Figure 3.8 Innervation is required for HH signaling in TDs.
A. IHC of denervated (Den) or sham-operated wild-type skin (Sham) showing TD epithelia (asterisk) and Merkel cells (green), 5 weeks after surgery. (Right) magnified views of TD areas (asterisk). Red indicates K17 expression.
B. Quantitation of the average size of K17+ TDs, as well as the abundance of K17+ cells and Merkel cells in the IFE, 3 and 5 weeks after denervation. Matched sham and denervated data from the same mice are connected by lines.
C. Denervation causes stable loss of nerve endings (red) from Merkel cells (green), 3 weeks after surgery.
D. Whole-mount LacZ staining showing that denervation inhibits induction of LacZ reporter expression in Gli1;LacZ mice. T, TAM; B, biopsy.

All superimposed bar graphs depict mean values. Scale bars represent 50µm. See also FIG 3.7, 3.9.
Figure 3.9 Denervation causes stable loss of epidermal innervation.
IHC showing stable loss of nerves, as assessed by Neurofilament staining (red), in Gli1;Ptch1 denervated skin (right panel) compared to matched sham control (left panel), approximately 4.5 weeks after denervation. Bottom panels are magnified views of the boxed areas in the top panels. Scale bars, 50 µm.
**Figure 3.10 Denervation inhibits TD-derived tumors.**

A. Whole-mount LacZ staining of Gli1;LacZ skin, showing that denervation performed after TAM does not have an immediate effect on labeled Gli1-expressing TD cells (blue), 2 weeks after induction.

B. IHC showing that denervation performed after TAM induction of Gli1;Ptch1 mice reduces the size and complexity of TD-derived tumors (white dotted lines). K8+ Merkel cells are labeled green (arrowheads). Yellow dotted lines indicate guard hairs.

C. Quantitation of TD-derived tumor size (left panel) and Merkel cell abundance (right panel), 2 and 5 weeks post-TAM. Matched sham and denervated data from the same mice are connected by lines.

D. H&E and quantitation showing that denervation does not affect the abundance of hair follicle-derived tumors.

E. IHC and quantitation showing that denervation does not affect K14-Cre\textsuperscript{ERT}-mediated recombination in the IFE.

F. Quantitation showing that denervation inhibits tumor formation in TDs (left), but not the formation of ectopic IFE buds (right) in K14;Ptch1 mice. All superimposed bar graphs depict mean values.

Data are represented as mean ± SEM. See also FIG 3.11.
Figure 3.11 A mechanosensory niche promotes tumorigenesis.

A. qPCR showing that DRG (blue bars) highly express all 3 HH ligands (Shh, Dhh, and Ihh) relative to epidermis (yellow bars). K14 and Tubulin b3 (Tubb3) are specificity controls for skin and neurons, respectively. All values are expressed as fold induction relative to that of DRG (for K14, left y axis) or epidermis (for all other genes, right y axis).

B. qPCR showing that K14;Ptc1 mice, relative to non-tumorigenic controls, upregulate Gli1 and Ptch2 in the skin, 5 weeks post-TAM.

C. IHC showing CD200 expression (red) throughout the hair follicle (left) and TD (right, dotted line), but not in the IFE. (Top right) TD marked by K17 (green) and CD200, or CD200 alone (bottom right). Strong red staining in the cornified IFE is due to autofluorescence.
D. (Left) IHC showing that CD200 (red) is highly expressed in hair follicle-derived tumors (blue dotted lines), but weakly expressed in ectopic IFE buds or hyperplastic IFE (white dotted lines) in $K14;Ptch1$ mice. (Right) IHC showing that CD200 is highly expressed in TD-derived tumors (yellow dotted lines), both 2 and 5 weeks post-TAM, in $Gli1;Ptch1$ mice. K17 (green) is indiscriminately upregulated in hyperplastic IFE, ectopic IFE buds, and tumors. All bottom panels are magnified views of the boxed areas.

E. qPCR showing that keratinocytes harboring $K5$ promoter-driven constitutive Cre and homozygous $Ptch1$ flox alleles upregulate canonical HH target genes as well as CD200.

F. qPCR showing that the Smoothened agonist purmorphamine (purm) upregulates CD200 in wild-type keratinocytes.

G. BCCs arise from multiple stem cell populations in the hair follicle and TD (filled regions). Tumors are also associated with the hair follicle infundibulum (beige), but do not arise efficiently from IFE stem cells (red X). Tumors associated with the middle bulge region were not observed in this study, possibly because of inefficient Cre-mediated recombination in this domain. Dotted lines indicate nerves. SHG, secondary hair germ.

H. IHC showing Merkel cells (arrowheads) expressing chromogranin A (green) and K20 (red) in a human BCC. (Inset) A magnified view of the region identified by the arrow.

Data are represented as mean ± SEM. The scale bars represent 50 µm. See also FIG 3.13 to 3.15.
Figure 3.12 HH target genes are active in Gli1;Ptch1 and K14;Ptch1 lesions.
In situ staining for GLI1, similar to IHC for K17, reveals that activation of downstream HH signaling occurs in both small ectopic downgrowths in K14;Ptch1 mice, as well as in hair follicle-associated tumors in Gli1;Ptch1 animals, both 5 weeks after TAM induction. Scale bars, 50 µm.
Figure 3.13 HH neutralizing antibody blocks anagen and HF tumorigenesis.
A. Depilated wild-type mice treated for 2 weeks with an Shh-neutralizing antibody (5E1, right) displayed a severe delay in anagen re-entry, compared to untreated mice (left), as previously reported (Wang et al., 2000).
B. Schematic for tamoxifen-induced Gli1;Ptc1 mice treated with either purified 5E1 antibody or a purified IgG1 isotype control. Biopsies (B) were collected both before and after antibody treatment (2 and 5 weeks post-TAM induction (T), respectively). Daily antibody injections were given throughout the final 2 weeks of the experiment.
C. Quantitation of TD-derived tumor cell abundance, both before and after antibody treatment. Yellow bars indicate data from animals treated with 5E1, whereas blue bars indicate data from animals treated with control antibody. n. s., not significant.
D. Fold-change in the abundance of TD-derived tumor cells between 2-5 weeks after tamoxifen induction, for animals treated with 5E1 (yellow) or IgG1 (blue) (p = 0.28) (n = 7 mice treated with 5E1, and 5 mice treated with IgG1). Data are represented as mean ± SEM.
E. Hair follicle tumor area for animals treated with 5E1 (yellow) or IgG1 (blue) (p=0.09) (n = 7 mice treated with 5E1, and 5 mice treated with IgG1).
Figure 3.14 MCs are associated with a subset of human BCCs

IHC staining, as indicated, identifying Merkel cells (arrows) in human BCCs. Merkel cells were observed both in superficial lesions (upper left) as well as in deeper tumor nests (upper right and lower images). Merkel cells were not observed within large tumor masses (not shown). Scale bars, 50 µm.
3.9 Reference List


Chapter IV – Cutaneous hyper-innervation via Neurotrophin-3 promotes ectopic Hedgehog activation and may enhance BCC tumorigenesis

4.1 Abstract

Basal cell carcinoma (BCC) is a common skin cancer caused by deregulated Hedgehog (HH) signaling, often due to mutations in the receptor Patched1 (PTCH1). Although mutations in HH pathway components drive BCC, we have recently identified a role for sensory nerves in promoting BCC tumor growth. Here, we generated a mouse model which displays ectopic innervation throughout the epidermis via expression of the nerve growth factor Neurotrophin-3 (NT-3). To test whether hyper-innervation promotes BCC tumorigenesis, we combined this model with Ptch1 deletion using two different Cre drivers. Our preliminary results suggest that hyper-innervation via NT-3 expression may promote increased BCC tumor growth in a sub-set of epidermal compartments.

4.2 Introduction

While the hair follicle (HF) and touch dome (TD) compartments readily form BCC tumors, the interfollicular epidermis (IFE) resists tumor formation [1-3]. When comparing these compartments in mice, one striking difference is the presence of sensory nerves which innervate the HF and TD, but are largely absent from the IFE [4]. Under normal conditions, these cutaneous sensory nerves secrete HH ligand to activate signaling [5, 6]. However, we have recently identified a potential promoting role for sensory nerves in BCC growth (see chapter III, [2]). Nerves have also been implicated in several other diseases. Surgical
denervation of skin causes clearing of psoriatic lesions [7]. Both surgical and chemical
denervation of autonomic nerve fibers reduces initiation and metastasis of prostate
adenocarcinoma [8]. Chemical denervation also improves survival in mouse models of
fibrosarcoma [9], gastric cancer [10], and pancreatic adenocarcinoma [11]. Based on these
results, we hypothesize that neural signaling promotes BCC tumor formation. In addition, we
predict that cells in non-neural niches, such as the IFE, are less likely to form BCCs and may
require additional mutations for transformation.

Innervation is regulated by secreted nerve growth factors. These factors, called
neurotrophins, bind to Trk receptors present on nerve terminals to promote nerve migration and
survival (reviewed in [12]). During epidermal development, newly formed HFs and TDs secrete
Neurotrophin-3 (NT-3), which promotes the survival of TrkC positive nerve fibers [13]. Mice
which overexpress NT-3 throughout the epidermis display ectopic innervation of the IFE, and
increased innervation of HFs and TDs [14]. In addition, NT-3 expressing mice have
accelerated HF morphogenesis [15]. However, this follicular phenotype is purely developmental
as adult hair cycling is not altered in NT-3 mice [15].

Here, we generated mice that express NT-3 under the control of the human Keratin 14
promoter (hK14-mNT-3) [14]. We confirmed previous reports that NT-3 expression causes
increased innervation of HFs and TDs, as well as ectopic innervation of the IFE. We also
observed a significant increase in TD size and the number of TD-associated MCs. NT-3
expressing mice also displayed an increase in HH target gene expression, with ectopic activation
in the HF canal or infundibulum.

To test whether ectopic innervation can impart BCC susceptibility, we combined NT-3
overexpression with Ptch1 deletion using two different Cre alleles. Here we demonstrate that
ectopic innervation does not affect BCC initiation or progression in the dorsal IFE compartment, but may promote tumorigenesis in ear and tail-skin. NT-3 expression also causes ectopic recombination in the hair canal of Gli1;Ptch1;NT-3 mice, resulting in infundibulum (INF)-derived BCC tumors in dorsal skin. In addition, preliminary results suggest that NT-3 expressing mice display increased BCC tumor initiation in the lower HF and TD.

During this study, we observed spontaneous regression of lower HF-derived BCC tumors in Gli1;Ptch1 mice 12-17 weeks after Ptch1 deletion (for more detail, see chapter V). Here, we show that hyper-innervation may partially protect against this phenomenon. Although lower follicle-derived tumors in both genotypes regress over time, ectopic INF-derived tumors in Gli1;Ptch1;NT-3 mice appear to resist regression.

### 4.3 Materials and Methods

#### 4.3.1 Animals

The following mice were used: Gli1^tm3(cre/ERT2)Alj (Gli1-Cre^ERT2)[16]; Tg(KRT14-cre/ERT2)20Efu (K14-Cre^ERT)[17]; Ptch1^tm1Hahn [18]; Gt(ROSA)26Sor^tm1Sor (ROSA26A-lacZ)[19]; hK14-mNT-3 (NT-3) [14]. To generate NT-3 animals, purified DNA containing mouse Neurotrophin-3 previously generated by Albers et al., was microinjected into fertilized eggs obtained by mating (C57BL/6 X SJL)F1 or C57BL/6 female mice with (C57BL/6 X SJL)F1 male mice [14]. Pronuclear microinjection was performed as described [20].

#### 4.3.2 Mouse manipulations

For all tumor and reporter experiments, animals were induced with tamoxifen during telogen at 8 weeks of age. Mice were treated with tamoxifen as follows: one dose at 5 mg per 40 grams body weight
for Gli1;NT-3;LacZ and Gli1;Ptc11;NT-3, and three daily doses, each 1 mg per 40 grams body weight, for K14;Ptc11;NT-3 mice. Biopsies were performed and harvested as previously described [21]. All studies were performed in accordance with regulations established by the University of Michigan Unit for Laboratory Animal Medicine.

4.3.3 Tissue staining

Biopsies were fixed for 1 hour in cold 3.7% paraformaldehyde, washed and incubated overnight in 30% sucrose at 4 degrees, before embedding in OCT mounting media. Frozen sections were stained using standard protocols with the following antibodies: rabbit anti-K17 (D73C7, 1:1,500, Cell Signaling); rat anti-K8 (TROMA-I, 1:500, Developmental Studies Hybridoma Bank); rat anti-CD200 eFluor660 (OX90, 1:2,000, eBioscience); goat anti-K14 (sc-17104, 1:1000, Santa Cruz); and rabbit anti-NF-L (C28E10, 1:500, Cell Signaling).

4.4 Results

4.4.1 Expression of Neurotrophin-3 causes hyper-innervation of the skin

We first tested the effect of hyper-innervation on epidermal homeostasis. For these experiments, we generated transgenic mice expressing mouse Neurotrophin-3 (NT-3) under the control of the human Keratin 14 promoter (hK14-mNT-3) (FIG 4.1A, [14]). These mice are reported to display increased innervation of the HF bulge [14]. Using flat-mount immunofluorescence (IF) staining for Neurofilament (NF), we confirmed an increase in NF+ fiber density surrounding the HF (FIG 4.1B). In addition, we observed ectopic innervation of the IFE, as seen by IF staining for NF in frozen sections (FIG 4.1B, yellow arrows).
4.4.2 NT-3 mice display enlarged touch domes with more Merkel cells

NT-3 overexpression is also reported to cause enlargement of mechanosensory TDs [14]. These specialized structures function to sense light touch, and rely on innervation for HH activation and maintenance [6]. To assess the effect of NT-3 over-expression on TDs, we harvested telogen skin biopsies from hK14-mNT-3 and wildtype littermates at 8 weeks. IF staining confirmed hyper-innervation and enlargement of the epidermal TD compartment, marked by Keratin 17 (K17) (FIG 4.1C). Interestingly, we also observed an increase in the number of MCs per TD, marked by Keratin 8 (K8) (FIG 4.1C). We quantified these differences across 15 non-consecutive sections and observed a significant increase in the number of K8+ MCs per cm of IFE (FIG 4.1D, p=0.03), while the increase in K17+ cells did not reach statistical significance (FIG 4.1D, p=0.10).

4.4.3 NT-3 mice display ectopic HH activation

Because sensory nerves secrete HH ligand [5], we predicted that hK14-mNT-3 mice would display increased HH activation throughout the epidermis. To address this question, we utilized a conditional LacZ reporter allele combined with tamoxifen (TAM) inducible Gli1-CreER T2 [16] (Gli1;LacZ), which labels HH-active cells upon TAM induction. For these experiments, we induced labeling at 8 weeks, during telogen, and collected skin biopsies after 4 days (FIG 4.2B). We also collected epidermal RNA from wildtype and hK14-mNT-3 mice and performed qPCR to assess expression of the HH target gene Gli1. We observed an increase in Gli1 expression in hK14-mNT-3 mice compared to wildtype controls (FIG 4.2C, p=0.06). To visualize TD and IFE labeling, we performed whole-mount LacZ staining. Whole mount staining highlighted a slight TD enlargement in Gli1;LacZ;NT-3 samples (FIG 4.2D).
of Gli1;LacZ;NT-3 TDs extended further around the guard follicle to the anterior side, which lacks labeling in Gli1;LacZ samples (FIG 4.2D). We also observed ectopic labeling outside of the TD compartment (FIG 4.2D, arrows). On-slide LacZ staining confirmed this ectopic labeling is present in the HF canal, or infundibulum of guard and non-guard follicles (FIG 4.2D, arrows). We observed no apparent increase in labeling of the HF bulge (bracket) or secondary hair germ (asterisk)(FIG 4.2D).

4.4.4 Hyper-innervation does not impart BCC susceptibility in K14;Ptch1 mice

To test whether ectopic innervation can impart BCC susceptibility onto the IFE, we utilized the tamoxifen (TAM) inducible K14-CreERT [22] allele in combination with homozygous Ptc1flx alleles [23] (K14;Ptch1). This system enables deletion of exons 8+9 of Ptc1 specifically in the basal cells of the IFE, with minimal recombination in the hair follicle (HF) [2, 21, 24](FIG 4.3A). We combined these alleles with our hK14-mNT-3 allele to generate K14;Ptch1;NT-3 mice. We induced mice with TAM at 8 weeks, and collected skin biopsies 5, 12, and 17 weeks after induction, as previously described [2](FIG 4.3B). We collected epidermal RNA from K14;Ptch1 and K14;Ptch1;NT-3 animals observed increased Gli1 expression 17 weeks after induction in K14;Ptch1;NT-3 mice (FIG 4.3C, n=1/1). Consistent with previous observations, after 5 weeks K14;Ptch1 mice did not develop BCC tumors (FIG 4.3D). After 12 weeks, lesions progressed slightly with more severe lesions deriving from the HF canal or infundibulum (FIG 4.3D). At 17 weeks post TAM, lesions do not appear more progressed, although changes in pigmentation were observed (FIG 4.3D). Surprisingly, we observed no significant differences in phenotype severity between K14;Ptch1 and K14;Ptch1;NT-3 mice at any time-point (n=1/1). We observed no difference in GLI1
immunostaining at this time-point (FIG 4.3E). This result was also surprising as it directly contradicts our Gli1 qPCR result. However, degradation of GLI1 protein could explain the difference in mRNA expression versus protein. Although ectopic IFE innervation is maintained 17 weeks after Ptch1 deletion, expression of BCC marker K17 is also not altered in K14;Ptch1;NT-3 lesions (FIG 4.3E). These preliminary results suggest that ectopic innervation via NT-3 expression does not impart BCC susceptibility onto the dorsal IFE.

We also collected ear and tail-skin samples 17 weeks post TAM induction to compare phenotype severity. Histology from these samples suggest that ear and tail-skin lesions in K14;Ptch1;NT-3 mice branch deeper into the dermis, when compared with K14;Ptch1 lesions (FIG 4.4, n=1/1). These preliminary results suggest that hyper-innervation could enhance tumorigenesis in specific contexts, although further characterization will be necessary to confirm these results.

4.4.5 Hyper-innervation may enhance HF BCC tumorigenesis

To assess whether hyper-innervation affects HF-derived tumorigenesis, we utilized TAM inducible Gli1-CreERT2 [16], which is active in the upper bulge and secondary hair germ (FIG 4.5A) in combination with Ptch1 flox alleles [23] (Gli1;Ptch1). These mice efficiently form HF-derived BCC-like tumors 5 weeks after induction with TAM (see chapter III, [2]). We induced Gli1;Ptch1;NT-3 and Gli1;Ptch1 mice at 8 weeks and collected skin biopsies after 5, 12, and 17 weeks (FIG 4.5B). After 5 weeks, we observed an increase in bulge-derived HF tumors in Gli1;Ptch1;NT-3 mice (FIG 4.5C, n=2/3). These preliminary results suggest that NT-3 expression may promote increased bulge-derived BCC tumorigenesis, although more samples will be needed to confirm whether this difference is significant. Between 12-17 weeks post
induction, we observed spontaneous regression of lower HF-derived tumors in \textit{Gli1;Ptch1;NT-3} and \textit{Gli1;Ptch1} mice (FIG 4.5C-D, \textit{n=1/1}). This preliminary data suggests a promoting role for follicular hyper-innervation in tumor growth and maintenance, although further characterization will be necessary to confirm these results.

\textbf{4.4.6 TD and INF-derived BCCs in Gli1;Ptch1;NT-3 mice resist spontaneous regression}

In addition to increased lower follicle-derived tumorigenesis, \textit{Gli1;Ptch1;NT-3} mice also display ectopic tumors derived from the hair canal, or infundibulum (INF) of non-guard follicles (FIG 4.5A, asterisks, \textit{n=2/3}). These tumors likely arise due to ectopic recombination activity of \textit{Gli1-Cre\textsuperscript{ERT2}}, as seen in our reporter study (FIG 4.2). We confirmed these tumors as being INF and not TD-derived based on their frequency and marker expression. While TD-derived tumors are rare, and only associated with large guard hair follicles, INF-derived tumors in \textit{Gli1;Ptch1;NT-3} mice were seen in consecutive HFs in sections and associated with non-guard follicles (FIG 4.5A). In addition, TD-derived tumors have associated Keratin 8 positive Merkel cells (MCs), which are absent from INF-derived tumors (FIG 4.6A). After 12 weeks, while lower HF-derived tumors have regressed; however, INF-derived tumors progress further (FIG 4.6D).

Because TDs are enlarged in \textit{NT-3} expressing mice, we also hypothesized that \textit{Gli1;Ptch1;NT-3} mice might display increased TD tumorigenesis. After 5 weeks, we observed larger TD-derived tumors in \textit{Gli1;Ptch1;NT-3} mice compared to \textit{Gli1;Ptch1} controls (FIG 4.6A). We quantified the TD-derived tumor area in 15 non-consecutive sections and observed an approximately 2-fold increase in tumor area in \textit{Gli1;Ptch1;NT-3} mice (FIG 4.6B, \textit{n=1/1}). Unlike bulge-derived tumors, TD tumors do not display spontaneous regression. After 17 weeks,
TD tumors in *Gli1;Ptch1;NT-3* and *Gli1;Ptch1* mice progress to large complex lesions, which branch deep into the dermis (FIG 4.6C). Despite the observed difference in tumor size at 5 weeks, TD tumors in *Gli1;Ptch1;NT-3* and *Gli1;Ptch1* mice appear indistinguishable at 17 weeks (n=1/1). In addition, these large lesions do not display any difference in BCC marker expression (FIG 4.6C, n=1/1).

**4.5 Discussion**

The mutations that drive BCC are known. However, cell of origin studies suggest that for tumor initiation, not all cells are created equal. The hair follicle (HF) and touch dome (TD) niches efficiently form tumors in response to loss of *Ptch1*, while the interfollicular epidermis (IFE) resists tumor formation [1-3]. In contrast, tumors driven by activating mutations in *Smoothened (Smo)* derive from the IFE but not HFs [25, 26]. One explanation for this differential susceptibility is the level of HH pathway activation. Work from Grachtchouk et al., showed that nearly all epidermal populations can make BCC tumors upon maximum HH activation [27]. But why do some cell populations require higher HH signaling than others to make tumors?

While initially investigating the cell of origin for *Ptch1*-driven BCC, we found that areas which normally display active HH signaling are the most efficient at making BCC tumors. These cells possess the necessary components (e.g. cilia) to activate HH. In addition, baseline HH activation could prime cells for BCC formation. During the telogen phase, these compartments include the upper bulge region of the HF, the secondary hair germ, and the TD [5]. HH ligand is predicted to come from two sources: the dermal papilla, which signals to the secondary hair germ, and sensory nerves, which signal to the upper bulge and TD [5, 6]. Unlike
the HF and TD, the IFE is not innervated, does not display active HH signaling, and does not efficiently form tumors [1-3, 5]. In addition, surgical denervation slows BCC tumor growth in the TD [2]. Based on these observations, we hypothesized that mimicking the HH-active neural environment of the HF/TD in the IFE would impart BCC susceptibility.

In this study, we were successful in creating a model that displays ectopic innervation throughout the IFE. However, despite this neural presence, we did not observe ectopic HH activation in the IFE. There are several potential explanations why these ectopic nerves were unable to activate signaling in the IFE. Although the IFE is expressing one required growth factor for nerve survival (NT-3), perhaps other target derived factors such are required for nerve ending maturation. A subset of nociceptive nerve endings in the IFE require both NGF and GDNF for full maturation [28]. Perhaps sensory endings also require additional factors in addition to NT-3. This may prevent the secretion of HH from ectopic nerve endings in our model.

Although ectopic innervation does not cause HH activation in the IFE, we did observe activation in the hair canal or infundibulum (INF). This suggests that ectopic nerves can secrete HH ligand in some contexts. Although INF innervation is largely absent in adult mice, this compartment does display initial innervation during development [4, 29, 30]. In comparison, the IFE never displays dense sensory innervation. Based on this observation, perhaps the INF is competent to form functional nerve endings, while the IFE is not.

In our model, ectopic innervation also did not impart BCC susceptibility onto the IFE. We predict that this is due to either: 1) lack of functionality of ectopic nerves, or 2) inability of the IFE to respond to neural signals. In contrast, our preliminary data indicate that hyper-innervation does enhance IFE tumorigenesis in ear and tail skin. Unlike the dorsal IFE, both ear
and tail IFE efficiently form tumors in response to *Ptch1* deletion [31]. This increased tumor-initiation capacity is predicted to be due to increased circulation, which may result in increased TAM concentrations and recombination efficiency [31]. In our own previous studies, we have also observed enhances tumorigenesis in ear and tail skin of *K14;Ptch1* mice. Because ear and tail skin appear competent to form BCC tumors upon *Ptch1* deletion, this data suggests that hyper-innervation can enhance tumorigenesis in BCC-susceptible compartments. In agreement with this hypothesis, we observed increased tumor growth in hyper-innervated hair follicles. *Gli1;Ptch1;NT-3* follicles also displayed ectopic INF-derived tumors which resisted spontaneous regression (FIG 4.6D). We also observed an increase in tumor initiation within the touch dome, although at later time-points TD tumors were indistinguishable between control and *NT-3* animals. This initial increased in tumorigenesis in the HF/TD compartments could also be a result of increased *Gli1-CreERT2* mediated recombination. Although we did not observe a significant increase in labeling in our reporter study (FIG 4.2D), we did see increased *Gli1* expression in our *NT-3* mice (FIG 4.2C). This increased expression could drive increased frequency of *Ptch1* deletion, promoting enhances BCC tumor initiation. More detailed analysis of our *Gli1;LacZ;NT-3* reporter samples could resolve this question.

Overall, our results suggest that hyper-innervation via *NT-3* expression enhances BCC tumorigenesis but is not sufficient to impart BCC susceptibility. However, it is possible that these conclusions are clouded by the limitations of our model. Although we observed ectopic innervation, we did not observe ectopic HH activation within the IFE. This suggests that ectopic nerves may not be fully functional when present in the IFE. Further characterization, including sensory evaluation will be necessary to confirm the functional capacity of these nerves.
4.6 Acknowledgements

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4.7 Author Contributions

S.C.P. and S.Y.W. conceived and performed experiments, wrote the manuscript and secured funding. A.A.D. provided expertise, technical support and feedback.
4.8 Figures

A

B

C

D

Figure 4.1: Neurotrophin-3 expression causes hyper-innervation and touch dome enlargement.
A. Neurotrophin-3 expression construct, mouse NT-3 is cloned downstream of human Keratin 14 promoter sequence
B. (Left) flat-mount and (right) frozen sections IF staining for Neurofilament (NF, red) in wildtype and NT-3 expressing mice. Ectopic innervation (arrows) along NT-3 expressing IFE.
C. IF staining of wildtype and NT-3 TDs. Neurofilament (left, red), Keratin 17 (right, red), K8+ Merkel cells (green)
D. Quantitation of K17+ cells (left) and MCs (right) in the IFE of wildtype and NT-3 mice. (*p≤0.05, WT (n=4), NT-3 (n=3)) Data are represented as mean ± SEM.
Figure 4.2: NT-3 mice display ectopic Hedgehog activity
A. Schematic showing area of activity (blue) for Gli1-CreERT2.
B. Schematic showing experimental timeline. Mice were induced with one IP injection of tamoxifen at 8 weeks, skin biopsies and RNA were collected 4 days post induction.
C. qPCR showing that Gli1 is more highly expressed in NT-3 mice (WT (n=3), NT-3 (n=3), p=0.06). Data are represented as mean ± SEM.
D. Whole mount and on-slide LacZ staining of Gli1;LacZ and Gli1;LacZ;NT-3 skin. Labeling of TDs (black lines), upper bulge (bracket), and secondary hair germ (*) is seen in all animals. (top inset) no labeling in IFE of Gli1;LacZ skin, ectopic labeling of Gli1;LacZ;NT-3 skin. (bottom inset) expanded labeling of TDs in Gli1;LacZ;NT-3 samples. Ectopic labeling (arrows) is present in the non-TD IFE and hair canal of Gli1;LacZ;NT-3 mice (WT (n=4), NT-3 (n=3)).
Figure 4.3 Ectopic innervation does not promote dorsal IFE-derived tumorigenesis

A. Schematic showing area of activity (blue) for K14-CreER<sup>T</sup>.
B. Schematic showing experimental timeline. Mice were induced with 3 IP injections of tamoxifen at 8 weeks, skin biopsies were collected 5, 12, and 17 weeks post induction.
C. qPCR showing increased NT-3 and Gli1 expression in K14;Ptc1;NT-3 mice relative to K14;Ptc1 mice. (K14;Ptc1 (n=1), K14;Ptc1;NT-3 (n=1))

D. H&E showing phenotype progression 5, 12, and 17 weeks post induction does not differ between K14;Ptc1;NT-3 and control animals.

E. IF staining showing increased NF+ innervation of K14;Ptc1;NT-3 lesions (right, red), but no difference in BCC markers K17 (left, red) or GLI1 (center, red).
Figure 4.4: *NT-3 expression enhances tumorigenesis in ear and tail skin*
Lesions in *K14;Ptch1;NT-3* ear and tail skin (bottom) appear more advanced, branching deeper into the dermis compared to *K14;Ptch1* control lesions (white bars) (*K14;Ptch1* (n=1), *K14;Ptch1;NT-3* (n=1)).
Figure 4.5: Hyper-innervation may enhance HF-derived tumor growth
A. Schematic showing area of activity (blue) for Gli1-Cre\textsuperscript{ERT2}.
B. Schematic showing experimental timeline. Mice were induced with one IP injection of tamoxifen at 8 weeks, skin biopsies were collected 5, 12, and 17 weeks post induction.
C. H&E showing tumor progression 5, 12, and 17 weeks post induction.
D. Quantitation showing HF-derived tumor area 5, 12, and 17 weeks post induction. Gli1;Ptch1;NT-3 animals display larger tumors at all 3 time-points. (Gli1;Ptch1;NT-3 n=2/1/1, Gli1;Ptch1 n=3/1/1, for 5, 12, and 17 weeks respectively) Data are represented as mean ± SEM.
Figure 4.6: *NT-3* expression causes ectopic infundibulum-derived tumors

A. (Left) H&E showing TD-derived tumors (bracket), and ectopic infundibulum-derived tumors (*) in *Gli1;Ptch1;NT-3* mice 5 weeks post induction. (Center) TD-derived tumors (K17, red) retain association with Merkel cells (K8, green, arrows). (Right) Infundibulum-derived tumors (yellow dashed) do not display associated Merkel cells.
**B.** Quantification of TD-derived tumor area. (*Gli1;Ptsch1 n=1, Gli1;Ptsch1;NT-3 n=1*)

**C.** (Left) H&E showing large TD-derived tumors in *Gli1;Ptsch1;NT-3* and *Gli1;Ptsch1* mice 17 weeks post induction. (Right) IF staining showing increased NF+ innervation (arrows) of *K14;Ptsch1;NT-3* lesions, but no difference in BCC markers K17 or GLI1.

**D.** H&E showing that INF-derived tumors (*) in *Gli1;Ptsch1;NT-3* mice resist regression at 12 weeks post TAM.
4.9 Reference List

Chapter V – Patched2 does not modulate basal cell carcinoma in an inducible Patched1 deletion model

5.1 Abstract

Basal cell carcinoma (BCC) is caused by deregulated Hedgehog signaling, typically due to mutations in the tumor suppressor Patched 1 (PTCH1). Under normal conditions, PTCH1 serves as the primary Hedgehog receptor; however, its paralog, Patched 2 (PTCH2), is thought to possess redundant properties. Previous findings have shown that in mouse embryonic skin, loss of both Ptch1 and Ptch2 causes a more severe developmental skin phenotype than loss of either receptor alone; however, whether loss of PTCH2 promotes BCCs in adult skin remains unclear. Our studies suggest that simultaneous loss of both PTCH receptors in adult mice does not cause increased Hedgehog (HH) activity or tumor growth.

5.2 Introduction

Using a variety of tissue specific Cre recombinase drivers, our lab characterized how different cell populations within the epidermis respond to Ptch1 deletion. Our results suggest that upon Ptch1 deletion, BCCs arise preferentially from cells within the hair follicle (HF) and mechanosensory touch dome (TD) (see chapter III). Surprisingly, we also found the interfollicular epidermis (IFE) to be resistant to BCC formation (see also chapter III). This confirms previous studies by Wang et al., and Kasper et al., who also reported HF-derived BCC tumors after Ptch1 deletion [1, 2]. Mutations in Smoothened (SMO) also drive a subset of human BCC tumors. In contrast to PTCH1 driven tumors, mouse models of SMO-driven BCC
suggest an IFE origin [3]. In addition, mice which express activated GLI2 form tumors from the HF and IFE compartments [4]. These conflicting results suggest variable susceptibility of the epidermis to BCC formation based on different driver mutations and level of HH activation.

Although PTCH1 is the primary HH receptor, another less characterized receptor is its paralog Patched 2 (PTCH2). PTCH2 has structural similarities to PTCH1, and shares sequence homology [5], although PTCH2 has a truncated C-terminus. Like Ptch1, Ptch2 is transcriptionally up-regulated in response to HH activation. As such, Ptch2 is highly expressed in HH-driven tumors such as BCC and medulloblastoma [6, 7]. Both Ptch receptors have ligand binding properties. This allows them to function in a negative feedback loop to repress signaling through ligand-sequestration [8]. Work from Adolphe et al., suggests that in the embryonic epidermis, loss of Ptch1 is partially compensated by expression of Ptch2, as HH activation and developmental disruption increases with additional loss of Ptch2 [9, 10]. This suggests PTCH2 can inhibit HH activation in the absence of PTCH1. In addition, dual loss of the PTCH receptors causes higher HH activation in vitro [11] and in vivo [9, 10] compared to loss of Ptch1 alone. Based on these studies, coupled with our observation that the IFE resists BCC formation, we hypothesized that PTCH2 could be inhibiting IFE-derived BCC in the absence of PTCH1.

In contrast, Ptch1 deletion in the HF/TD is sufficient to induce BCC formation. This could be due to the presence of nerve-derived HH ligand. In vitro studies have highlighted a primary role for PTCH2 in mediating HH response in the absence of PTCH1 [11, 12]. Using mouse embryonic fibroblasts (MEFs) and neutralized embryoid bodies (NEBs), mouse embryonic stem cells that have aggregated to resemble early caudal neural tube, Alfaro et al., highlighted the ability of Ptch1/-/- cells to activate target genes in response to endogenous and recombinant Sonic Hedgehog (SHH) ligand [12]. This response is at least in part mediated by
PTCH2, as \(Ptch1^{-/-}; Ptch2^{-/-}\) NEBs have a higher level of HH activation than \(Ptch1^{-/-}\) cells [12]. In addition, dual loss of the PTCH receptors causes expanded HH activation in the neural tube, compared to loss of \(Ptch1\) alone [13]. Based on these results, we hypothesize that in HF/TDs lacking PTCH1, nerve-derived HH ligand binds and inhibits PTCH2, allowing maximum HH activation and BCC tumor growth.

To investigate whether PTCH2 inhibits BCC in the absence of PTCH1, we deleted \(Ptch1\) in specific epidermal compartments using two Cre recombinase drivers in \(Ptch2^{-/-}\) mice. Here we demonstrate that loss of \(Ptch2\) does not affect BCC initiation or progression in the IFE, HFs or TDs of \(Ptch1^{loxp/loxp}\) mice. During this study, we also observed spontaneous regression of HF-derived tumors between 12-17 weeks after induction with tamoxifen. Evidence of regression has been observed in human BCC tumors and is predicted to be immune-mediated [14-16]. However, our samples showed no evidence of immune infiltration. We believe this regression could be hair cycle mediated, although further studies will be necessary to confirm this hypothesis. Regression was also not altered by loss of \(Ptch2\). We also observed that TD-derived tumors are resistant to regression. Instead, TD tumors progress to characteristic BCC-like tumors after 17 weeks. Together, these results indicate that PTCH2 does not play an inhibitory role in BCC tumorigenesis in adult mice.

5.3 Materials and Methods

5.3.1 Animals

The following mice were used: \(Gli1^{tm3(cre/ERT2)Alj}\) (\(Gli1^{-Cre_{ERT2}}\)) [17]; \(Tg(KRT14-cre/ERT)20Efu\) (\(K14-Cre^{ERT}\)) [18]; \(Ptch1^{tm1Hahn}\) [19]; \(Ptch2^{-/-}\) [13].
5.3.2 Mouse manipulations

For all tumor experiments, animals were induced with tamoxifen during telogen at 8 weeks of age. Mice were treated with tamoxifen as follows: one dose at 5 mg per 40 grams body weight for \textit{Gli1;Ptch1;Ptch2}, and three daily doses, each 1 mg per 40 grams body weight, for \textit{K14;Ptch1;Ptch2} mice. Biopsies were performed and harvested as previously described [20]. All studies were performed in accordance with regulations established by the University of Michigan Unit for Laboratory Animal Medicine.

5.3.3 Tissue staining

Biopsies were fixed for 1 hour in cold 3.7% paraformaldehyde, washed and incubated overnight in 30% sucrose at 4 degrees, before embedding in OCT mounting media. Frozen sections were stained using standard protocols with the following antibodies: rabbit anti-K17 (D73C7, 1:1,500, Cell Signaling); rat anti-K8 (TROMA-I, 1:500, Developmental Studies Hybridoma Bank); rat anti-CD200 eFluor660 (OX90, 1:2,000, eBioscience); and goat anti-K14 (sc-17104, 1:1000, Santa Cruz).

5.3.4 Quantitation

To quantitate hair follicle-associated tumors, for each \textit{Gli1;Ptch1;Ptch2} sample, we measured tumor area from 3 non-overlapping 10x images of representative H&E stained sections (1cm in length, ~10um thick). We outlined tumors in Photoshop from 10x images, and recorded tumor area in pixels.
5.4 Results

5.4.1 Loss of Ptch2 does not induce IFE-derived BCC tumors in K14;Ptch1 mice

We first sought to test whether PTCH2 protects the interfollicular epidermis (IFE) from BCC formation in the absence of PTCH1. For these experiments, we utilized the tamoxifen (TAM) inducible K14-Cre\textsuperscript{ERT} [21] allele in combination with homozygous Ptch1\textsuperscript{floX} alleles [22] (K14;Ptch1). This system enables deletion of exons 8+9 of Ptch1 specifically in the basal cells of the IFE, with minimal recombination in the hair follicle (HF) [20, 23, 24](FIG 5.1A). We combined these alleles with 1 or 2 mutant alleles of Ptch2 (Ptch2\textsuperscript{+/-} or Ptch2\textsuperscript{-/-})[13]. We induced mice with TAM at 8 weeks, and collected skin biopsies 5 and 10 weeks after induction, as previously described [23](FIG 5.1B). Consistent with our previous study, after 5 weeks K14;Ptch1;Ptch2\textsuperscript{+/-} mice did not develop BCC tumors (FIG 5.1D). This suggests that losing 1 copy of Ptch2 in combination with Ptch1 deletion does not affect BCC formation in the IFE. After 10 weeks, K14;Ptch1;Ptch2\textsuperscript{+/-} mice display hyperplastic epidermis with small lesions resembling early stage benign follicular hamartoma (BFH) (FIG 5.1D). More extensive lesions are present, but are attached to the infundibulum, or HF canal (FIG 5.1D). Surprisingly, we observed no significant differences between K14;Ptch1;Ptch2\textsuperscript{+/-} and K14;Ptch1;Ptch2\textsuperscript{-/-} mice at 5 or 10 weeks after TAM (FIG 5.1D). We also harvested ear and tail skin biopsies at 10 weeks, and again observed no obvious differences between genotypes (FIG 5.1E). We confirmed Ptch2 deletion and HH activation by performing qPCR on epidermal RNA collected 10 weeks post TAM. Although we observed complete loss of Ptch2 expression, there was no significant difference in expression of HH target gene, Gli1 (FIG 5.1C). Immunofluorescence staining for BCC marker K17 also did not differ between genotypes (FIG 5.1D). This suggests PTCH2 does not inhibit IFE-derived BCC in the absence of PTCH1 in adult mice.
5.4.2 Loss of Ptch2 does not promote BCC tumorigenesis in Gli1;Ptch1 mice

To test whether deletion of Ptch2 augments HF-derived tumor growth, we utilized Gli1-Cre\textsuperscript{ERT2} [25], which drives recombination in the upper and lower bulge, secondary hair germ (SHG), and the touch dome (TD) [26](FIG 5.2A). We combined this allele with homozygous \textit{Ptch1}\textsuperscript{flax} alleles [22] (Gli1;Ptch1), and 1 or 2 mutant alleles of Ptch2 (Ptch2\textsuperscript{+/-} or Ptch2\textsuperscript{-/-})[13]. Similar to our previous studies using Gli1;Ptch1 mice, Gli1;Ptch1;Ptch2\textsuperscript{+/-} mice form microscopic HF-derived tumors 5 weeks after induction (FIG 5.2D). HF-derived tumors in Gli1;Ptch1; Ptch2\textsuperscript{-/-} mice were indistinguishable from Gli1;Ptch1;Ptch2\textsuperscript{+/-} mice (FIG 5.2D). Although Ptch2 expression is absent in mutant mice, expression of HH target gene \textit{Gli1} (FIG 5.2C) and immunofluorescence staining for BCC markers (K17, GLI1, CD200) were indistinguishable between genotypes (FIG 5.2E). These results suggest that loss of Ptch2 does not enhance BCC tumorigenesis in the HF.

5.4.3 Lower HF-derived BCC tumors undergo spontaneous regression

Evidence of partial regression is observed in several skin tumors including BCC (reviewed in [27]). In a prospective study of 400 randomly selected BCC tumors, Curson and Weedon observed evidence of regression in 6% of tumors [14]. In a subsequent study of 62 BCC tumors, Hunt et al., observed histological evidence of previous or active regression in 41 (66%) tumors [15]. These regressing human BCC tumors also showed evidence of lymphatic infiltration, suggesting immune-mediated regression [15]. Cytokine profiling of regressing BCCs highlighted an increase in IFN-\gamma expression [16]. These results, coupled with increased CD3+ and CD4+ T-cells and \textit{IL-2R} expression in actively regressing BCCs, suggests a role for activated T-cells in human BCC regression [15]. Although partial regression is seen in a sub-set
of BCC tumors, full regression is very rare. As such, we believe the regression observed in our model is not a representation of human BCC biology and instead a potential limitation of our system.

There are several fundamental differences in mouse and human hair cycling. Mouse hair cycling occurs in waves, with sub-populations cycling in synchrony. This synchronization is controlled by Bone Morphogenic Protein 2 and 4 (BMP2/4) expression [28]. BMP signaling from the dermal macro-environment provides an inhibitory signal to prevent telogen follicles from anagen induction. This inhibition can be overcome by epidermal Wnt activation and expression of the BMP-inhibitor, Noggin [28-31]. This inductive signal spreads until the anagen wave encounters a population of follicles which are refractory, due to high expression of BMP [28]. In contrast, human follicles cycle independently, and have a drastically longer anagen phase compared to mice [32, 33]. The anagen phase of human scalp follicles can last between 2-6 years [33]. This explains the difference in hair length between humans and mice. However, other hair follicle types, such as facial hair in humans, cycle more frequently [33].

At 12 weeks post TAM administration, we observed spontaneous regression of HF-derived tumors in all Gli1;Ptch1;Ptch2 mice regardless of Ptch2 status. HF tumor area regression ranged from $\downarrow53$-$99\%$ (FIG 5.2F). After 17 weeks, 4 of 7 mice showed additional regression ($\downarrow13$-$55\%$), while 3 mice displayed tumor progression ($\uparrow16$-$31\%$) (5.2F). Regression/progression did not correlate with Ptch2 status. Although immune infiltration is the proposed mechanism of spontaneous regression in human BCC, there was no apparent histological evidence of immune infiltration in regressed samples.

There are several alternative explanations for regression in our model. The first explanation could be an indirect effect relating to the overall health of the mice. Indirect effects
due to Gli1-Cre\textsuperscript{ERT2}-mediated loss of \textit{Ptch1} in the digestive tract [34], could contribute to health decline. The mice which displayed the strongest regression (\(\downarrow\)97-99\%) at 12 weeks post TAM, were in poor health and did not survive the biopsy procedure, suggesting that overall animal health could contribute to tumor regression. Another potential mechanism for spontaneous HF tumor regression is hair follicle cycling. In the \textit{Gli1;Ptch1} model, tumors are derived from the HF bulge and secondary hair germ (SHG). Both compartments undergo major remodeling throughout the hair cycle [35-37]. Perhaps, tumor cells retain their ability to respond to the strong dermal BMP signals present throughout mouse hair cycling, and regress as follicles re-enter the telogen phase. We examined the hair cycle status at each time-point, but observed no correlation between HF tumor area and hair cycle stage (data not shown). However, because the timing of hair cycling in adult mice is variable (reviewed in [33, 38]), it is unclear how many cycles occurred between our experimental time-points. To address whether hair cycling or induces HF tumor regression, mice would need to be carefully monitored between biopsies, and samples taken at different stages of the hair cycle.

5.4.4 Touch dome-derived tumors resist spontaneous regression

In addition to HF-derived BCCs, \textit{Gli1;Ptch1;Ptch2} mice develop tumors derived from the touch dome (TD), a mechanosensory organ that senses light touch. Our previous study highlighted the TD as a “hot spot” for BCC formation in \textit{Gli1;Ptch1} mice [23]. The TD is densely innervated by sensory nerves, which secrete HH ligand to activate signaling within the TD [39]. These nerves also support BCC growth, as surgical denervation mitigates tumor growth [23]. TDs are located caudally to a subset of HFs called tylotrich or guard hair follicles. A recent study by Marshall et al., suggests TDs undergo major remodeling throughout the hair
cycle [40]. However, despite their association with hair follicles TD-derived tumors appear to resist spontaneous regression. At 5 weeks post TAM in Gli1;Ptc1 mice, the TD compartment begins to grow downward, displacing underlying Merkel cells (MCs) (FIG 5.3A) [23]. By 17 weeks post induction, HF derived tumors are hardly visible (FIG 5.3B). However, TD tumors become advanced and develop changes in pigmentation, as seen in a sub-set of human BCCs [41-44]. Pigmented TD tumors are macroscopically visible, appearing as dark raised dots present throughout the skin (FIG 5.3B).

5.5 Discussion

Mouse models have highlighted conflicting cellular origins for BCC. While SMO-M2 expression drives tumors derived from the IFE [3], Ptc1 deletion is not sufficient to induce IFE tumors [1, 2, 23]. In contrast, Ptc1 deletion causes HF and TD-derived tumors after only 5 weeks [23], while SMO-M2 expression in the HF bulge does not yield tumors [3]. In the embryonic epidermis, Ptc1 deletion is compensated by expression of its homologue, Ptc2 [10]. As such, deletion of both PTCH receptors causes higher HH activation and a more severe developmental phenotype then loss of either receptor alone [9, 10].

Based on these results, we hypothesized that in the adult IFE, PTCH2 could be partially compensating for the loss of Ptc1. This could explain the milder phenotype observed in K14;Ptc1 mice (FIG 5.1D). We have seen previously that Ptc2 is up-regulated in the absence of Ptc1 [23]. However, our results suggest that deletion of Ptc2 in conjunction with Ptc1 does not impart BCC susceptibility onto the IFE (FIG 5.1D). In addition, loss of Ptc2 did not increase the level of HH activation above Ptc1 deletion alone (FIG 5.1C). This suggests that in our model, PTCH2 does not inhibit HH activation or BCC formation in the absence of PTCH1.
These results directly oppose published results from Adolphe et al., who observed a striking developmental phenotype upon dual loss of the PTCH receptors during embryogenesis [10]. There are several potential explanations as to why embryonic skin might respond differently to loss of Ptch1/2 compared to adult skin. Firstly, the microenvironment of the epidermis during development is strikingly different than adult skin. During development, the epidermis is more proliferative, and undergoes drastic morphological changes. As such, differential response to oncogenic signaling is not entirely surprising. Secondly, while the tamoxifen dosages used in this study promote ~15% recombination via K14-Cre<sup>ERT</sup> throughout the adult IFE (see chapter III). Embryonic deletion of Ptch1 using K5-Cre is much more efficient [10]. This difference in efficiency of Ptch1 deletion could also account for differential tumor response. In addition, a recent study by Roberts et al., highlighted the ability of PTCH receptors to non-cell autonomously inhibit SMO [45]. Based on this observation, in our model cells which escape Ptch1 deletion may inhibit HH signaling in adjacent Ptch1/Ptch2 null cells. Adolphe et al., also observed more severe transformation in adult K14-Cre<sup>ERT2</sup>;Ptch1<sup>loxo/lox</sup>;Ptch2<sup>tm1/tm1</sup> compared to K14-Cre<sup>ERT2</sup>;Ptch1<sup>loxo/lox</sup> mice 4 weeks after tamoxifen treatment [10]. There are several possible explanations for why these results diverge from ours. Firstly, it is important to note the differences in alleles used in these studies. Adolphe et al., employed K14-Cre<sup>ERT2</sup> [46], which utilizes the human Keratin 14 promoter cloned upstream of Cre<sup>ERT2</sup>. While the K14-Cre<sup>ERT</sup> used in our study preferentially targets the IFE, this promoter is more broadly active throughout the epidermis and hair follicles [47]. This broader recombination and epidermal disruption could account for different tumor phenotypes, although the phenotype observed in K14-Cre<sup>ERT2</sup>;Ptch1<sup>loxo/lox</sup> mice appears similar to what we have previously observed in our K14;Ptch1 model. Additionally, the Ptch1<sup>loxo/lox</sup> mice used by
Adolphe et al., target deletion of exon 3 of *Ptch1* [48], while our model targets exons 8+9 [22]. Differences could also be attributed to the use of two different targeted alleles of *Ptch2* (*Ptch2*^tm1/tm1^ [49] vs. *Ptch2*^−/−^ [13]). Divergent results could also be the result of experimental design differences, as Adolphe et al., employed topical tamoxifen administration to induce *Ptch1* deletion [10]. This requires shaving of dorsal skin prior to TAM administration. Perhaps shaving induced micro-wounds to the dorsal skin, creating an environment which is more susceptible to tumor formation, as seen in previous studies [2, 20]. Although there are several potential explanations, further experiments will be necessary to confirm why our observations do not match those observed by Adolphe et al., [10].

Unlike the IFE, the HF/TD compartments efficiently form tumors in response to *Ptch1* deletion. These compartments also display active HH signaling under normal conditions. This is due to HH ligand secreted by the dermal papilla (DP) and sensory nerves [26]. Because *Ptch2* is a target of the HH pathway, *Ptch2* is upregulated in BCC tumors [6, 23]. However, despite *Ptch2* expression in BCC, loss of *Ptch2* does not affect BCC tumor growth in our model (FIG 5.2D). In addition, *Ptch2* deletion does not increase HH activation in the absence of *Ptch1* (FIG 5.2C). There are two potential explanations for this result, 1) it is possible that loss of *Ptch1* in the TD/HF compartments where HH ligand is present, achieves maximum HH activation which is not enhanced by loss of *Ptch2* or 2) residual *Ptch1* expression in non-recombined cells compensates for loss of *Ptch1/2* in tumor cells. The first possibility has been proposed by Adolphe et al., in a recent publication where the level of HH activation in *Ptch1/2* null cells is equal to wildtype hair follicle cells in the bulb which receive a high concentration of HH ligand [9]. The second stems from observations made by Roberts et al., where *Ptch1/2* null cells co-cultured with cells expressing *Ptch1* display lower HH activation [45].