee, T.M., Kaur, G., Cedeno, D.C., Doan, L.T., Atwood, A.X. (2021). PI3K promotes basal cell carcinoma growth through kinase-induced p21 degradation. *Frontiers in Oncology*. In review.

Chow, R.Y., Levee, T.M., Kaur, G., Cedeno, D.C., Doan, L.T., Atwood, A.X. (2021). mTOR promotes basal cell carcinoma growth through atypical PKC. *Experimental Dermatology 30*(3): 358-366.

Daugaard, I., Sanders, K.J., Idica, A., Vittayarukskul, K., Hamdorf, M., Krog, J.D., **Chow, R.**, Jury, D., Hansen, L.L., Hager, H., Lamy, P., Choi, C.L., Agalliu, D., Zisoulis, D.G., Pedersen, I.M. (2017). miR-151a induces partial EMT by regulating E-cadherin in NSCLC cells. *Oncogenesis 6*(7): e366.

Wood, T.R.*, **Chow, R.Y**.*, Hanes, C.M.*, Zhang, X., Kashiwagi, K., Shirai, Y., Trebak, M., Loegering, D.J., Saito, N., and Lennartz, M.R. (2013). PKC-ε pseudosubstrate and catalytic activity are necessary for membrane delivery during IgG-mediated phagocytosis. *Journal of Leukocyte Biology 94*(1): 109-22.

PRESENTATIONS

American Association for Cancer Research: Washington, D.C. April 2010
"Vitamin D Receptor Resensitizes Resistant Breast Cancer Cells to Tamoxifen"

American Society for Biochemistry and Molecular Biology: New Orleans, LA April 2009
"Effects of Vitamin D on Tamoxifen Resistant Breast Cancer Cell Growth"

American Society for Cell Biology: San Francisco, CA December 2008
"Inhibition of tamoxifen-resistant breast cancer cell proliferation by Vitamin D3"

National Conference of Undergraduate Research: Salisbury, MD April 2008
"Phytochemical Screening and *in vitro* Acetylcholinesterase Inhibitory Activity of Three Native California Plant Species"

TEACHING EXPERIENCE

Cell Biology, UCI: Teaching Assistant Fall 2016, 2018, 2019

- *Encouraged students to use their critical thinking skills by designing comprehensive questions for their quizzes and exams*
- *Held office hours to help students understand difficult lecture content*

Teaching Assistant: Scientific Writing, UCI: Teaching Assistant Spring 2018, 2019

- *Helped to facilitate in-class group discussions*
- *Held office hours to aid students in the scientific writing process and to help with idea development*
- *Worked in a team with other TAs to help design assignments and exam questions*

Developmental and Cell Biology Lab, UCI: Teaching Assistant Spring 2016

- *Facilitated two lab sections*
- *Encouraged team work by having students work in lab groups*
- *Stimulated students to exercise creativity in their experimental designs*
- *Supervised students as they performed experiments in the lab*
- *Met with students 1-on-1 to discuss final lab report critiques and write-up*

HONORS AND AWARDS

Dominican University Academic Scholar	2010
Dominican University Outstanding Biology Student Award	2006- 2010
National Dean's List	2006- 2010
Honors Program Scholar	2006- 2010
Robert C. Byrd Scholarship	2006- 2010
Dominican University Trustee Scholarship	2006- 2010
Dominican Valedictorian Honorarium Scholarship	2006- 2010

PROFESSIONAL AND ACADEMIC AFFILIATIONS

American Association for Cancer Research	2010- 2011, 2020-2021
American Society for Cell Biology	2008- 2009, 2015-2016
Alpha Chi Honor Society	2008- 2010
Beta Beta Beta Honor Society	2008- 2010
Alpha Lambda Delta Honor Society	2007- 2010

ABSTRACT OF THE DISSERTATION

Identification of Alternative HH regulators in Basal Cell Carcinoma

by

Rachel Y. Chow

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2021

Associate Professor Scott Atwood, Chair

Basal cell carcinoma is the most prevalent cancer and it has been well-documented to be driven predominantly via overactivation of the Hedgehog signaling pathway. Although vismodegib, a SMO inhibitor, has been proven to be highly effective in treating BCCs, advanced forms of BCCs often possess inherent resistance while many that initially respond to drug treatment develop drug resistance over time. This highlights the importance of identifying targets that are either downstream of SMO or alternative drivers of BCCs in order to bypass SMO-inhibitor resistance. Here, we use a combination of RNA-sequencing and immunofluorescence staining to identify and validate the overexpression of other potential pathways that are upregulated in BCCs. This analysis highlighted the expression of mTOR, PI3K, and SRC. We found that upon pharmacological inhibition of mTOR and PI3K in our *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mouse BCC tumor model that although BCC growth is significantly inhibited, HH signaling is not. This implies that both the mTOR and PI3K pathway may work either downstream of or in parallel with HH signaling. Furthermore, our data suggests that mTOR is affecting BCC growth via aPKC independent of HH

signaling whereas PI3K is likely driving BCC growth via aPKC- and AKT-driven p21 degradation. However, when SRC was pharmacologically inhibited, it reduced both BCC growth and HH signaling. We were able to demonstrate that SRC promotes BCC growth via aPKC-dependent phosphorylation and activation of GLI1. Together, these findings identify alternative means of being able to target and treat BCCs and, potentially, resistant BCCs as well.

CHAPTER 1

Introduction

Basal Cell Carcinoma

Basal cell carcinoma (BCC) is the most common of cancers, resulting in approximately 5 million new cases and 3,000 deaths annually in the U.S. alone (Nguyen et al., 2019; Mohan & Chang, 2014). BCCs make up nearly 80% of non-melanoma skin cancers, with squamous cell carcinoma making up the other 20%. BCCs are locally invasive malignancies that arise predominantly from basal cells in the hair follicle bulge, touch dome, and interfollicular epidermis (IFE) (Peterson et al., 2015; Tan et al., 2018) and are driven by abnormal Hedgehog (HH) signaling (Atwood et al., 2015; Sekulic and Hoff, 2016). They become evident by a change in the skin, such as by growth or as a sore that doesn't heal. These abnormalities are also accompanied by one of the following characteristics: 1) a pearly white, skin-colored or pink bump, 2) a brown, black or blue lesion, 3) a flat, scaly, reddish patch, or 4) a white, waxy, scar-like lesion (The Mayo Clinic, 2019).

Not all BCCs appear phenotypically similar, and therefore, can be classified simplistically into three subtypes based on their histology- nodular, superficial, and infiltrative. However, a majority of BCCs appear to be a blend of these three subtypes (Raasch et al., 2006). Common risk factors include chronic sun exposure, radiation therapy, use of immune-suppressing drugs, fair skin and light hair, increasing age, exposure to arsenic, a personal or family history, and inherited syndromes that can cause skin cancer such as Gorlin-Goltz or Xeroderma Pigmentosum (The Mayo Clinic, 2019; Lear et al., 2007; Jaju et al., 2016). BCC tumors have been found more frequently on individuals with higher incidences of solar keratoses, caused by prolonged and repeated sun exposure. Additionally, chronic cutaneous sun damage can be used to predict those who will be affected by more than one BCC. (Richmond-Sinclair et al., 2010). Since chronic sun exposure is one of the major factors contributing to BCC development, BCCs are most commonly

found in areas with the most exposure, such as the face, chest, arms, and legs (Montagna et al., 2017).

As UV exposure and other determinants have the potential to cause a number of random mutations, this can lead to different forms of BCCs. Roughly 1-10% of patients have locally advanced BCC, whereas those with metastatic BCC make up about 0.5% of the cases (Mohan et al., 2014). Their potential for metastasis is low and most cases can be treated with local therapies such as vismodegib, sonidegib, imiquimod, photodynamic therapy, cryotherapy, surgical excision, or a combination of these methods. However, recurrences of BCC tumors are frequent, with 50% risk within five years and 90% in ten years (Montagna et al., 2017). As the rate of recurrence is quite high, it is important to unveil new therapies to prevent BCC return and to combat the new mutations that lead to these recurrences.

Hedgehog Signaling and Basal Cell Carcinoma

HH signaling is essential for the development of all vertebrates and drives proliferation, migration, and differentiation of progenitor cells to pattern organs (Varjosalo and Taipale, 2008). In the absence of a HH ligand, 12-transmembrane cholesterol receptor Patched1 (PTCH1) is active and suppresses 7-transmembrane receptor Smoothened (SMO) activity. This allows Suppressor of Fused (SUFU) to inhibit GLI from translocating into the nucleus to activate target genes. However, in the presence of a HH ligand, PTCH1 is suppressed, allowing for SMO activity and SUFU repression. This allows for GLI to be activated and translocated into the nucleus to activate genes involved in proliferation, migration, and invasion. Additionally, GLI1 auto-regulates HH signaling by transregulating *PTCH1*, resulting in a positive feedback loop (Yoon et al., 2002).

As BCCs are driven by aberrant activation of the HH pathway, mutations along this pathway are of no surprise. Inactivating PTCH1 mutations make up roughly 73% of BCC cases,

with activating mutations in SMO making up 20%, and loss-of-function SUFU mutations making up about 8% (Bonilla et al., 2016). These mutations, in addition to mutations in other cancer-related genes such as *P53* (Bonilla et al., 2016), in the epidermal basal cells result in the aberrant and uncontrolled activation of the HH pathway which leads to BCC development and growth. Additionally, this type of inappropriate HH pathway activation also drives growth of a variety of cancers including brain, pancreatic, prostate, and small cell lung cancer that account for up to 25% of all human cancer deaths (Epstein et al., 2008).

Drug Resistant Basal Cell Carcinoma

SMO antagonists such as vismodegib are FDA-approved to treat advanced and metastatic BCC (Atwood et al., 2012). However, nearly 60% of advanced tumors display inherent vismodegib resistance and 20% of tumors that do respond acquire drug resistance every year (Chang and Oro, 2012). The majority of vismodegib resistance is driven by SMO mutations that are either within or immediately adjacent to the drug binding pocket or at a more distant site. These mutations have the ability to either block drug interaction or inappropriately activate the protein even in the presence of inhibitor (Atwood et al., 2015; Sharpe et al., 2015). SMO mutations account for about 50% of resistant BCC cases (Atwood et al., 2015; Ridky and Cotsarelis, 2015) while a smaller proportion possesses SUFU inactivation mutations and/or GLI2 amplification (Sharpe et al., 2015). This illustrates a critical need to identify therapeutic targets downstream of SMO or targets that contribute to non-canonical HH signaling in order to suppress HH pathway activity.

More recently, there has been evidence of resistant BCCs switching from HH to Ras/MAPK pathway dependence in the absence of cilia. Additionally, data suggests that Ras/MAPK pathway activation can lead to resistance against both canonical and non-canonical HH pathway inhibitors

while resulting in sensitivity to MEK inhibitors. This supports a switch from a GLI1-dependent to a GLI1-independent switch (Kuonen et al., 2019).

Non-Canonical HH Signaling

No system is ever straight-forward, especially in cancers. In addition to canonical signaling, there exists non-canonical signaling and crosstalk between different signaling pathways. This poses a challenge because cancers which possess non-canonical HH signaling are more resistant to SMO inhibition, decreasing the efficacy of SMO antagonists (Pietrobono et al., 2019). To date, there are a number of identified pathways and players that have been demonstrated to regulate the HH pathway. By being able to understand how these non-canonical regulators affect the HH pathway, specifically in the context of BCC, it will give us a better understanding of how we can better treat both sensitive and drug-resistant BCCs.

PI3K/AKT/mTOR Signaling

The PI3K/AKT/mTOR signaling pathway is an important pathway involved in various cellular processes such as proliferation, metabolism, survival, and differentiation in both normal physiology and in pathological conditions such as cancer (Yu and Cui, 2016). Riobó et al. first demonstrated the involvement of PI3K signaling in HH regulation by showing that PI3K signaling is essential for GLI transcriptional activity in NIH 3T3 cells. They demonstrated that AKT antagonizes PKA, thereby preventing proteosomal degradation of Gli2 and facilitating its activation and nuclear localization (Riobó et al., 2006). Singh et al. showed that overexpression of AKT in large cell lymphoma increases GLI1 expression by inhibiting GSK3β (Singh et al., 2009) and that activation of the PI3K/AKT pathway enhances GLI1 protein stability in pancreatic and ovarian cancers (Singh et al. 2017). Similarly, AKT has been shown to stimulate GLI1 nuclear

localization and transcriptional activity in human prostate cancer, melanoma, and glioma cells (Stecca et al., 2007).

Downstream of PI3K signaling is mTOR, which has been demonstrated to have conflicting results in terms of whether it functions upstream or downstream of the HH pathway. In esophageal adenocarcinoma, activated mTOR signaling promotes GLI1 transcriptional activity through S6K1-1 mediated phosphorylation of GLI1, releasing it from its endogenous inhibitor, SUFU (Wang et al., 2012). In glioblastoma multiforme, mTORC2 inactivates GSK3β, thereby preventing ubiquitination of GLI2. This promotes GLI2 stability and its nuclear localization (Miati et al., 2017). However, studies in neuroblastoma demonstrate that inhibition of the mTOR/S6K1 pathway suppresses cancer growth but does not affect GLI1 expression (Diao et al., 2014). Additionally, in pancreatic and ovarian cancer cells, HH signaling has been shown to induce DYRK1B expression, which leads to activation of the mTOR/AKT pathway (Singh et al., 2017).

RAS/RAF/MEK/ERK Signaling

The RAS/RAF/MEK/ERK cascading pathway activates transcription factors and regulates gene expression in processes which include apoptosis, proliferation, and differentiation (Li et al., 2016). Activation of the RAS/RAF/MEK/ERK pathway, predominantly via mutations or alterations in RAS, RAF, and MEK, results in the overactivation of extracellular signal-regulated kinase 1 and 2 (ERK 1/2) and mitogen-activated protein kinase (MAPK), which ultimately leads to tumor growth (Samatar & Poulikakos, 2014). The first evidence of the RAS/RAF/MEK/ERK pathway positively regulating the HH pathway was described in NIH 3T3 cells by Riobó et al. (2006a), who demonstrated that constitutively active MEK1 upregulates GLI1 activity and this activity is abolished upon MEK1/2 inhibition. Following this, a number of reports demonstrated a relationship between HH and RAS/RAF/MEK/ERK signaling, especially in instances of cancer.

In pancreatic adenocarcinoma (PDAC), characterized by high incidences of activating *KRAS* mutations, it has been shown that HH signaling is important for both cancer initiation and progression (Morton et al., 2007). In a human PDAC cell line, HPDE-c7, $KRas^{V12}$ leads to an increase in GLI1 protein levels and transcriptional activity, and this increase is insensitive to SMO inhibition. However, when MEK1/2 is inhibited, GLI1 protein stability and HH pathway activation are decreased (Ji et al., 2007). In a mouse model of PDAC, oncogene $KRAS^{G12D}$ coordinates with a dominant active form of GLI2 which lacks the repressive N-terminal domain to initiate PDAC growth.

In human melanoma cells, oncogenes $NRAS^{Q61K}$ and $HRAS^{V12G}$ prevent SUFU's sequestration of GLI1 in the cytoplasm. This enhances GLI1 transcriptional activity and nuclear localization (Stecca et al., 2007). In gastric cancer, KRAS increases GLI transcriptional activity and ultimately tumor proliferation (Seto et al., 2009). Similarly, in colon cancer cells, the RAS/RAF pathway promotes GLI1 and GLI2 transcriptional activity (Mazumdar et al., 2011).

PKC Signaling

Protein kinase C is a family of serine/threonine kinases that consists of three classes: classical (isoforms α, βI, βII, and ɣ), calcium-independent (isoforms δ, ε, η, and θ), and atypical (isoforms ζ and ι/λ). PKC signaling regulates a variety of cellular responses including proliferation, differentiation, apoptosis, and the inflammatory response (Frey et al., 2000, Kim et al., PLos One 2013). As PKC is involved in a multitude of signaling cascades, it is unsurprising that PKCs have been shown to play a role in HH regulation.

Atwood et al. demonstrated that aPKC-ι/λ can phosphorylate and activate GLI1 in BCC, indicating that aPKC-ι/λ is capable of promoting BCC growth and progression by regulating the HH signaling pathway. Additionally, in a lung squamous cell carcinoma model, aPKC-ι/λ has been shown to directly phosphorylate and activate SOX2, a well-known stem-associated transcription

factor. Activation of SOX2 results in the production of HH ligand which then feeds into the HH signaling pathway (Justilien et al., 2014). This provides additional evidence in which aPKC-ι/λ can contribute to the HH pathway. Despite its importance, little is known about how aPKC-ι/λ activity is regulated in these contexts. Based on reports from other labs, potential activators may include CDC42, PARD6A, SRC, Ceramide, PDK1, mTOR, and PI3K. Previous *in vitro* and *in vivo* work show aPKC-ι/λ has a moderate level of basal activity that is repressed by Par6 (Atwood et al., 2007). Cdc42 interacts with Par6 and partially relieves this repression through unknown mechanisms. aPKC-ι/λ is initially phosphorylated co-translationally by mTOR on aPKC-ι/λ's turn motif, and then subsequently phosphorylated by PDK1 on aPKC-ι/λ's activation loop (Tobias et al., 2016). Both phosphorylations are required maturation steps for aPKC-ι/λ kinase activity, but there may be overlap with other kinases during aPKC-ι/λ maturation. SRC also phosphorylates the kinase domain of aPKC-ι/λ, and mutation of the SRC phosphosite significantly reduces aPKC-ι/λ activity in cell culture (Wooten et al., 2001). Ceramide has also been shown to regulate aPKC-ι/λ activity *in vitro* and aPKC-ι/λ localization in cell culture by binding aPKC-ι/λ's kinase domain, however, both activation and inhibition was observed to occur depending on the concentration of ceramide (Wang et al., 2009; 1999). Finally, PI3K has been shown to activate aPKC-ι/λ through generation of phosphoinositides that bind aPKC-ι/λ's pseudosubstrate and preventing autoinhibition (Ivey et al., 2014; Standaert et al., 1997).

The role of other PKC isoforms, however, is somewhat controversial. In HEK293T cells, constitutive activation of PKC-α has been shown to reduce GLI1 transcriptional activity, whereas overactivation of PKC-δ enhances it independently of MAPK signaling (Neill et al.,2003). Contrastingly, Cai et al. (2015) found that in hepatoma Hep3B cells, PKC-α upregulates GLI1 transcriptional activity via MEK1/2-ERK1/2 activation while both wild-type and constitutively active PKC-δ negatively regulates both *Gli1* and *PTCH1*. And lastly, Riobo et al. (2006b) showed that

PKC-δ and MEK-1 mediation of phorbol esters results in the overexpression of GLI1 in NIH3T3 cells.

TGF-β Signaling

The transforming growth factor β (TGF-β) signaling pathway is important for adult tissue homeostasis and embryo development (Pietrobono et al., 2019). However, its role in carcinogenesis can be complicated because of its ability to act as both a tumor suppressor or oncogene depending on the type and stage of cancer (David and Massague, 2018). Dennler et al. was the first group to demonstrate that TGF-β can regulate GLI transcriptional activity independent of SMO. They showed in human fibroblasts and keratinocytes, and in PDAC and breast cancer cells, that elevation of GLI1 and GLI2 expression is dependent on TGF-β via a SMAD3-dependent mechanism (Dennler et al., 2007). Their subsequent study showed that GLI2 is a direct target of TGF-β, resulting in substantial increase of GLI2 expression via SMAD3 and β-catenin cooperation on the GLI2 promoter (Dennler et al., 2009).

Several mouse models have highlighted the necessity of TGF-β signaling in HH-dependent cancers. In a SMO-driven BCC model, inhibition of TGF-β by TβRI antagonist SD208 dramatically reduced tumor load and promoted lymphatic infiltration, suggesting the importance of TGF-β. A similar phenomenon is seen in human BCCs that possess both HH and TGF-β hyperactivation (Fan et al., 2010). Additionally, in a pancreatic cancer model, both TGF-β and KRAS signaling appear to be necessary for SMO-independent GLI1 activation (Nolan-Stevaux et al., 2009).

GLI activation by TGF-β also plays a prominent role in epithelial to mesenchymal transition (EMT) and invasion in various tumors. In a metastatic breast cancer cell line, MDA-MB-231, TGF-β requires SMO-independent GLI2 induction in order to encourage the expression of PTHrP, a predominant factor in bone metastasis (Johnson et al., 2011). SMO does not appear to be detectable in these cells and thus SMO inhibition by cyclopamine has no effect on GLI2. However,

using the repressor form of GLI2 to inhibit HH signaling suppresses both endogenous and TGF-β-stimulated PTHrP, reducing bone metastasis in mice. This suggests that GLI2 works downstream of TGF-β to drive metastasis (Johnson et al., 2011). In melanoma, GLI2 levels are associated with mesenchymal features and phenotype both *in vitro* and *in vivo*. And therefore, GLI2 silencing has been shown to prevent bone metastasis and reduce invasion into the extracellular matrix (Alexaki et al., 2010).

The relationship between TGF-β and HH signaling has also been associated with cancer stemness and chemoresistance. In colorectal cancer, TGF-β secreted by cancer-associated fibroblasts and hypoxia-inducible factor (HIF-1α) coordinate to induce GLI2 expression independent of HH signaling (Tang et al., 2018). This TGF-β/HIF-1α/GLI2 signature has been associated with cancer stemness and chemoresistance in colorectal cancer patients, with patients commonly relapsing after treatment. Interestingly, inhibition of both GLI and TGF-β restores chemosensitivity and reduces the presence of cancer stem cells (Tang et al., 2018). In ovarian cancers that are enriched with cancer stem cells expressing high levels of GLI2 and elements of the TGF-β pathway, GLI2 inhibition sensitizes these tumors to cisplatin treatment and dramatically suppresses tumor growth (Steg et al., 2012). Lasty, in resistant BCCs, TGF-β cooperates with AP-1 to activate nuclear myocardin-related transcription factor (nMRTF) to amplify Gli1 activity non-canonically (Yao et al., 2020).

AMPK

5′ Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a serine/threonine kinase that plays a crucial role in cellular energy homeostasis. It is activated by elevated AMP/ATP ratios and responds to changes in nutrient supply and environmental conditions (Pietrobono et al., 2019). Li et al. (2015) demonstrated that activated AMPK decreases GLI1 transcriptional activity and protein stability via serine/threonine phosphorylation (Ser102, Ser408, Thr1074).

However, Di Magno et al. (2016) shows in human medulloblastoma that only phosphorylation at Ser408 by AMPK is necessary for GLI1 degradation and reduced HH-driven cell growth. Additionally, AMPK increases the cytoplasmic localization of GLI1, promoting its interaction with β-TRCP, an E3-ubiquitin ligase, leading to proteasomal degradation of GLI1 (Zhang et al., 2017).

AMPK can also metabolically reprogram adipocytes towards glycolysis, resulting in increased glucose uptake. This leads to HH pathway activation downstream of SMO (Teperino et al., 2012). Additionally, AMPK can mediate non-canonical HH signaling which results in polyamine metabolism, and targeting this phenomenon has been shown to inhibit HH-dependent proliferation of medulloblastoma *in vivo* and *in vitro* (D'Amico et al., 2015).

DYRK1 and 2

Dual-specificity tyrosine phosphorylation-regulated kinases (DYRKs) are a family of serine, threonine, and tyrosine kinases that play essential roles in cell proliferation and differentiation (Boni et al., 2020). The DYRK family consists of five members, but only DYRK1 and DYRK2 have been shown to be associated with the HH pathway. DYRK1A and DYRK1B play dual roles in GLI regulation. Overexpression of DYRK1A has been shown to promote GLI1 nuclear translocation (Mao et al., 2002; Shimokawa et al., 2008) via direct phosphorylation of GLI1 in the N-terminus (Schneider et al., 2015; Ehe et al., 2017). Conversely, Schneider et al. also demonstrated that DYRK1A can repress megakaryoblastic leukemia 1 (MLK1), a transcriptional coactivator, thereby inducing GLI1 degradation independent of upstream PTCH1/SMO signaling. The functionality of DYRK1A seems to be dependent on GLI1 expression levels and is cell-type specific. DYRK1B can also act as either an activator or repressor. It is capable of both inhibiting GLI2 function and promoting the formation of the GLI3 repressor form (Lauth et al., 2010). However, other reports have shown that DYRK1B can increase GLI1 activity and that its inhibition of its activity can repress GLI1 expression in both sensitive and SMO-inhibitor resistant cells (Gruber et al., 2016).

Another study in fibroblasts demonstrated that DYRK1B can promote AKT-mediated GLI1 stability (Singh et al., 2017). Unlike DYRK1A and DYRK1B, DYRK2 is mainly an inhibitor. It can phosphorylate GLI2 at conserved serine residues to promote its proteasomal degradation (Varjosalo et al., 2008).

Potential BCC Therapies

Although it is clear that inhibition of HH signaling is necessary for suppressing and inhibiting the growth of certain cancers, most efforts have been made in mainly targeting HH at the level of SMO. Such inhibitors include vismodegib (GCD-0449), sonidegib (NPV-LDE-225), glasdegib (PF-04449913), taladegib (LY2940680), BMS-833923, saridegib (IPI-926), and cyclopamine (Pietrobono and Stecca, 2018). Of these, vismodegib and sonidegib have been FDA approved to treat locally advanced and metastatic BCCs and have been quite successful in treating not only BCCs, but also medulloblastoma and other HH-dependent cancers (Rodon et al., 2014; Wagner et al., 2015; Stathis et al., 2017). However, the acquisition of resistance due to SMO mutations poses a challenge to these therapies. Therefore, it is important to develop therapies that target HH signaling either at the level of GLI or through non-canonical activators of the HH pathway.

In recent years, there have been attempts at developing GLI inhibitors in order to bypass the dilemma of SMO mutations. GANT58, GANT61 (Lauth et al., 2007), and Glabrescione B (Infante et al., 2015) have been shown to interfere with GLI's ability to bind DNA, whereas HPI1-4 (Hyman et al., 2009) and ATO (Kim et al., 2010; Beauchamp et al., 2011) have been shown to regulate GLI processing and activation. However, all of these drugs, with the exception of ATO which showed to be cytotoxic *in vitro* and is not specific for GLI (Yedjou & Tchounwou, 2007), have proven to be unfit clinical candidates. Therefore, since directly targeting GLI does not seem

plausible at the moment, the most ideal method for disrupting GLI-dependent cancers may be to target non-canonical activators of GLI in addition to using SMO inhibitors.

Since the PI3K-AKT-mTOR pathway has been shown to be involved in enhancing GLI1 stability, nuclear localization, and transcriptional activity, it poses as a promising therapeutic target for HH-dependent cancers. In pancreatic cancer stem cells, combined treatment with SMO inhibitor sonidegib and PI3K/mTOR inhibitor NVP-BKM120 resulted in reduced survivability and tumorigenicity (Sharma et al., 2015). Similarly, a combination of GLI inhibitor GANT61 and mTOR inhibitor rapamycin resulted in an additive effect in decreasing pancreatic cancer cell viability and cancer stem cell regeneration. This combination also reduced in vivo growth of pancreatic cancer xenografts (Miyazaki et al., 2016). In esophageal adenocarcinoma cell lines and mouse xenografts, combination of SMO inhibitor vismodegib and mTOR inhibitor everolimus resulted in a dramatic decrease in HH signaling, suggesting that PI3K/mTOR could be a major component of the development of SMO-inhibitor resistance in esophageal cancers (Wang et al., 2012). When comparing expression profiles of sonidegib- sensitive and -resistant medulloblastomas, a number of PI3K target genes were enriched only in the resistant samples. This also suggests that PI3K activation may contribute to the development of resistance. In fact, dual treatment with NVP-BKM120 and sonidegib delayed the onset of resistance and tumor regrowth (Buonamici et al., 2010). This same treatment in glioblastomas induces mitotic catastrophe and apoptosis (Gruber-Filbin et al., 2013). Additionally, PI3K inhibitor GDC-0941 has been shown to result in significant growth inhibition in vismodegib-resistant tumors (Dijgraaf et al., 2011). Taken together, SMO inhibition combined with PI3K/mTOR inhibitors may prove promising in preventing the onset of resistance in HH-dependent tumors.

The RAS-RAF-MEK pathway has been demonstrated to provide compensatory GLI activation independent of SMO, making it a favorable target for HH-dependent cancer treatment.

Several studies have illustrated a synergistic effect between SMO and MEK inhibitors. In prostate cancer characterized by hyperactivation of MAPK signaling, dual-administration of SMO inhibitor SANT-1 and MEK1 inhibitor PD325901 reduced cancer cell growth more dramatically than with a single treatment (Gioeli et al., 2011). Similarly, in cholangiocarcinioma cells, treatment with both SMO inhibitor cyclopamine and MEK inhibitor U0126 displayed heightened reduction in cell proliferation and survival (Jinawath et al., 2007). In medulloblastomas, overactivation of the MAPK signaling pathway has been associated with the acquisition of vismodegib resistance. It does so by promoting a small subpopulation of SMO-inhibitor resistant cells that are driven by the RAS-RAF-MEK pathway for proliferation (Zhao et al., 2015). Similarly, inhibition of MEK1/2 in resistant PDAC cells displayed reduced GLI activation (Ji et al., 2007).

Although studies on PKCs have provided some conflicting evidence of HH regulation, aPKC-ι/λ has been demonstrated to be a promising target for HH-dependent and drug resistant cancers. Treatment of allografted BCC tumors and resistant BCC cells with aPKC inhibitor PSI resulted in reduced HH signaling and tumor cell proliferation (Atwood et al., 2013). Additionally, Mirza et al. (2017) showed that combined inhibition of aPKC-ι/λ with PSI and histone deacetylase (HDAC) with vorinostat demonstrated cooperative inhibition of BCC growth.

Since the TGF-β pathway has been shown to activate GLI independently of SMO, perhaps combined treatment targeting both SMO and TGF-β could help eradicate HH-dependent tumors. Dennler et al. (2007) demonstrated that inhibition of TGF-β can reduce GLI2 expression in SMO-inhibitor resistant PDAC cells which express high levels of GLI, resulting in repression of cancer cell growth. Additionally, combination treatment with TGF-β inhibitor SD208 and GLI inhibitor GANT61 has the ability to reverse drug resistance in colorectal cancer and prevent cancer stem cell regrowth after chemotherapy treatment (Tang et al., 2018).

Other more nonconventional modes of inhibiting HH signaling involve targeting cytoskeletal remodeling. Whitson et al. (2018) demonstrate that inhibition of MLK1 blocks RhoA-dependent serum growth factor (SRF)/MLK1-driven upregulation of GLI1, a pathway known to promote drug resistance in BCCs.

Summary

The aim of this dissertation is to identify alternative regulators of HH signaling in BCC in order to shed light on other potential therapies for sensitive and resistant BCCs. Our lab previously reported that aPKC can directly phosphorylate and activate HH signaling at the level of GLI1 (Atwood et al., 2013), so this project initially started out as a screen to determine if targeting potential aPKC activators could ultimately reduce BCC growth and HH signaling. Potential activators included CDC42, PARD6A, SRC, Ceramide, PDK1, mTOR, and PI3K. Our initial screen only looked at whether or not inhibiting these potential targets affect HH signaling and/or BCC growth. The most promising targets are discussed in Chapters 2-4. In Chapter 2, we demonstrate that inhibition of mTOR reduces BCC growth but not HH signaling. However, aPKC activity does appear to be attenuated, suggesting that mTOR may be affecting BCC growth potentially through another aPKC target independent of GLI1. In Chapter 3, similar to Chapter 2, we show that PI3K inhibition decreases BCC growth but not HH signaling *in vivo*. However, two PI3K downstream targets, aPKC and AKT, showed a reduction in activity whereas their substrate, p21, displayed an increase in protein stability. This suggests that PI3K promotes BCC growth via p21 degradation. Lastly, in Chapter 4, we provide evidence that SRC affects both HH signaling and BCC growth via an aPKC mechanism. Overexpression of SRC alone increases HH signaling whereas an inactive form of SRC reduces HH to the levels of control. Additionally, when SRC phosphorylation sites on aPKC are mutated, HH signaling is significantly reduced. This suggests that SRC can promote BCC

growth possibly through direct phosphorylation and activation of aPKC. Taken together, we've been able to provide evidence of BCC reduction via inhibition of mTOR, PI3K and SRC. Targeting these other proteins and pathways may provide promising therapies for sensitive and advanced BCCs.

CHAPTER 2

mTOR promotes basal cell carcinoma growth through atypical PKC

CONTRIBUTIONS

This project was conceived by Scott Atwood and Rachel Chow. Scott Atwood supervised the research. Rachel Chow performed the experiments. Taylor Levee, Gurleen Kaur, and Daniel Cedeno quantified and immunostained the tumor data. Linda Doan collected and annotated the human clinical samples. Scott Atwood and Rachel Chow wrote the manuscript. All authors analyzed and discussed the results and commented on the manuscript. The work described in this chapter was published in *Experimental Dermatology* (Chow et al., 2021).

SUMMARY

Advanced basal cell carcinomas (BCCs) are driven by the Hedgehog (HH) pathway and often possess inherent resistance to SMO inhibitors. Identifying and targeting pathways that bypass SMO could provide alternative treatments for patients with advanced or metastatic BCC. Here, we use a combination of RNA-sequencing analysis of advanced human BCC tumor-normal pairs and immunostaining of human and mouse BCC samples to identify an mTOR expression signature in BCC. Pharmacological inhibition of mTOR activity in BCC cells significantly reduces cell proliferation without affecting HH signaling. Similarly, treatment of the *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mouse BCC tumor model with everolimus reduces tumor growth. aPKC, a downstream target of mTOR, shows reduced activity, suggesting that mTOR promotes tumor growth by activating aPKC and demonstrating that suppressing mTOR could be a promising target for BCC patients.

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 (Varjosalo et al., 2008). Smoothened (SMO) inhibitors such as vismodegib are commonly used to suppress tumor growth in advanced cases where tumors are surgically non-resectable. 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 & Oro, 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).

Normally, HH ligand binds to the cholesterol transporter Patched1 (PTCH1), derepressing the G-protein coupled receptor SMO and allowing for activation of the GLI transcription factors to translocate into the nucleus and activate target genes involved in proliferation, migration, and invasion (Varjosalo et al., 2008). Most patients who develop BCCs possess inactivating *PTCH1* (~70%) or activating *SMO* (~20%) mutations which drive uncontrolled HH signaling (Bonilla et al., 2016), making SMO a natural target to treat a majority of cases. Resistance to SMO inhibitors primarily occurs through secondary mutations in SMO that either prevent drug binding or result in constitutive activation even when drug is bound (Atwood et al., 2015; Sharpe et al., 2015). Recent work on circumventing SMO inhibition has concentrated on shutting down GLI activation, where preclinical targeting of aPKC (Atwood et al., 2015; Atwood et al., 2013), HDAC1 (Mirza et

al., 2017; Gruber et al., 2017), MKL1 (Whitson et al., 2018), and MEKK2/3 (Lu et al., 2018) have all shown some efficacy.

Targeting other GLI responsive signaling pathways is an area of growing interest, however, untangling their myriad interactions to define their mechanism of action is complex. For instance, loss of primary cilia in advanced BCCs can, in some cases, shut down HH signaling and concomitantly increase MAPK pathway activation, resulting in a switch from BCC to squamous cell carcinoma (Kuonen et al., 2019). This mutual antagonism between RAS/MAPK and HH signaling can drive SMO inhibitor resistance and MAPK inhibitors can suppress tumor cell growth when the RAS/MAPK pathway is dominant (Zhao et al., 2015). mTOR is another major oncogenic player that has been associated with uncontrolled proliferation, resistance of cell death, evasion of immune destruction, and dysregulated cell metabolism (Kim et al., 2017). Whether mTOR acts upstream, alongside, or downstream of the HH pathway in BCC and by what mechanism is complicated by disparate results in other cancers. In esophageal adenocarcinoma, mTOR functions through S6K1 to phosphorylate GLI1 and promote its transcriptional activity (Wang et al., 2012). However, studies in neuroblastoma demonstrate that inhibition of the mTOR/S6K1 pathway suppresses cancer growth but does not affect GLI1 expression (Diao et al., 2014). Additionally, in pancreatic and ovarian cancer cells, HH signaling has been shown to induce DYRK1B expression, which leads to activation of the mTOR/AKT pathway (Singh et al., 2017).

Here, we provide evidence that an mTOR signature is significantly enriched in both human and mouse BCCs. We demonstrate that *in vitro* and *in vivo* inhibition of mTor results in significant reduction in BCC growth independent of aPKC-mediated activation of HH signaling. Our results suggest that mTOR operates downstream of GLI1 and may be a viable target to treat advanced BCC patients.

RESULTS

mTOR pathway expression is significantly enriched in advanced human BCCs

To identify alternative pathways that drive BCC growth, we reanalyzed our bulk-level RNA sequencing data from 14 tumor-normal pairs of advanced BCC patients whose tumors were surgically non-resectable (Atwood et al., 2015). 1602 genes were upregulated by two-fold or more when differential gene expression was averaged across all 14 samples (Figure 2.A). KEGG analysis of upregulated genes indicated the expected cancer-related terms such as cell cycle, genes involved in BCC, and the HH signaling pathway (Figure 2.1B). Interestingly, the mTOR pathway showed significant enrichment along with the related PI3K-AKT and HIF-1 pathways (Figure 2.1B). mTOR-related pathways were even more prominent with Kinase Enrichment Analysis (Rouillard et al., 2016) of the upregulated genes where MAPK, AKT, GSK3B, PLK1 and S6K kinase terms showing significant enrichment, along with the expected CDKs (Figure 2.1C). Three of the tumors (7, 8, 13) showed similar strong differential gene expression compared with the rest of the cohort. When analyzing the 1412 genes that were commonly upregulated by two-fold or more in these three tumors and the 429 commonly upregulated genes from the rest of the samples, the mTOR pathway remained significantly enriched in both datasets, suggesting that promotion of the mTOR pathway is a common event in BCC. When we analyzed gene expression of the mTOR pathway, mTORC1 complex components and downstream targets showed significant upregulation in most tumors such as RPTOR, RPS6KA1, and EIF4EBP1, whereas mTOR gene expression itself was significantly increased in only a subset of tumors (Figure 2.1D).

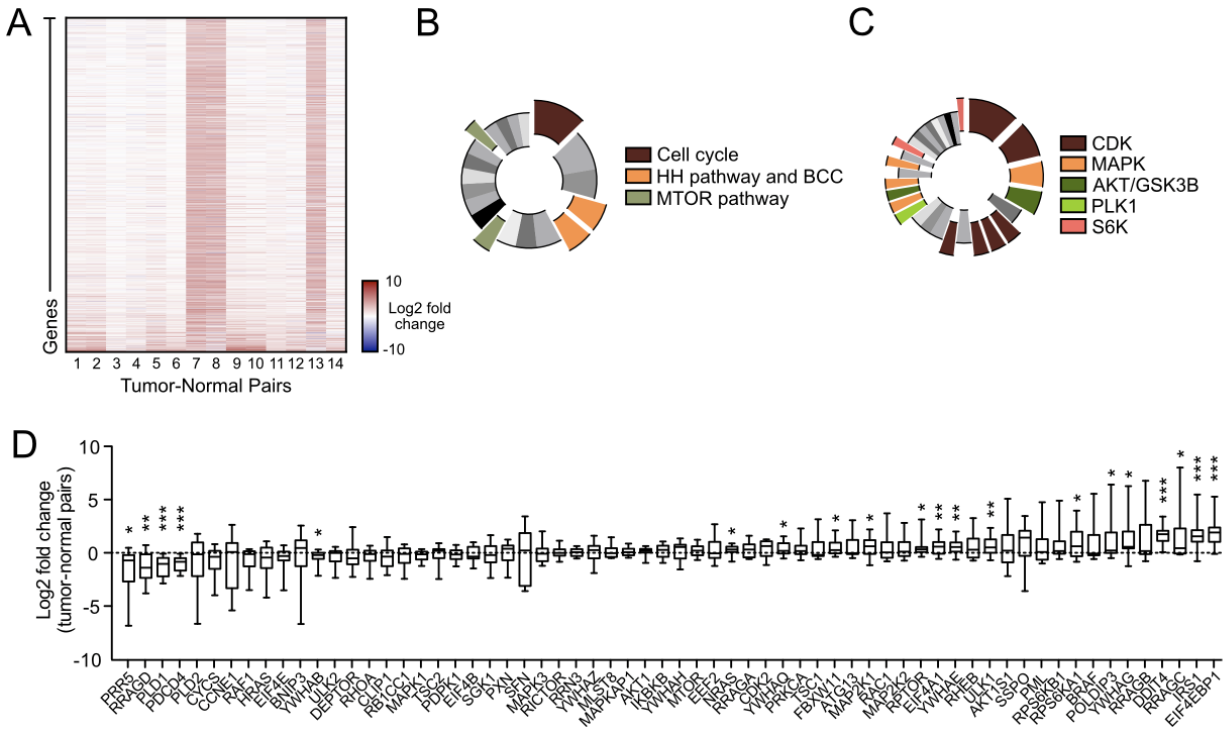
A
Genes
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Tumor-Normal Pairs
10
Log2 fold change
-10
B
Cell cycle
HH pathway and BCC
MTOR pathway
C
CDK
MAPK
AKT/GSK3B
PLK1
S6K
D
Log2 fold change (tumor-normal pairs)
10
5
0
-5
-10
PRR5
RRAGD
PDCL
PDCL4
PTD2
CYCS
CCNE1
RALA
HRAS
EIF4E
ENPP3
YWHAB
ULK2
DEPTOR
RHOA
CTTN
RB1CC1
MAPK1
TSC2
PDPK1
EIF4B
SGK1
PXN
SFN
MAPK3
RICTOR
RRN3
YWHAZ
MLST8
MAPKAP1
AKT1
IKBKB
YWHAH
MTOR
EIF4E2
ENPA5
RRAGA
CDK2
YWHAQ
PRKCA
TSC1
FBXW11
ATG13
MAPK1
RAC1
MAPK3
RPTOR
EIF4A1
YWHAE
RHEB
ULK1
AKT1S1
SSPO
PML
RPS6KB1
RPS6KA1
BRAF
PDCD4
YWHAG
RRAGB
DDIT4
RRAGC
IRS1
EIF4EBP1

Figure 2.1. The mTOR pathway is differentially expressed in advanced human BCCs. **A)** Heat map of differentially expressed genes upregulated by 2-fold or more in advanced human BCCs compared to patient-matched normal skin. **B)** KEGG analysis of differentially expressed genes showing significant terms as indicated. Cell cycle, $p = 3.10 \times 10^{-8}$; BCC, $p = 1.03 \times 10^{-4}$; HH signaling pathway, $p = 2.49 \times 10^{-4}$; mTOR signaling pathway, p = 0.00228; PI3K-AKT signaling pathway, p = 0.00675; HIF-1 signaling pathway, p = 0.0132. **C)** Kinase Enrichment Analysis of differentially expressed genes showing significant kinases as indicated. In descending significance to color codes: CDK2, $p = 4.80 \times 10^{-12}$; CDK1, $p = 1.13 \times 10^{-8}$; MAPK14, $p = 2.59 \times 10^{-6}$; GSK3B, $p = 5.42 \times 10^{-6}$; CDK15, $p = 3.88 \times 10^{-4}$; CDK14, $p = 4.39 \times 10^{-4}$; CDK18, $p = 4.94 \times 10^{-4}$; CDK11A, $p = 6.23 \times 10^{-4}$; PLK1, p = 0.00296; MAPK1, p = 0.00460; AKT1, p = 0.00534; MAP3K10, p = 0.00641; MAPK9, p = 0.00828; RPS6KA5, p = 0.0123; RPS6KA1, p = 0.0332. **D)** Box and whisker plots of differentially expressed mTOR pathway components in advanced human BCCs compared to patient-matched normal skin. Box represents 25^{th} to 75^{th} percentiles. Whiskers represent minimum and maximum data points. Bar represents mean. Significance was determined by unpaired two-tailed *t* test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

mTOR is upregulated in human and mouse BCCs

To validate mTOR expression at the protein level in BCC tumors, we immunostained both human and mouse BCC tumor samples and compared them to normal epidermis. Human BCC tumors showed significant enrichment of mTOR immunostaining in the cytoplasm of nodular human BCC tumor cells compared to normal epidermis (Figure 2.2A-B). However, individual tumor immunostaining showed a large variation in protein expression with some tumors not showing enrichment compared to normal epidermis, a similar pattern to the RNA-seq data analysis (Figure 2.1D). To analyze mTor expression in mice, we utilized a *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mouse model in which BCC tumors predominantly arise from *Gli1*-positive regions within the hair follicle bulge and secondary hair germ (Peterson et al., 2015). BCC tumors were allowed to grow for five weeks post-tamoxifen treatment and formed predominantly from the hair follicle regions. mTor immunostaining showed significantly increased expression in the cytoplasm of tumor cells compared to either the normal epithelium or normal hair follicle (Figure 2.2C-D). A similar variation in staining was observed in both mouse and human tumors. Collectively, these results indicate that the mTOR pathway is overexpressed in a subset of both human and mouse BCCs.

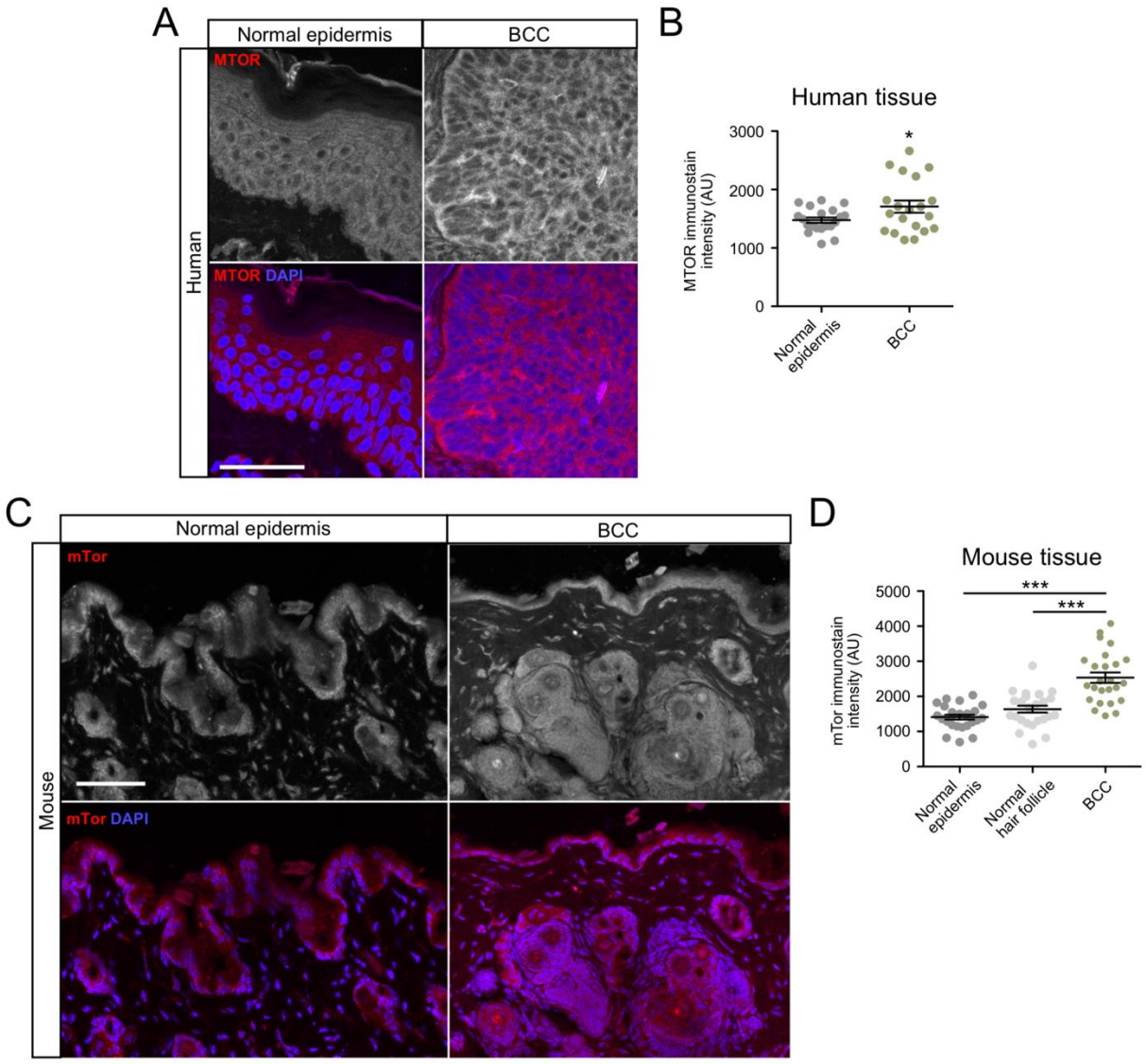

Figure 2.2. mTOR is overexpressed in human and mouse BCC. A) Immunofluorescent staining of indicated markers in human normal epidermis and nodular BCC. Scale bar, 50 μm. **B)** Quantification of mTOR immunostain intensity (n = 5 different points of measurement from 4 individual samples). AU, arbitrary units. **C)** Immunofluorescent staining of indicated markers in normal epidermis, normal hair follicle, or BCC derived from *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mice. Scale bar, 50 μm. **D)** Quantification of mTor immunostain (n = 5 different points of measurement from 5 mice). Error bars represent SEM. Significance was determined by unpaired two-tailed *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

mTor inhibition suppresses murine BCC cell growth but not HH signaling

We next wanted to assay whether mTor inhibition suppresses HH signaling and tumor cell growth. We treated ASZ001 mouse BCC cells with three different mTor inhibitors that are in various stages of clinical use: everolimus, rapamycin, and OSI-027. Everolimus and rapamycin act as allosteric inhibitors, while OSI-027 acts as a competitive ATP inhibitor (Benjamin et al., 2011; Leung et al., 2015; Rehan et al., 2017). Rapamycin and OSI-027 treatments did not result in significant changes in HH signaling as assayed by *Gli1* mRNA expression, whereas everolimus treatment resulted in a slight but not significant increase in *Gli1* expression (Figure 2.3A). As mTor expression has previously been shown to be HH-dependent in ASZ001 cells (Kim et al., 2018), our results reinforce the idea that mTor does not operate upstream of the HH pathway in BCC. Despite not significantly influencing HH signaling, treatment with all three mTor inhibitors resulted in a decrease in BCC cell growth over time (Figure 2.3B-D). A reduction in BCC cells undergoing everolimus and rapamycin treatment can be seen as early as two days after initial drug exposure, whereas OSI-027 treatment required at least four days to see a significant effect on tumor cell growth compared to DMSO vehicle control. An increase in cleaved Casp3 and decrease in Mki67 immunostaining was observed upon everolimus treatment (Figure 2.4), suggesting that mTor inhibition promotes apoptosis and a decrease in proliferation of tumor cells. Together, these data demonstrate that mTor inhibition can suppress BCC cell growth without altering *Gli1* expression, suggesting that mTor operates downstream of, or in parallel to, the HH pathway.

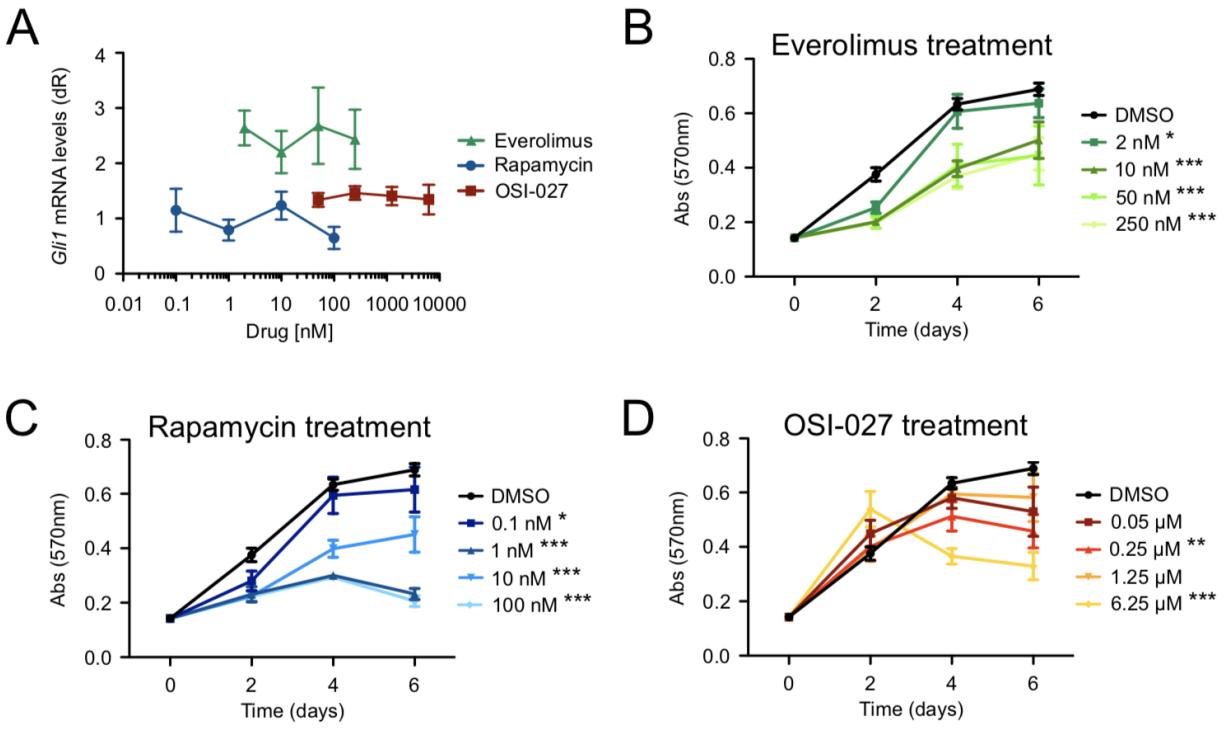

Figure 2.3. mTor inhibition suppresses BCC cell growth but not HH signaling. A) Gli1 mRNA levels of ASZ001 cells treated with DMSO or varying concentrations of Rapamycin, OSI-027, or Everolimus (n = 3 experiments). dR, delta reporter signal normalized to passive reference dye. **B-D)** MTT assay of ASZ001 cells treated with **B)** Everolimus**, C)** Rapamycin, or **D)** OSI-027 (n = 3 experiments). Abs, absorbance. Error bars represent SEM. Significance was determined by two-way ANOVA test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

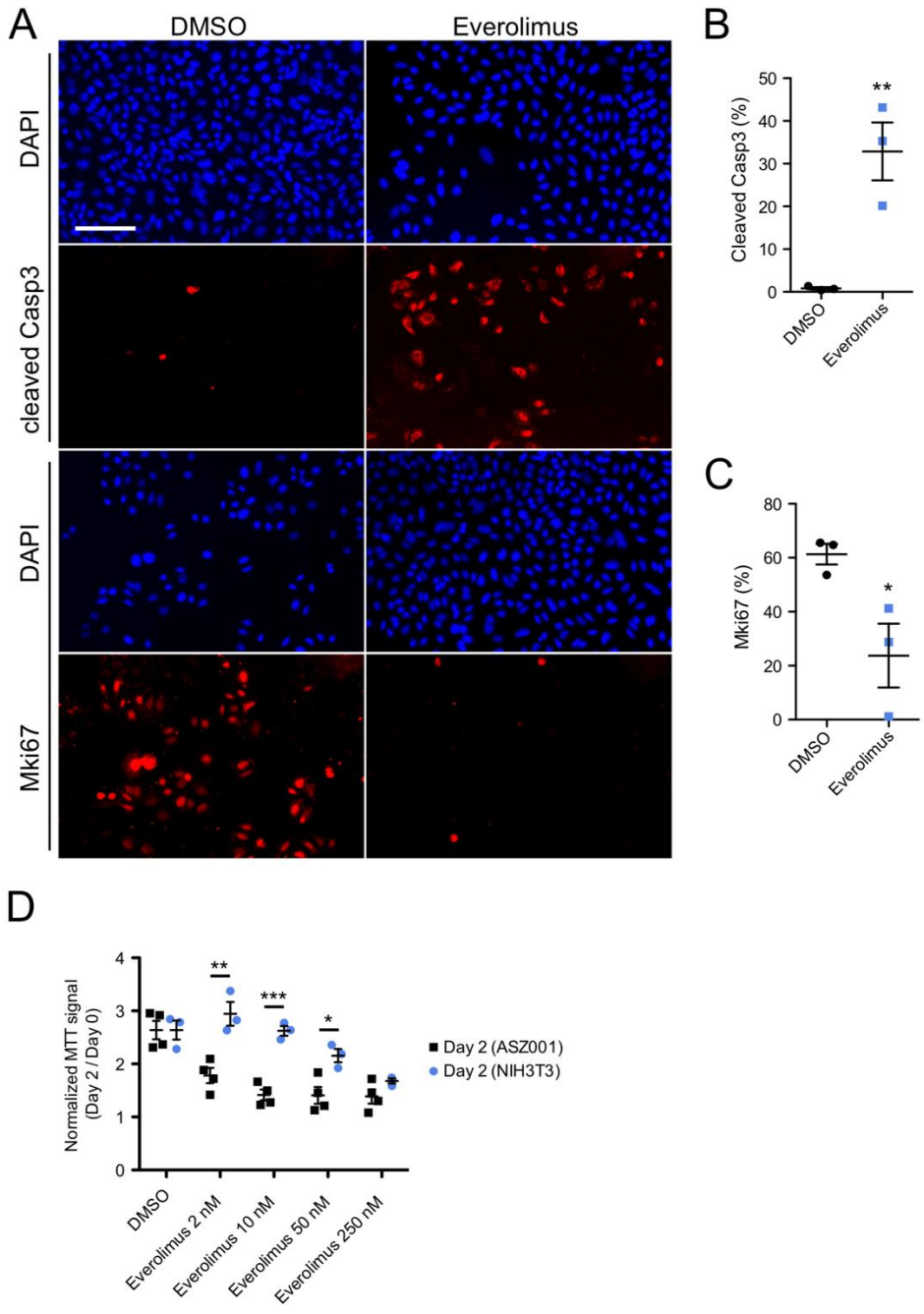
A
DMSO
Everolimus
DAPI
cleaved Casp3
DAPI
Mki67
B
Cleaved Casp3 (%)
50
40
30
20
10
0
**
DMSO
Everolimus
C
Mki67 (%)
80
60
40
20
0
*
DMSO
Everolimus
D
Normalized MTT signal
(Day 2 / Day 0)
4
3
2
1
0
**
***
*
Day 2 (ASZ001)
Day 2 (NIH3T3)
DMSO
Everolimus 2 nM
Everolimus 10 nM
Everolimus 50 nM
Everolimus 250 nM

Figure 2.4. mTor inhibition promotes apoptosis and reduces cell proliferation. A) Immunofluorescence staining of ASZ001 cells treated with DMSO or Everolimus and immunostained for the indicated markers. Scale bar, 100 μm. **B)** Quantification of cleaved Casp3 signal. n = 3 experiments. **C)** Quantification of Mki67 signal. n = 3 experiments. **D)** MTT assay of ASZ001 (n = 4 experiments) or NIH3T3 (n = 3 experiments) cells treated with DMSO or Everolimus. Error bars represent SEM. Significance was determined by unpaired two-tailed *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

mTor functions independently of aPKC to suppress murine BCC tumors

To explore whether mTor inhibition can be used as an effective BCC therapeutic, we grew BCC tumors in *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mice for 5 weeks after tamoxifen injection and intraperitoneally injected either DMSO or 3 mg/kg everolimus once a day for seven days. We used everolimus despite a slight increase in *Gli1* expression in ASZ001 mouse BCC cells because it is FDA approved, has been shown to be effective in treating certain cancers and other diseases (MacKaeigan et al., 2015; Klawitter et al., 2015; Yang et al., 2017; Viana et al., 2018), and showed a therapeutic window where BCC cells were adversely affected compared to normal NIH3T3 fibroblasts (Figure 2.4). Histological staining of the dorsal skin of everolimus-treated mice showed a significant reduction in total tumor area compared to DMSO controls (Figure 2.5A-B). Gli1 immunostains showed a downward trend in expression that is not significant (Figure 2.5C-D, Figure 2.6), corresponding to the quantitative PCR data from mouse BCC cells and reinforcing that mTor does not function upstream of the HH pathway.

To further delineate mTor's mode of action, we assayed that status of aPKC, a Gli1 kinase that is necessary for high sustained Gli1 activity (Atwood et al., 2013). mTor has been shown to phosphorylate and activate aPKC at residue T560 in mouse embryonic fibroblasts (Lin & Gao, 2014), while aPKC phosphorylates and activates Gli1 at residue T304 (Atwood et al., 2013; Drummond et al., 2018). We observe a slight increase in p-T304 Gli1 expression, along with an increase in total aPKC immunostaining in everolimus-treated mouse BCC tumors and in everolimus-treated mouse BCC cells (Figure 2.5C, E-F, Figure 2.6). However, p-T560 aPKC immunostaining is reduced, along with a concomitant reduction in p-T410 aPKC, an activation site that is thought to be phosphorylated by Pdpk1 (Tobias et al., 2015) (Figure 2.5C, G-H, Figure 2.6). Despite a significant reduction in the ratio of phosphorylated aPKC to total aPKC (Figure 2.5I), total aPKC is elevated in everoliumus-treated tumors, which may limit the contribution of

aPKC in this context. Overall, our data suggests that mTor is likely promoting tumor growth independent of Gli1 and potentially through another aPKC target.

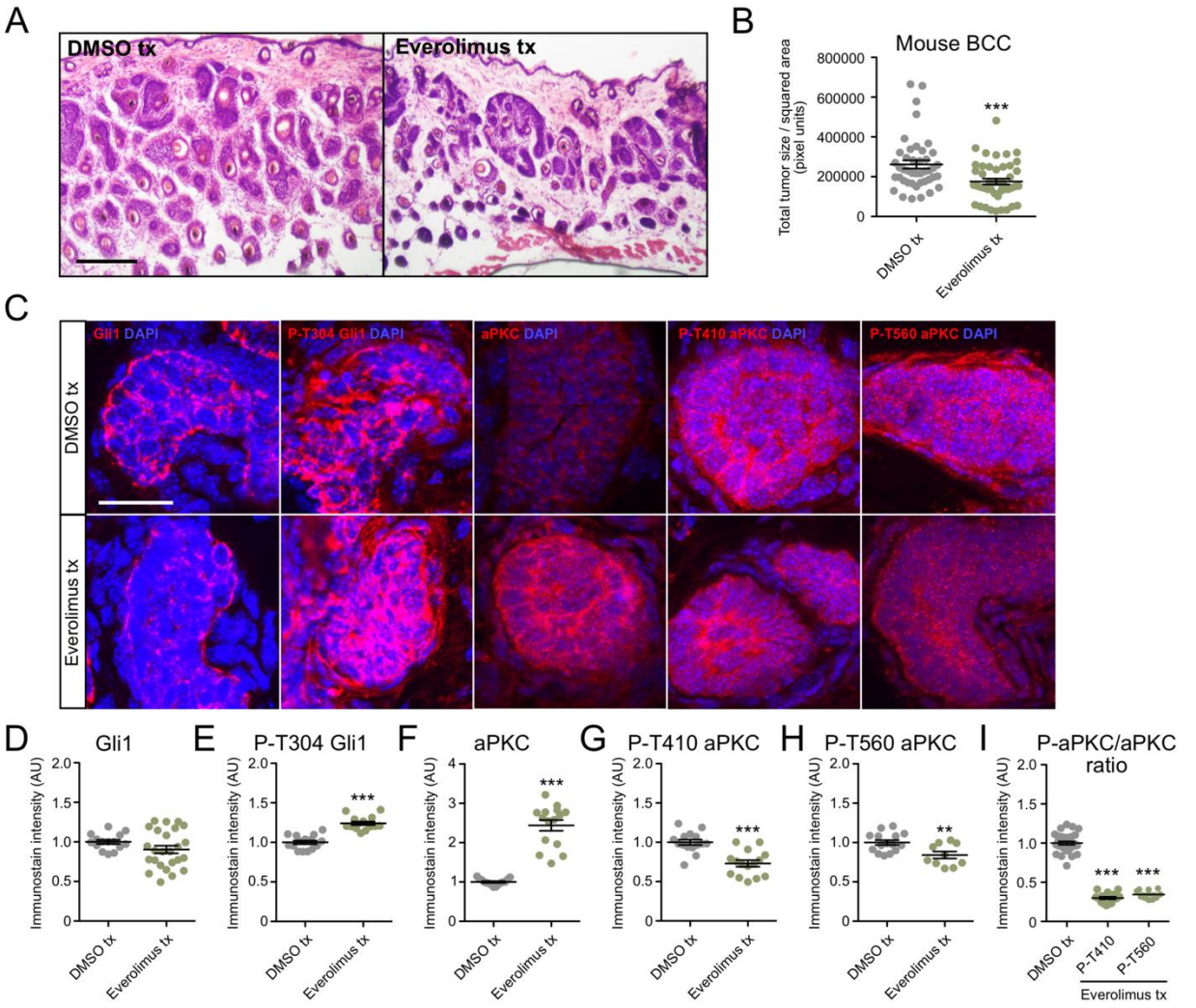

Figure 2.5. mTor inhibition suppresses murine BCC growth and aPKC activity. A) Hematoxylin and eosin staining of dorsal back skin collected from DMSO- or Everolimus-treated *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mice. Scale bar, 50 μm. **B)** Quantification of total tumor size per square area (n > 250 tumors from 5 mice). tx, treatment. **C)** Immunofluorescent staining of DMSO- or Everolimus-treated *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mice for the indicated markers. Scale bar, 25 μm. D-I) Quantification of immunostains (n = 5 tumors from 3 mice) for **D)** Gli1, **E)** phosphorylated T304 Gli1, **F)** aPKC, **G)** phosphorylated T410 aPKC, **H)** phosphorylated T560 aPKC, and **I)** the ratio of phosphorylated aPKC over total aPKC protein. AU, arbitrary units. Error bars represent SEM. Significance was determined by unpaired two-tailed *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

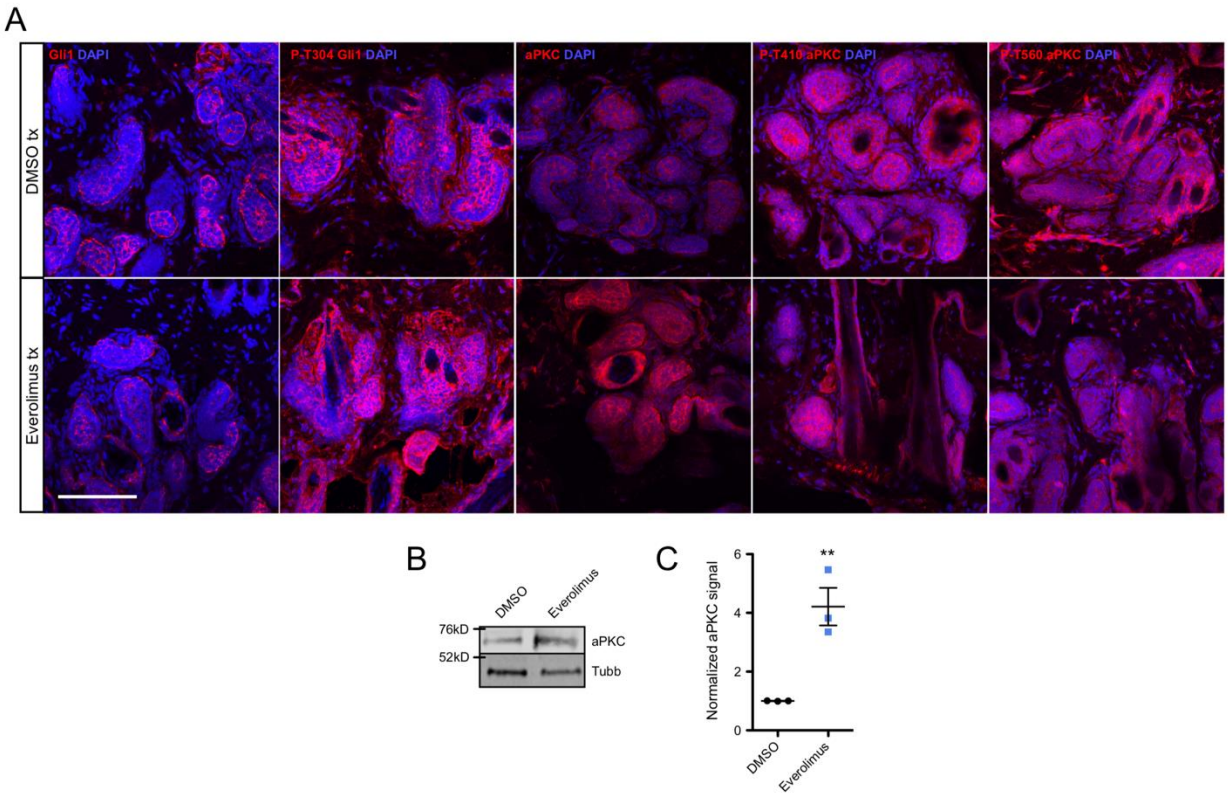

Figure 2.6. mTor inhibition suppresses aPKC activity. A) Larger immunofluorescent images of DMSO- or Everolimus-treated *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mice for the indicated markers. Scale bar, 100 μm. **B)** Western blot and **C)** quantification of DMSO- or Everolimus-treated mouse BCC cells (n = 3 experiments). Error bars represent SEM. Significance was determined by unpaired two-tailed *t* test. **, $p < 0.01$.

DISCUSSION

How mTOR functions in BCC and interacts with the HH pathway is unclear given reports that it can operate upstream, downstream, or parallel to the pathway in various cancers. For instance, in glioblastoma multiforme, mTOR inactivates GSK3b to prevent GLI2 ubiquitination, thereby promoting GLI2 stability and nuclear translocation (Miati et al., 2017). This is likely not the case in BCC as we show mTOR inhibition does not significantly alter GLI1 expression. Our findings are more consistent with models where mTOR acts downstream of the HH pathway, such as in *Ptch1*$^{+/-}$/*SKH-1* BCCs where HH signaling promotes Sox9 expression to enhance mTor activity and tumor growth (Kim et al., 2018). In fact, *SOX9* is significantly enriched in our bulk-level RNA sequencing data of advanced BCC patients (Supplementary Table 1).

Our data and others (Kim et al., 2018) suggest that mTOR acts downstream of the HH pathway to promote tumor growth, but mTOR's mechanism of action is less clear. mTOR may converge on Cyclin D1 (CCND1) to directly promote BCC cell growth, which is also a target of the HH pathway (Oliver et al., 2003), as mTOR inhibition disrupts CCND1/CDK2 complexes (Law et al., 2006). Another possibility is that mTOR affects BCC growth via AKT1, an mTOR target that functions downstream of HH signaling in BCCs (Sarbassov et al., 2005; Kim et al., 2016). mTOR phosphorylates AKT1 at S473 (Law et al., 2006), and ASZ001 mouse BCC cells treated with intraconazole, a SMO inhibitor, reduces p-S473 AKT1 expression. Our data suggests mTOR phosphorylates and activates aPKC in BCC, but does not alter GLI1 phosphorylation, suggesting that another aPKC target may be responsible for continued tumor growth (Reina-Campos et al., 2019).

Cancer is heterogeneous and BCCs are no exception (Bonilla et al., 2016; Atwood et al., 2015; Sharpe et al., 2015). Our bioinformatics analysis and subsequent immunostaining suggests not all tumors possess an mTOR profile, where a subset of tumors show strong upregulation while

others display a more modest mTOR signature, reinforcing the wide range of mTOR expression seen in BCC patients (Kim et al., 2018). This is not surprising as other signaling pathways are known to regulate BCC in conjunction with HH signaling, such as the WNT (Yang et al., 2008), NOTCH (Eberl et al., 2018; Shi et al., 2017), and Hippo pathways (Debaugnies et al., 2018), and may make upregulation of the mTOR pathway dispensable in some tumors. As such, combination therapy may be an important step going forward to therapeutically treat advanced BCC patients. For SMO antagonist-resistant patients, assaying mTOR pathway expression levels may serve as a biomarker for the efficacy of mTOR inhibitor therapy. Alternatively, mTOR inhibitors may be used in conjunction with SMO antagonists as a way to prevent drug resistance, a phenomenon seen in a HH-induced medulloblastoma mouse model where combination therapy with the SMO antagonist LDE225 and PI3K inhibitor BKM120 delayed the development of drug resistance (Buonamici et al., 2010).

CHAPTER 3

PI3K promotes basal cell carcinoma growth through kinase-induced p21 degradation

CONTRIBUTIONS

This project was conceived by Scott Atwood and Rachel Chow. Scott Atwood supervised the research. Rachel Chow performed the experiments. Taylor Levee, Gurleen Kaur, and Daniel Cedeno quantified and immunostained the tumor data. Linda Doan collected and annotated the human clinical samples. Scott Atwood, Ung Seop Jeon, and Rachel Chow wrote the manuscript. All authors analyzed and discussed the results and commented on the manuscript. The work described in this chapter has been submitted to *Frontiers in Oncology* (Chow et al., 2021).

SUMMARY

Basal cell carcinoma (BCC) is a locally invasive epithelial cancer that is primarily driven by the Hedgehog (HH) pathway. Advanced BCCs is a critical subset of BCCs that frequently acquires resistance to Smoothened (SMO) inhibitors and identifying pathways that bypass SMO could provide alternative treatments for patients with advanced or metastatic BCC. Here, we use a combination of RNA-sequencing analysis of advanced human BCC tumor-normal pairs and immunostaining of human and mouse BCC samples to identify a PI3K pathway expression signature in BCC. Pharmacological inhibition of PI3K activity in BCC cells significantly reduces cell proliferation and HH signaling. However, treatment of *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mouse BCCs with the PI3K inhibitor BKM120 results in a reduction of tumor cell growth with no significant effect on HH signaling. Downstream PI3K components aPKC and AKT1 showed a reduction in active protein, whereas their substrate, cyclin-dependent kinase inhibitor p21, showed a concomitant increase in protein stability. Our results suggest that PI3K promotes BCC tumor growth by kinase-induced p21 degradation without altering HH signaling.

INTRODUCTION

The Hedgehog (HH) pathway is an evolutionarily conserved signaling pathway that plays an essential role in vertebrate embryogenesis and adult tissue homeostasis (Armas-Lopez et al., 2017). Aberrant activation of the HH pathway results in uncontrolled proliferation and differentiation that leads to tumorigenesis in various tissues, with medulloblastoma (Taylor et al., 2002; Raffel et al., 1997), rhabdomyosarcoma (Tostar et al., 2006), and basal cell carcinoma (BCC) (Reifenberger et al., 2005) commonly displaying mutations in HH pathway components. BCC is a locally invasive epithelial cancer that represents the most prevalent cancer in the United States, with more than four million cases estimated each year (Nguyen et al., 2019). While most cases of BCC are characterized by low mortality and metastasis that can be easily excised (Kauvar et al., 2015), advanced BCCs display elevated invasiveness, metastasis, and mortality (Lear et al., 2014). As the initiation and progression of BCCs predominantly depend on deregulation of the canonical HH pathway via activation of the seven-pass transmembrane protein Smoothened (SMO) (Pietrobono et al., 2019), such dependency has led to the development of vismodegib and other SMO inhibitors for the treatment of locally advanced and metastatic BCCs (Von Hoff et al., 2009). Yet, despite vismodegib demonstrating feasibility and efficacy in clinical trials against HH-driven medulloblastoma (Gajjar et al., 2013; Robinson et al., 2015), it has failed to achieve adequate success in treating advanced BCCs with 57% of patients displaying inherent resistance to the treatment (Sekulic et al., 2012) and 21% of treated patients developing secondary resistance after 56 weeks (Chang et al., 2012). Thus, it is vital to elucidate the mechanisms by which resistant BCCs evade SMO inhibition, as well as develop alternative therapeutic strategies that would effectively undermine such mechanisms.

In the normal vertebrate cell state, ion-driven cholesterol transporter Patched1 (PTCH1) actively depletes cholesterol from the membrane of the primary cilium and thus inhibits the

cholesterol-dependent activation of SMO (Zhang et al., 2018). This inhibition allows Suppressor of Fused homolog (SUFU) to sequester the Glioma-associated oncogene (GLI) transcription factors (Humke et al., 2010) and facilitate their post-translational proteolytic processing into repressor forms (Wang et al., 2000). The canonical HH pathway signaling initiates with the binding of HH ligands to PTCH1, which inhibits its activity and allows the activation of SMO via cholesterylation (Xiao et al., 2017; Deshpande et al., 2019). Activated SMO in turn induces the disassociation of the SUFU-GLI complex and facilitates the nuclear localization of the activator forms of GLI (Tukachinsky et al., 2010), which results in the expression of HH target genes. Uncontrolled activation of the HH pathway in BCC patients has been observed to occur primarily through inactivating mutations in PTCH1 (73%) or activating mutations in SMO (20%) (Bonilla et al., 2016). Yet, inspection of BCC patients with inherent and secondary resistance to vismodegib has revealed that the majority of mutations are within the SMO gene and either incite constitutive activity or deter inhibitor binding (Atwood et al., 2015). Current efforts to circumvent BCC chemoresistance are focused on perturbing the oncogenic activity of GLI, either through directly inhibiting the GLI proteins or inhibiting the molecules that modulate GLI activity (Peer et al., 2019). Recent studies have demonstrated the potency of inhibiting GLI (Kim et al., 2013), DYRK1B (Gruber et al., 2016), HDAC1 (Gruber et al., 2018), BRD4 (Tang et al., 2014), MLK1 (Whitson et al., 2018), and aPKC (Atwood et al., 2013) in attenuating resistant BCCs in preclinical studies.

Recently, various studies have highlighted the critical interconnections between the HH pathway and other signaling pathways in promoting the persistence and chemoresistance of cancer. In BCC, progression and therapeutic resistance have been linked with molecular crosstalk between the HH pathway and other developmental signaling pathways such as WNT (Yang et al., 2008), Notch (Eberl et al., 2018), TGF-b (Yao et al., 2020), and RAS/MAPK (Zhao et al., 2015) pathways. The phosphoinositide 3-kinase (PI3K) pathway is another developmental signaling

pathway that has been demonstrated to interact with the HH pathway in colon (Cai et al., 2015), pancreatic, and ovarian carcinomas (Singh et al., 2017). In addition, the PI3K pathway functions in therapeutic resistance against SMO inhibitors in medulloblastoma (Buonamici et al., 2010) and esophageal adenocarcinoma (Wang et al., 2012). Combinatory inhibition of both PI3K and HH signaling pathways in preclinical studies on medulloblastoma have demonstrated favorable efficacy in attenuating SMO inhibitor-resistant tumors (Buonamici et al., 2010).

The canonical PI3K pathway initiates with the activation of PI3K by receptor tyrosine kinases, which subsequently activates AKT via phosphorylation facilitated by phosphoinositides (Vanhaesebroeck et al., 2010). Activated AKT in turn phosphorylates and regulates the activities of a wide array of signaling proteins that are associated with proliferation and differentiation of the cell (Engelman et al., 2006). The PI3K/AKT pathway has been shown to interact with the HH pathway through multiple mechanisms that are largely independent of canonical HH signaling, with components of the PI3K/AKT pathway coinciding primarily upstream of GLI (Brechbiel et al., 2014). In embryonic fibroblasts, upregulation of the PI3K/AKT pathway signaling promotes HH signaling by antagonizing the inhibitory function of PKA on GLI2 (Riobó et al., 2006). Additionally, upregulation of the PI3K/AKT pathway signaling has been shown to promote HH signaling and tumor cell proliferation in esophageal (Wei et al., 2011) and breast cancers (Ramaswamy et al., 2012). However, the PI3K/AKT pathway has been shown to promote tumor cell growth with no effect on GLI1 activity in neuroblastomas (Diao et al., 2014), suggesting that PI3K/AKT either operates in parallel to or downstream of the HH pathway in this context. Thus, variation in how PI3K/AKT operates with respect to HH signaling confounds our ability to generally apply its function across distinct cancers.

Here, we demonstrate that the PI3K pathway signaling is upregulated in bulk-level RNA-sequencing data of 14 matched tumor-normal pairs. Human and mouse BCC tumors show a

significant increase in PI3K protein expression, and PI3K is essential for both BCC tumor cell growth and HH signaling. However, our data shows disparate results between BCC cells and *in vivo* tumors, where PI3K inhibition has no effect on GLI1 activity despite suppressing tumor growth. Finally, we show that PI3K likely functions in BCC tumors by promoting aPKC- and AKT1-depedent degradation of cyclin-dependent kinase inhibitor p21 to maintain cell cycle progression. Our results suggest that the PI3K pathway functions in parallel to or downstream of the HH pathway to promote BCC tumor growth.

RESULTS

PI3K/AKT pathway is upregulated in advanced BCC tumors

To assess alternative pathways that may drive BCC tumor growth, we reanalyzed our bulk-level RNA-sequencing (RNA-seq) data of 14 matched tumor-normal pairs of advanced and SMO inhibitor-resistant BCC samples (Chow et al., 2021; Atwood et al., 2015). Differential gene expression analysis across the 14 tumor-normal pairs identified 1602 genes that were upregulated by two-fold or more in the resistant BCC tumors compared to their normal skin counterparts (Chow et al., 2020). Database analysis of the upregulated genes with the Kyoto Encyclopedia of Genes and Genomes (KEGG) showed the expected upregulation of the cell cycle, HH pathway, and BCC-associated genes (Figure 3.1A). Another term that was significantly enriched was the PI3K/AKT pathway (Figure 3.1A). Analogously, Kinase Enrichment Analysis (KEA) (Lachmann et al., 2009) linked many of the upregulated genes with kinases that are closely associated with PI3K/AKT signaling, such as MAPK, AKT, GSK3b, CSNK, S6K, and PRKCB (Figure 3.1B). Close analysis of the PI3K pathway gene expression showed many components and downstream targets significantly upregulated in most tumors, including GRB2, PLCG1, and RPS6KA1 (Figure 3.1C).

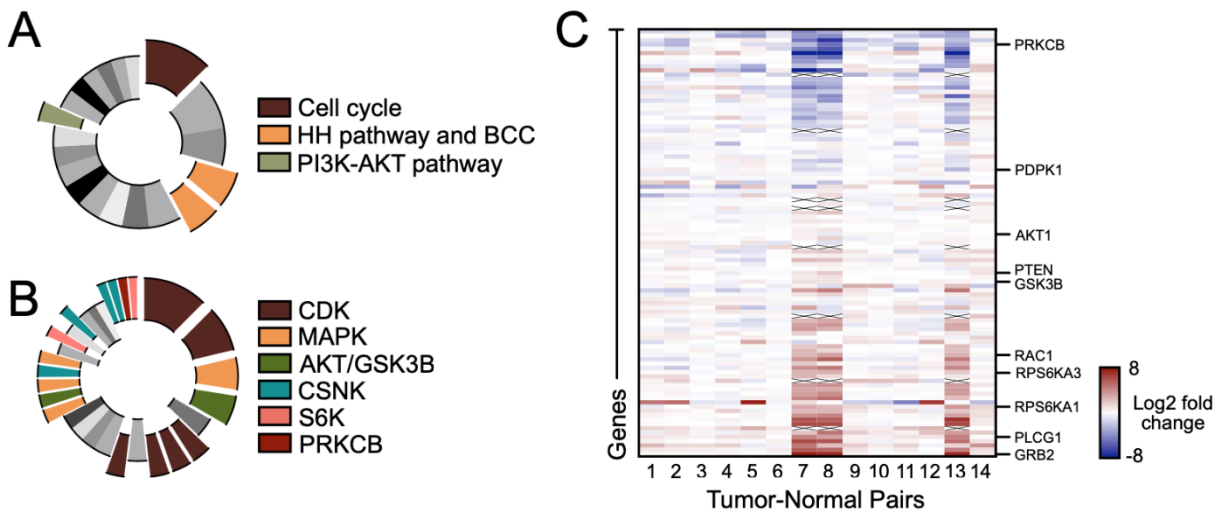

Figure 3.1. **PI3K/AKT pathway is upregulated in advanced BCC tumors. A)** KEGG analysis of the upregulated genes in advance BCC tumors highlighting the significant indicated terms. **B)** Kinase Enrichment Analysis of differentially expressed genes showing significant kinases as indicated. **C)** Heat map of the differentially expressed PI3K pathway genes in advanced human BCCs compared to patient-matched normal skin. X mark, absence of data.

PIK3CA is upregulated in human and mouse BCC tumors

To validate whether PI3K pathway upregulation in BCC tumors is consistent at the protein level, we measured the expression of the catalytic subunit PIK3CA in human nodular BCC tumors and normal epidermis using immunofluorescence staining. We observed that tumors displayed significantly enhanced expression of PIK3CA compared to normal epidermis (Figure 3.2A-B). To analyze Pik3ca expression in mice, we utilized a *Ptch1*$^{fl/fl}$*; Gli1-Cre*^{ERT2} mouse model in which BCC tumors arise from the hair follicle and the secondary hair germ (Peterson et al., 2015). BCC tumors were grown for five weeks post-Cre induction and formed predominantly from the hair follicle regions. Similar to our observations in human BCC tumors, we also observed a significant increase in Pik3ca expression in mouse BCC tumors compared to both normal epithelium and the hair follicle (Figure 3.2C-D). Together, these results suggest that PI3K pathway activity is upregulated in human and mouse BCC tumors.

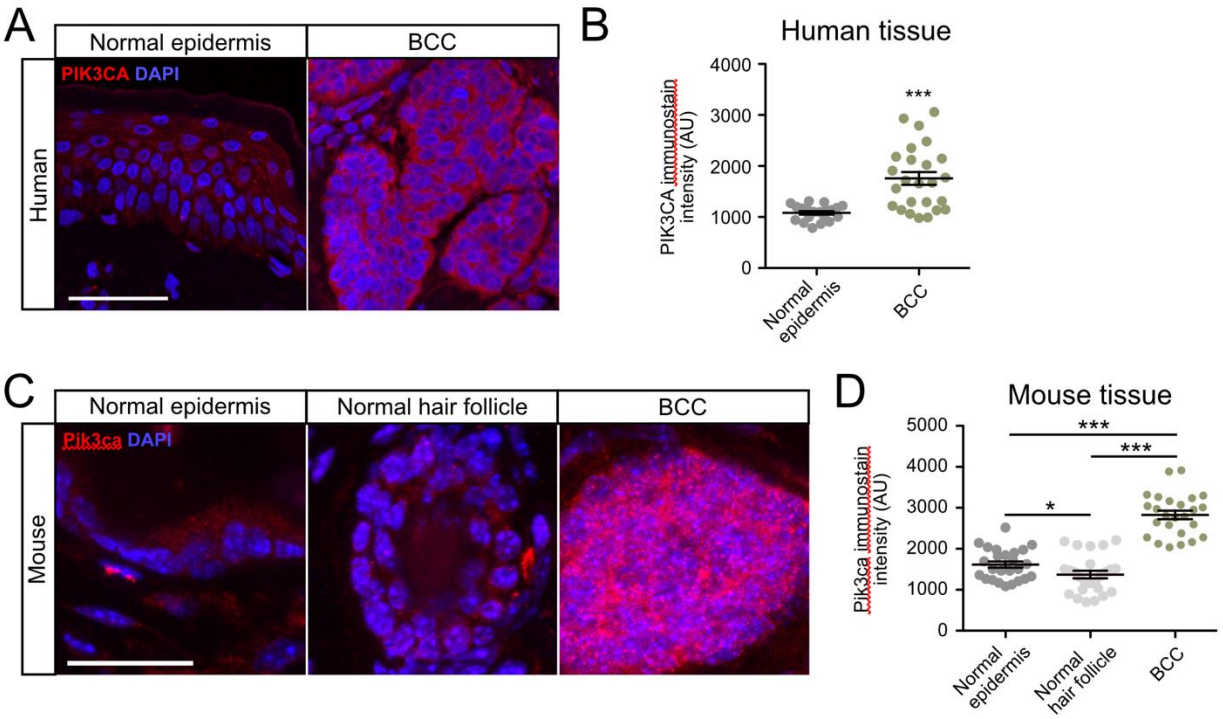

Figure 3.2. **PIK3CA is upregulated in human and mouse BCC tumors. A)** Immunofluorescence staining of PI3KCA (red) and DAPI counterstain (blue) in human normal epidermis and nodular BCC tumors. Scale bar, 50 mm. **B)** Quantification of PI3KCA immunofluorescence intensity (five points of measurement per sample, n=4 samples). AU, arbitrary unit. Error bar, SEM. **C)** Immunofluorescence staining of Pi3kca (red) and DAPI counterstain (blue) in mouse normal epithelium, normal hair follicle, and BCC tumors. Scale bar, 25 mm. **D)** Quantification of Pi3kca immunofluorescence intensity (five points of measurement per animal, n=5 mice). AU, arbitrary unit. Error bars, SEM. Significance was determined by unpaired two-tailed *t* test. *, $p < 0.05$; ***, $p < 0.001$.

Inhibition of PI3K suppresses growth and HH signaling in BCC cells *in vitro*

To assess whether upregulation of the PI3K pathway signaling affects growth and HH signaling in BCCs, we assayed ASZ001 mouse BCC cells with two PI3K inhibitors, BKM120 and LY294002. BKM120 acts as an allosteric inhibitor of PI3K (Burger et al., 2011) while LY294002 acts as an ATP-competitive inhibitor (Walker et al., 2000). Treatment of ASZ001 cells with BKM120 and LY294002 both significantly decreased HH signaling as assayed by *Gli1* mRNA expression (Figure 3.3A). Additionally, treatment of ASZ001 cells with BKM120 and LY294002 both resulted in complementary and dose-dependent reduction of tumor cell growth over time (Figure 3.3B-C). A significant increase in Casp3-mediated apoptosis was also observed upon BKM120 inhibition, with the proliferation marker Mki67 trending downward (Figure 3.3D-F). Together, these results show that the PI3K pathway promotes BCC cell growth upstream of the HH pathway.

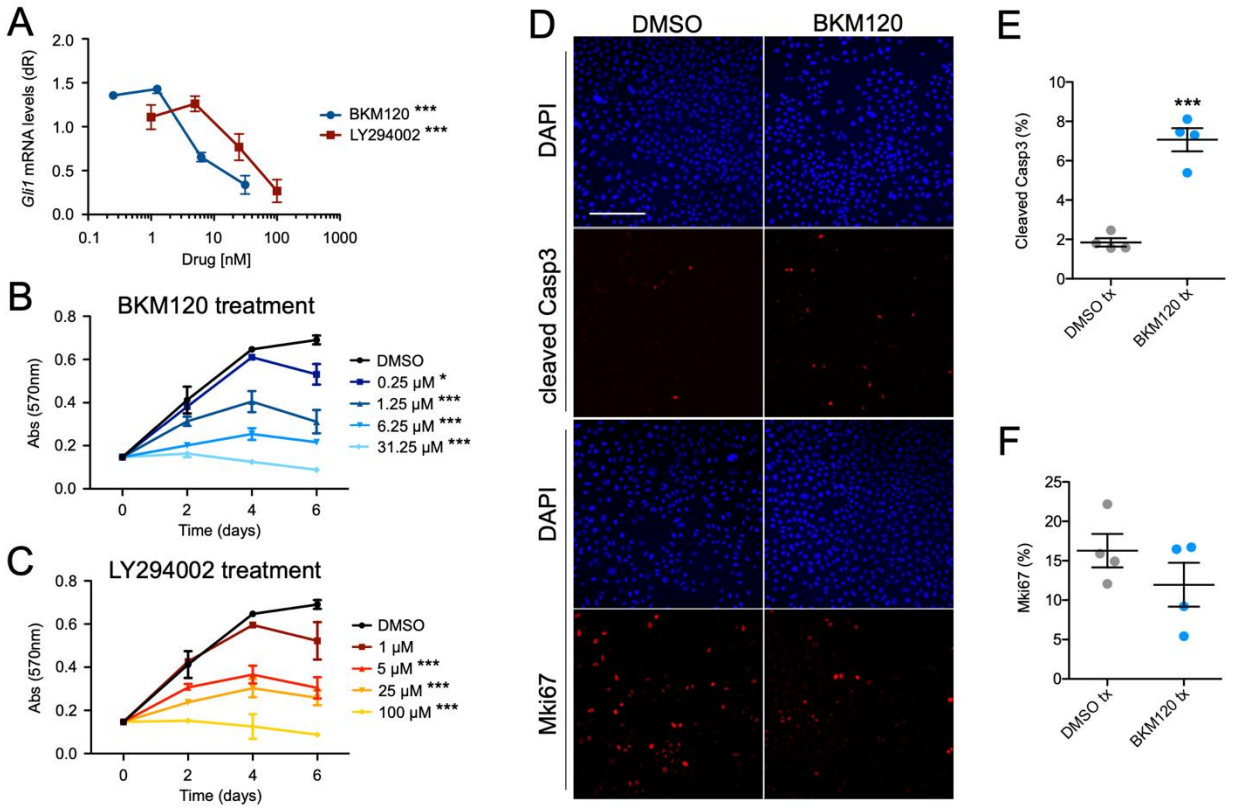

Figure 3.3. **Inhibition of PI3K suppresses BCC cell growth and HH signaling. A)** *Gli1* mRNA expression in ASZ001 cells treated with DMSO or varying concentrations of BKM120 or LY294002 (n = 3 experiments). dR, delta reporter gene normalized to passive reference dye. Error bar, SEM. Significance was determined by one-way ANOVA test. ***P<0.001. **B-C)** MTT assay of ASZ001 cells treated with DMSO or varying concentrations of **B)** BKM120 or **C)** LY294002 (n = 3 experiments). Abs, absorbance. Error bar, SEM. Significance was determined by two-way ANOVA test. *, p < 0.05; **, p < 0.01; ***, p < 0.001. **D)** Immunofluorescence staining of the indicated markers in ASZ001 cells treated with DMSO or BKM120. Scale bar, 200 μm. **E)** Quantification of cleaved Casp3 signal (n = 4 experiments). **F)** Quantification of Mki67 signal (n = 4 experiments). Error bars, SEM. Significance was determined by unpaired two-tailed *t* test. ***, p < 0.001.

Inhibition of PI3K suppresses growth but not HH signaling in BCC tumors *in vivo*

To evaluate whether inhibition of PI3K can serve as an effective therapeutic strategy in attenuating BCC tumors, we generated BCC tumors in the *Ptch1*$^{fl/fl}$*; Gli1-Cre*^{ERT2} mouse model and intraperitoneally injected either DMSO or 10 mg/kg of BKM120 daily for seven days. Histological staining of the dorsal skin of BKM120-treated mice showed a significant reduction in total tumor size compared to DMSO controls (Figure 3.4A-B). Interestingly, Gli1 protein expression was not altered in BKM120-treated mice (Figure 3.4C-D), suggesting that the PI3K operates downstream or in parallel to the HH pathway *in vivo*, a result that is similar to mTOR inhibition (Chow et al., 2021). The discrepancy between our *in vitro* and *in vivo* results may indicate that the tumor microenvironment alters how the PI3K pathway functions in relation to the HH pathway.

To further define how the PI3K pathway functions *in vivo*, we assayed that status of aPKC, a Gli1 kinase that is necessary for high sustained Gli1 activity (Atwood et al., 2013). Atypical PKCs are activated downstream of PI3K by PDK1-dependent phosphorylation at T410 in a variety of cell types (Chou et al., 1998; Le Good et al., 1998). While we observed a slight increase in total aPKC immunostaining in BKM120-treated mouse BCC tumors, phosphorylation at T410 was significantly reduced (Figure 3.4C-D), indicating suppressed kinase activity. Although aPKC phosphorylates and activates Gli1 at residue T304 (Atwood et al., 2013), we observed no change in p-T304 Gli1 expression (Figure 3.4C-D), reinforcing the possibility of PI3K's role outside of HH signaling and suggesting that aPKC likely exerts its effects on another substrate. Akt1 is also activated downstream of PI3K by PDK1-dependent phosphorylation at T308 (Alessi et al., 1997), and we found a significant reduction in both total and p-T308 Akt1 expression in BKM120-treated mouse BCC tumors (Figure 3.4E-F). As both aPKC and Akt1 facilitate the degradation of cyclin-dependent kinase inhibitor p21 by phosphorylating T145 (Rössig et al., 2001; Zhou et al., 2001; Scott et al., 2002), we assayed p21 protein expression and found a substantial increase in p21

stability and a corresponding decrease in p21 phosphorylation (Figure 3.4E-F). Altogether, our data suggests PI3K likely facilitates BCC tumor growth by promoting cell cycle progression through aPKC- and Akt1-mediated p21 degradation.

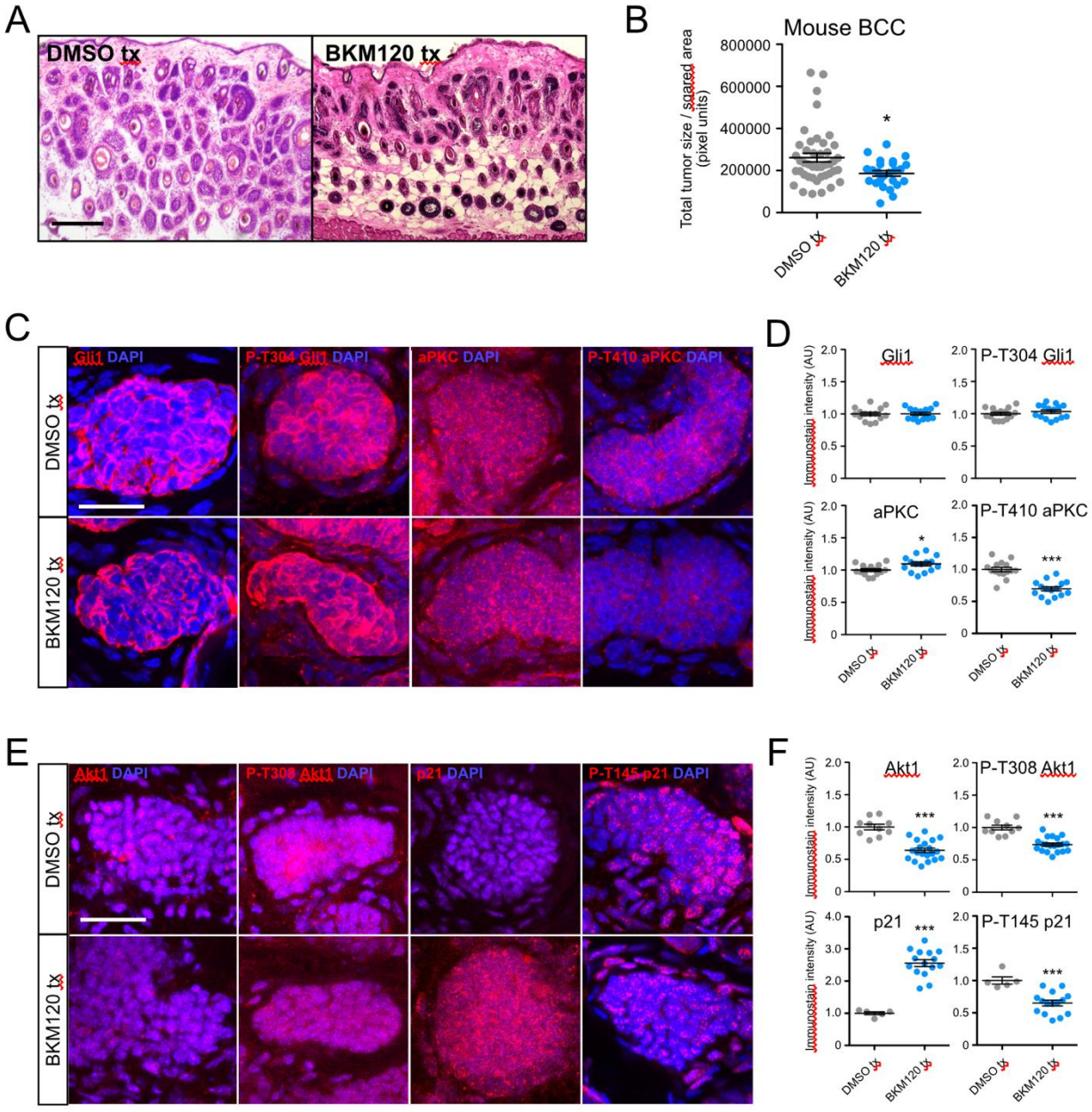
A
DMSO tx
BKM120 tx
B
Mouse BCC
Total tumor size / squared area (pixel units)
800000
600000
400000
200000
0
*
DMSO tx
BKM120 tx
C
DMSO tx
BKM120 tx
Gli1 DAPI
P-T304 Gli1 DAPI
aPKC DAPI
P-T410 aPKC DAPI
D
Gli1
P-T304 Gli1
aPKC
P-T410 aPKC
Immunostain intensity (AU)
2.0
1.5
1.0
0.5
0
*
***
DMSO tx
BKM120 tx
E
DMSO tx
BKM120 tx
Akt1 DAPI
P-T308 Akt1 DAPI
p21 DAPI
P-T145 p21 DAPI
F
Akt1
P-T308 Akt1
p21
P-T145 p21
Immunostain intensity (AU)
4.0
3.0
2.0
1.0
0
***
DMSO tx
BKM120 tx

Figure 3.4. PI3K inhibition suppresses murine BCC growth and stabilizes p21. A) Hematoxylin and eosin staining of dorsal back skin collected from *Ptch*$^{fl/fl}$*; Gli1-Cre*^{ERT2} mice treated with DMSO or BKM120. Tx, treatment. Scale bar, 50 mm. **B)** Quantification of total tumor size per square area (n>250 tumors from 5 mice). Tx, treatment. **C)** Immunofluorescence staining of indicated markers (red) and DAPI counterstain (blue) in *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} tumors treated with DMSO or BKM120. Scale bar, 25 mm. **D)** Quantification of immunofluorescence intensity of indicated markers (five points of measurement per animal, n=3 mice). AU, arbitrary unit. **E)** Immunofluorescence staining of indicated markers (red) and DAPI counterstain (blue) in *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} tumors treated with DMSO or BKM120. Scale bar, 25 mm. **F)** Quantification of immunofluorescence intensity of indicated markers (five points of measurement per animal, n=3 mice). Error bars, SEM. Significance was determined by unpaired two-tailed *t* test. *, $p < 0.05$; ***, $p < 0.001$.

DISCUSSION

PI3K appears to operate at distinct levels within the HH pathway depending on context. For instance, upregulation of the PI3K/AKT pathway promotes HH signaling in embryonic fibroblasts (Riobó et al., 2006), esophageal cancer (Wei et al., 2011), and breast cancer (Ramaswamy et al., 2012). Alternatively, the PI3K/AKT pathway promotes tumor cell growth independent of GLI1 activity in neuroblastomas (Diao et al., 2014), suggesting that PI3K/AKT either operates in parallel to or downstream of the HH pathway in this context. Our results show HH signaling is dependent on PI3K signaling in BCC cells grown in culture, but not in BCC tumors. This discrepancy between cell culture and three-dimensional (3D) growth conditions is a relatively common phenomenon and has been shown for the AKT-mTOR pathway, where inhibition of AKT resulted in elevated ERK signaling in cell culture but reduced signaling in 3D culture (Riedl et al., 2017). PI3K likely operates downstream or in parallel to the HH pathway in BCC, similar to our results for mTOR in *Ptch1*$^{fl/fl}$*; Gli1-Cre*^{ERT2} BCCs (Chow et al., 2021) and consistent with models where mTOR acts downstream of the HH pathway in *Ptch1*$^{+/-}$*/SKH-1* BCCs (Kim et al., 2018).

p21 is a potent universal cyclin-dependent kinase inhibitor that is activated downstream of p53 upon DNA damage or other cellular stresses and promotes G1 cell cycle arrest, which can lead to senescence or apoptosis (Georgakilas et al., 2017). When p53 is present, p21 and p53 can act together to help correct DNA damage and preserve genome stability. However, when p53 is disrupted, p21 can promote genomic instability and escape from senescence (Galanos et al., 2016). p21 degradation is facilitated by aPKC- and AKT1-dependent phosphorylation at T145 (Rössig et al., 2001; Zhou et al., 2001; Scott et al., 2002). As aPKC and AKT1 are both overexpressed in BCC and are required for tumor growth (Atwood et al., 2013; Kim et al., 2016), our results suggest that BCCs activate both kinases downstream of PI3K to promote cell cycle progression and continued tumor growth. PI3K inhibition significantly suppresses aPKC and Akt1

activity, likely leading to enhanced p21 stability, suppression of proliferation, and enhanced apoptosis.

Targeting the PI3K pathway in the clinic may be a viable option for BCC patients. In addition to the present study showing BKM120 efficacy on *Ptch1*$^{fl/fl}$*; Gli1-Cre*^{ERT2} BCCs, inhibition of the PI3K/AKT/mTOR pathway has been shown to suppress irradiated *Ptch1*$^{+/-}$*; Krt14*^{CreER2}*; p53*$^{fl/fl}$ BCCs with XL765, but not XL147 or GDC-0941 (So et al., 2014). In addition, suppressing mTOR activity using everolimus can suppress *Ptch1*$^{fl/fl}$*; Gli1-Cre*^{ERT2} BCCs through an aPKC-dependent process (Chow et al., 2021) and has been used in the clinic for compassionate treatment of BCCs in elderly patients who refused surgery and did not respond to alternative treatments (Eibenschutz et al., 2013). Combination therapy may also be crucial to treat advanced BCC patients. For instance, PI3K/mTOR inhibition can delay therapeutic resistance against SMO inhibitors in mouse models of cancer, including BKM120 treatment in HH-mediated medulloblastoma (Buonamici et al., 2010) or treatment with the mTOR inhibitor everolimus in esophageal adenocarcinoma (Wang et al., 2012). In addition, SMO inhibitor-resistant mouse medulloblastoma are still sensitive to PI3K inhibition (Dijkgraaf et al., 2011). And combination therapy with the GLI inhibitor GANT61 and PI3K/mTOR inhibitor PI103 synergistically inhibited tumors in a HH-driven rhabdomyosarcoma mouse model (Graab et al., 2015). Altogether, PI3K pathway-targeted therapies, solely or in combination with HH pathway inhibitors, may broaden our repository for treating advanced and SMO inhibitor-resistant BCCs.

CHAPTER 4

Src promotes basal cell carcinoma growth via phosphorylation and activation of atypical PKC

CONTRIBUTIONS

This project was conceived by Scott Atwood and Rachel Chow. Scott Atwood supervised the research. Rachel Chow performed the experiments. Taylor Levee immunostained and quantified the tumor data. Scott Atwood and Rachel Chow wrote the manuscript. All authors analyzed and discussed the results and commented on the manuscript. The work described in this chapter has not been published.

SUMMARY

Basal cell carcinomas (BCCs) are very prominent epithelial cancers driven by the Hedgehog (HH) signaling pathway. Advanced BCCs are known to possess inherent resistance to Smoothened (SMO) inhibitors. Therefore, it is important to identify and target alternative drivers of BCC that can bypass SMO in order to provide novel therapies for patients with advanced or metastatic BCCs. Here, we show with immunostaining that Src and p-Src are elevated in a *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mouse BCC tumor model, which suggests that active Src may play a role in BCC tumorigenesis. Pharmacological inhibition of Src activity in BCC cells significantly reduces both HH signaling and tumor cell proliferation. Additionally, overexpression of Src alone dramatically increases *Gli1* expression whereas kinase-dead Src K299M completely abrogates this expression. Similarly, treatment of BCC mice with KX01 results in reduced tumor growth and HH signaling as well. aPKC, which is known to directly phosphorylate and activate GLI1, displays reduced activity upon Src inhibition, which subsequently results in reduced HH signaling. Upon mutating known Src phosphosites on aPKC, we observe that HH signaling is affected as well. This suggests that Src promotes BCC growth via aPKC activation.

INTRODUCTION

Basal cell carcinoma (BCC) is a very common form of epithelial cancer which arises from the basal layer of the epidermis and is driven predominantly by overstimulation of the Hedgehog (HH) signaling pathway (Atwood et al., 2015; Sekulic and Hoff, 2016). In a normal cell state, cholesterol transporter Patched1 (PTCH1) is active and suppresses the activity of transmembrane receptor Smoothened (SMO). This then allows Suppressor of Fused (SUFU) to sequester and inhibit Glioma-associated oncogene (GLI) in the cytoplasm. However, in the presence of a HH ligand, PTCH1 is inhibited, allowing for SMO to become activated. This subsequently leads to the dissociation of SUFU-GLI (Tukachinsky et al., 2010), resulting in the activation of GLI transcription factors to enter into the nucleus to activate target genes involved in proliferation, migration and invasion (Varjosalo et al., 2008). In cases of BCC, most patients possess inactivating mutations in PTCH1 (~73%), activating mutations in SMO (20%), or loss-of-function mutations in SUFU (~8%) (Bonilla et al., 2016).

As a majority of the driving mutations are upstream in the HH pathway, SMO inhibitors, such as vismodegib, have been a popular form of treatment to combat most forms of BCCs, including advanced and metastatic (Atwood et al., 2012). However, nearly 60% of advanced BCCs possess an inherent resistance to vismodegib treatment and roughly 20% of tumors that initially respond to treatment acquire drug resistance each year (Chang and Oro, 2012). This illuminates how critical it is to identify alternative therapeutics that are either downstream of SMO or contribute to non-canonical activation of the HH pathway.

Src is a proto-oncogene that is overexpressed and highly activated in a variety of cancers. It is a non-receptor tyrosine kinase that interacts with a number of receptor tyrosine kinases known to contribute to events such as angiogenesis, invasion, migration, proliferation, apoptosis, and differentiation, all of which are characteristic of the development of metastasis (Irby &

Yeatman, 2000). Such factors include: vascular endothelial growth factor (VEGF) (Brown & Cooper, 1996), epidermal growth factor receptor (EGFR) (Tice et al, 1999; Luttrell et al., 1994; Mao et al., 1997), fibroblast growth factor receptor (FGFR) (LaVallee et al., 1998), platelet-derived growth factor receptor (PFGFR) (Courtneidge et al., 1993), colony stimulating factor-1 receptor (CSF-1R) (Courtneidge et al., 1993; Levitzki 1996), and human epidermal growth factor receptor 2 (HER2)/*neu* (Rahimi et al., 1998). In HER2-positive breast cancer cells, Src was demonstrated to coordinate with HER2 to induce PI3K-mediated progression of disease (Nagata et al., 2014). In non-small cell lung cancer cells, inhibition of EGFR resulted in decreased activity of Src and its downstream target STAT3 (Kloth et al., 2003). In colon cancer, Src expression and activity has been associated with advancement and malignancy of disease (Talamonti et al., 1991). Additionally, a clinical study was conducted in order to evaluate the expression of Src in human epithelial cancers. What they revealed was that Src is overexpressed in malignant melanomas, squamous cell carcinomas, and BCCs (Lee et al., 2010). Although we have evidence of Src being overexpressed in BCCs, we do not yet know the role that it plays in BCC tumorigenesis or HH signaling.

Here, we utilize a *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mouse BCC tumor model to confirm Src overexpression and to help elucidate Src's contribution to BCC growth. We demonstrate that inhibition of Src activity *in vitro* and *in vivo* results in an increase in both HH activity and BCC cell growth. Our results also propose that Src drives BCC progression in a non-canonical manner via activation of aPKC, and thus Gli1. This suggests that inhibiting Src may serve as a promising therapeutic in targeting HH-driven BCC growth.

RESULTS

Src and p-Src are upregulated in mouse BCCs

Since Src has already been shown to be overexpressed in human BCCs (Lee et al., 2010), we wanted to confirm that the same phenomenon occurs in mouse BCCs. To analyze Src expression in mice, we utilized a *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mouse model in which BCC tumors predominantly arise from *Gli1*-positive regions within the hair follicle bulge and secondary hair germ (Peterson et al., 2015). Mice were treated with tamoxifen to induce BCC tumorigensis and the tumors were allowed to grow for five weeks, where they formed mainly in the hair follicle region. Src immunostaining illustrated a significant increase in expression in the BCC tumors compared to normal epithelium and the hair follicles (Figure 4.1A-B). Additionally, to assess Src activity in these BCC's, we immunostained for p-Src, a common marker used to determine activation (Kim et al., 2017). Similar to Src expression, p-Src expression is also elevated in the tumors compared to normal epithelium and hair follicle (Figure 4.1A, C). Together, these data suggest that Src activity is elevated in mouse BCCs.

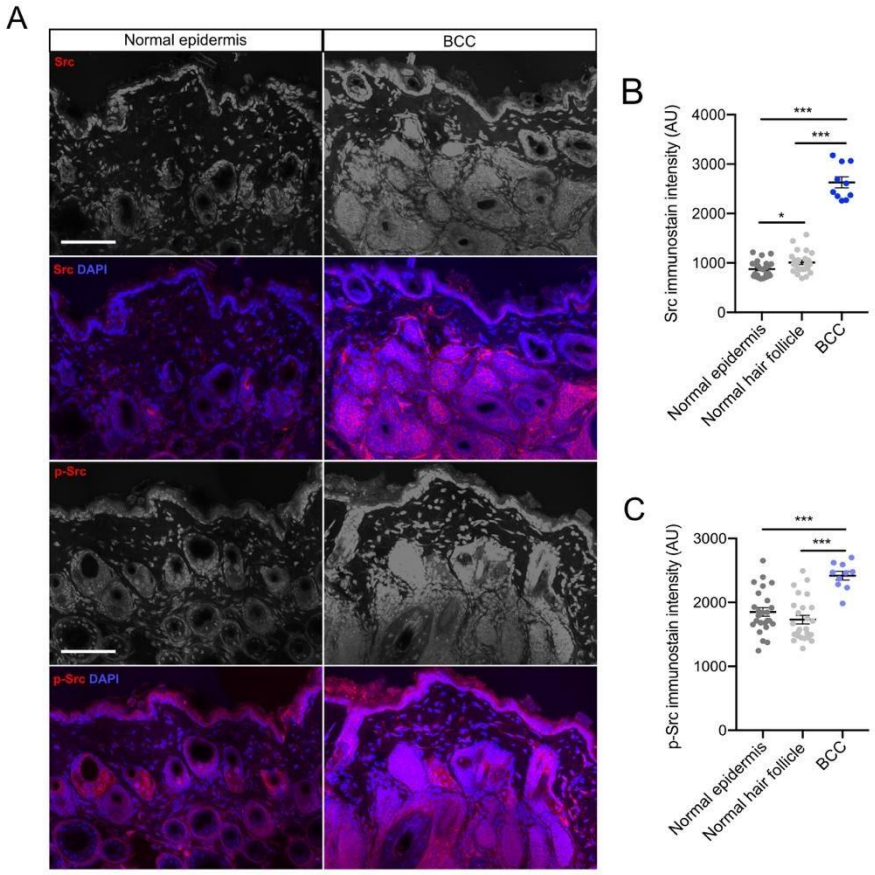

Figure 4.1. Src and p-Src are overexpressed in mouse BCC. A) Immunofluorescent staining of indicated markers in normal epidermis, normal hair follicle, or BCC derived from *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mice. Scale bar, 50 μm. **B and C)** Quantification of Src and p-Src immunostains (n = 5 different points of measurement from 5 mice). Error bars represent SEM. Significance was determined by unpaired two-tailed *t* test. *, $p < 0.05$; ***, $p < 0.001$.

Src inhibition suppresses HH signaling and BCC cell growth *in vitro*

We next wanted to determine if Src inhibition suppresses HH signaling and tumor cell growth. A BCC cell line, ASZ001, was treated with three different Src inhibitors: dasatinib, saracatinib, and KX01. Dasatinib and saracatinib are both ATP-competitive inhibitors (Schittenhelm et al., 2006; Nam et al., 2013) whereas KX01 targets the peptide substrate site of Src (Anbalagan et al., 2012). Treatment with dasatinib and saracatinib resulted in either no change or a slight increase in HH signaling as assayed by *Gli1* mRNA expression, whereas KX01 treatment resulted in a dose-dependent decrease in HH signaling (Figure 4.2A). As dasatinib and saracatinib are promiscuous and can bind to other Src family members (Schittenhelm et al., 2006; Nam et al., 2013), the results seen in Figure 4.2A may be a consequence of off-target effects. Therefore, we decided to proceed with only KX01 since it has been shown to be highly specific to Src (Anbalagan et al., 2012). Next, we assayed BCC cell growth in the presence of KX01 and saw that BCC cell growth is also reduced in a dose-dependent manner (Figure 4.2B). Additionally, cell growth in NIH-3T3 cells was not stunted upon KX01 treatment (Figure 4.2C), suggesting that KX01 is capable of specifically targeting BCC cells without affecting surrounding normal cells. We also observed a decrease in Mki67 and a dramatic increase in cleaved Casp3 immunostaining in BCC cells treated with KX01 (Figure 4.2D), suggesting that a decrease and proliferation an increase in apoptosis is attributing to the total decrease in BCC cells growth upon Src inhibition. Next, to affirm that Src activity is necessary for HH signaling and, therefore, BCC growth, we overexpressed wild-type and kinase-dead (K299M) Src and saw a dramatic increase in *Gli1* expression with wild-type Src overexpression and a complete inhibition of HH signaling with Src K299M (Figure 4.2E). Together, these results demonstrate that Src activity is necessary and promotes BCC cell growth upstream of the HH pathway.

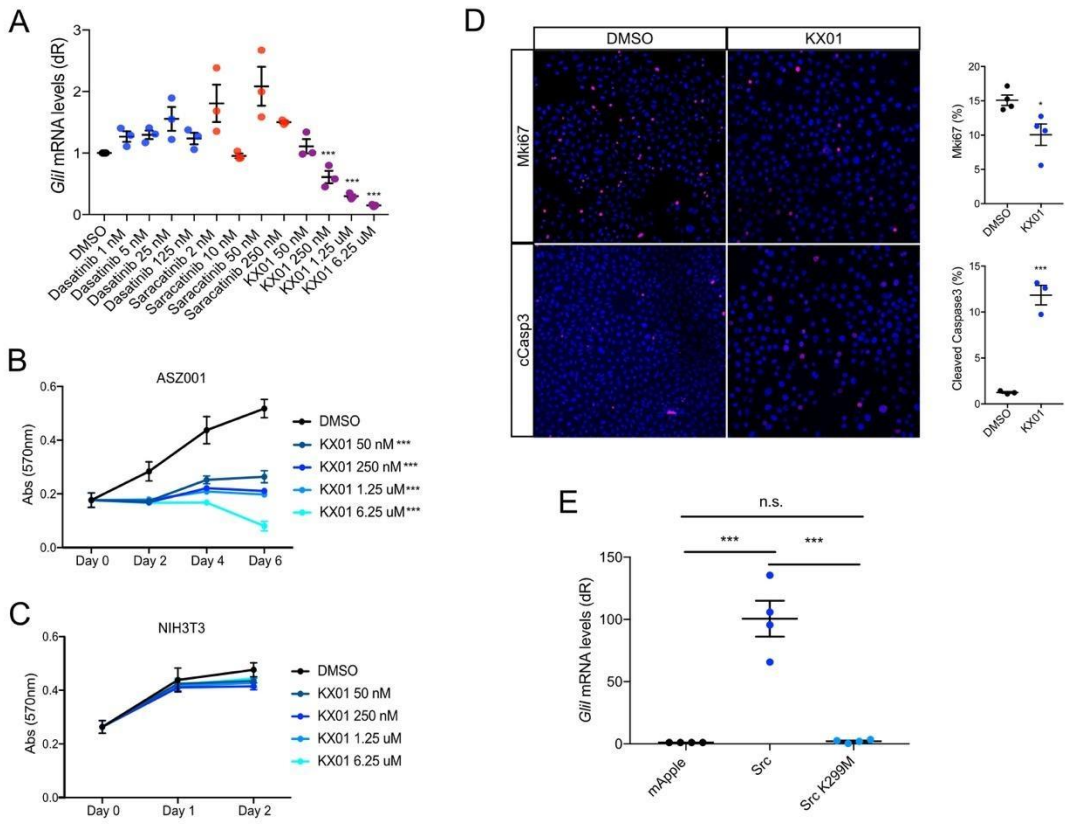

Figure 4.2. Src inhibition suppresses HH signaling and BCC cell growth. A) Gli1 mRNA levels of ASZ001 cells treated with DMSO or varying concentrations of Dasatinib, Saracatinib, or KX01 (n = 3 experiments). dR, delta reporter signal normalized to passive reference dye. Significance was determined by one-way ANOVA test. **B and C)** MTT assay of **B)** ASZ001 and **C)** NIH3T3 cells treated with KX01. Abs, absorbance. Significance was determined by two-way ANOVA test. **D)** Immunofluorescence staining and quantification of Mki67 (n = 4 experiments) and cleaved Casp3 (n = 3 experiments) signal in ASZ001 cells treated with DMSO or KX01. **E)** Gli1 mRNA levels of ASZ001 overexpressing mApple (control), Src, or Src K299M (n = 4 experiments). Significance was determined by unpaired two-tailed *t* test. Error bars, SEM. *, $p < 0.05$; ***, $p < 0.001$.

Src inhibition suppresses BCC growth and HH signaling *in vivo*

To evaluate whether Src inhibition could be used as an efficacious therapeutic strategy, we initiated BCC tumor growth in *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mice for 5 weeks and, subsequently, intraperitoneally injected with either DMSO or 5mg/kg KX01 for seven consecutive days. Histological staining of the dorsal skin of KX01-treated mice showed a significant reduction in total tumor size compared to DMSO controls (Figure 4.3A- B). Additionally, Gli1 protein levels were also dramatically reduced as indicated by immunostaining (Figure 4.3C-D). These data are in support of our *in vitro* data (Figure 4.2) which suggests that Src is likely acting upstream of HH signaling to encourage BCC growth.

To verify the effectiveness of KX01 *in vivo*, we immunostained for p-Src and p-Fak to confirm that Src activity is indeed being suppressed upon KX01 treatment. pT416-Src represents an active form of Src (Kim et al., 2017) whereas pT861-Fak is used as a readout of Src activity (Schweppe et al., 2009). As expected, our quantification indicates that both p-Src and p-Fak are significantly downregulated upon KX01 treatment (Figure 4.3C-D), affirming that KX01 is specific and can be effective in treating BCCs.

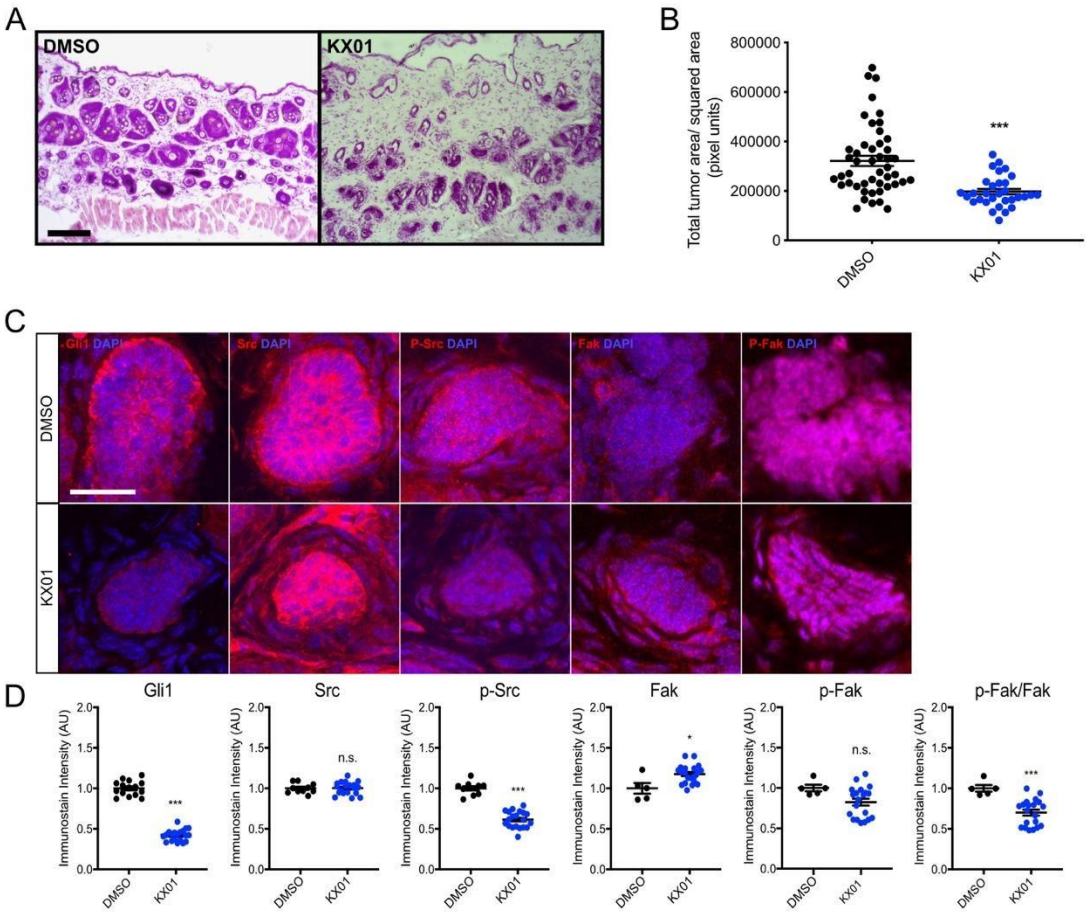

Figure 4.3. Inhibition of Src suppresses murine BCC growth. A) Hematoxylin and eosin staining of dorsal back skin collected from DMSO- or KX01-treated *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mice. Scale bar, 50 μm. **B)** Quantification of total tumor size per square area (n > 250 tumors from 5 mice). **C)** Immunofluorescence staining of indicated markers (red) and DAPI counterstain (blue) in *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} tumors treated with DMSO or KX01. Scale bar, 25 mm. **D)** Quantification of immunofluorescence intensity of indicated markers (five points of measurement per animal, n=3 mice). AU, arbitrary unit. Error bars, SEM. Significance was determined by unpaired two-tailed *t* test. *, $p < 0.05$; ***, $p < 0.001$.

Src phosphorylates and activates aPKC to upregulate HH signaling

To further understand the mechanism of how Src is regulating HH signaling, we assayed the status of aPKC, which is known to directly phosphorylate and activate Gli1 at residue T304 to perpetuate HH activity (Atwood et al., 2013). Immunostaining of p-T410, p-T560 aPKC, and total aPKC, which have commonly been used as readouts for aPKC activity (Tobias et al., 2015; Lin & Gao, 2014), indicates a decrease in aPKC activation upon KX01 treatment (Figure 4.4A-B). Furthermore, we also see a dramatic reduction in p-T304 Gli1 (Figure 4.4A-B), suggesting that Src works through aPKC to activate Gli1. To validate this, we expressed either WT aPKC kinase domain (KiDo), aPKC Y265F KiDo, aPKC Y280F KiDo, or aPKC Y334F KiDo in ASZ001 cells. The Y265, Y280, and Y334 residues of aPKC have been previously shown to be directly phosphorylatable by Src (Wooten et al., 2001); therefore, we introduced non-phosphorylatable mutants to determine if aPKC phosphorylation by Src is necessary for HH signaling. Quantitative PCR analysis of *Gli1* expression indicates that aPKC Y265F KiDo significantly reduces Gli1 expression compared to wild-type. This proposes that phosphorylation of the Y265 residue of aPKC is important for HH activity, and thus, BCC growth. Altogether, our data suggests that Src phosphorylates and activates aPKC in order to encourage enhanced HH signaling and tumor growth.

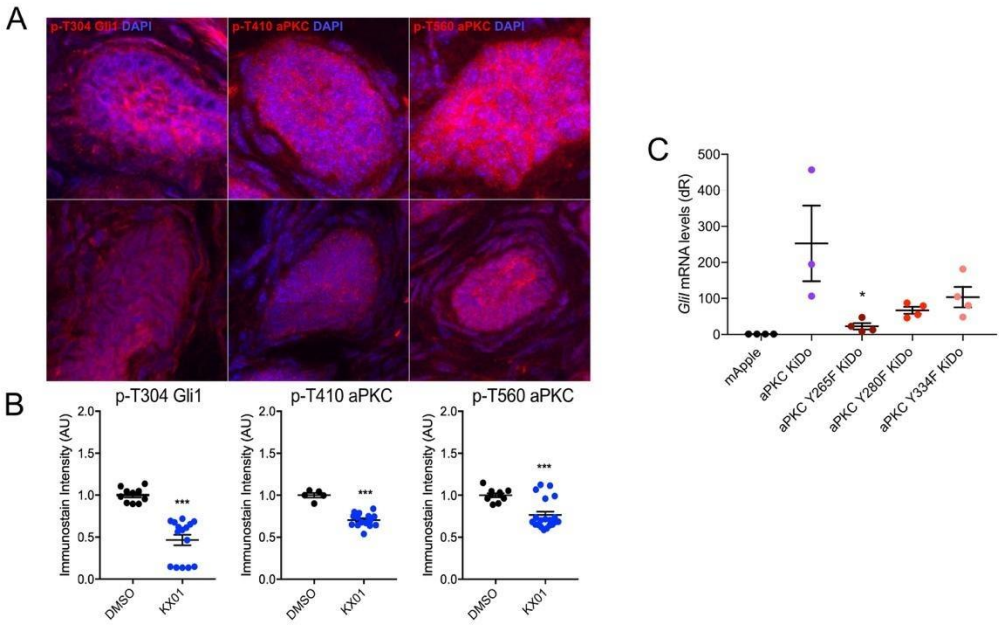

Figure 4.4. Src inhibition suppresses aPKC activity and HH signaling. **A)** Immunofluorescence staining of indicated markers (red) and DAPI counterstain (blue) in *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} tumors treated with DMSO or KX01. Scale bar, 25 mm. **B)** Quantification of immunofluorescence intensity of indicated markers (five points of measurement per animal, n=3 mice). AU, arbitrary unit. C) Gli1 mRNA levels of ASZ001 cells overexpressing mApple, aPKC KiDo, aPKC Y265F KiDo, aPKC Y280F KiDo, or aPKC Y334F KiDo (n = 4 experiments). Significance was determined by unpaired two-tailed *t* test. Error bars, SEM. *, $p < 0.05$; ***, $p < 0.001$.

DISCUSSION

Whether Src positively or negatively regulates HH signaling is a matter that is up for debate. Our data shows that HH signaling is reduced upon Src inhibition *in vitro* and *in vivo* (Figure 4.2A and 4.3C-D) and is upregulated when Src is overexpressed (Figure 4.2E). This supports the notion that Src positively regulates HH signaling. However, work by Bershteyn et al. would claim otherwise. It has already been well-accepted that the primary cilium is a necessary organelle for transducing HH signaling (Huangfu & Anderson, 2005) and Bershteyn et al. (2010) demonstrates that Missing-in-Metastasis (MIM) is necessary for ciliogenesis in dermal papilla cells. When MIM is knocked down, Src activity is upregulated, resulting in hyperphosphorylation of Cortactin and ultimately, cilia disassembly. Therefore, they propose a mechanism in which Src inhibits ciliogenesis and, thus, HH signaling downstream of MIM. As with most biological systems, whether Src upregulates or downregulates HH is probably context dependent.

Very little is known about the relationship between Src and HH signaling other than what was above mentioned. Therefore, to get a glimpse into other possible relationships, we can turn to a few studies on SFKs and the HH pathway. In spermatogonial stem cells (SSCs), HH signaling promotes the expression of glial cell-derived neurotrophic factor (GDNF) (Inaba et al., 2016), which then can activate SFKs and AKT to promote SSC self-renewal and survival (Oatley & Brinster, 2012). In a neuronal model, HH stimulates the activity of SFK members non-canonically in order to mediate axon guidance. They show that SFKs are required for HH-mediated axon guidance but not for Gli transcriptional activity (Yam et al., 2009). Another group showed that the SFK Hck phosphorylates several tyrosine residues of Gli1, relieving Gli1's inhibition by Sufu and leading to its transactivation. They also demonstrate that *Hck* happens to be a direct Gli1 target in HH-responsive NIH3T3 cells and cerebellum granular neuron precursor (CGNP) cells, resulting in a Gli1/Hck positive feedback loop to perpetuate HH signaling. Our data most closely

resembles that of the latter study. Although we do not currently know if Src can directly phosphorylate Gli1, our data suggests that Src may phosphorylate and activate aPKC, which has been previously shown to directly phosphorylate and activate Gli1 (Atwood at al., 2013). And similar to Hck in the previously mentioned study, *aPKC* is a direct target of Gli1, thereby forming a Gli1/aPKC positive feedback loop.

As vismodegib treatment has already been proven to be an effective therapeutic for BCCs (Atwood et al., 2012), it may be advantageous to start treating BCCs with a combined treatment of vismodegib and KX01. This would target both canonical and non-canonical HH pathway activation, increasing the likelihood of fully eradicating BCC tumor cells. Furthermore, it would be interesting to also examine the effects of Src inhibition on vismodegib-resistant BCCs. Studies on ovarian and lung cancers have demonstrated that Src inhibition can actually restore drug sensitivity to resistant tumors, suggesting that Src may play a crucial role in the development of resistance (Pengetnze et al, 2003; Zhang et al., 2014). Additionally, since resistant BCC tumors have developed ways to evade the effects of SMO inhibition, targeting HH signaling via noncanonical means may be a viable route. However, for both drug-sensitive and resistant tumors, there is a possibility that vismodegib treatment and/or Src inhibition may not be sufficient in completely eliminating BCC cells. This may be the case for subpopulations of BCCs in which alternative pathways may be supporting BCC growth independently of HH signaling. Such instances may occur in tumors where either mTOR or PI3K are drivers of a particular BCC subpopulation (Chow et al., 2021; Chow et al, unpublished). In these cases, mTOR and PI3K are able to encourage BCC growth without upregulating HH signaling. All in all, Src has been shown to be a promising target in combating BCCs, but more work needs to be done to identify all drivers of BCC carcinogenesis and progression in order to successfully extinguish all forms of BCCs.

Chapter 5

Conclusions

HH- independent BCC growth and progression

For as long as BCCs have been studied, the HH pathway was thought to be the sole driver of BCC growth and progression. However, our research, in conjunction with other recent studies, suggest that alternative pathways can promote BCC growth in parallel and independently of HH signaling. When RNA-sequencing was performed on a large collection of BCC tumors, Hippo/ YAP pathway genes were found significantly upregulated in BCCs (Bonilla et al., 2016). Additionally, Maglic et al. (2018) demonstrated that the Hippo pathway regulator, YAP, is required for BCC development and tumor growth. When YAP is depleted in BCC tumors, the JAK- JUN pathway, a well-established tumor-driving cascade, is impaired. However, manipulation of YAP does not affect canonical Wnt or HH signaling. This suggests that Hippo signaling is a HH-independent promoter of BCC tumorigenesis, possibly functioning in parallel with the HH pathway to perpetuate BCC growth.

Our findings in both Chapters 2 and 3 also support parallel, or possibly downstream, athways that function in conjunction with HH signaling in BCCs. In Chapter 2, we demonstrate that mTOR is overexpressed in both human and mouse BCCs and that its inhibition results in a decrease in BCC cell growth *in vitro* and *in vivo* without affecting HH signaling. This implies that mTOR functions either downstream of or in parallel with the HH pathway. Additionally, we show that mTOR inhibition suppresses aPKC activation, and although aPKC is known to directly phosphorylate and activate GLI1, we don't observe any reduction of GLI1 phosphorylation upon mTOR inhibition. These data suggest that mTOR is regulating BCC growth via aPKC independently of HH signaling. Moreover, aPKC is a well-established oncogene, affecting a plethora of other pathways which influence invasion, tumor growth, and survival (Fields & Regala, 2009), so additional work is needed to determine which downstream targets of aPKC are being affected in an mTOR-dependent manner. In Chapter 3, we also demonstrate that PI3K is overexpressed in

both human and mouse BCCs and that PI3K inhibition suppresses BCC growth without affecting HH signaling *in vivo*. This, too, suggests that PI3K likely operates either downstream of or in parallel with the HH pathway. We show that PI3K inhibition results in a decrease in aPKC and AKT phosphorylation, both targets of PI3K, and that they possibly converge onto p21. Therefore, our data suggests that PI3K promotes BCC tumor growth by kinase-induced p21 degradation.

Application of R26SmoM2; *Gli1-Cre^{ERT2}* mouse model for studying drug resistance

SMO W535L is a common mutation found in sporadic and drug-resistant BCC tumors. This residue is located outside the drug-binding pocket and mutation at this site is believed to change the conformation, making the drug-binding pocket inaccessible to inhibitors. This allows for continued growth even in the presence of drugs (Liao et al., 2020; Danial et al., 2015; Atwood et al., 2015). Our lab has recently started using a *R26SmoM2*; *Gli1-Cre^{ERT2}* (SmoM2) mouse model, which possesses the W535L mutation, in order to study BCC drug resistance.

Since we've been able to show that Src inhibition can effectively repress HH signaling and reduce BCC tumor burden, we next wanted to evaluate whether Src inhibition could be used as an effective therapeutic to treat drug resistant BCCs. We initiated BCC tumor growth in SmoM2 mice for 16 weeks and, subsequently, intraperitoneally injected with either DMSO, 50mg/kg vismodegib, or 5mg/kg KX01 for seven consecutive days. Histological staining of the dorsal skin of vismodegib-treated mice confirmed that this mouse model is indeed resistant to SMO-inhibitor treatment and KX01-treated mice showed no significant reduction in total tumor size compared to DMSO controls (Figure 5.1 A- B). Therefore, our preliminary data suggests that KX01 treatment alone cannot reduce tumor burden.

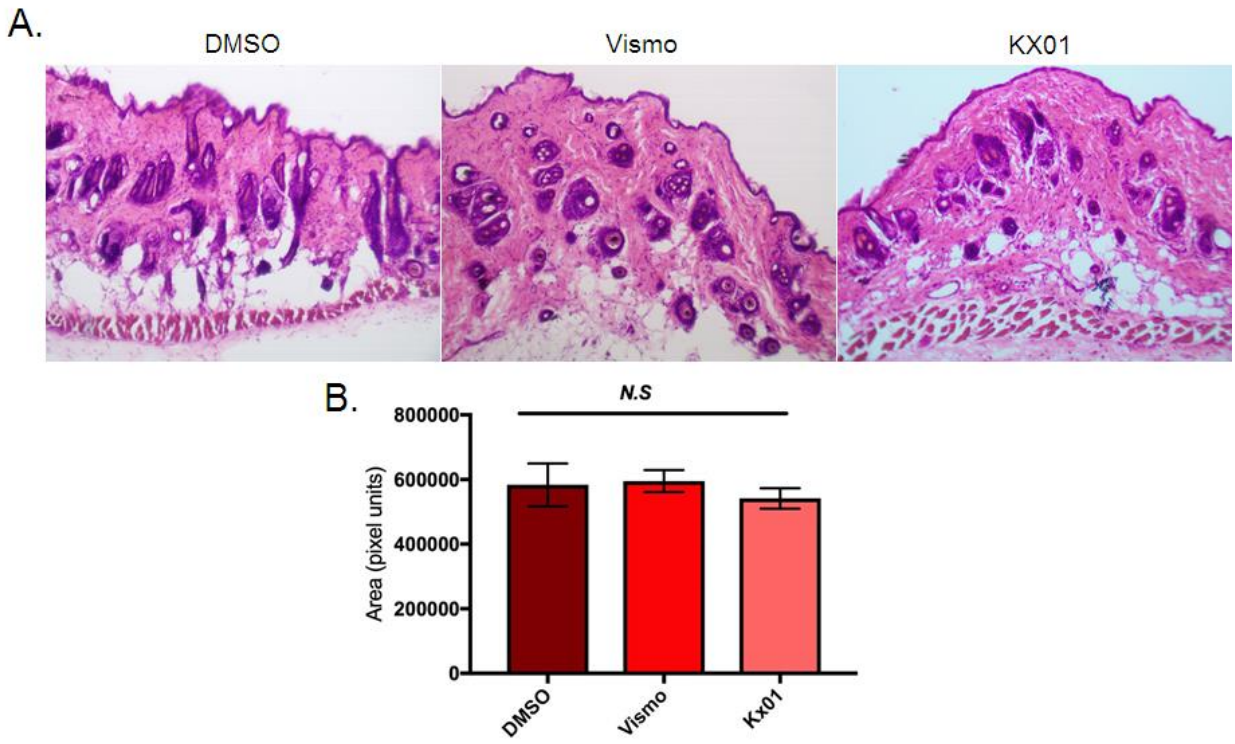

Figure 5.1. Inhibition of Src does not suppress drug-resistant BCC growth. A) Hematoxylin and eosin staining of dorsal back skin collected from DMSO- or KX01-treated *R26SmoM2*; *Gli1-Cre*^{ERT2} mice. Scale bar, 50 μm. **B)** Quantification of total tumor size per square area (n > 150 tumors from 3 mice). AU, arbitrary unit. Error bars, SEM.

Studies on ovarian and lung cancers have shown that Src overexpression has been linked to disease progression and the development of drug resistance. And as these studies have demonstrated that Src inhibition can restore drug sensitivity to resistant tumors (Pengetnze et al., 2003; Zhang et al., 2014), we propose treating SmoM2 mice with both vismodegib and KX01 to see if this same type of phenomenon is possible in BCCs. If so, it would suggest that Src plays an important role in the development of SMO-inhibitor resistance in BCCs and that combination treatment could serve as a promising therapy for patients with resistant tumors.

Additionally, as our work suggests that inhibition of both mTOR and PI3K can reduce BCC growth independently of HH signaling, it would be advantageous for us to examine how inhibiting both of these pathways affects drug resistance as well. If inhibiting mTOR and/or PI3K can reduce tumor burden in SmoM2 mice, it would provide promising therapies in the battle against BCC drug resistance.

Alternative mechanisms of resistance in BCCs

A majority of drug resistance in BCCs has been attributed predominantly to SMO mutations (Atwood et al., 2015; Ridky and Cotsarelis, 2015), and to a lesser extent, SUFU inactivation mutations and abnormal GLI2 amplification (Sharpe et al., 2015). Although this portrays a need to identify therapeutic targets that are either downstream of SMO or targets that contribute to non-canonical activation of HH signaling, more research needs to be allocated towards targeting the small subpopulations of BCC cells that are inherently resistant and insensitive to HH inhibitors. More recent work done by a few labs have identified two alternative pathways that could be driving BCC progression following drug treatment and dictating response to drugs.

Sánchez-Danés et al. (2018) identified a persistent BCC cell population that is driven by Wnt signaling. They showed that vismodegib treatment induces tumor regression by promoting tumor differentiation. However, even after treatment, a small population of slow-cycling tumor cells persists. These persistent cells display reduced proliferation but no change in apoptosis following treatment, suggesting that the cells are entering quiescence and ultimately contributing to the emergence of drug resistance. *Lgr5*, a Wnt target gene, is highly expressed in non-treated BCCs and its expression persists, albeit to a slightly lesser degree, with vismodegib treatment (Sánchez-Danés et al., 2018). They demonstrated that these Lgr5+ cells undergo a phenotypic switch from proliferative to quiescent upon vismodegib treatment. However, once vismodegib treatment is discontinued, these cells switch back to a proliferative state. As Wnt inhibition alone

cannot reduce tumor burden in mice (Youssef et al., 2012; Yang et al., 2008), they combined vismodegib treatment with Lgr5 lineage ablation in *K14CreER/ Ptch1cKO/Lgr5-DTR-GFP* mice and showed that there is almost a complete elimination of the drug resistant cells (99.5%) and a significant reduction in tumor burden (Sánchez-Danés et al., 2018). Their data suggests that HH inhibition alone promotes the maturation of a drug-resistant, persistent tumor cell population but that dual inhibition of both HH and Wnt pathways can eliminate a majority of the Lgr5+ cells, preventing tumor relapse. Similarly, work by Biehs et al. (2018) also demonstrated that BCC cells undergo an identity switch upon vismodegib treatment. They show that the original transcription program of BCC, which resembles that of the hair follicle bulge, switches to that of the stem cells of the IFE and isthmus. Therefore, these tumor cells are becoming quiescent and are able to evade drug treatment. This identity switch is also associated with rapid activation of the Wnt pathway. Ultimately, they, too, demonstrated that combined treatment with vismodegib and a Wnt pathway inhibitor in a different *Ptch1-Trip53* mouse model can drastically reduce tumor burden (Biehs et al., 2018). Together, these two groups demonstrated that BCC drug resistance is a result of tumor cell persistence following vismodegib treatment.

Another pathway that has been shown to promote BCC tumor persistence is the Notch signaling pathway (Eberl et al., 2018). However, even though Notch 1/2 mutations are among the most commonly detected in BCCs and normal skin, the Notch pathway is not a driver of BCC tumorigenesis (Bonilla et al., 2016; Jayaraman et al., 2014; Martincorena et al., 2015). Eberl et al. provide supporting evidence that the 3-dimensional architecture of BCCs dictates its drug response and that this response can be adjusted by modulating Notch. Tumors from *Ptch1*$^{fl/fl}$; *Gli1-Cre*^{ERT2} mice consist of peripheral HH^{+++}/Notch^{-} basal cells and HH^{++}/Notch^{+} suprabasal cells with vismodegib-induced apoptosis only occurring in the HH^{++}/Notch^{+} suprabasal cells whereas the peripheral HH^{+++}/Notch^{-} basal cells display some type of survival advantage. This is consistent

with previous reports which show that inhibition of HH signaling results in reduced proliferation and induced apoptosis (Eberl et al., 2018; Hutchin et al., 2005; Williams et al., 2003). Additionally, Shi et al. (2017) show that the Notch pathway is normally suppressed in BCC cells and that upon stimulation of this signaling pathway, apoptosis is induced. So building upon previous studies, Eberl et al. have been able to determine that activating Notch can cause existing tumors to regress while inhibiting this pathway promotes tumor persistence. Also, when Notch signaling is blocked in these BCC tumors, there is an increase in tumor persistence, and they demonstrate with *SmoM2;Gli1-Cre*^{ERT2} mice that this persistence is independent of HH-driven drug resistance. Together, they conclude that Notch inhibition can lead to tumor persistence without causing drug resistance. Even if they do not consider Notch inhibition as a mechanism of drug resistance, others may still think of this as a mode of resistance depending on how one defines what drug resistance means. However, regardless of terminology, Notch modulation has the ability to dictate how BCCs respond to drug treatment.

Summary

As SMO-inhibitor drug resistance is a common dilemma and recurring issue in BCCs, we sought out to unveil alternative targets for combating and overcoming resistance. In Chapters 2 and 3, we demonstrated that both mTOR and PI3K could both be working either downstream of or in parallel with HH signaling. This makes both of these pathways encouraging targets as an alternative means for treating normal BCCs and as a novel means for treating resistant BCCs. Additionally, in Chapter 4, we showed that Src is a non-canonical promoter of HH signaling via its activation of aPKC. This, too, makes Src a promising therapeutic target for both drug-sensitive and -resistant BCCs. Furthermore, it would be interesting to see if any of these pathways are further upregulated in resistant BCCs compared to normal BCCs, suggesting that they play a role in the development of drug resistance. This would shed light and provide a better understanding

of alternative modes of drug resistance development. As such, more work needs to be done to paint an entire picture of all potential regulators of BCC pathogenesis and progression.

REFERENCES

Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., & Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Current Biology 7*(4): 261-269.

Alexaki, V. I., Javelaud, D., Van Kempen, L. C., Mohammad, K. S., Dennler, S., Luciani, F., Hoek, K. S., Juarez, P., Goydos, J. S., Fournier, P. J., Sibon, C., Bertolotto, C., Verrecchia, F., Saule, S., Delmas, V., Ballotti, R., Larue, L., Saiag, P., Guise, T. A., & Mauviel, A. (2010). GLI2-mediated melanoma invasion and metastasis. *Journal of the National Cancer Institute 102*(15): 1148–1159.

Anbalagan, M., Carrier, L., Glodowski, S., Hangauer, D., Shan, B., & Rowan, B. G. (2012). KX-01, a novel Src kinase inhibitor directed toward the peptide substrate site, synergizes with tamoxifen in estrogen receptor α positive breast cancer. *Breast Cancer Research and Treatment 132*(2): 391-409.

Armas-López, L., Zúñiga, J., Arrieta, O., & Ávila-Moreno, F. (2017). The Hedgehog-GLI pathway in embryonic development and cancer: implications for pulmonary oncology therapy. *Oncotarget 8*(36): 60684-703.

Atwood, S. X., Li, M., Lee, A., Tang, J. Y., & Oro, A. E. (2013). GLI activation by atypical protein kinase C i/l regulates the growth of basal cell carcinoma. *Nature 494*(7438): 484-8.

Atwood, S. X., Sarin, K. Y., Whitson, R. J., Li, J. R., Kim, G., Rezaee, M., Ally, M. S., Kim, J., Yao, C., Chang, A. L., Oro, A. E., & Tang, J. Y. (2015). Smoothened variants explain the majority of drug resistance in basal cell carcinoma. *Cancer 27*(3): 342-53.

Atwood, S. X., Chabu, C., Penkert, R. R., Doe, C. Q., & Prehoda, K. E. (2007). Cdc42 acts downstream of Bazooka to regulate neuroblast polarity through Par-6 aPKC. *Journal of Cell Science 120*: 3200–3206.

Beauchamp, E. M., Ringer, L., Bulut, G., Sajwan, K. P., Hall, M. D., Lee, Y. C., Peaceman, D., Ozdemirli, M., Rodriguez, O., Macdonald, T. J., Albanese, C., Toretsky, J. A., & Uren, A. (2011). Arsenic trioxide inhibits human cancer cell growth and tumor development in mice by blocking Hedgehog/GLI pathway. *Journal of Clinical Investigation 121*(1): 148–160.

Benjamin, D., Colombi, M., Moroni, C., and Hall, M.N. (2011). Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discov. 10*: 868-880.

Bershteyn, M., Atwood, S. X., Woo, W. M., Li, M., & Oro, A. E. (2010). MIM and cortactin antagonism regulates ciliogenesis and hedgehog signaling. *Developmental Cell 19*(2): 270-283.

Biehs, B., Dijkgraaf, G., Piskol, R., Alicke, B., Boumahdi, S., Peale, F., Gould, S. E., & de Sauvage, F. J. (2018). A cell identity switch allows residual BCC to survive Hedgehog pathway inhibition. *Nature 562*(7727): 429–433.

Boni, J., Rubio-Perez, C., Lopez-Bigas, N., Fillat, C., & de la Luna, S. (2020). The DYRK family of kinases in cancer: molecular functions and therapeutic opportunities. *Cancers 12*(9): 2106-2132.

Bonilla, X., Parmentier, L., King, B., Bezrukov, F., Kaya, G., Zoete, V., Seplyarskiy, V. B., Sharpe, H. J., McKee, T., Letourneau, A., Ribaux, P. G., Popadin, K., Basset-Seguin, N., Ben Chaabene, R., Santoni, F. A., Andrianova, M. A., Guipponi, M., Garieri, M., Verdan, C., Grosdemange, K., Sumara, O., Eilers, M., Aifantis, I., Michielin, O., de Sauvage, F. J., Antonarakis, S. E., & Nikolaev, S. I. (2016). Genomic analysis identifies new drivers and progression pathways in skin basal cell carcinoma. *Nature Genetics 48*(4): 398-406.

Brechbiel, J., Miller-Moslin, K., & Adjei, A. A. (2014). Crosstalk between hedgehog and other signaling pathways as a basis for combination therapies in cancer. *Cancer Treatment Reviews 40*(6): 750-9.

Brown, J. A., Yonekuba, Y., Hanson, N., Sastre-Perona, A., Basin, A., Rytlewski, J. A., Dolgalev, I., Meehan, S., Tsirigos, A., Beronja, S., & Schober, M. (2017). TGF-β-Induced Quiescence Mediates Chemoresistance of Tumor-Propagating Cells in Squamous Cell Carcinoma. *Cell Stem Cell 21*(5): 650–664.

Brown, M.T. & Cooper, J.A. (1996) Regulation, substrates and functions of src. *Biochimica et Biophysica Acta 1287*(2-3): 121-49.

Buonamici, S., Williams, J., Morrissey, M., Wang, A., Guo, R., Vattay, A., Hsiao, K., Yuan, J., Green, J., Ospina, B., Yu, Q., Ostrom, L., Fordjour, P., Anderson, D. L., Monahan, J. E., Kelleher, J. F., Peukert, S., Pan, S., Wu, X., Maira, S. M., García-Echeverría, C., Briggs, K. J., Watkins, D. N., Yao, Y. M., Lengauer, C., Warmuth, M., Sellers, W. R., & Dorsch, M. (2010). Interfering with resistance to smoothened antagonists by inhibition of the PI3K pathway in medulloblastoma. *Science Translational Medicine 2*(51), 51ra70.

Burger, M. T., Pecchi, S., Wagman, A., Ni, Z. J., Knapp, M., Hendrickson, T., Atallah, G., Pfister, K., Zhang, Y., Bartulis, S., Frazier, K., Ng, S., Smith, A., Verhagen, J., Haznedar, J., Huh, K., Iwanowicz, E., Xin, X., Menezes, D., Merritt, H., Lee, I., Wiesmann, M., Kaufman, S., Crawford, K., Chin, M., Bussiere, D., Shoemaker, K., Zaror, I., Maira, S. M., & Voliva, C. F. (2011). Identification of NVP-BKM120 as a Potent, Selective, Orally Bioavailable Class I PI3 Kinase Inhibitor for Treating Cancer. *Cell Stem Cell 16*(4): 400-12.

Cai, X., Yu, K., Zhang, L., Li, Y., Li, Q., Yang, Z., Shen, T., Duan, L., Xiong, W., & Wang, W. (2015). Synergistic inhibition of colon carcinoma cell growth by Hedgehog-Gli1 inhibitor arsenic trioxide and phosphoinositide 3-kinase inhibitor LY294002. *Onco Targets and Therapy* 8: 877-83.

Chang, A. L. & Oro, A. E. (2012). Initial assessment of tumor regrowth after vismodegib in advanced Basal cell carcinoma. *Archives of Dermatology 148*(11): 1324-5.

Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S., & Toker, A. (1998). Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. *Current Biology 8*(19): 1069-77.

Chow, R., Levee, T. M., Kaur, G., Cedeno, D. P., Doan, L. T., & Atwood, S. X. (2021). mTOR promotes basal cell carcinoma growth through atypical PKC. *Experimental Dermatology 30*(1): 358-366.

Courtneidge, S. A., Fumagalli, S., Koegl, M., Superti-Furga, G., & Twamley-Stein, G. M. (1993). The Src family of protein tyrosine kinases: regulation and functions. *Development (Cambridge, England). Supplement*, 57–64.

D'Amico, D., Antonucci, L., Di Magno, L., Coni, S., Sdruscia, G., Macone, A., Miele, E., Infante, P., Di Marcotullio, L., De Smaele, E., Ferretti, E., Ciapponi, L., Giangaspero, F., Yates, J. R., 3rd, Agostinelli, E., Cardinali, B., Screpanti, I., Gulino, A., & Canettieri, G. (2015). Non-canonical hedgehog/AMPK-mediated control of polyamine metabolism supports neuronal and medulloblastoma cell growth. *Developmental Cell*, *35*(1): 21–35.

Danial, C., Sarin, K. Y., Oro, A. E., & Chang, A. L. (2016). An Investigator-Initiated Open-Label Trial of Sonidegib in Advanced Basal Cell Carcinoma Patients Resistant to Vismodegib. *Clinical Cancer Research 22*(6): 1325–1329.

David, C. J. & Massagué, J. (2018). Contextual determinants of TGFβ action in development, immunity and cancer. *Nature Review Molecular Cell Biology 19*(7): 419–435.

Debaugnies, M., Sanchez-Danes, A., Rorive, S., Raphael, M., Liagre, M., Parent, M.A., Brisebarre, A., & Salmon, I. (2018). YAP and TAZ are essential for basal and squamous cell carcinoma initiation. *EMBO Reports 19*(7): e45809.

Dennler, S., André, J., Alexaki, I., Li, A., Magnaldo, T., ten Dijke, P., Wang, X. J., Verrecchia, F., & Mauviel, A. (2007). Induction of sonic hedgehog mediators by transforming growth factor-beta: smad3-dependent activation of Gli2 and Gli1 expression in vitro and in vivo. *Cancer Research 67*(14): 6981–6986.

Dennler, S., André J., Verrecchia, F., & Mauviel, A. (2009). Cloning of the human GLI2 promoter: transcriptional activation by transforming growth factor-beta via SMAD3/beta-catenin cooperation. *Journal of Biological Chemistry 284*(46): 31523–31531.

Deshpande, I., Liang, J., Hedeen, D., Roberts, K. J., Zhang, Y., Ha, B., Latorraca, N. R., Faust, B., Dror, R. O., Beachy, P. A., Myers, B. R., & Manglik, A. (2019). Smoothened stimulation by membrane sterols drives Hedgehog pathway activity. *Nature 571*(7764): 284-8.

Di Magno, L., Basile, A., Coni, S., Manni, S., Sdruscia, G., D'Amico, D., Antonucci, L., Infante, P., De Smaele, E., Cucchi, D., Ferretti, E., Di Marcotullio, L., Screpanti, I., & Canettieri, G. (2016). The energy sensor AMPK regulates Hedgehog signaling in human cells through a unique Gli1 metabolic checkpoint. *Oncotarget*, *7*(8): 9538–9549.

Diao, Y., Rahman, M. F., Villegas, V. E., Wickström, M., Johnsen, J. I., & Zaphiropoulos, P. G. (2014). The impact of S6K1 kinase on neuroblastoma cell proliferation is independent of GLI1 signaling. *BMC Cancer* 14: 600.

Dijkgraaf, G. J., Alicke, B., Weinmann, L., Januario, T., West, K., Modrusan, Z., Burdick, D., Goldsmith, R., Robarge, K., Sutherlin, D., Scales, S. J., Gould, S. E., Yauch, R. L., & de Sauvage, F. J. (2011). Small molecule inhibition of GDC-0449 refractory smoothened mutants and downstream mechanisms of drug resistance. *Cancer Research 71*(2): 435-44.

Drummond, M. L., Li, M., Tarapore, E., Nguyen, T. T. L., Barouni, B. J., Cruz, S., Tan, K. C., Oro, A. E., & Atwood, S. X. (2018). Actin polymerization controls cilia-mediated signaling. *Journal of Cell Biology 217*(9): 3255-66.

Eberl, M., Mangelberger, D., Swanson, J. B., Verhaegen, M. E., Harms, P. W., Frohm, M. L., Dlugosz, A. A., & Wong, S. Y. (2018). Tumor Architecture and Notch Signaling Modulates Drug Response in Basal Cell Carcinoma. *Cancer Cell 33*(2): 229-43.

Ehe, B. K., Lamson, D. R., Tarpley, M., Onyenwoke, R. U., Graves, L. M., & Williams, K. P. (2017). Identification of a DYRK1A-mediated phosphorylation site within the nuclear localization sequence of the hedgehog transcription factor GLI1. *Biochemical and Biophysical Research Communications 491*(3): 767–772.

Eibenschutz, L., Colombo, & D., Catricalà, C. (2013). Everolimus for compassionate use in multiple Basal cell carcinomas. *Case Reports in Dermatological Medicine* 2013.

Engelman, J. A., Luo, J., & Cantley, L. C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nature Reviews Genetics 7*(8): 606-19.

Epstein, E. H. Basal cell carcinoma: attack of the hedgehog. *Nature Reviews Cancer 8*(1): 743-754.

Fan, Q., He, M., Sheng, T., Zhang, X., Sinha, M., Luxon, B., Zhao, Q., & Xie, J. (2010). Requirement of TGFbeta signaling for SMO-mediated carcinogenesis. *Journal of Biological Chemistry 285*(47): 36570–36576.

Fields, A. P. & Regala, R. P. (2007). Protein kinase C iota: human oncogene, prognostic marker and therapeutic target. *Pharmacological Research 55*(6): 487–497.

Frey, M. R., Clark, J. A., Leontieva, O., Uronis, J. M., Black, A. R., & Black, J. D. (2000). Protein kinase C signaling mediates a program of cell cycle withdrawal in the intestinal epithelium. *The Journal of Cell Biology 151*(4): 763-777.

Gajjar, A., Stewart, C. F., Ellison, D. W., Kaste, S., Kun, L. E., Packer, R. J., Goldman, S., Chintagumpala, M., Wallace, D., Takebe, N., Boyett, J. M., Gilbertson, R. J., & Curran, T. (2013). Phase I study of vismodegib in children with recurrent or refractory medulloblastoma; a pediatric brain tumor consortium study. *Clinical Cancer Research 19*(22): 6305-12.

Galanos, P., Vougas, K., Walter, D., Polyzos, A., Maya-Mendoza, A., Haagensen, E. J., Kokkalis, A., Roumelioti, F. M., Gagos, S., Tzetis, M., Canovas, B., Igea, A., Ahuja, A. K., Zellweger, R., Havaki, S., Kanavakis, E., Kletsas, D., Roninson, I. B., Garbis, S. D., Lopes, M., Nebreda, A., Thanos, D., Blow, J. J., Townsend, P., Sørensen, C. S., Bartek, J., & Gorgoulis, V. G. (2016). Chronic p53-independent p21 expression causes genomic instability by deregulating replication licensing. *Nature Cell Biology 18*(7): 777-89.

Georgakilas, A. G., Martin, O. A., & Bonner, W. M. (2017). p21: A Two-Faced Genomic Guardian. *Trends in Molecular Medicine 23*(4): 310-19.

Gioeli, D., Wunderlich, W., Sebolt-Leopold, J., Bekiranov, S., Wulfkuhle, J. D., Petricoin, E. F., III, Conaway, M., & Weber, M. J. (2011). Compensatory pathways induced by MEK inhibition are effective drug targets for combination therapy against castration-resistant prostate cancer. *Molecular Cancer Therapeutics 10*(9): 1581–1590.

Graab, U., Hahn, H., & Fulda, S. (2015). Identification of a novel synergistic lethality of combined inhibition of hedgehog and PI3K signaling in rhabdomyosarcoma. *Oncotarget 6*(11): 8722-35.

Gruber, W., Hutzinger, M., Elmer, D. P., Parigger, T., Sternberg, C., Cegielkowski, L., Zaja, M., Leban, J., Michel, S., Hamm, S., Vitt, D., & Aberger, F. (2016). DYRK1B as therapeutic target in Hedgehog/GLI-dependent cancer cells with Smoothened inhibitor resistance. *Oncotarget 7*(6): 7134-48.

Gruber, W., Peer, E., Elmer, D. P., Sternberg, C., Tesanovic, S., Del Burgo, P., Coni, S., Canettieri, G., Neureiter, D., Bartz, R., Kohlhof, H., Vitt, D., & Aberger, F. (2018). Targeting class I histone deacetylases by the novel small molecule inhibitor 4SC-202 blocks oncogenic hedgehog-GLI signaling and overcomes smoothened inhibitor resistance. *International Journal of Cancer 142*(5): 968-75.

Gruber-Filbin, M., Dabral, S. K., Pazyra-Murphy, M. F., Ramkissoon, S., Kung, A. L., Pak, E., Chung, J., Theisen, M. A., Sun, Y., Franchetti, Y., Sun, Y., Shulman, D. S., Redjal, N., Tabak, B., Beroukhim, R., Wang, Q., Zhao, J., Dorsch, M., Buonamici, S., Ligon, K. L., Kelleher, J. F., & Segal, R. A. (2013). Coordinate activation of Shh and PI3K signaling in PTEN-deficient glioblastoma: new therapeutic opportunities. *Nature Medicine 19*(11): 1518–1523.

Huangfu, D. & Anderson, K. V. (2005). Cilia and hedgehog responsiveness in the mouse. *PNAS 102*(32): 11325-11330.

Humke, E. W., Dorn, K. V., Milenkovic, L., Scott, M. P., & Rohatgi, R. (2010). The output of Hedgehog Signaling is controlled by the dynamic association between Suppressor of Fused and Gli proteins. *Genes & Development 24*(7): 670-82.

Hutchin, M. E., Kariapper, M. S., Grachtchouk, M., Wang, A., Wei, L., Cummings, D., Liu, J., Michael, L. E., Glick, A., & Dlugosz, A. A. (2005). Sustained Hedgehog signaling is required for basal cell carcinoma proliferation and survival: conditional skin tumorigenesis recapitulates the hair growth cycle. *Genes & Development 19*(2),:214–223.

Hyman, J. M., Firestone, A. J., Heine, V. M., Zhao, Y., Ocasio, C. A., Han, K., Sun, M., Rack, P. G., Sinha, S., Wu, J. J., Solow-Cordero, D. E., Jiang, J., Rowitch, D. H., & Chen, J. K. (2009). Small-molecule inhibitors reveal multiple strategies for Hedgehog pathway blockade. *Proc. Natl. Acad. Sci. U.S.A. 106*(33): 14132–14137.

Inaba, M., Yamashita, Y. M., & Buszczak, M. (2016). Keeping stem cells under control: new insights into the mechanisms that limit niche-stem cell signaling within the reproductive system. *Molecular Reproduction & Development 83*(8): 675-683.

Infante, P., Mori, M., Alfonsi, R., Ghirga, F., Aiello, F., Toscano, S., Ingallina, C., Siler, M., Cucchi, D., Po, A., Miele, E., D'Amico, D., Canettieri, G., De Smaele, E., Ferretti, E., Screpanti, I., Uccello, B., Botto, M., Botta, B., Gulino, A., & Di Marcotullio, L. (2015). Gli1/DNA interaction is a druggable target for Hedgehog-dependent tumors. *EMBO Journal* 34: 200–217

Irby, R. B. & Yeatman, T. J. (2000). Role of Src expression and activation in human cancer. *Oncogene 19*: 5636-5642.

Ivey, R. A., Sajan, M. P., and Farese, R. V. (2014). Requirements for Pseudosubstrate Arginine Residues During Auto-inhibition and Phosphatidylinositol-3,4,5-(PO4)3-dependent Activation of Atypical PKC. *Journal of Biological Chemistry 289*(36): 25021-25130.

Jaju, P. D., Ransohoff, K. J., Tang, J. Y. & Sarin, K. Y. Familial skin cancer syndromes: Increased risk of nonmelanotic skin cancers and extracutaneous tumors. (2016). *Journal of the American Academy of Dermatology 74*(3), 437–451.

Jayaraman, S. S., Rayhan, D. J., Hazany, S., & Kolodney, M. S. (2014). Mutational landscape of basal cell carcinomas by whole-exome sequencing. *The Journal of investigative Dermatology 134*(1): 213–220.

Ji, Z., Mei, F. C., Xie, J., & Cheng, X. (2007). Oncogenic KRAS activates hedgehog signaling pathway in pancreatic cancer cells. *Journal of Biological Chemistry 282*(19): 4048–14055.

Jinawath, A., Akiyama, Y., Sripa B., & Yuasa, Y. (2007). Dual blockade of the hedgehog and ERK1/2 pathways coordinately decreases proliferation and survival of cholangiocarcinoma cells. *Journal of Cancer Research and Clinical Oncology 133*(4): 271–278.

Johnson, R. W., Nguyen, M. P., Padalecki, S. S., Grubbs, B. G., Merkel, A. R., Oyajobi, B. O., Matrisian, L. M., Mundy, G. R., & Sterling, J. A. (2011). TGF-beta promotion of Gli2-induced expression of parathyroid hormone-related protein, an important osteolytic factor in bone metastasis, is independent of canonical Hedgehog signaling. *Cancer Research 71*(3): 822–831.

Justilien, V., Walsh, M. P., Ali, S. A., Thompson, E. A., Murray, N. R., & Fields, A. P. (2014). The PRKCı and SOX2 oncogenes are coamplified and cooperate to activate Hedgehog signaling in lung squamous cell carcinoma. *Cancer Cell 25*(2): 139–151.

Kauvar, A. N., Cronin, T. Jr., Roenigk, R., Hruza, G., & Bennett, R. (2015). Consensus for nonmelanoma skin cancer treatment: basal cell carcinoma, including a cost analysis of treatment methods. *Dermatologic Surgery 41*(5): 550-71.

Kim, A. L., Back, J. H., Chaudhary, S. C., Zhu, Y., Athar, & M., Bickers, D. R. (2018). SOX9 Transcriptionally Regulates mTOR-Induced Proliferation of Basal Cell Carcinomas. *Journal of Investigative Dermatology 138*(8): 1716-25.

Kim, A. L., Back, J. H., Zhu, Y., Tang, X., Yardley, N. P., Kim, K. J., Athar, M., & Bickers, D. R. (2016). AKT1 Activation is Obligatory for Spontaneous BCC Tumor Growth in a Murine Model that Mimics Some Features of Basal Cell Nevus Syndrome. *Cancer Prevention Research 9*(10): 794-802.

Kim, H., Zamel, R., Bai, X. H., & Liu, M. (2013). PKC activation induces inflammatory response and cell death in human bronchial epithelial cells. *PLOS One 8*(1): e64182.

Kim, J., Aftab, B. T., Tang, J. Y., Kim, D., Lee, A. H., Rezaee, M., Kim, J., Chen, B., King, E. M., Borodovsky, A., Riggins, G. J., Epstein, E. H. Jr., Beachy, P. A., & Rudin, C. M. (2013). Itraconazole and arsenic trioxide inhibit Hedgehog pathway activation and tumor growth associated with acquired resistance to smoothened antagonists. *Cancer Cell 23*(1): 23-4.

Kim, L.C., Cook, R.S., & Chen, J. (2017). mTORC1 and mTORC2 in cancer and the tumor microenvironment. *Oncogene 36*: 2191-2201.

Kim, J., Lee, J. J., Kim, J., Gardner, D., & Beachy, P. A. (2010). Arsenic antagonizes the Hedgehog pathway by preventing ciliary accumulation and reducing stability of the Gli2 transcriptional effector. *Proc. Natl. Acad. Sci. U.S.A. 107*(30): 13432–13437.

Kim, S., Min, A., Lee, K. H., Yang, Y., Kim, T. Y., Lim, J. M., Park, S. J., Nam, H. J., Kim, J. E., Song, S. H., Han, S. W., Oh, D. Y., Kim, J. H., Kim, T. Y., Hangauer, D., Lau, J. Y. N., Im, K., Lee, D. S., Bang, Y. J., & Im, S. A. (2017). Antitumor effect of KX-01 through inhibiting Src family kinases and mitosis. *Cancer Research and Treatment 49*(3): 643-655.

Klawitter, J., Nashan, B., & Christians, U. (2015). Everolimus and sirolimus in transplantation-related but different. *Expert Opin Drug Saf. 14*(7): 1055-1070.

Kloth, M. T., Laughlin, K. K., Biscardi, J. S., Boerner, J. L., Parsons, S. J., & Silva, C. M. (2003). STAT5b, a Mediator of Synergism between c-Src and the Epidermal Growth Factor Receptor. *The Journal of Biological Chemistry 278*(3): 1671–1679.

Kuleshov, M. V., Jones, M. R., Rouillard, A. D., Fernandez, N. F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S. L., Jagodnik, K. M., Lachmann, A., McDermott, M. G., Monteiro, C. D., Gundersen, G. W., & Ma'ayan, A. (2016). Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids Research 44*(W1): W90-7.

Kuonen, F., Huskey, N.E., Shankar, G., Jaju, P., Whitson, R.J., Rieger, K.E., Atwood, S.X., Sarin, K.Y., & Oro, A.E. (2019). Loss of primary cilia drives switching from hedgehog to Ras/MAPK pathway in resistant basal cell carcinoma. *Journal of Investigative Dermatology 139*(7): 1439-1448.

Lachmann, A. & Ma'ayan, A. (2009). KEA: kinase enrichment analysis. *Bioinformatics 25*(5): 684-6.

Lauth, M., Bergström, A., Shimokawa, T., & Toftgård, R. (2007). Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists. *Proc. Natl. Acad. Sci. U.S.A. 104*(20): 8455–8460.

Lauth, M., Bergström, A., Shimokawa, T., Tostar, U., Jin, Q., Fendrich, V., Guerra, C., Barbacid, M., & Toftgård, R. (2010). DYRK1B-dependent autocrine-to-paracrine shift of Hedgehog signaling by mutant RAS. *Nature Structural Molecular Biology 17*(6): 718–725.

LaVallee, T. M., Prudovsky, I. A., McMahon, G. A., Hu, X., & Maciag, T. (1998). Activation of the MAP kinase pathway by FGF-1 correlates with cell proliferation induction while activation of the Src pathway correlates with migration. *The Journal of Cell Biology 141*(7): 1647–1658.

Law, M., Forrester, E., Chytil, A., Corsino, P., Green, G., Davis, B., Rowe, T., & Law, B. (2006). Rapamycin disrupts Cyclin/Cyclin-Dependent Kinase/p21/Proliferating Cell Nuclear Antigen Complexes and Cyclin D1 reverses rapamycin action by stabilizing these complexes. *Cancer Res 66*(2): 1070-1080.

Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., & Parker, P. J. (1998). Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science 281*(5385): 2042-5.

Lear, J. T., Corner, C., Dziewulski, P., Fife, K., Ross, G. L., Varma, S., & Harwood, C. A. (2014). Challenges and new horizons in the management of advanced basal cell carcinoma: a UK perspective. *British Journal of Cancer 1111*(8): 1476-81.

Lear, W., Dahlke, E., & Murray, C.A. (2007). Basal Cell Carcinoma: Review of Epidemiology, Pathogenesis, and Associated Risk Factors. *Journal of Cutaneous Medicine and Surgery 11*(1):19-30.

Lee, J. H., Pyon, J.K., Kim, D. W., Lee, S. H., Nam, H. S., Kim, C. H., Kang, S. G., Lee, Y. J., Park, M. Y., Jeong, D. J., & Cho, M. K. (2010). Elevated c-Src and c-Yes expression in malignant skin cancers. *Journal of Experimental & Clinical Cancer Research 29*: 116-122.

Leung, E. Y., Askarian-Amiri, M., Finlay, G.J., Rewcastle, G.W., & Baguley, B.C. (2015)/ Potentiation of growth inhibitory responses of the mTOR inhibitor everolimus by dual mTORC1/2 inhibitors in cultured breast cancer cell lines. *PLoS One 10*(7): e0131400.

Levitzki A. (1996). SRC as a target for anti-cancer drugs. *Anticancer Drug Design 11*(3):175-82.

Li, L., Zhao, G. D., Shi, Z., Qi, L. L., Zhou, L. Y., & Fu, Z. X. (2016). The Ras/Raf/MEK/ERK signaling pathway and its role in the occurrence and development of HCC. *Oncology Letters 12*(5): 3045-3050.

Li, Y. H., Luo, J., Mosley, Y. Y., Hedrick, V. E., Paul, L. N., Chang, J., Zhang, G., Wang, Y. K., Banko, M. R., Brunet, A., Kuang, S., Wu, J. L., Chang, C. J., Scott, M. P., & Yang, J. Y. (2015). AMP-activated protein kinase directly phosphorylates and destabilizes hedgehog pathway transcription factor GLI1 in medulloblastoma. *Cell reports*, *12*(4): 599–609.

Liao, S., Floyd, C., Verratti, N., Leung, L., & Wu, C. (2020). Analysis of vismodegib resistance in D473G and W535L mutants of SMO receptor and design of novel drug derivatives using molecular dynamics simulations. *Life Sciences 244*: 117302.

Lin, X. & Gao, T. (2014). mTORC2 phosphorylates protein kinase Cz to regulate its stability and activity. *EMBO Rep. 15*(2): 191-198.

Lu, J., Liu, L., Zheng, M., Li, X., Wu, A., Wu, Q., Liao, C., Zou, J., & Song, H. (2018). MEKK2 and MEKK3 suppress hedgehog pathway-dependent medulloblastoma by inhibiting GLI function. *Oncogene 37*: 3864-3878.

Luttrell, D. K., Lee, A., Lansing, T.J., Crosby, R. M., Jung, K. D., Willard, D., Luther, M., Rodriguez, M., Berman, J., & Gilmer, T.M. (1994). Involvement of pp60c-src with two major signaling pathways in human breast cancer. *Proceedings of the National Academy of Sciences of the United States of America 91*(1): 83-87.

Ma, Y., Zhang, P., Wang, F., Yang, J., Yang, Z., & Qin, Hbon. The relationship between early embryo development and tumourigenesis. *J Cell Mol Med.* 2010; 14(12): 2697-2701.

MacKeigan, J.P. & Krueger, D.A. (2015). Differentiating the mTOR inhibitors everolimus and sirolimus in the treatment of tuberous sclerosis complex. *Neuro Oncol. 17*(12): 1550-1559.

Maglic, D., Schlegelmilch, K., Dost, A. F., Panero, R., Dill, M. T., Calogero, R. A., & Camargo, F. D. (2018). YAP-TEAD signaling promotes basal cell carcinoma development via a c-JUN/AP1 axis. *The EMBO Journal 37*(17): e98642.

Mao, J., Maye, P., Kogerman, P., Tejedor, F. J., Toftgard, R., Xie, W., Wu, G., & Wu, D. (2002). Regulation of Gli1 transcriptional activity in the nucleus by Dyrk1. *The Journal of Biological Chemistry 277*(38): 35156–35161.

Mao, W., Irby, R., Coppola, D., Fu, L., Wloch, M., Turner, J., Yu, H., Garcia, R., Jove, R., & Yeatman, T. J. (1997). Activation of c-Src by receptor tyrosine kinases in human colon cancer cells with high metastatic potential. *Oncogene 15*(25): 3083–3090.

Martincorena, I., Roshan, A., Gerstung, M., Ellis, P., Van Loo, P., McLaren, S., Wedge, D. C., Fullam, A., Alexandrov, L. B., Tubio, J. M., Stebbings, L., Menzies, A., Widaa, S., Stratton, M. R., Jones, P. H., & Campbell, P. J. (2015). Tumor evolution. High burden and pervasive positive selection of somatic mutations in normal human skin. *Science (New York, N.Y.) 348*(6237): 880–886.

Mazumdar, T., DeVecchio, J., Agyeman, A., Shi, T., & Houghton, J. A. (2011). The GLI genes as the molecular switch in disrupting Hedgehog signaling in colon cancer. *Oncotarget 2*(8): 638–645.

Miati, S., Mondal, S., Satyavarapu, E.M., Mandal, C. (2017). mTORC2 regulates hedgehog pathway activity by promoting stability to Gli2 protein and its nuclear translocation. *Cell Death and Disease 8*(7): e2926.

Mirza, M.A. Fry, N.M. Urman, S.X. Atwood, J. Roffey, G.R. Ott, B. Chen, A.S. Brown, S.Z. Aasi, T. Hollmig, M.A. Ator, B.D. Dorsey, B.R. Ruggeri, C.A. Zificsak, M. Sirota, J.Y. Tang, A. Butte, E. Epstein, K.Y Sarin, A.E. Oro, A.E. (2017). Combined inhibition of atypical PKC and histone deacetylase 1 is cooperative in basal cell carcinoma. *JCI Insight 2*(21): e97071.

Miyazaki, Y., Matsubara, S., Ding, Q., Tsukasa, K., Yoshimitsu, M., Kosai, K., & Takao, S. (2016). Efficient elimination of pancreatic cancer stem cells by hedgehog/GLI inhibitor GANT61 in combination with mTOR inhibition. *Molecular Cancer 15*(1):49.

Mohan, S. V. & Chang, A. L. S. (2014). Advanced basal cell carcinoma: epidemiology and therapeutic innovations. *Current Dermatology Reports 3*(1): 40-45.

Montagna, E. & Lopes, O. S. (2017). Molecular basis of basal cell carcinoma. *Brazilian Annals of Dermatology 92*(4): 517-520.

Morton, J. P., Mongeau, M. E., Klimstra, D. S., Morris, J. P., Lee, Y. C., Kawaguchi, Y., Wright, C. V. E., Hebrok, M., & Lewis, B. C.. (2007). Sonic hedgehog acts at multiple stages during pancreatic tumorigenesis. *Proc. Natl. Acad. Sci. U.S.A. 104*(12): 5103–5108.

Nagata, Y., Lan, K. H., Zhou, X., Tan, M., Esteva, F. J., Sahin, A. A., Klos, K. S., Li, P., Monia, B. P., Nguyen, N. T., Hortobagyi, G. N., Hung, M. C., & Yu, D. (2004). PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell 6*(2): 117–127.

Nam, H. J., Im, S. A., Oh, D. Y., Elvin, P., Kim, H. P., Yoon, Y. K., Min, A., Song, S. H., Han, S. H., Kim, T. Y., & Bang, Y. J. (2013). Antitumor activity of saracatinib (AZD0530), a c-Src/Abl kinase inhibitor, alone or in combination with chemotherapeutic agents in gastric cancer. *Molecular Cancer Therapeutics 12*(1): 16-26.

Neill, G. W., Ghali, L. R., Green, J. L., Ikram, M. S., Philpott, M. P., & Quinn, A. G. (2003). Loss of protein kinase Calpha expression may enhance the tumorigenic potential of Gli1 in basal cell carcinoma. *Cancer Research 63*(15): 4692–4697.

Nguyen, T. T. L., Tarapore, E., & Atwood, S. X. (2019) Common Skin Diseases – Skin Cancer. In Imaging technologies and transdermal delivery in skin disorders. Wiley. ISBN: 978-3-52734460-4.

Nolan-Stevaux, O., Lau, J., Truitt, M. L., Chu, G. C., Hebrok, M., Fernández-Zapico, M. E., & Hanahan, D. (2009). GLI1 is regulated through Smoothened-independent mechanisms in neoplastic pancreatic ducts and mediates PDAC cell survival and transformation. *Genes & Development 23*(1) 24–36.

Oatley, J. M. & Brinster, R. L. (2012). The germline stem cell niche unit in mammalian testes. *Physiological Reviews 92*(2): 577-595.

Oliver,T. G., Grasfeder, L. L., Carroll, A. L., Kaiser, C., Gillingham, C. L., Lin, S. M., Wickramasinghe, R., Scott, M. P., & Wechsler-Reya, R. J.. Transcriptional profiling of the sonic hedghehog response: a critical role for N-myc in proliferation and neuronal precursors. *PNAS* 2003; 100(12): 7331-7336.

Peer, E., Tesanovic, S., & Aberger, F. (2019). Next-Generation Hedgehog/GLI Pathway Inhibitors for Cancer Therapy. *Cancers 11*(4): 538.

Pelullo, M., Zema, S., Nardozza, F., Checquolo, S., Screpanti, I., & Bellavia, D. (2019). Wnt, Notch, and TGF-β Pathways Impinge on Hedgehog Signaling Complexity: An Open Window on Cancer. *Frontiers in Genetics*, *10*: 711.

Pengetnze, Y., Steed, M., Roby, K. F., Terranova, P. F., & Taylor, C. C. (2003). Src tyrosine kinase promotes survival and resistance to chemotherapeutics in a mouse ovarian cancer cell line. *Biochemical and Biophysical Research Communications 309*(2): 377–383.

Peterson, S. C., Eberl, M., Vagnozzi, A. N., Belkadi, A., Veniaminova, N. A., Verhaegen, M. E., Bichakjian, C. K., Ward, N. L., Dlugosz, A. A., & Wong, S. Y. (2015). Basal cell carcinoma preferentially arises from stem cells within hair follicle and mechanosensory niches. *Cell Stem Cell 16*(4): 400-12.

Pietrobono, S., and Stecca, B. (2018). Targeting the oncoprotein smoothened by small molecules: focus on novel acylguanidine derivatives as potent smoothened inhibitors. *Cells 7*(12):272.

Pietrobono, S., Gagliardi, S., & Stecca, B. (2019). Non-canonical Hedgehog Signaling Pathway in Cancer. Activation of GLI Transcription Factors Beyond Smoothened. *Frontiers in Genetics* 10: 556.

Raasch, B. A., Buettner, P. G., & Garbe, C. (2006). Basal cell carcinoma: histological classification and body-site distribution. *The British Journal of Dermatology 155*(2): 401–407.

Raffel, C., Jenkins, R. B., Frederick, L., Hebrink, D., Alderete, B., Fults, D. W., & James, C. D. (1997). Sporadic medulloblastomas contain PTCH mutations. *Cancer Research 47*(5): 842-5.

Rahimi, N., Hung, W., Tremblay, E., Saulnier, R., & Elliott, B. (1998). c-Src kinase activity is required for hepatocyte growth factor-induced motility and anchorage-independent growth of mammary carcinoma cells. *The Journal of Biological Chemistry 273*(50): 33714–33721.

Ramaswamy, B., Lu, Y., Teng, K. Y., Nuovo, G., Li, X., Shapiro, C. L., & Majumder, S. (2012). Hedgehog signaling is a novel therapeutic target in tamoxifen-resistant breast cancer aberrantly activated by PI3K/AKT pathway. *Cancer Research 72*(19): 5048-59.

Rehan, M. (2017). An anti-cancer drug candidate OSI-027 and its analog as inhibitors of mTOR: computation insights into the inhibitory mechanisms. *J Cell Biochem. 118*(12): 4558-4567.

Reifenberger, J., Wolter, M., Knobbe, C. B., Köhler, B., Schönicke, A., Scharwächter, C., Kumar, K., Blaschke, B., Ruzicka, T., & Reifenberger G. (2005). Somatic mutations in PTCH, SMOH, SUFUH, and TP53 genes in sporadic basal cell carcinomas. *British Journal of Dermatology 152*(1): 43-51.

Reina-Campos, M., Diaz-Meco, M.T., & Moscat, J. (2019). The dual roles of the atypical protein kinase Cs in cancer. *Cancer Cell 36*(3): 218-235.

Richmond-Sinclair, N. M., Pandeya, N., Williams, G. M., Neale, R. E., van der Pols, J. C., & Green, A. C. (2010). Clinical signs of photodamage are associated with basal cell carcinoma multiplicity and site: A 16 year longitudinal study. *International Journal of Cancer 127*(11): 2622-2629.

Ridky, T.W., and Cotsarelis, G. (2015). Vismodegib resistance in basal cell carcinoma: not a smooth fit. *Cancer Cell 27*: 315-316.

Riedl, A., Schlederer, M., Pudelko, K., Stadler, M., Walter, S., Unterleuthner, D., Unger, C., Kramer, N., Hengstschläger, M., Kenner, L., Pfeiffer, D., Krupitza, G., & Dolznig, H. (2017). Comparison of cancer cells in 2D vs 3D culture reveals differences in AKT-mTOR-S6K signaling and drug responses. *Journal of Cell Signaling 130*(1): 203-18.

Riobò, N. A., Haines, G. M., & Emerson, C. P., Jr. (2006a). Protein kinase C-delta and mitogen-activated protein/extracellular signal-regulated kinase-1 control GLI activation in hedgehog signaling. *Cancer Research 66*(2): 839–845.

Riobó, N. A., Lu, K., Ai, X., Haines, G. M., & Emerson, C. P. Jr. (2006b). Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. *PNAS USA 103*(12): 4505-10.

Robinson, G. W., Orr, B. A., Wu, G., Gururangan, S., Lin, T., Qaddoumi, I., Packer, R. J., Goldman, S., Prados, M. D., Desjardins, A., Chintagumpala, M., Takebe, N., Kaste, S. C., Rusch, M., Allen, S. J., Onar-Thomas, A., Stewart, C. F., Fouladi, M., Boyett, J. M., Gilbertson, R. J., Curran, T., Ellison, D. W., & Gajjar, A. (2015). Vismodegib Exerts Targeted Efficacy Against Recurrent Sonic Hedgehog-Subgroup Medulloblastomas: Results from Phase II Pediatric Brain Tumor Consortium Studies PBTC-025B and PBTC-032. *Journal of Clinical Oncology 33*(24): 2646-54.

Rodon, J., Tawbi, H. A., Thomas, A. L., Stoller, R. G., Turtschi, C. P., Baselga, J., Sarantopoulos, J., Mahalingam, D., Shou, Y., Moles, M. A., Yang, L., Granvil, C., Hurh, E. Rose, K. L., Amakye, D. D., Dummer, R., Mita, A. C. (2014). A phase I, multicenter, open-label, first-in-human, dose-escalation study of the oral smoothened inhibitor Sonidegib (LDE225) in patients with advanced solid tumors. *Clinical Cancer Research 20*(7): 1900–1909.

Rössig, L, Jadidi, A. S., Urbich, C., Badorff, C., Zeiher, A. M., & Dimmeler, S. (2001). Akt-dependent phosphorylation of p21(Cip1) regulations PCNA binding and proliferation of endothelial cells. *Molecular and Cell Biology 21*(16): 5644-57.

Rouillard, A.D., Gundersen, G.W., Fernandez, N.F., Wang, Z., Monteiro, C.D., McDermott, M.G., & Ma'ayan, A. The harmonizome: a collection of processed datasets gathered to serve and mine knowledge about genes and proteins. *Database- Oxford 2016*; baw100.

Rutkowski, S., Bode, U., Deinlein, F., Ottensmeier, H., Warmuth-Metz, M., Soerensen, N., Graf, N., Emser, A., Pietsch, T., Wolff, J.E., Kortmann, R.D., and Kuehl, J. (2005). Treatment of early childhood medulloblastoma by postoperative chemotherapy alone. *N Engl J Med. 352*(10):978–986.

Samatar, A. A. & Poulikakos, P. I. (2014). Targeting RAS-ERK signalling in cancer: promises and challenges. *Nature Reviews Drug Discovery 13*(12): 928–942.

Sánchez-Danés, A., Larsimont, J. C., Liagre, M., Muñoz-Couselo, E., Lapouge, G., Brisebarre, A., Dubois, C., Suppa, M., Sukumaran, V., Del Marmol, V., Tabernero, J., & Blanpain, C. (2018). A slow-cycling LGR5 tumour population mediates basal cell carcinoma relapse after therapy. Nature *562*(7727): 434–438.

Sarbassov, D.D., Guertlin, D.A., Ali, S.M., & Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science 307*, 1098-1101.

Schittenhelm, M. M., Shiraga, S., Schoeder, A., Corbin, A. S., Griffith, D., Lee, F. Y., Bokemeyer, C., Deininger, M. W. N., Druker, B. J., & Heinrich, M. C. (2006). Dasatinib (BMD-354835), a dual SRC/ABL kinase inhibitor, inhibits the kinase activity of wild-type, juxtamembrane, and activation loop mutant KIT isoforms associated with human malignancies. *Cancer Research 66*(1): 473-481.

Schneider, P., Bayo-Fina, J. M., Singh, R., Kumar Dhanyamraju, P., Holz, P., Baier, A., Fendrich, V., Ramaswamy, A., Baumeister, S., Martinez, E. D., & Lauth, M. (2015). Identification of a novel actin-dependent signal transducing module allows for the targeted degradation of GLI1. *Nature Communications 6*: 8023.

Schweppe, R. E., Kerege, A. A., French, J. D., Sharma, V., Grzywa, R. L., & Haugen, B. R. (2009). Src-focal adhesion kinase complex as a novel therapeutic target in papillary and anaplastic thyroid cancer. *The Journal of Clinical Endocrinology & Metabolism 94*(6): 2199- 2203.

Scott, M. T., Ingram, A., & Ball, K. L. (2002). PDK1-dependent activation of atypical PKC leads to degradation of the p21 tumour modifier protein. *The EMBO Journal 21*(24): 6771-80.

Sekulic, A., and Hoff, Von, D. (2016). Hedgehog Pathway Inhibition. *Cell 164*: 831.

Sekulic, A., Migden, M. R., Oro, A. E., Dirix, L., Lewis, K. D., Hainsworth, J. D., Solomon, J. A., Yoo, S., Arron, S. T., Friedlander, P. A., Marmur, E., Rudin, C. M., Chang, A. L., Low, J. A., Mackey, H. M., Yauch, R. L., Graham, R. A., Reddy, J. C., & Hauschild, A. (2012). Efficacy and safety of vismodegib in advanced basal-cell carcinoma. *New England Journal of Medicine 366*(23): 2171-9.

Set,o M., Ohta, M., Asaoka, Y., Ikenoue, T., Tada, M., Miyabayashi, K., Mohri, D., Tanaka, Y., Ijichi, H., Tateishi, K., Kanai, F., Kawabe, T., & Omata, M. (2009). Regulation of the hedgehog signaling by the mitogen-activated protein kinase cascade in gastric cancer. *Molecular Carcinogenesis 48*(8): 703–712.

Sharma, N., Nanta, R., Sharma, J., Gunewardena, S., Singh, K. P., Shankar, S., & Srivastava, R.K. (2015). PI3K/AKT/mTOR and sonic hedgehog pathways cooperate together to inhibit human pancreatic cancer stem cell characteristics and tumor growth. *Oncotarget 6*(31): 32039–32060.

Sharpe, H.J., Pau, G., Dijkgraad, G.J, Basset-Seguin, N., Modrusan, Z., Januario, T., Tsui, V., Durham, A.B., Dlugosz, A.A., Haverty, P.M., Bourgon, R., Tang, J.Y., Sarin, K.Y., Dirix, L., Fisher, D.C., Rudin, C.M., Sofen, H., Migden, M.R., Yauch, R.L., & de Sauvage, F.J. (2015). Genomic analysis of smoothened inhibitor resistance in basal cell carcinoma. *Cancer Cell 27*(3): 327-341.

Shi, F.T., Yu, M., Bell, R.H., Wang, E., Akhoundsadegh, N., Leung, G., Haegert, A., Carr, N., Shapiro, J., & McElwee, K.J. (2017). Notch signaling is significantly suppressed in basal cell carcinomas and activation induces basal cell carcinoma cell apoptosis. *Molecular Medicine Reports 15*(4): 1441-1454.

Shi, X., Zhan, X., & Wu J. (2015). A positive feedback loop between Gi1 and tyrosine kinase Hck amplifies shh signaling activities in medulloblastoma.

Shimokawa, T., Tostar, U., Lauth, M., Palaniswamy, R., Kasper, M., Toftgård, R., & Zaphiropoulos, P. G. (2008). Novel human glioma-associated oncogene 1 (GLI1) splice variants reveal distinct mechanisms in the terminal transduction of the hedgehog signal. *The Journal of Biological Chemistry 283*(21): 14345–14354.

Singh, R., Dhanyamraju, P.K., & Lauth, M. (2017). DYRK1B blocks canonical and promotes non-canonical Hedgehog signaling through activation of the mTOR/AKT pathway. *Oncotargets 8*(1): 833-845.

Singh, R. R., Cho-Vega, J. H., Davuluri, Y., Ma, S., Kasbidi, F., Milito, C., Lennon, P., (2009). Drakos, E., Medeiros, L. J., Luthra, R., & Vega, F. Sonic hedgehog signaling pathway is activated in ALK-positive anaplastic large cell lymphoma. *Cancer Research 69*(6) 2550–2558.

So, P. L., Langston, A. W., Daniallinia, N., Herbert, J. L., Fujimoto, M. A., Khaimskiy, Y., Aszterbaum, M., & Epstein, E. H. Jr. (2006). Long-term establishment, characterization, and manipulation of cell lines from mouse basal cell carcinoma tumors. *Experimental Dermatology 15*(9): 742-50.

So, P. L., Wang, G. Y., Wang, K., Chuang, M., Chiueh, V. C., Kenny, P. A., & Epstein, E. H. Jr. (2014). PI3K-AKT signaling is a downstream effector of retinoid prevention of murine basal cell carcinogenesis. *Cancer Prevention Research 7*(4): 407-17.

Standaert, M. L., Galloway, L., Karnam, P., Bandyopadhyay, G., Moscat, J., & Farese, R. V. (1997). Protein kinase C-zeta as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes. Potential role in glucose transport. *Journal of Biological Chemistry 272*(48): 30075–30082.

Stathis, A., Hess, D., von Moos, R., Homicsko, K., Griguolo, G., Joerger, M., Mark, M., Ackermann, C. J., Allegrini, S., Catapano, C. V., Xyrafas, A., Enoiu, M., Berardi, S., Gargiulo, P., Sessa, C., & Swiss Group for Clinical Cancer Research (SAKK). (2017). Phase I trial of the oral smoothened inhibitor sonidegib in combination with paclitaxel in patients with advanced solid tumors. *Investigational New Drugs 35*(6), 766–772.

Stecca, B., Mas, C., Clement, V., Zbinden, M., Correa, R., Piguet, V., Beermann, F., & Altaba, A. R. (2007). Melanomas require HEDGEHOG-GLI signaling regulated by interactions between GLI1 and the RAS-MEK/AKT pathways. *Proc. Natl. Acad. Sci. U.S.A.* 104(14): 5895–5900.

Steg, A. D., Bevis, K. S., Katre, A. A., Ziebarth, A., Dobbin, Z. C., Alvarez, R. D., Conner, M., & Landen, C. N. (2012). Stem cell pathways contribute to clinical chemoresistance in ovarian cancer. *Clinical Cancer Research 18*(3): 869–881.

Talamonte, M. S., Roh, M. S., Curley, S. A., & Gallick, G. E. (1993). Increase in activity and level of pp60c-src in progressive stages of human colorectal cancer. *The Journal of Clinical Investigation 9*(1): 53-60.

Tan, S. T., Ghaznawie, M., Heenan, P. J., & Dosan, R. (2018). Basal cell carcinoma arises from interfollicular layer of epidermis. *Journal of Oncology 2018*.

Tang, Y., Gholamin, S., Schubert, S., Willardson, M. I., Lee, A., Bandopadhayay, P., Bergthold, G., Masoud, S., Nguyen, B., Vue, N., Balansay, B., Yu, F., Oh, S., Woo, P., Chen, S., Ponnuswami, A., Monje, M., Atwood, S. X., Whitson, R. J., Mitra, S., Cheshier, S. H., Qi, J., Beroukhim, R., Tang, J. Y., Wechsler-Reya, R., Oro, A. E., Link, B. A., Bradner, J. E., & Cho, Y. J. (2014). Epigenetic targeting of Hedgehog pathway transcriptional output through BET bromodomain inhibition. *Nature Medicine 20*(7): 732-40.

Tang, Y. A., hen, Y., Bao, Y., Mahara, S., Yatim, S. M. J. M., Oguz, G., Lee, P. L., Feng, M., Cai, Y., Tan, E. Y., Fong, S. S., Yang, Z., Lan, P., Wu, X., & Yu, Q. (2018). Hypoxic tumor microenvironment activates GLI2 via HIF-1α and TGF-β2 to promote chemoresistance in colorectal cancer. *PNAS USA 115*(26): 5990-5999.

Taylor, M. D., Liu, L., Raffel, C., Hui, C. C., Mainprize, T. G., Zhang, X., Agatep, R., Chiappa, S., Gao, L., Lowrance, A., Hao, A., Goldstein, A. M., Stavrou, T., Scherer, S. W., Dura, W. T., Wainwright, B., Squire, J. A., Rutka, J. T., & Hogg, D. (2002). Mutations in SUFU predispose to medulloblastoma. *Nature Genetics 31*(3): 306-10.

Teperino, R., Amann, S., Bayer, M., McGee, S. L., Loipetzberger, A., Connor, T., Jaeger, C., Kammerer, B., Winter, L., Wiche, G., Dalgaard, K., Selvaraj, M., Gaster, M., Lee-Young, R. S., Febbraio, M. A., Knauf, C., Cani, P. D., Aberger, F., Penninger, J. M., Pospisilik, J. A., … Esterbauer, H. (2012). Hedgehog partial agonism drives Warburg-like metabolism in muscle and brown fat. Cell, 151(2): 414–426.

The Mayo Clinic. (2019). *Basal cell carcinoma*. https://www.mayoclinic.org/diseases-conditions/basal-cell-carcinoma/symptoms-causes/syc-20354187.

Tice, D. A., Biscardi, J. S., Nickles, A. L., & Parsons, S. J. (1999). Mechanism of biological synergy between cellular Src and epidermal growth factor receptor. *Proceedings of the National Academy of Sciences of the United States of America 96*(4): 1415–1420.

Tobias, I. S., Kaulich, M., Kim, P. K., Simon, N., Jacinto, E., Dowdy, S. F., King, C. C., & Newton, A. C. (2016). Protein kinase Cz exhibits constitutive phosphorylation and phosphatidylinositol-3,4,5-triphosphate independent regulation. *Biochemical Journal 473*(4): 509-523.

Tostar, U., Malm, C. J., Meis-Kindblom, J. M., Kindblom, L. G., Toftgård, R., & Undén, A. B. (2006). Deregulation of the hedgehog pathway: a possible role for the PTCH and SUFU genes in human rhabdomyoma and rhabdomyosarcoma development. *Journal of Pathology 208*(1): 17-25.

Tukachinsky, H., Lopez, L. V., & Salic, A. (2010). A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and disassociation of SuFu-Gli protein complexes. *Journal of Cell Biology 191*(2): 415-28.

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., & Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signaling. *Nature Reviews Molecular Cell Biology 11*(5): 329-41.

Varjosalo, M. & Taipale, J. (2008). Hedgehog: functions and mechanisms. *Genes & Development 22*: 2454-2472.

Viana, S.D., Reis, F., & Alves, R. (2018). Therapeutic use of mTOR inhibitors in renal diseases: advances, drawbacks, and challenges. *Oxid Med Cell Longev. 2018*, 3693625.

Von Hoff, D. D., LoRusso, P. M., Rudin, C. M., Reddy, J. C., Yauch, R. L., Tibes, R., Weiss, G. J., Borad, M. J., Hann, C. L., Brahmer, J. R., Mackey, H. M., Lum, B. L., Darbonne, W. C., Marsters, J. C. Jr., de Sauvage, F. J., & Low, J. A. (2009). Inhibition of the hedgehog pathway in advanced basal-cell carcinoma. *New England Journal of Medicine 361*(12): 1164-72.

Wagner, A. J., Messersmith, W. A., Shaik, M. N., Li, S., Zheng, X., McLachlan, K. R., Cesari R., Courtney, R., Levin, W. J., El-Khoueiry, A. E. (2015). A phase I study of PF-04449913, an oral hedgehog inhibitor, in patients with advanced solid tumors. *Clinical Cancer Research 21*(5): 1044–1051.

Walker, E. H., Pacold, M. E., Perisic, O., Stephens, L., Hawkins, P. T., Wymann, M. P., & Williams, R. L. (2000) Structural determinants of phosphoinositide 3-kinase inhibition by wortmannin, LY294002, quercetin, myricetin, and staurosporine. *Molecular Cell 6*(4): 909-19.

Wang, G., Krishnamurthy, K., Umapathy, N. S., Verin, A. D., & Bieberich, E. (2009). The carboxyl-terminal domain of atypical protein kinase Czeta binds to ceramide and regulates junction formation in epithelial cells. *Journal of Biological Chemistry 284*(21): 14469–14475.

Wang, B., Fallon, J. F., & Beachy, B. A. (2000). Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell 100*(4): 423-34.

Wang, Y., Ding, Q., Yen, C. J., Xia, W., Izzo, J. G., Lang, J. Y., Li, C. W., Hsu, J. L., Miller, S. A., Wang, X., Lee, D. F., Hsu, J. M., Huo, L., Labaff, A. M., Liu, D., Huang, T. H., Lai, C. C., Tsai, F. J., Chang, W. C., Chen, C. H., Wu, T. T., Buttar, N. S., Wang, K. K., Wu, Y., Wang, H., Ajani, J., & Hung, M. C. (2012). The crosstalk of mTOR/S6K1 and Hedgehog pathways. *Cancer Cell 21*(3): 374-87.

Wang, Y. M., Seibenhener, M. L., Vandenplas, M. L., & Wooten, M. W. (1999). Atypical PKC zeta is activated by ceramide, resulting in coactivation of NF-kappaB/JNK kinase and cell survival. *Journal of Neuroscience Research 55*: 293–302.

Wei, L. & Xu, Z. (2011) Cross-signaling among phosphoinositide-3 kinase, mitogen-activated protein kinase and sonic hedgehog pathways exists in esophageal cancer. *International Journal of Cancer 129*(2): 275-84.

Whitson, R. J., Lee, A., Urman, N. M., Mirza, A., Yao, C. Y., Brown, A. S., Li, J. R., Shankar, G., Fry, M. A., Atwood, S. X., Lee, E. Y., Hollmig, S. T., Aasi, S. Z., Sarin, K. Y., Scott, M. P., Epstein, E. H. Jr., Tang, J. Y., & Oro, A. E. (2018). Noncanonical hedgehog pathway activation through SRF-MKL1 promotes drug resistance in basal cell carcinoma. *Nature Medicine 24*(3): 271-81.

Williams, J. A., Guicherit, O. M., Zaharian, B. I., Xu, Y., Chai, L., Wichterle, H., Kon, C., Gatchalian, C., Porter, J. A., Rubin, L. L., & Wang, F. Y. (2003). Identification of a small molecule inhibitor of the hedgehog signaling pathway: effects on basal cell carcinoma-like lesions. *Proceedings of the National Academy of Sciences of the United States of America 100*(8) 4616–4621.

Wooten, M. W., Vandenplas, M. L., Seibenhener, M. L., Geetha, T., & Diaz-Meco, M. T. (2001). Nerve growth factor stimulates multisite tyrosine phosphorylation and activation of the atypical protein kinase C's via a src kinase pathway. *Molecular Cell Biology 21*(24): 8414-8427.

Xiao, X., Tang, J. J., Peng, C., Wang, Y., Fu, L., Qiu, Z. P., Xiong, Y., Yang, L. F., Cui, H. W., He, X. L., Yin, L., Qi, W., Wong, C. C., Zhao, Y., Li, B. L., Qiu, W. W., & Song, B. L. (2017). Cholesterol Modification of Smoothened Is Required for Hedgehog Signaling. *Molecular Cell 66*(1): 154-62.

Yam, P. T., Langlois, S. D., Morin, S., & Charron, F. (2009). Sonic hedgehog guides axons through a noncanonical, src-family-kinase-dependent signaling pathway. *Neuron 62*(2): 349-362.

Yang, M-T., Lin, Y-C., Ho, W-H., Liu, C-L., & Lee, W-T. (2017). Everolimus is better than rapamycin in attenuating neuroinflammation in kainic acid-induced seizures. *Journal of Neuroinflammation 14*(1): 15.

Yang, S. H., Andl, T., Grachtchouk, V., Wang, A., Liu, J., Syu, L. J., Ferris, J., Wang, T. S., Glick, A. B., Millar, S. E., & Dlugosz, A. A. (2008). Pathological responses to oncogenic Hedgehog signaling in skin are dependent on canonical Wnt/beta3-catenin signaling. *Nature Genetics 40*(9): 1130-5.

Yao, C. D., Haensel, D., Gaddam, S., Patel, T., Atwood, S. X., Sarin, K. Y., Whitson, R. J., McKellar, S., Shankar, G., Aasi, S., Rieger, K., & Oro, A. E. (2020). AP-1 and TGFb cooperatively drives non-canonical Hedgehog signaling in resistant basal cell carcinoma. *Nature Communications 11*(1): 5079.

Yedjou, C. G., and Tchounwou, P. B. (2007). In-vitro cytotoxic and genotoxic effects of arsenic trioxide on human leukemia (HL-60) cells using the MTT and alkaline single cell gel electrophoresis (Comet) assays. *Molecular and Cellular Biochemistry 301*(1-2): 123–130.

Yoon, J. W., Kita, Y., Frank, D. J., Majewski, R. R., Konicek, B. A., Nobrega, M. A., Jacob, H., Walterhouse, D., & Lannaccone, P. (2002). Gene expression profiling leads to identification of GLI1-binding elements in target genes and a role for multiple downstream pathways in GLI1-induced cell transformation. *Journal of Biological Chemistry 277*(7): 5548-5555.

Youssef, K. K., Lapouge, G., Bouvrée, K., Rorive, S., Brohée, S., Appelstein, O., Larsimont, J. C., Sukumaran, V., Van de Sande, B., Pucci, D., Dekoninck, S., Berthe, J. V., Aerts, S., Salmon, I., del Marmol, V., & Blanpain, C. (2012). Adult interfollicular tumour-initiating cells are reprogrammed into an embryonic hair follicle progenitor-like fate during basal cell carcinoma initiation. *Nature Cell Biology 14*(12): 1282–1294.

Yu, J. S. & Cui, W. (2016). Proliferation, survival and metabolism: the role of PI3K/AKT/mTOR signalling in pluripotency and cell fate determination. *Development 143*(17): 3050–3060.

Zhang, Y., Bulkley, D. P., Xin, Y., Roberts, K. J., Asarnow, D. E., Sharma, A., Myers, B. R., Cho, W., Cheng, Y., & Beachy, P. A. (2018). Structural Basis for Cholesterol Transport-like Activity of the Hedgehog Receptor Patched. *Cell 175*(5): 1352-64.

Zhang, R., Huang, S. Y., Ka-Wai Li, K., Li, Y. H., Hsu, W. H., Zhang, G. J., Chang, C. J., & Yang, J. Y. (2017). Dual degradation signals destruct GLI1: AMPK inhibits GLI1 through β-TrCP-mediated proteasome degradation. *Oncotarget 8*(30): 49869–49881.

Zhang, K., Wang, X., & Wang, H. (2014). Effect and mechanism of Src tyrosine kinase inhibitor sunitinib on the drug-resistance reversal of human A549/DDP cisplatin-resistant lung cancer cell line. *Molecular Medicine Reports 10*(4): 2065–2072.

Zhao, X., Ponomaryov, T., Ornell, K. J., Zhou, P., Dabral, S. K., Pak, E., Li, W., Atwood, S. X., Whitson, R. J., Chang, A. L., Li, J., Oro, A. E., Chan, J. A., Kelleher, J. F., & Segal, R. A. (2015). RAS/MAPK Activation Drives Resistance to Smo Inhibition, Metastasis, and Tumor Evolution in Shh Pathway-Dependent Tumors. *Cancer Research 75*(17): 3623-35.

Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H., & Hung, M. C. (2001). Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nature Cell Biology 3*(3): 245-52.

Zibat, A., Missiaglia, E., Rosenberger, A., Pritchard-Jones, K., Shipley, J., Hahn, H., & Fulda, S. (2010). Activation of the hedgehog pathway confers a poor prognosis in embryonal and fusion gene-negative alveolar rhabdomyosarcoma. *Oncogene 29*(48): 6323–6330.

APPENDIX

Materials and Methods

Ethics statement

Human clinical studies were approved by the Ethics Committee of the University of California Irvine. All human studies were performed in strict adherence to the Institutional Review Board (IRB) guidelines of the University of California Irvine.

Data Availability Statement

The dataset that supports the findings of these studies are available in GEO at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE58375, reference number GSE58375.

RNA Sequencing Analysis

RNA-seq data were obtained from patient-matched advanced human BCC patients (Atwood et al., 2015) whose tumors were surgically non-resectable. RNA-Seq data were aligned as previously described (Atwood et al., 2015). The NCBI Reference Sequence (RefSeq) databases were used as reference annotations to calculate values of reads per kilobase of transcript per million mapped reads for known transcripts (RPKM). RPKM values were then log2-transformed and heat map analysis was used to visualize the differential gene expression. Pathway enrichment terms from RNA-seq data were obtained using Enrichr (Kuleshov et al., 2016).

Human Samples

Written informed consent was obtained for all archived human samples and was reviewed by the University of California Irvine IRB. Human normal epidermis and BCC samples were collected from UC Irvine Medical Center. Paraffinized samples were sectioned with a rotary microtome (Leica RM2155) at 7 μm for analysis. Samples were deparaffinized as described by Abcam and antigen retrieval was performed using a Tris-EDTA buffer (10 nM Tris base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) at 60°C overnight.

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 kinase domain of *PRKCI* (aPKC-KiDo), Y265F kinase domain *PRKCI* (aPKC Y265F KiDo), Y280F *PRKCI* (aPKC Y280F KiDo), Y334F *PRKCI* (aPKC Y334F KiDo), wild-type *Src* , and kinase dead K299M *Src* (Src K299M). 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 Rapamycin (0.1 nM, 1 nM, 10 nM, and 100 nM) (Fisher Scientific), OSI-027 (5 µM, 10 µM, 25 µM, and 100 µM) (Fisher Scientific), Everolimus (2 nM, 10 nM, 50 nM, and 250 nM) (Fisher Scientific), LY294002 (1 mM, 5 mM, 25 mM, and 100 mM; Fisher Scientific), BKM120 (250 nM, 1.25 mM, 6.25 mM, and 31.25 mM; Fisher Scientific), Dasatanib (1 nM, 5 nM, 25 nM, and 125 nM), Saracatinib (2 nM, 10 nM, 50 nM, 250 nM), or KX01 (50 nM, 250 nM, 1.25 µM, and 6.25 µM) 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 (RT-qPCR) was performed using the iTaq Univer 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 GATGCT CAGTGT 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.

Growth assay

ASZ001 or NIH3T3 cells were seeded at 2000 cells/well into 96-well plates. After 48 hours, cells were treated with DMSO or varying concentrations of Rapamycin, OSI-027, Everolimus, LY294002, BKM120, Dasatanib, Saracatinib, or KX01 (refer to HH assay) for the indicated amount 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.

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. 5 weeks later, mice were treated with either DMSO Everolimus (3mg/kg), BKM120 (10mg/kg), or KX01 (5mg/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, 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. Five mice were used for each treatment condition.

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 total tumor size per square area. More than 50 tumors were measured in each of 5 mice.

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: mTor (rabbit, Cell Signaling Technology 2983S, 1:400), PI3K (rabbit, Abcam ab40776, 1:100), Src (rabbit, Cell Signaling Technology 2108, 1:400), p-Y418 Src (rabbit, Abcam ab4816, 1:500), Gli1 (rabbit, Santa Cruz Biotechnology sc-20687, 1:500), Gli1 p-T304 (rabbit, 1:200) (Drummond et al., 2018), Krt14 (chicken, Fisher Scientific 50-103-0174, 1:5000), aPKC (rabbit, Santa Cruz Biotechnology sc-216, 1:500), aPKC p-T410 (rabbit, Santa Cruz Biotechnology sc-12894-R, 1:200), aPKC p-T560 (rabbit, Abcam ab62372, 1:300), AKT (rabbit, Cell Signaling 4691S, 1:400), p-T308 AKT (rabbit, Cell Signaling 13038S, 1:400), p21 (rabbit, Cell Signaling 2947S, 1:250), p-T145 p21 (rabbit, GeneTex GTX32376, 1:250), FAK (rabbit, 1:500), and p-T861 FAK (rabbit, Fisher Scientific 44626G, 1:250). 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, Everolimus (10 nM), BKM120 (6.25 μM), or KX01 (1.25 μM) 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, 1:10,000). The coverslips were imaged using an EVOS fluorescence microscope.

Protein Immunoblotting

ASZ001 cells were seeded to confluency, serum-starved, and treated with DMSO or Everolimus (10 nM) for 24 hours. Cells were collected and lysed in SDS sample preparation buffer (100 mM Tris-HCl, pH 6.8; 1 M DTT, 4% SDS, 20% glycerol, and 0.2% bromophenol blue). Samples were loaded onto a 4-20% gradient polyacrylamide gel (BioRad) and transferred onto nitrocellulose membrane (Genesee Scientific). Membranes were immunoblotted with antibodies against aPKC (Santa Cruz Biotech, 1:1000) and β-tubulin (DSHB, 1:2000) in 1x TBST, incubated with secondary antibodies donkey anti-mouse Alexa 680 or donkey anti-rabbit Alexa 790 (Jackson, 10,000), and then imaged using the LI-COR Odyssey system.

Statistics

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