NEW MOUSE MODELS TO AID STUDIES OF THE ROLE OF PRIMARY CILIA IN LIMB PATTERNING AND BONE DEVELOPMENT

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CELL BIOLOGY

ABSTRACT

Hedgehog (Hh) signaling is required for many developmental processes, and causes several diseases and cancers. Described in chapter one below, vertebrate Hh signaling is dependent on the primary cilium (PC), a small organelle that extends from the surface of most mammalian cell types. A better understanding of how the PC modulates Hh signaling is important, as defects in the structure or function of PC result in severe human disorders termed the ciliopathies. For example, patients with Meckel–Gruber Syndrome have extra digits and neural tube defects, which are Hh both related phenotypes. In chapter two we sought to further understand the relationship between Hh mediated limb patterning and cilia loss on different cell populations in the limb. Using multiple Cre lines to delete the ciliogenic gene Ifit88 in the limb, no patterning defects were observed. This was due to the fact that cilia remained on many cells days after Cre induction in vivo. Follow up in vitro studies revealed prompt genetic recombination of Ifit88 and Kif3a, however these proteins were detectable for more than 96 hours. These data indicate that protein and PC loss were prolonged beyond the window of Hh patterning. To complement this study, in chapter three, a Cre line was generated using the Alx4 promoter. This is the first Cre line known to exclusively affect the anterior limb. We used it to develop a fate map of Alx4 expressing cells and noted that they do not contribute to the ulna, and that Alx4 is expressed in the developing kidney. Finally, in chapter
four, we developed a mouse expressing a fluorescent ciliary protein, Sstr3::GFP, for direct visualization of PC in vivo. Interestingly, with this model we were able to observe ciliary movement within live renal tubules, and robust labeling of PC in many organs. The CiliaGFP mouse will allow for real-time evaluation of cilia in live samples, and will facilitate the evaluation of ciliary integrity over time. In sum, these studies have provided insights into the temporal requirements of cilia during limb patterning and have yielded useful tools for in vivo analysis of cilia function in multiple tissues.

Keywords: Cilia, limb, bone, Cre, Mouse, Hedgehog, Gardener Mouse, CiliaGFP
DEDICATION

For my loving Father. Ever an inspiration and whip cracker. I remember you telling me that I was not allowed to get any B’s in school; you never got B’s so I could not either. It got me here.
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I need to acknowledge those that bore the burden of my studies. In the beginning there was Kelly Dunham, and she was still there in the end- ON ON. My lab-mates Corey Williams and Mandy Croyle, you helped me cope. I must also thank Erica Brown who would not let me swear, Jake White who loaned me a dollar once, and Michael Ceballos who introduced me to hipster music. Kevin Vanderwall, my own personal artist, I am thankful for your generosity and curiosity. Alecia Gross and lab, you were inviting and fun when I needed it the most. Laura Cotlin I hope I grow up to be like you. GMZ. James Machamer, the best friend who hated me. Jim, you have made me proud, and you made me realize that I am not the only perpetually lost soul. It was always you and me against the world. I’d also like to thank Brad, I was not always easy to work with. And finally, the man who won my heart: Marcus Furlow. You will never understand how grateful I am to have you in my life, you will never understand how much you have meant to me, nor how you have kept me sane.
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**Introduction:** Primary cilia are solitary, nonmotile, microtubule based structures that extend from nearly every cell type in the mammalian body. Despite an indispensable role in development, the precise functions of primary cilia are not well defined. One developmental pathway that is altered in cilia mutants, and requires functional cilia, is the Hedgehog (Hh) pathway. Indeed, cilia mutants present with several phenotypes that are linked to Hh signaling defects. The objective of my studies has been to use the limb bud as a model system to explore the functional requirement for cilia in limb development. A better understanding of the mechanisms responsible for the defects observed in cilia mutants will be critical in defining the role of cilia in limb patterning and bone development. While it is known that removal of cilia throughout the limb bud results in extensive polydactyly and bone defects, we do not know the source of these extra digits or the means by which they arise.

**Cilia structure and defects:** Primary cilia are immotile, microtubule based structures that are built and maintained by the process of Intraflagellar transport (IFT) (for details see [1-3]). IFT is mediated by large protein complexes that facilitate transport along the bundle of microtubules that make up the axoneme. Large protein complexes travel along these microtubules, Complex A mediates retrograde transport, while complex B participates in anterograde transport. The kinesin-II motor proteins Kif3a and Kif3b are associated with retrograde movement of complex B, and the cytoplasmic dynein motor DHC2...
is responsible for the retrograde movement of complex A. The components of the IFT particle and other proteins destined for the cilium congregate at the base of the cilium where it is thought that various ciliary cargoes such as receptors, signaling effectors, and structural components are loaded onto the IFT particle and transported along the axoneme into the cilium [1-3].

The roles and importance of motile cilia are well established, including functions in mucus movement in the trachea, and sperm locomotion. The significance of primary cilia found in nearly all cells types in mammals, however, has only recently been acknowledged [4]. Initial presumptions that primary cilia were vestigial were proven false when mutations in ciliogenic genes were found to be pathogenic and often prenatally lethal [5-8]. The growing list of human diseases associated with defects in ciliary biogenesis or signaling, termed the ciliopathies, further supports the indispensable nature, and clinical importance of primary cilia. Common phenotypes associated with cilia dysfunction are cystic kidneys, hydrocephalus, blindness, anosmia, left-right body axis defects, neural tube closure and patterning defects, and polydactyly [4, 9]. Primary cilia differ from motile cilia in their microtubule substructure (Figure 1a and 1b). In addition, primary cilia have been shown to participate in multiple developmentally significant pathways, for example Planar Cell Polarity (PCP) and Hedgehog (Hh), whose disregulation contributes to disease phenotype [10-13].

**Hedgehog signaling:** Hedgehog signaling is initiated when one of the three mammalian ligands, Sonic (Shh), Indian (Ihh), or Desert Hedgehog (Dhh), binds to the transmembrane receptor Patched1 (Ptch) which is found within the ciliary membrane (the
related protein, Patched2 seems to be dispensable for Hh signaling) (for detailed information see Figure 1 and [14-18]). Concomitant with its residence in the cilium Ptch represses the activating capability of another integral membrane protein, Smoothened (Smo). Once ligand binding occurs, Ptch internalizes from the ciliary membrane and the repression of Smo is alleviated [19]. Smo then translocates into the cilium to activate the pathway through the transcription factors Gli1-3 which reside therein. Gli1, acts as an activator, and a read-out of pathway activity due to its own upregulation [20]. Genetic analysis suggests that Gli2 functions as either an activator or repressor depending on post-translational modifications. The extent of in vivo processing and repression, as well as the regulatory steps involved in Gli2 activation are not yet fully understood, but each appears to be dependent on functional IFT/Cilia [21-23]. The processing of Gli3 into a

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**Figure 1: Ciliary substructure and Hedgehog Signaling.** (a) Primary, immotile, cilia have a 9+0 axoneme with nine microtubule doublets. (b) Motile cilia require a central pair of microtubules as well as radial spokes to generate movement. (-Hh) In the absence of Hh ligand the receptor Ptch in the ciliary membrane represses Smo. Gli Activators accumulate in the cilia and Gli Repressor keeps target genes off. (+Hh) In the presence of ligand Ptch is internalized and degraded, Smo enters the cilium, Gli Activator function is enhanced while Gli Repressor formation is inhibited. Target genes (Gli1, Ptch, others) are activated.

Note: Adapted from “The Primary Cilium as a Complex Signaling Center” by NF Berbari, AK O’Connor, CJ Haycraft, BK Yoder, 2009, Current Biology, Volume 19, Issue 13, Pages R526–R535. Copyright 2009 Elsevier Ltd, adapted with permission.
repressor is well documented; in the absence of Hh ligand Gli3 undergoes phosphorylation, ubiquitination, and partial degradation by the proteasome forming Gli3R [22, 24, 25]. The processing of Gli3 is inhibited during Hh signaling allowing the activator molecules (Gli1 and 2) to carryout transcription of target genes. Hh signaling is altered in cilia mutants because the cells are defective in Gli processing and activation, improper localization of pathway components, and failed signal reception [4, 13, 24].

Ptch acts as the primary mechanism for cell-autonomous pathway modulation. As shown in Figure 1, Ptch binds Hh and then is internalized, acting to sequester and decrease the amount of diffusible ligand [26]. In addition, Ptch is upregulated in response to signaling which further reduces free ligand when bound [18]. These actions serve as a concentration detection system for a cell. When the Hh signal is initially received the cellular response is robust as most of the Ptch receptors become ligand bound. Thus as more Ptch is expressed either the signal remains robust (indicating high concentrations of Hh), or the signal is progressively reduced due to the Ptch:ligand ratio indicating that the morphogenic source is some distance away [16]. It is hypothesized that the amount of time a cell is exposed to detectable levels of Hh translates into higher and more lasting expression of Gli1 activator allowing for secondary and tertiary gene target activation [16]. This allows for dynamic changes in the expression profile of a cell based on the Hh signal in concentration and time, and as a consequence cells adopt different fates (i.e. an anterior vs posterior digit). Thus, this negative regulation of the pathway allows a cell to assess the concentration of the ligand and to respond accordingly [18].
Limb development and patterning: Limb development is a complex process dependent on multiple signaling pathways (for details see [27-30]). The limb is patterned in its three axes by signals emanating from distinct regions of the limb bud. The dorsal-ventral (DV) axis is established by Wnt signals from the dorsal ectoderm [31-33]. The proximal-distal (PD) axis is regulated by Fgf signals produced by the apical ectodermal ridge (AER), a specialized group of cells that runs along the most apical boundary of the limb bud spanning from anterior to posterior [34-36]. The anterior-posterior (AP) axis is specified by Hh signaling initiating in a mesenchymal population of cells in the most posterior region of the limb bud in the zone of polarizing activity (ZPA) [35, 37, 38]. In mammals these signals give rise to the stylopod (humerus/femur), zeugopod (radius-ulna/tibia-fibula), and autopod with five digits numbered I-V from the anterior (thumb) to posterior [39]. Many of the details known regarding limb patterning have been resolved through molecular and genetic manipulations; however, exactly how all of these signals are received and interpreted on a cellular level to form a properly patterned limb is still under investigation.

PD outgrowth is controlled by the production of several Fibroblast Growth Factor (Fgf) molecules in the AER [34]. Fgf10, produced by the mesenchyme underlying a polarized epithelium, is thought to specify the AER and in response the ectodermal cells aggregate and produce Fgf8. Rapid proliferation of the cells directly adjacent to the AER in a region called the ‘progress zone’ is maintained by Fgf8 (and later Fgf4). The progress zone is associated with proliferation keeping a population of cells dividing for outgrowth. Two models have been proposed for PD specification; in one the AER specifies cells to become more distal elements after longer amounts of time under its influence.
This ‘progressive specification’ model claims that the cells are malleable and that their fate is determined over time [40, 41]. The other model suggests that the AER only causes outgrowth of pre-specified cell populations of the early limb bud that already know their fate and simply need to expand [42]. Despite this debate, it is known that Fgf8 induces the expression of Shh from the posterior limb mesenchyme which then induces Fgf4 expression in the AER. Together these signals positively feed forward on each other with the AER maintaining the ZPA and the ZPA maintaining the AER. In addition, local expression of the Bone Morphogenic Proteins (BMPs) negatively influences the AER, as implanted BMP beads are capable of inhibiting AER signaling and inducing apoptosis [43, 44]. Thus, BMP expression in the developing limb serves to regulate the region and extent of Fgf signaling. The BMPs themselves are negatively influenced by Shh activity as the BMP antagonist Gremlin (Grem) is upregulated by Hh signaling [45]. Ultimately these overlapping signaling pathways strictly control limb patterning- for example many of these molecules are ectopically expressed or absent in mice with patterning or digit anomalies [46-48].

AP patterning is established by the expression of Shh, a morphogen that is secreted from the ZPA in the posterior limb bud. Early in limb specification the expression of dHAND serves to antagonize Gli3 and promotes Shh expression in the ZPA [49]. The ZPA was shown to posses AP patterning capabilities upon its transplantation onto the anterior side of the limb resulting in the mirror image duplication of digits. Shh was subsequently identified as the specific polarizing factor in the ZPA through similar experiments [37]. Shh patterns the limb by regulating the expression and activity of many genes known to affect cell fate and limb morphogenesis (e.g.: HoxD genes, Gli1-3, Ptch,
BMPs, Grem, Fgfs, and Alx4). One of the most influential targets of Shh signaling is the transcription factor Gli3. Gli3 acts predominantly as a repressor (Gli3R) when proteolytically processed in the absence of Shh [50]. The formation of Gli3R is inhibited by Shh signaling and as such a reciprocal gradient of Shh and Gli3R occurs across the AP limb field. Mis-expression of many patterning genes is noted in Shh mutants, as well as in models of polydactyly, suggesting a role for Shh mediated gene expression in regulating digit number [46-48]. Indeed, Shh null mice develop only a rudimentary zeugopod and an autopod with a single digit that resembles digit I (the thumb), whereas Gli3 mutant mice present with severe polydactyly and digits whose identities are uncertain [51, 52].

The distinct location of Shh expression causes a gradient of pathway activity throughout the limb which dictates both the number and identity of the digits [21]. It is believed that cells interpret the concentration and length of exposure to Shh yielding distinct digit identities, converting from the ‘default’ anterior fate to progressively more posterior fate with prolonged Shh exposure [53]. Fate mapping studies indicate that cells that did once reside within the ZPA expand and contribute to digits IV and V as well as parts of digit III [54]. Further, the diffusion of Shh can stimulate pathway activity as far away as digit II as determined by downstream gene expression [55, 56]. Together this allows for the division of the developed limb into three distinct domains: a Shh independent domain (digit I, presumed), a Shh receiving domain (digits II - V), and a Shh expressing domain (digits IV and V). The Shh independent domain is not derived from cells that were part of the ZPA nor does it receive ligand. The Shh responding domain of cells includes those that were brief and early residents of the ZPA but who primarily receive diffusible ligand. Finally, the Shh producing domain is derived directly from cells that were
part of the ZPA late into development. Together, it is believed that the amount of Shh signal a cell receives, both in concentration and length of exposure, is the determining factor of its specific gene expression profile which in turn determines the fate of the cell [17].

**Endochondral bone formation:** Endochondral bone formation (EBF) is another developmental process dependent on proper Hh signaling, however Ihh is the primary ligand (for details see Figure 2 and [57-60]). In the developing limb where a bone will form the mesenchymal cells condense and differentiate into Sox9 expressing chondrocytes [61]. The cells at the periphery of the condensation differentiate into perichondrium which later gives rise to the bone collar. The cells in the center of the condensation become chondrocytes which progressively differentiate and lay a cartilage matrix for the eventual mineral deposition by osteoblasts. The cells at the most polar ends of a developing long bone (the epiphyses) are chondrocytes that are small, round, rarely divide, and are referred to as resting chondrocytes. The cells closest to the primary spongiosa are induced to proliferative by Ihh and divide longitudinally forming columns causing the anlagen to lengthen. As the cells progress away from the epiphyses due to continual proliferation, they then differentiate into pre-hypertrophic chondrocytes. These cells are large, round, and express Ihh. The ligand diffuses towards the resting chondrocytes to induce proliferation, and to the perichondrium causing the expression of the parathyroid hormone related protein (PTHrP). PTHrP is highly expressed in the epiphysis and inhibits proliferation. The reciprocal signals of PTHrP and Ihh on the resting chondrocytes maintains a pool of chondrocytes and the appropriate rate
of proliferation to allow the long bones to lengthen throughout development [57-60].

As shown in Figure 2, the chondrocytes continue to differentiate, progressing from pre-hypertrophic to hypertrophic expressing a matrix rich in type 10 collagen, and vascular endothelial growth factor which induces vessel sprouting from the perichondrium into the anlagen along the central length of the forming bone (diaphysis) [57-60]. The hyper-
trophic cells will apoptose and osteoblasts circulate in through the newly formed vessels. The osteoblasts will lay the final mineralized bone matrix upon the former cartilage matrix. At this time Ihh also induces Hh and Wnt signaling within the perichondrium initiating osteoblast differentiation and the production of a ring of bone around the chondrocyte center. The perichondrium is the primary responder of the Ihh signal from the pre-hypertrophic chondrocytes, as indicated by Ptch and Gli1 expression [62].

**Limb mutants:** Together these developmental processes make the limb an ideal model system in which to analyze the role of primary cilia in Hh related patterning and bone development. As described above Hh signaling regulates the activity of Gli3, the expression of the Hox genes and other transcription factors, maintains the AER, regulates digit number, plays a critical role in chondrocyte differentiation and proliferation impacting bone growth [28, 45-48]. Each of these processes requires intact Hh signaling, and ultimately the primary cilium. Indeed, hallmark phenotypes of cilia mutants include phenotypes associated with defects in these specific aspects of limb development and resemble Gli3 and Ihh null mutants [13, 63].

Multiple seminal studies have contributed to a simplified view of limb development. Gli3 mutant animals present with severe polydactyly having ~8 digits of unidentifiable pattern (Figure 3b) [52]. This has been shown to be independent of Shh (Gli3;Shh compound mutants are identical to Gli3 mutants) and is thought to be due to the loss of Gli3R and the expansion of ‘posteriorizing’ genes [64]. Due to early lethality, EBF has not been thoroughly evaluated in Gli3 mutants; however, no defects are readily identifiable at late embryonic stages. Shh null embryos on the other hand have a reduction of dig-
it number, often only having a single rudimentary digit on the hindlimb (Figure 3c) [51]. It is hypothesized that this is caused by elevated levels of Gli3R. Shh mutants are prenatally lethal and fail to develop limbs thus the skeletal consequences of congenic Shh loss have not been explored; however, the Shh ligand has no known function in bone development. Indian hedgehog mutants on the other hand have properly patterned digits, yet the animals are severely runted, having defects in chondrocyte maturation and development leading to a lack of mineralized bone (Figure 3d) [65]. Without Ihh chondrocytes would predominately have Gli3R likely causing the developmental defects. Indeed, removal of Gli3 in Indian null animals partially rescues the bone phenotypes [62]. Interestingly, pan-limb cilia mutant animals (Prx1Cre;IFT88fl/fl) present with both defects in patterning, having up to 8 unpatterned digits, and bone development as they are runted and have no bone collar (Figure 3e) [63]. These mutants phenocopy the predicted outcome of a compound Gli3 and Ihh null animal which is thought to be the result of the inability of cells to receive any Shh or Ihh, nor generate Gli3R.

**Figure 3: Summary of limb phenotypes**

![Summary of limb phenotypes](image)

<table>
<thead>
<tr>
<th>Wild Type</th>
<th>Gli3-/-</th>
<th>Shh-/-</th>
<th>Ihh-/-</th>
<th>Cilia null</th>
</tr>
</thead>
<tbody>
<tr>
<td>✓ Stylopod, Zeugopod, and Autopod</td>
<td>✓ Stylopod, Zeugopod, and Autopod</td>
<td>✓ Malformed stylopod, Fused zeugopod</td>
<td>✓ Stylopod, Zeugopod, and Autopod, all truncated</td>
<td>✓ Stylopod, Zeugopod, and Autopod, all truncated</td>
</tr>
<tr>
<td>✓ Pentadactyly</td>
<td>✓ Polysactyly</td>
<td>✓ One digit I</td>
<td>✓ Pentadactyly</td>
<td>✓ Plyadactyly</td>
</tr>
<tr>
<td>✓ AP polarity in autopod</td>
<td>✓ Normal ossification</td>
<td>✓ Ossification not evaluated</td>
<td>✓ Accelerated ossification</td>
<td>✓ Ossification not evaluated</td>
</tr>
<tr>
<td>✓ Normal ossification</td>
<td>✓ Shh independent</td>
<td>✓ Rescued by Gli3 removal</td>
<td></td>
<td></td>
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</tbody>
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**Purposed Research:** The Hedgehog signaling pathway is required for many aspects of development. This potent morphogen has also been linked to various developmental disorders and cancers when reactivated postnatally [66, 67]. In addition, Hh signaling is dependent on the primary cilium, a small cellular antenna found on most mammalian cell types. The series of disorders associated with defects in cilia function or formation are called the ciliopathies [4]. It is now known that several of these ciliopathy phenotypes are caused by Hh disturbances including the polydactyly seen in Bardet–Biedl patients, and the neural tube defects seen in Meckel–Gruber Syndrome [9]. Thus, a more complete understanding of how cilia function in the Hh pathway will provide insights into disease mechanisms and possible therapies.

In the following chapter, we set out to address what impact regional cilia deletion has on limb patterning and bone development. My goal was to assess the relative contribution of the Shh producing and responding cell populations to the polydactyly seen in whole limb cilia mutants (Figure 3). By perturbing cilia on distinct populations of cells during limb development -leaving signaling intact throughout the rest of the limb- we hope to discern the spatial requirements for primary cilia in limb patterning and EBF. We targeted the sonic producing and responding cells with two previously characterized Cre lines Shh::GFPCre and Gli1::CreER respectively [54, 56]. Of note however, there was no Cre line currently available that would affect only the anterior, sonic independent limb bud cells (see chapter 3). We did not observe patterning defects, however, because of protracted cilia deletion and protein stability. This study provides important information regarding the considerations involved in time sensitive developmental studies.
In our limb bud studies (Chapter 2) there was no Cre line available that would exclusively affected the anterior, Shh independent cells of the limb. Thus, in chapter three, we describe the generation of the Gardener Mouse a Cre line that affects the anterior the limb bud. We used the Alx4 promoter [68] known to regulate expression in the anterior limb, to generate the Gardener Mouse and fate mapped the Alx4 descendents. Importantly, when used in combination with the Shh and Gli1 Cre lines, the Gardener Mouse will allow for regional Cre deletion throughout the limb bud and will aid future limb studies.

Finally in chapter four, we generated a mouse expressing a fluorescent ciliary protein to allow for the immediate assessment of primary cilia in vivo. We call this model the Cilia^{GFP} mouse, and evaluate the expression and utility for in vivo imaging of cilia in many tissues. Further, when used in ciliary loss studies like those described in chapter two, this model will be useful and important for assessing the dynamics of cilia loss and the resultant phenotypes over time. In sum, this dissertation describes cilia loss in the limb; and two new mouse models generated to complement the limb studies and facilitate other advancements in cilia biology.
CHAPTER 2: DELAYED CILIA LOSS RESULTS IN SKELETAL BUT NOT PAT-TERNING DEFECTS IN THE LIMB BUD

AMBER K. O’CONNOR; CHING-FANG CHANG, ROSA SERRA, AND BRADLEY K. YODER
Abstract

The Hedgehog signaling pathway is required for many developmental processes including limb patterning and endochondral bone formation. Additionally, Hh signaling has been linked to the primary cilium, a small organelle that extends from the surface of most mammalian cell types. Defects in many of the proteins required for cilia function or formation, for example Ift88 and Kif3a, result in severe human disorders termed the ciliopathies. It is now known that several of these ciliopathy phenotypes are caused by perturbation of the Hh pathway. Here we analyze the consequences of conditional deletion of the Ift88 gene and cilia loss on different cell populations in the mouse limb bud. We determined that the structure of the cilia remained for many days beyond Cre expression \textit{in vivo}. Interestingly, despite the retention of ciliary structures skeletal malformations were seen, suggesting that ciliary signaling was indeed disrupted. Follow up \textit{in vitro} studies revealed that despite prompt genetic recombination, ciliary proteins IFT88 and Kif3a persist in culture for up to 96 hours. Together our data suggest that the temporal dynamics of gene deletion, protein turnover, ciliary ablation, and loss of ciliary function need to be well defined in order to assess and interpret the consequence of conditional cilia loss during development.
Introduction

The Hedgehog signaling pathway is required for many developmental processes including axis specification, neural tube and limb patterning, and endochondral bone formation [17, 18, 69]. Signaling initiates when one of the secreted ligands Sonic (Shh), Indian (Ihh), or Desert (Dhh) bind to the transmembrane receptor Patched (Ptch) in the cilia membrane. Ptch antagonizes another transmembrane effector, Smoothened (Smo), such that upon degradation of Ptch, Smo facilitates the accumulation of the Gli transcription factors and the inhibition of Gli repressor (GliR) formation. In the absence of ligand Gli2 and Gli3 are proteolytically processed into transcriptional repressors (GliR) whose activity keeps hedgehog target genes off. Gli1 and Gli2 are generally considered pathway activators (GliA) and become functional in response to ligand binding and Smo derepression. In this way, downstream target DNA is regulated at Gli binding sites, which are bound by either GliA or GliR. Gli1 and Ptch are primary pathway targets involved in feedback and are used as readouts of pathway activity [17, 18, 69].

Anterior-posterior (AP) limb patterning is a well characterized Shh dependent process. Shh is secreted from the Zone of Polarizing Activity (ZPA), a small mesenchymal population of cells at the posterior margin of the limb [70-73]. This distinct source of Shh expression causes asymmetric pathway activity across the limb where GliA is abundant in the posterior near the ZPA, and GliR activity predominates in the anterior side of the limb. Thus, a reciprocal gradient of GliR to GliA is formed across the AP axis of the limb field and it is believed that the ratio of these factors dictates the identity of the digits through the regulation of other transcription factors and signaling molecules [74]. A second Hh mediated process is endochondral bone formation (EBF) [63,
EBF is regulated by Ihh produced by a subset of cells, the prehypertrophic chondrocytes and signals over a long range to coordinate chondrocyte proliferation and differentiation. Briefly, EBF is the process by which mesenchyme cells condense and differentiate into chondrocytes forming an anlagen of the future bone. Ihh signaling from the chondrocytes in the center of the anlage cause the mesenchymal cells along the periphery to condense around the cartilage forming the perichondrium which is later mineralized forming the bone collar. In addition, Ihh signals to the less mature chondrocytes stimulating proliferation; while the parathyroid hormone related protein (PTHrP) from the resting chondrocytes antagonizes the Ihh signal to slow the rate of chondrocyte proliferation and differentiation [62, 65, 77]. Together the proliferative effects of Ihh and the contrasting PTHrP effects modulate the rate of proliferation and differentiation maintaining the growth plate within the developing bone and result in the formation of bones of appropriate length and morphology.

Interestingly, many phenotypes seen in animals with defects in Ihh and Shh signaling activity are also found in mice with mutations affecting cilia structure and function [63, 76, 78]. Primary cilia are small organelles that extend from the surface of most mammalian cell types including mesenchymal and epithelial cells, as well as those involved in bone formation: chondrocytes, osteoblasts, and osteocytes. Defects in proteins required for cilia function or formation, such as Ift88 or Kif3a, result in severe human disorders termed the ciliopathies and it is now known that many of their phenotypes are related to the Hh pathway [4, 9]. Indeed, most of the signaling machinery required for Hh signaling including Ptch, Smo, and the Gli transcription factors, localize transiently in the primary cilium. Furthermore, pathway activation and full repression requires the cili-
um presumably, at a minimum, for proper localization of the receptor and efficient modulation. Formation of the pathway repressors also requires a functional cilium as a reduction in the level of Gli3R is seen in cilia mutants [13, 79]. Together this makes the primary cilium a unique organelle critical for Hh signaling and an important modulator of both pathway activation and repression. In mice, phenotypes associated with ciliary mutations range from midgestational lethality to cystic kidneys, liver, and pancreas, as well as Hh related defects in tooth and neural tube closure and patterning, polydactyly, cerebellum development and ossification of the skeleton. These phenotypes are also seen in human ciliopathy patients [4, 62].

Here we set out to analyze the consequences of removing cilia from specific cells in the limb bud using the Cre lox system to delete the intraflagellar transport protein Ift88 which is required for ciliogenesis [63]. It was our goal to delete cilia on the Shh producing and responding cells in the limb and to assess any patterning defects. Unexpectedly, despite observing deletion of the floxed allele, we did not observe defects in digit patterning typical of cilia mutants. Subsequent analysis indicated that cilia persisted on Cre affected cells in vivo for nearly six days after Cre activity. While patterning abnormalities were not observed skeletal phenotypes related to defects in EBF indicated that there was sufficient cilia loss to affect later developmental processes. In vitro analysis confirmed the stability of Ift88 and the cilium for several days after Cre activation and deletion of the floxed sequence. These data indicate that primary cilia are remarkably stable, and that their deletion in vivo and in vitro must be carefully monitored. In addition, careful examination of cilia loss is necessary when interpreting results obtained during development.
Results

Cre expression and cilia loss on the Shh producing and responding cells of the limb bud

To evaluate which cells contribute to the polydactyly associated with ciliary dysfunction in the limb bud we used the Shh::GFPCre and Gli1::CreER lines to disrupt cilia on the Shh producing and responding cells. To first determine the efficiency of cilia loss in the Shh::GFPCre and Gli1::CreER models we used the mTmG fluorescent Cre reporter as well as the GFPCre fusion protein. Cre recombination in the mTmG reporter line causes a change in fluorescence from red (membrane tomato) to green (membrane GFP) allowing for the assessment of Cre activity. As expected, the Shh::GFPCre expressing cells were limited to the posterior margin of the limb bud at the ZPA at embryonic day 11.5 (e11.5, Figure1A). Analysis of the cilia on effected cells, using immunofluorescence with the ciliary marker Arl13b revealed that a high percent of cilia still remain on affected cells at e11.5 (Figure 1C). This was despite Shh::GFPCre expression starting at e9.25.

We conducted a similar evaluation of the Shh responding cells in the early limb bud. To generate as comparable data, we analyzed the limbs of Gli1::CreER animals injected with tamoxifen at e9.5 and harvested at e11.5. Shh responding cells (Gli1Cre expressing) encompass a much broader domain of the limb than the Shh producing cells (Figure 1A versus 1B) at the same developmental stage, while still being restricted to the posterior half of the limb. As observed in the Shh::GFPCre samples, analysis of cilia on
the Gli1Cre+/GFP+ Shh responding cells at e11.5 revealed that many of the cells maintained primary cilia (Figure 1D).

To assess whether primary cilia were gone later in limb development, we evaluated cells in the limbs at e16.5 (injecting the Gli1::CreER animals at e9.5 and evaluating cilia at e16.5). Sections of the forelimbs were again stained with the Arl13b antibody. Even 7 days after the onset of Cre activity, Arl13b positive cilia are seen on many GFP+ cells (Figure 1E and F arrows). This suggests that the time it takes for Cre to be expressed and excise the DNA, followed by the time required for IFT88 to turn over and disturb cilia maintenance, may be protracted in vivo, even in proliferative cells.

**Effect of Shh::GFPCre activity during limb development in IFT88^{FL/FL} animals**

Surprisingly, mice whose Ift88 gene has been disrupted on Shh producing cells throughout the animals are born at expected Mendelian ratios and had no evidence of compromised survival. In fact, the only outward phenotype seen in these animals is a malformation of digits IV and V on all limbs (Figure 2B). The digits curve posteriorly and fold into the palm suggesting a defect in bone development (Supplemental Movies 1 & 2). This phenotype is seen with 100% penetrance, on all limbs, and was identifiable by e16.5 (Supplemental Figure 1B). Interestingly, these animals maintain a proper pentadactylous pattern suggesting that either the initial pulse of Shh signaling is sufficient for patterning (see Zhu et al) or that the cilia were not affected rapidly enough to disrupt limb patterning. Together these data indicate that the eventual disruption of IFT88 on the Shh producing cells using the Shh::GFPCre is not lethal, does not impact limb patterning, but does cause malformations of digits IV and V.
As suggested by the malformation of digits IV and V, and due to the known requirement of Ihh signaling in bone development, we assessed possible defects in EBF caused by the loss of cilia on the Shh::GFPCre descendants. Using the Rosa26 (R26) β-galactosidase Cre reporter to determine the exact region of the limb that was affected, we were able to resolve a unique and distinct expression domain that included all of digit V and only half of digit IV (β-gal, Supplemental Figure 1A and B). Interestingly the differentiation of chondrocytes into mineralized tissue (alizarin red positive) was altered specifically in these digits (Figure 2B and inset). At P5 chondrocytes (alcian blue positive) still surround some of the more distal phalanges and are asymmetrically located along the anterior edge of digit IV (Figure 2B inset). The Cre expression pattern seen in the Shh::GFPCre limbs explains the developmental asymmetry seen in digit IV. Cells in the anterior half of digit IV are unaffected and retain their cilia, they are able to detect Ihh, undergo differentiation and apoptosis, leaving mineralized tissue. In contrast, along the posterior margin of the same phalange which lacks cilia, the cells cannot detect Ihh, and remain chondrocytes (Supplemental Figure 1C and Figure 2D). Data to support this idea was obtained from R26 mice were the majority of chondrocytes remaining on the posterior edge of digit IV were β-gal positive, and thus had been affected by Cre, while no chondrocytes persisted along the anterior margin (Supplemental Figure 1D). Because all of digit V is affected by Cre, the digit is wholly mutant and rudimentary, and lacks proper rigidity. Together this data suggests that the a sufficient number of Shh producing cells lose the ability to detect and respond to Hh ligand causing regional defects in EBF altering the biomechanical properties of the bone resulting in malformation of the affected digits.
To better characterize the delay in bone formation and determine if there were any changes in cell identity at the molecular level we performed histological and in situ hybridization (ISH) analyses on the Shh::GFPCre autopods. Evaluation of limbs at postnatal day 5 using a histological stain to reveal the proteoglycan rich chondrocytes (safranin O, orange), and the mineralized bone (fast green, blue) reveals that the posterior margin of the bone collar is disorganized and discontinuous in mutant animals, and contains large nodules of chondrocytes (Figure 2C vs. D, arrows). ISH was used to define the molecular characteristics of the ectopic chondrocytes. It was found that within the ectopic nodules, there were cells positive Collagen type two a (ColII) and/or ColX (Figure 2F and H, arrows), something not seen in wild type animals (Figure 2E and G). This phenotype resembles that of Ihh−/− animals and supports the hypothesis that the chondrocytes lack the ability to sense Ihh and fail to differentiate properly resulting in a cilia null skeletal phenotype.

**Effect of Gli1::CreER induction during limb development in IFT88FL/FL animals**

To assess the consequences of disrupting cilia on the Shh responding cells we utilized the Gli1::CreER model to evaluate any defects incurred by Cre induction/cilia loss between e8.5-e10.5 during limb patterning. No patterning defects were noted when Cre activity was induced at e8.5 (Supplemental Figure 2C) presumably due to the fact that Gli1 expression in the limb has not yet been initiated (Supplemental Figure 2A). Interestingly though, no patterning defects were observed in animals where Cre activity was induced at e9.5 or e10.5 (Supplemental Figure 2F and H). As discussed above this is likely due to the fact that cilia are intact at the onset of Shh signaling and because of the low
levels of recombination having occurred during the critical time frames (Supplemental Figure 2D). Interestingly, the loss of cilia from the various sonic responding cells in the animal does result in prenatal lethality, however the cell type or organs responsible for the lethality are not known.

Despite proper patterning of the autopod, there were defects in zeugopod development, specifically in the ulna. Fate mapping data have shown that the ulna is populated in large part by Shh responding cells [56]. In litters injected at e9.5, mutant ulnae appear to be severely delayed in mineralization, including a complete lack of alizarin red (calcification) staining at e18.5 in extreme cases (Figure 3B). In addition, the ulnae are shorter than would be expected compared to the radius which is normally the shorter of the two bones (Figure 3A’ and B’). Due to the dichotomy in size of the radius versus the ulna the zeugopod curves posteriorly creating a bowed appearance (Figure 3B and B’). Lingering chondrocytes are seen along the diaphysis of the ulna and the growth plate is disorganized (Figure 3D arrows and inset). Using ColII (Figure 3C and D) and ColX ISH markers (Figure 3E and F) we again observed coexpression of these two genes in mutant domains. Indeed, the residual chondrocytes along the diaphysis and the majority of the cells at the ephesial ends were positive for both markers (Figure 3D and 3F arrows). These data support the idea that the chondrocytes lacking cilia are unable to receive the normal developmental cues required for regular and progressive differentiation. This causes the disruption and disorganization of the growth plate resulting in alterations in the biomechanical properties of the developing skeleton.
In vitro analysis of ciliary stability in CreER models

The data thus far suggest that cilia must be ablated early in limb bud development to affect digit patterning. Due to this requirement, we were unable to affect the patterning phase of limb development. Our results further indicate that the temporal dynamics of IFT protein and cilia loss must be monitored and considered when analyzing developmental phenotypes. To better assess ciliary dynamics we analyzed the timeline of DNA recombination and protein loss in vitro.

We first conducted time course experiments on chondrocytes isolated from Ift88$^{F/F}$; CAGGCreER mice [80] to assess ciliary turnover. CAGGCreER is a ubiquitously expressed tamoxifen inducible Cre. The chondrocytes were isolated and treated with 4-Hydroxy Tamoxifen (4-OHT) over several days with samples being taken at 24 hour intervals. Gene deletion was detectable at 48 hours and nearly complete by 96 hours (Figure 4A). Similar to the DNA, the IFT88 protein was detectable by western blotting even at 96 hours after Cre induction (Figure 4B) indicating a lengthy delay between Cre induction and protein loss. To determine if the delay in cilia loss was in part due to the inefficiency in Cre induction we assessed gene deletion and cilia loss using an Adenovirus expressing Cre (AdCre) in Ift88$^{F/F}$ chondrocytes. We found that Ift88 deletion was highly efficient using the AdCre, with deletion of the gene being nearly complete within 24 hours of transduction (Figure 4C). Despite the rapid loss of the ift88 floxed allele, the IFT88 protein was readily detectable 96 hours after Cre addition (Figure 4D).

To evaluate whether this is a universal feature of cilia, we analyzed a second ciliogenic gene, Kif3a using mouse embryonic fibroblasts (MEFs). Kif3a$^{F/F}$; CAGGCreER MEFs were grown to confluence and then treated with 4-OHT in low se-
rum conditions with samples being harvested every 24 hours. Within the first 24 hours in culture the cells showed recombination, however some of the floxed allele remained (Figure 4E). By 48 hours after treatment the Kif3a gene appears to be completely deleted from the cells in culture (Figure 4E). Why this deletion was more robust than was seen with the Ift88^{F/F} ; CAGGCreER cells (Figure 4A) may be due to the cell type, or a difference in the efficiency of deletion at the genomic level as the proliferation rates and serum conditions were both kept very low. In conjunction with this analysis the Kif3a protein was analyzed over time and was shown to be reduced compared to control untreated MEFs at 24 hours, and 48 hours (Figure 4F lanes 1-4). However, the Kif3a protein levels did not diminish completely and remain detectable at 96 hours after the addition of 4-OHT (Figure 4F lane 8).

Discussion

Our goal was to evaluate whether the Shh producing or responding cells contribute to the polydactyly associated with ciliary dysfunction in the limb bud. This was accomplished using the well characterized Shh::GFPCre and Gli1::CreER lines. Combined with the floxed Ift88 allele we attempted to ablate primary cilia early in limb development on specific cell populations. Or data suggests that the rapid development of the embryo and the temporal dynamics of cilia loss precluded this analysis using this approach. For example, the onset of Shh (Shh::GFPCre) expression in the limb initiates at about e9.25, and evaluation of primary cilia on the Shh producing cells ~42 hours after the onset of Cre expression (at e11.5) revealed persistent Arl13b positive primary cilia. However, even if cilia loss occurred rapidly there would have been an initial pulse of
Shh, as in our system cilia loss was inherently downstream of the initiation Hh signaling. Together this data suggests that our goal of removing primary cilia, and regionally disrupting Shh signaling, during the early stages of limb development was not feasible using this paradigm. Importantly, despite this we did note cilia related phenotypes later in development indicating cilia ablation did occur.

These data on the temporal dynamics of IFT disruption in vitro and in vivo have critical implications for this and other studies. We have found that despite deletion of the floxed ift88 allele within 24 hours of Cre activity as determined by PCR; that two ciliogenic proteins persisted in vitro for more than 96 hours after Cre induction, and cilia remained on cells for up to seven days after the onset of Cre activity in vivo. These data have important implications when evaluating developmental processes. During embryonic development patterning and organogenesis are occurring rapidly [81] and the experimental system must conform to strict temporal control to be a proper evaluation of a given event. Interestingly, to our knowledge, no comprehensive studies have been conducted on cilia loss in vivo during limb development. How long do ciliary proteins take to turnover in vivo? What impact does the rate of proliferation have on ciliary stability? How stable is the microtubule structure relative to intact ciliary signaling? Can retrograde movement (IFTA) occur independently of anterograde movement (IFTB) for efficient turnover of the organelle? It is also possible that Hh signaling can occur independently of IFT88, while the ciliary structure remains, and that IFT has no direct role in Hh signaling. Our in vivo studies suggest that even in an environment of rapid cell division cilia are remarkably stable and it is likely that some developmental processes are resistant to moderate changes in IFT88 levels and may not be affected.
Our studies support other recent discoveries in limb patterning and bone development. Firstly, in 2008 Zhu et al. published their studies of a dual role for Shh in limb patterning, one role being early in limb development to establish pattern, and the other to sustain growth and survival of the pre-patterned limb [82]. This suggests that due to the initial Shh signal in the limbs of our animals they would indeed develop properly patterned autopods. Further, it would appear that neither phase of Shh signaling was inhibited in these studies as there was no loss of distal structures due to defective outgrowth.

We found that the process disrupted in our models was that of endochondral bone formation with abnormal differentiation of the chondrocytes, poor ossification, and malformation of the affected bones. All of which are defects noted upon cilia disruption throughout the mesenchyme of the limb [63] and specifically in chondrocytes [83]. Our results suggest that disturbances in chondrogenic development are cell autonomous; being that the unaffected chondrocytes within the affected anlagens did carry on with development, hypertrophy, and apoptosis. In addition to developmental roles for cilia in bone, there is mounting evidence that cilia are required for bone homeostasis. For example, it is known that activation of the pathway leads to increased bone mass, and that Gli3R antagonizes Runx2 activity, suggesting that cilia loss may mimic a Runx2 gain of function phenotype [84]. These studies underscore the need to understand the function of primary cilia, and encourage us to look toward modulation of cilia and IFT as a possible axis of therapeutic intervention in the skeleton.

Together this data suggests that the interpretations made of the phenotypes associated with cilia loss have to be done with temporal dynamics in mind, especially in the context of development. Indeed, despite the fact that cilia appear to linger on the cells in
this study, their functionality remains in question. We considered the possibility that while the structural remnants of the cilia were still present that they might no longer be functional. Unfortunately, there is no de facto analysis to assess ciliary function. Until there is a way to assess whether or not signaling is impaired in the time between gene deletion and cilia loss, we must be cautious in our interpretations.

Materials and Methods:

Mice The mouse alleles and transgenes used were as follows:, Gt(Rosa)26Sor<sup>tm4(ACTB-tdTomato,-EGFP)Luo</sup> (mTmG), Shh<sup>tm1(EGFP/cre)Cjt</sup> (Shh::GFPCre), Gli1<sup>tm3(cre/ERT2)Alj</sup> (Gli1::CreER), Kif3a<sup>tm2Gsn</sup> (Kif3a<sup>F/F</sup>), Tg(CAG-cre/Esr1*)<sup>5Amc</sup> (CAGGCreER), and Gt(Rosa)26Sor (R26) all obtained from Jackson Laboratories, Bar Harbor ME. The Ift88<sup>tm1Bky</sup> (Ift88<sup>F/F</sup>) mice were generated in our lab and reported previously [63]. Animals were maintained in AAALAC accredited facilities in accordance with IACUC regulations at the University of Alabama at Birmingham.

Timed matings were set up before lights out and vaginal plugs assessed the following morning. Noon on the date of the vaginal plug was designated e0.5. For strains requiring tamoxifen induction, tamoxifen (Sigma, T5648) was dissolved in corn oil at 20mg/mL and a single 6mg dose was given to pregnant females via IP injection at the times indicated. Embryos were harvested into PBS and imaged, or processed as indicated below, embryos >e16.5 were decapitated before being processed.

Fluorescence Analysis Whole images of embryos with inherent fluorescent signal were taken immediately after isolation in PBS or within 24 hours of isolation after
having been stored in 4% paraformaldehyde (PFA) at 4°C. Whole fluorescent embryo images were taken on a Zeiss Lumar v12 equipped with an Axiocam MRm using AxioVision. For wholemount cilia analysis at e11.5, forelimbs were removed and fixed in 4% PFA for 10 min. The samples were blocked and permeabilized in 1% bovine serum albumin (BSA), 1% Donkey Serum, in PBS + 0.1% Triton for 30 minutes at room temperature (RT). Primary antibodies were mixed in blocking solution and applied overnight at 4°C. After washing, secondary antibodies were applied for 1hr at RT washed in PBS and then samples were incubated in 50% glycerol in PBS and Hoechst for 5’ at RT, followed by storage and imaging in 70% glycerol in PBS. For analysis of cilia on sections, embryos were fixed in 4% PFA overnight at 4°C, rinsed in PBS and immersed in 30% sucrose in PBS at 4°C. Embryos were then oriented in OCT, frozen in a dry ice ethanol bath, and stored at -80°C until sectioned. 12-15um sections were processed as above through secondary antibody, the slides were washed in PBS, incubated in PBS containing Hoechst for 5 min at RT, and coverslips were affixed using Immu-mount (Thermo #9990402). Confocal images were obtained using a Perkin Elmer ERS 6FE spinning disk confocal module on a Nikon TE-2000-U, using Velocity software. Primary antibodies were diluted as follows: anti-IFT88 1:1500 ([85]) and anti-Arl13b 1:2000 (gift from Dr. Tamara Caspary). Secondary antibodies included: Alexa Fluor-594 -Oregon green -647 conjugated donkey anti-rabbit IgG (Invitrogen #A11037, O6381, A21244). Nuclei were visualized by Hoechst (Invitrogen#33342).

**βgal Staining** Wholemount beta-galactosidase staining was performed as outlined in [86]. Briefly, fresh fixative was prepared (4% PFA, 0.2% Glutaraldehyde, 0.02%
Igapal, 0.01% Sodium deoxycholate), samples were kept on ice for fixation and three 15 min washes. Staining solution (0.1 M Phosphate buffer pH 7.3, 2 mM MgCl, 5 mM each K ferri- and ferro-cyanide, 1 mg/ml X-gal) was added to samples until sufficient staining occurred. Samples were then imaged whole, or dehydrated and embedded in wax to be sectioned and counter-stained.

**Skeletal Staining** For mouse skeleton visualization (animals from e14-p7), skin, viscera, and as much tissue as possible were removed from samples before being fixed in 95% EtOH for 3 days. After fixation samples were incubated in acetone for 24 hours before staining in 0.3% Acian blue in 70% EtOH and 0.1% Alzarin Red in 95% EtOH for 2 days. Destaining was done in 1% KOH followed by progressive clearing in glycerol/KOH and stored in 100% glycerol. Images were obtained on an Olympus SZX12 with an Optronics color camera and Picture Frame software.

**Histology** Histological analysis was performed following standard protocols. Briefly, samples were isolated fixed in 4% PFA before being transferred to 70% EtOH and being dehydrated through Xylene and embedded in wax. 6 m sections were cut and mounted. For staining, samples were rehydrated and stained using Hematoxalyn (Fisher SH26) & Eosin (Sigma HT110132), Safrannin O (Sigma S8884) & Fast Green (Fisher BP123), or processed for *In situ* hybridization (ISH). After staining, slides were dehydrated and coverslips were affixed with Permount (Fisher SP15). Samples from >p3 mice were first fixed in 4% PFA overnight, then decalcified before being dehydrated and embedded.
**In Situ Hybridization**  ISH on histological sections were done as in [86]. Briefly, once sectioned samples are rehydrated before being post fixed in PFA for 10 minutes, then treated with Proteinase K at 1 ug/ml for 10 minutes, and acetylated using acetic anhydride in 0.1 M Triethanolamine. Digoxigenin labeled probe (previously described [63, 83]) was added overnight, and washed in SSC/Formamide. RNaseA treatment (20 ug.ml) was followed by SSC washes and anti-DIG-AP Fab fragments (Roche, #10937240) were applied in a maleic acid buffer (100 mM maleic acid, 250 mM NaCl, bring to pH 7.5 with NaOH) overnight. Color was developed using BM Purple (Roche, #11 442074 001). Black and white images were captured on a Nikon Eclipse TE200 and a photomertrics cascade camera using Metamorph software.

**µCT**  Excised autopods were scanned using the Scanco µCT40 desktop cone-beam micro-CT scanner (Scanco Medical AG, Brüttisellen, Switzerland). The samples were placed in a 30mm scanning holder and scanned at the following settings: 15µm resolution, 70kVp, 114µA with an integration time of 200ms. Scans were automatically reconstructed into 2-D slices and all slices were analyzed using the µCT Evaluation Program (v5.0A, Scanco Medical). A region of interest (ROI) was drawn on each of the slices to include all the bone. The threshold for bone was set at 129. 3-D reconstruction was performed using all the outlined slices.

**Cell Culture**  MEFs were generated as previously described [81, 87]. Fourteen day old embryos from Kif3a<sup>Fli/Fli</sup>; CAGGCreER crosses were harvested and grown in
DMEM with High Glucose, 0.05 mg/ml Penicillin/Streptomycin, 2 mM l-Glutamine, 0.2 mM β-mercaptoethanol, and 10% Fetal Bovine Serum (FBS). Where noted the FBS was reduced to 0.5% to induce cilia formation. 1.6 x10^5 cells/cm^2 were seeded into 6 well culture dishes, allowed to adhere and become confluent overnight before being treated. Chondrocytes were harvested from newborn Ift88^{F/F} ; CAGGCreER rib cages and cultured as previously described [80, 88]. Cells were plated at a density of 2x10^5 cells/cm^2. Culture medium contained DMEM/F12, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 mg/ml L-ascorbate-2-phosphate (Fluka), 50 U penicillin, and 50 mg streptomycin and no serum during treatments. 4-OHT was added to media and then applied to cells at 1-10μM.

**Immunoblotting** Cells were scraped into cold lysis buffer [89] with Complete Protease Inhibitor (Roche #11 873 580 001) and disrupted by manual homogenization. The lysates were incubated on ice for 30 min before centrifugation and discarding of the insoluble fraction. Protein concentrations were determined by the Bradford assay (Bio-Rad). Equal amounts of protein (Figure 4B and D), or equal volumes of lysate (Figure 4F) were resolved on a denaturing 10% Tris–HCl gel (Bio-Rad Laboratories) and transferred to PVDF (Millipore). Membranes were blocked in PBS with 0.1% Tween-20 and 5% milk for 1 h and incubated with primary antibody overnight at 4°C. Membranes were probed with horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h at room temperature. Secondary antibodies were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce) and bands were visualized using Blue Ultra Autorad Film (Bioexpress). The following primary antibodies and dilutions were used:
anti-IFT88 [85] 1:1000, Kif3a (Sigma #K3513) 1:2000, GAPDH (abcam #8245). Secondary antibodies were HRP conjugated anti-rabbit (Biorad #31460) and anti-mouse (Pierce #185843) both at 1:1000.

References


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Figure Legends

Figure 1) Cre expression and cilia retention on the Shh producing and responding cells of the limb bud. Whole mount analysis of Cre activity (A-B), Immunofluorescence analysis of cilia (C-F). Cilia were identified with Arl13b (purple) staining in the limb bud of e11.5 IFT88^{Fl/Fl}; Shh::GFPCre (C) and IFT88^{Fl/Fl}; Gli1::CreER limbs (D). At e16.5 Arl13b (purple) cilia were labeled in IFT88^{Fl/Fl}; Shh::GFPCre (E) and IFT88^{Fl/Fl}; Gli1::CreER limbs (F). Cre affected cells (green) with cilia are indicated by arrows. Nuclei in blue, anterior is up.

Figure 2) Shh::GFPCre activity during limb development in IFT88^{FL/FL} animals causes malformation. Alcian blue and alizarin red skeletal staining of the forelimbs of neonatal IFT88^{Fl/W} (A) and IFT88^{Fl/Fl} (B) Shh::GFPCre animals. The inset in B shows higher magnification. Safranin O and Fast Green staining of dIV sections in IFT88^{Fl/W} (C) and IFT88^{Fl/Fl} (D) Shh::GFPCre animals. In situ hybridization analysis on sections from IFT88^{Fl/W} (E and G) and IFT88^{Fl/Fl} (F and H) Shh::GFPCre animals. In situ hybridization analysis using a Collagen II (E and F) and Collagen X (G and H) riboprobes were conducted on digit IV sections harvested at p5. Anterior is up and C-H sections are of digit 4 focused on the distal region of the proximal phalange.

Figure 3) Limb defects in Gli1::CreER IFT88^{FL/FL} animals. Alcian blue and Alizarin red skeletal staining of the forelimbs of e16.5 IFT88^{Fl/W} (A) and IFT88^{Fl/Fl} (B) Gli1::CreER animals after injection the pregnant females with tamoxifen at e9.5 of gesta-
tion. The inset (A’ and B’) shows higher magnification of the ulna. (C-F) *In situ* hybridization analysis on sections from IFT88^Fl/W (C and E) and IFT88^Fl/Fl (D and F) Gli1::CreER animals injected at e9.5. *In situ* hybridization analysis using a Collagen II (C and D) and Collagen X riboprobe (E and F). Anterior is up.

**Figure 4)** Gene deletion and protein loss *in vitro*. Ift88; CAGGCreER chondrocytes treated with 4-OHT (A-B). Genotyping of treated cells at 48h, 72h, and 96h; versus untreated control cells at 96h and Ift88^F/W tail DNA (A). IFT88 protein levels in treated cells were assessed at 48h, 72h, and 96h versus untreated control cells (B). Ift88 chondrocytes transduced using an Adenovirus carrying Cre (C-D). Genotyping of chondrocytes 24hours after transduction versus Ift88^F/W tail DNA (C). IFT88 protein levels in transduced cells were assessed at 24h, 48h, 72h, and 96 hours (D). Kif3a; CAGGCreER MEFs treated with 4-OHT (E-F). Genotyping of MEFs at 24h, 48h, 72h, and 96 hours after treatment versus control tail DNA (E). Kif3a protein levels were analyzed in MEFs at 24h, 48h, 72h, and 96 hours after treatment versus untreated conditional MEFs. GAPDH was used as a loading control (F).

**Supplemental Figure 1)** Betagal analysis in Shh::GFPCre limbs. β-gal staining in IFT88^Fl/Fi Flh::GFPCre Rosa26 e14.5 (A) and e16.6 (B) whole forelimbs. Sections of IFT88^Fl/Fi Shh::GFPCre Rosa26 limbs showing dIV and V (C) and dIV at higher magnification (D). Cre activity is shown in blue, chondrocytes in orange (Safrannin O).
Supplemental Figure 2) Limb patterning in Gli1::CreER; IFT88^{FL/FL} animals. mTmG analysis showing the expanse of Cre activity (green) at e10.5 after injection of tamoxifen at e8.5 (A) and at e9.5 (D) in whole limb buds. Skeletal staining of autopods from e16.5 animals after the pregnant females were injected with tamoxifen at e8.5 (B and C), e9.5 (E and F), or at e10.5 (G and H). IFT88^{FL/W} Gli1::CreER control animals (B, E, and G) have the same phenotype as mutant IFT88^{FL/FL} Gli1::CreER (C, F, and H) animals.

Supplemental Movie 1) Micro computed tomography analysis of WT autopod. Adult IFT88^{FL/W}; Shh::GFPCre limbs analyzed by micro computed tomography. Serial stacks were converted into movies with rotation to allow for fine observation of the malformed digits IV and V in mutant animals.

Supplemental Movie 2) Micro computed tomography analysis of mutant autopod. Adult IFT88^{FL/FL}; Shh::GFPCre limbs analyzed by micro computed tomography. Serial stacks were converted into movies with rotation to allow for fine observation of the malformed digits IV and V in mutant animals.
Figure 1

(A) F/F Shh::GFPCre

(B) F/F Glil1::CreER

(C) F/F Shh::GFPCre c11.5

(D) F/F Glil1::CreER c11.5

(E) F/F Shh::GFPCre c16.5

(F) F/F Glil1::CreER c16.5
Figure 2

F/W Shh::GFPCre  F/F Shh::GFPCre

Skeletal

A

B

SO/FG

C

D

Col II

E

F

Col X

G

H
Figure 3
Figure 4

A

Ift88F1/F1; CAGGCCreER+ chondrocytes

96h 48h 72h 96h F/W

4-OHT

Flox WT Delta

B

96h 48h 72h 96h

4-OHT

IB: α IFT88

C

24h F/W AdCre

Flox WT Delta

D

Ift88F1/F1 chondrocytes + Cre via Adenovirus

24h 48h 72h 96h AdCre

IB: α IFT88

E

100 bp 24h 48h 72h 96h F/Δ Δ/Δ

4-OHT

Flox Delta

F

Kif3aF1/F1; CAGGCCreER+ MEFs

4-OHT

IB: α Kif3a

IB: α GAPDH
Supplemental Figure 1

F/F Shh::GFPCre

A

e14.5

B

e16.5

C

R26+ p0

D

R26+ p0
Supplemental Movie 1
Micro computed tomography analysis of WT autopod

F/W Shh::GFPCre
Supplemental Movie 2
Micro computed tomography analysis of mutant autopod

F/F Shh::GFPCre
CHAPTER 3: THE GARDENER MOUSE: THE GENERATION OF AN ALX4CREER TRANSGENIC MOUSE FOR STUDIES OF THE CELLS IN THE ANTERIOR LIMB BUD

AMBER K. O’CONNOR, ROBERT A. KESTERSON, AND BRADLEY K. YODER

In preparation for Genesis
Format adapted for dissertation
Abstract

There are multiple Cre mouse models for studies of the limb bud, however no models exist that target the cells exclusively in the anterior limb. Here we describe the Gardener Mouse, a new mouse expressing CreER\textsuperscript{T2} regulated by the Alx4 promoter. Using the mTmG Cre reporter line, we characterized the domain and fate of the Alx4 descendents. Cre activity was seen in the anterior limb bud where it recapitulated the endogenous gene expression domain. Labeled cells were shown to contribute to digits I-IV depending on when Cre activity was initiated. In the zeugopod, the descendants were restricted to the radius and anterior wrist; however Alx4::CreER cells in the epidermis showed no specific distribution. Alx4::CreER activity was also detected in the nephric duct and ureteric bud and thus many kidney tubules showed mosaic epithelial labeling. To our knowledge there have not been any studies of spatial Alx4 expression or its role in kidney development making this an important for renal Alx4 characterization. The development of the Gardener Mouse will facilitate recombination based studies in the limb and kidney, and will be useful for following the fate of Alx4 descendents in mutant backgrounds.
Introduction

Limb development is a complex process controlled by multiple signaling pathways and depends on strict coordination. The limb is patterned in its three axes by signals emanating from distinct regions of the limb bud (recent reviews: [90] [29]). The dorsal-ventral (DV) axis is established by Wnt signals from the dorsal ectoderm [91]. The proximal-distal (PD) axis is regulated by Fibroblast growth factor (Fgf) signals produced by the apical ectodermal ridge (AER) [36], a specialized group of cells that runs along the most apical boundary of the limb bud spanning from anterior to posterior. Finally, the anterior-posterior (AP) axis is specified by sonic hedgehog (Shh) signaling initiating in the Zone of Polarizing Activity (ZPA) [37], a population of mesenchymal cells in the posterior region of the limb bud. In mammals these signals give rise to the limb with a stylopod (humerus/femur), zeugopod (radius-ulna/tibia-fibula), and autopod with five digits numbered I-V from the anterior (thumb) to posterior [92]. Many of the details known regarding limb patterning have been resolved through molecular and genetic manipulations in mouse models; however, exactly how all of these signals are received and coordinated on a cellular level to form a properly patterned limb is still being elucidated.

AP patterning in the limb bud is established by the secretion of the morphogen Shh from the ZPA in the posterior margin of the limb bud [37]. Shh patterns the limb by regulating the expression and activity of many genes known to affect cell fate and development. One of the most critical targets of Shh signaling is the transcription factor Gli3 [93]. Gli3 functions as a repressor (Gli3R) when proteolytically processed. The formation of Gli3R is inhibited by Shh signaling and as such a reciprocal gradient of Shh
secretion/signaling and Gli3R activity occurs across the AP limb field [21]. The distinct expression domains of Shh and Gli3 then set up the expression domains of downstream patterning genes including many of the hox genes and the transcription factor aristaless-like homeobox 4 (Alx4) [46]. Alx4, is expressed only in the anterior region of the limb bud, and requires Gli3R for its proper distribution [94]. Fine tuning of the expression domains of the genes involved in limb development is critical for proper digit pattern and number. Analysis of Shh and Gli3 mutant animals has lead to a simplified explanation that Shh (and other posterior genes) promotes digit formation [51], while Gli3 (as well as other anterior genes) restricts the number to five [50]. This is supported by the Shh null animal, which expresses only anterior genes (i.e. Gli3 and Alx4), and one digit arises. Conversely, in a Gli3 mutant where Shh signaling and posterior genes (i.e. Gli1, Hox genes) are expanded, extra digits form.

Determining where embryonic cells end up in the developed animal, or fate mapping, is a powerful tool for elucidating the consequences of disturbances in development. In the limb bud, the descendants of the ZPA or cells under the influence of the ZPA in the posterior region have been fate mapped using Shh::GFPCre and Gli1CreER respectively ([54], [56]). These studies show that ZPA descendants contribute to digit V and parts of digits IV and III. The Shh responding cells of the limb bud, those that express Gli1CreER, contribute to a much broader range owing to the diffusion of the Shh ligand. Descendants of the cells that once expressed Gli1 encompass the posterior two-thirds of the limb bud contributing to the ulna and all digits except for digit I. Due to the fact that none of the cells in digit I have been shown to receive any Shh signal from the ZPA, and because in Shh null animals retain only digit I, it is referred to as the sonic independent
digit. The Shh::GFPCre and Gli1CreER mouse lines have been important tools in analyzing how genetic mutations alter cell fate and for assessing the consequence of disrupting patterning genes. However, similar fate mapping studies have not been reported for cells in the anterior limb in large part due to the lack of an appropriate Cre line to mark these cells. Here we describe the Gardener Mouse, a transgenic line expressing CreER\textsuperscript{T2} under the Alx4 promoter [68]. Alx4::CreER activity is detected in the anterior limb bud and epithelial components of the nephron. This new line can be used to indelibly label Alx4 expressing cells to assess cell fates in normal and mutant backgrounds, or to mediate the deletion of floxed alleles in the anterior limb bud or kidney to analyze gene function.

**Results & Discussion**

The Aristaless-like 4 (Alx4) gene encodes a homeodomain containing transcription factor expressed in mesenchymal components of a number of developing tissues. These include, the limb bud, skin appendages (hair, whiskers, teeth), craniofacial skeleton, mammary stromal cells, and the mesonephric tubules and nephric duct in early urogenital development ([46], [95], [96], [68]). In the limb bud, Alx4 is specifically expressed in cells in the anterior margin where it is thought to help confine the Shh expression domain to the posterior margin. In Alx4 mutants the ZPA is duplicated in the anterior limb leading to the formation of an extra preaxial digit (polydactyly) and the absence of the tibia. Alx4 deficient mice also have alopecia, abnormal craniofacial structures, defects in body wall closure, and abnormal mammary ductal branching([97], [98]).
In an effort to generate a transgenic CreER T2 line to target the anterior limb, we used a 17kb genomic fragment from the Alx4 promoter region, including the first noncoding exon and a portion of exon 2 encoding the first 13 amino acids (Figure 1A Xho-EagI fragment). This was subcloned into a tamoxifen inducible CreER T2 expression vector (Figure 1B). Two independent Alx4::CreER transgenic lines (Tg(Alx4-cre/ERT2)1Bky) were established that had indistinguishable expression patterns, hereafter the line is referred to as the “Gardener Mouse” in honor of its green thumbs (Figure 2).

Limb

To characterize the expression pattern and Cre activity of the Alx4::CreER transgene, the Gardener Mouse was mated to the Cre reporter line mTmG (Gt(ROSA)26Soertm4(ACTB-tdTomato,-EGFP)Luo) that expresses GFP after Cre mediated deletion of a Tomato coding sequence [99]. To assess the spatial activity of Cre in the Gardener Mouse, tamoxifen (TM) was administered to pregnant mice at different gestational stages and the embryos were isolated 24 hours after injection. As Alx4 plays an important role in limb development beginning at about embryonic day 9.0 (e9.0), we began the analysis at e8.5. In agreement with lack of expression of endogenous Alx4 at e8.5, we did not observe Alx4::CreER activity in embryos isolated a day later (TMe8.5-e9.5) (Figure 2A). In contrast, injection at e9.5 showed strong CreERT2 induced GFP expression in the anterior region of the fore and hindlimbs (Figure 2D). In addition, a few GFP positive cells were seen in the dorsal ectoderm and inside the visceral cavity of the embryos, described below. At subsequent time points (TMe10.5-e11.5 and TMe11.5-e12.5), larger domains
of recombination were observed throughout the anterior portion of the limbs (Figure 2G and 2J). The hindlimbs appear to have a broader domain of transgene expression at TMe9.5-e10.5 and TMe10.5-e11.5 (Figure 2D and G, Supplemental Figure 1B and C insets), with GFP expressing cells found within nearly half of the hindlimb. Analysis at TMe12.5-e13.5 resulted in cutaneous, sporadic, GFP+ cells in the anterior limb indicating a dramatic reduction in transgene expression (Supplemental Figure 1E). Together these data indicate that the Gardener Mouse exclusively labels the anterior limb and thus will be a useful resource for studies of limb patterning and development.

We next analyzed the fate of the Alx4 descendants in the limb bud with the mTmG reporter later in development. Induction of Cre activity by TM injection was done as above and the subsequent fate of the cells was analyzed at e16.5. Although the TMe8.5-e9.5 embryos did not show any CreER\(^T2\) activity after 24 hours (Figure 2A), TMe8.5-e16.5 indicated recombination in the limb, as well as in the kidney, and skin (Figure 2B and 2C, Figure 3C and 3C’, and data not shown). This labeling is due to the persistence of TM for 24-48 hours in the circulatory system such that when the transgene did eventually activate deletion occurred. Early injection, TMe8.5-e16.5 labeled the most anterior parts of the limb, digits I-III, very few cells in digit IV, the anterior wrist, and radius (Figure 2B and 2C). Analysis of TMe9.5-e16.5 and TMe10.5-16.5 limbs showed a range of GFP+ contribution, encompassing digit I and II and portions of digit III (Figure 2E-F and 2H-I). Importantly the Alx4 descendents exclusively occupy the radius as no GFP+ cells are seen in the ulna (Figure 2F, 2I, and 2L). Induction of Cre at e11.5 resulted in cells contributing almost exclusively to anterior structures at e16.5. Digit I has high levels of contribution with little to no GFP+ cells in more posterior digits (Figure 2K and
Further, the cells labeled at this late time point are located more proximally than at the other time points, with some muscle cells in the stylopod being positive (Figure 2K and 2L). Importantly the GFP+ cells contribute to most all of the cell types in the limb; muscle and connective tissues, chondrocytes and the perichondrium, the dermis and epidermis all contain Alx4 descendents (Figure 2C, 2F, 2I, 2L and inset).

In sum, this new line can be used to label Alx4 expressing cells, to assess cell fates in normal and mutant backgrounds, or to mediate the deletion of floxed alleles in the anterior limb bud; together allowing for the evaluation of the complex genetic interactions required for maintaining pentadactyly.

**Kidney**

In 2005 when Kuijper and colleagues first assessed Alx4 expression under the control of this 17kb promoter fragment regulating β-galactosidase (β-gal) expression, they noted reporter activity in the nephric duct and mesonephric tubules [68]. In agreement with these observations two GFP+ streaks of cells, one on either side of the neural tube at the level of the forelimb running towards the tail, were noted (Figure 3A arrow). Sectioning of the embryos confirmed GFP expression in the epithelial cells of the developing nephric duct (Figure 3B). To analyze the contribution of these Alx4 descendents to the kidney, we evaluated the kidneys of the mice described above. TMe8.5-e16.5 showed GFP+ foci within the kidney (Figure 3C). Sections revealed the positive cells to be lining the tubules in the nephrogenic zone as well as in the more mature nephrons in the medulla (Figure 3C’). At TMe9.5-e16.5 the same puncta were observed at the gross level (Figure 3D), and sections confirmed that several tubules and budding nephrons had
contribution of GFP+, Alx4, descendants (Figure 3D’). No whole nephron structures were completely derived from cells that had expressed Alx4; however, it appears that only epithelial cells were positive. Later injections (TMe10.5 and e11.5, Figure 3E-3F’) revealed similar labeling. Throughout this window of development (TMe8.5–e11.5) each kidney appeared speckled with GFP+ spots, while the adrenal glands and surrounding tissues were negative for GFP/Alx4 expression. This inducible, and tubule/epithelial specific Cre will facilitate the analysis of cell fate and kidney development in mutant and wild type contexts.

Skin and Cranium

While Alx4 is known to be expressed and participate in hair follicle cycling [100], neither the lines generated by Kuijper et al. or the Gardener Mouse recapitulated any pilosebaceous expression. This suggests that the enhancer elements required for follicle expression are not within the first 17kb of the promoter. Sporadic expression was seen in the epidermis (Supplemental Figure 2A and 2B) that did not correlate with developing follicles or the dermal condensates (Supplemental Figure 2B). To determine if Alx4 was expressed later during follicle cycling, the Gardener Mouse line was bred onto the R26 β-gal reporter allele (Gt(ROSA)26Sor<sup>tm1Sor</sup>) [101]. At 3 months of age, tamoxifen was administered over the course of 5 days, and on the 5<sup>th</sup> day the ventral pelage of the mouse was depilatated. This paradigm would label any cells expressing Alx4 throughout the 5 day injection period before synchronization, and for ∼2 days after hair removal (early anagen stages [102]). Histological sections subjected to β-gal staining revealed that the Gardener Mouse’s transgene was only activated in the neck region of the sebaceous gland.
in an unknown cell type (Supplemental Figure 2C and 2D, arrows), no expression was seen in the dermal papilla, or along the follicle proper.

Alx4 is also known to be expressed during craniofacial and tooth development; however, there was no evidence that the Gardener Mouse transgene was active in these regions of the embryo. In addition, as our studies were focused mainly on embryonic development we did not evaluate transgene expression in the mammary glands [95].

Methods

Mice

Mouse alleles and transgenic lines used, were as follows: Gt(ROSA)26Sor
\textsuperscript{tm4}(ACTB-tdTomato,-EGFP)Luo (mTmG), and Gt(ROSA)26Sor
\textsuperscript{tm1Sor} (R26) obtained from Jackson Laboratories (Bar Harbor ME), and Tg(Alx4-cre/ERT2)1Bky (Alx4::CreER, Gardener Mouse) mice were generated in our lab. Animals were maintained in AAALAC accredited facility in accordance with IACUC regulations at the University of Alabama at Birmingham.

Timed pregnancies were set up before lights out and vaginal plugs assessed the following morning. Noon on the date of the plug being observed was designated e0.5. For tamoxifen induction, tamoxifen (Sigma, T5648) was dissolved in corn oil at 20 mg/mL and a single 6mg dose was given to pregnant females via IP injection at the times indicated. Embryos were harvested into PBS and imaged, or processed as indicated below, embryos >e15.5 were killed by decapitation before being processed. Adult TM administration was done following the same paradigm with consecutive injections over a 5 day period.
Generation of the Gardener Mouse: The Alx4::CreER transgene was generated by replacing the β-galactosidase gene from the Alx4-LacZ construct described previously by Kuijper et al. ([68]) with the CreER\textsuperscript{T2} sequence. This was accomplished using the XhoI restriction endonuclease site located 17kb upstream of the Alx4 start codon, and an EagI restriction endonuclease site located 13 codons downstream of the start, within exon 2 (Figure 1A). After ligation, the promoter was in frame creating a fusion of the first 13 codons of Alx4 and CreER\textsuperscript{T2}(Figure 1B). To generate a fragment for pronuclear injection p372 was digested with XhoI and BfuI retaining ~500bp of downstream vector sequence.

**Immunofluorescence**

Embryos with fluorescent signal were imaged immediately after isolation or within 24 hours of isolation after having been stored in 4% PFA at 4°C. Whole embryo images were taken on a Zieiss Lumar v12 fluorescence stereomicroscope equipped with an Axiocam MRm camera using AxioVision. For analysis of GFP in sections, embryos were fixed in 4% PFA overnight at 4°C, rinsed in PBS and immersed in 30% sucrose/PBS at 4°C. Embryos/tissues were then oriented in OCT, frozen in a dry ice ethanol bath, and stored at -80°C until sectioned. Sections 12-15um thick were cut and incubated in PBS+Hoechst (Invitrogen#33342) for 5’ at RT, coverslips were affixed using Immu-mount (Thermo #9990402). Confocal images were obtained using a Perkin Elmer ERS 6FE spinning disk confocal module on a Nikon TE-2000-U, using Velocity 6.1
software. No antibodies were used for enhancing either the tomato or GFP signals from the mTmG reporter.

**β-galactosidase Staining**

β-galactosidase staining was performed as outlined in [86]. Briefly, freshly frozen skin samples were embedded in OCT and frozen, sectioned, and fixed in 4% PFA, 0.2% glutaraldehyde, 0.02% NP-40, 0.01% NaDC and were kept on ice throughout fixation. The samples were washed three times for 5 minutes in PBS, and the staining solution was added to samples and allowed to develop until sufficient staining occurred. Samples were then counterstained with eosin, dehydrated, and mounted with permount (Fisher #SP15-500).

**References**


Acknowledgements

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Figure Legends

Images with asterisks (*) are representative and will not necessarily be part of the final version of the manuscript. In addition, Figure 2I and 3D’ are intentionally blank, this data is still being acquired.

Figure 1: The generation of an Alx4::CreER transgene: (A) a schematic representation of the genomic Alx4 promoter. (B) The XhoI-EagI fragment was cloned upstream of CreERT2 in frame creating a fusion protein. Exons denoted by black boxes, translational start site denoted by an arrow.

Figure 2: Alx4 descendents occupy the anterior limb: (A-L) Immunofluorescence analysis of Cre recombination (green) in Gardener Mice. Tamoxifen (TM) was administered as described in the methods, whole embryos (A, D, G, J) were isolated 24hr after injection and imaged. Embryos were also isolated at e16.5, (B, E, H, K) whole limbs and (C, F, I, L) sections were imaged. Higher magnification of the boxed region in L is shown below. Arrows indicate Alx4 expressors/descendents. m = muscle, e = epidermis, d = dermis, c = chondrocyte.
Figure 3: Alx4 is expressed in the developing kidney. (A-B) Immunofluorescence analysis of an e11.5 embryo exposed to TM at e8.5. (A) The lateral flank below the forelimb of a whole embryo showing Alx4 Cre activity (green). (B) Horizontal sections below the forelimb show the mesonephric duct (green epithelia) lateral to the dorsal aorta (da). (C-F) Images of whole kidneys and sections showing Alx4 expression (green) in puncta covering the whole kidney (C, D, E, F) and in the developing nephrons (C’, D’, E’, F’) when injected with tamoxifen as indicated and harvested at e16.5.

Supplemental Figure 1: The Alx4 expression domain in the limb changes over time: (A-E) Immunofluorescence analysis of Cre recombination (green) in the Gardener Mouse in 24hour increments. Pregnant mothers were injected with tamoxifen at the indicated times and whole embryos were harvested 1 day later. Magnified views of the fore- (upper) and hindlimbs (lower) are shown to the right of each embryo.

Supplemental Figure 2: Alx4::CreER expression in the skin. (A) Whole embryo images of a TMe9.5-16.6 embryo with sporadic epidermal recombination (green), arrows. (B) A magnified view of dorsal skin from a TMe11.5-16.6 showing Alx4 expression (green). (C-D) Skin sections from Alx4::CreER ; R26 mice were treated with TM, depilated, and allowed to recover for ~4 weeks. Beta-galactosidase positive cells (blue) are only seen in the neck of the sebaceous glands (arrows). sg = sebaceous gland, dp = dermal papilla.
Figure 1

A

Xhol

17 Kb

B

1.7 Kb promoter

Fagl

Xhol

Bful

Bful

p372

2: CreERT2
CHAPTER 4: DEVELOPMENT OF THE CILIA<sup>GFP</sup> MOUSE, A MODEL FOR VISUALIZING CILIA<em> IN VIVO</em>

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Abstract:

Cilia are found on nearly every cell type in the mammalian body, and have been historically classified as either motile or immotile. Motile cilia are important for fluid and cellular movement; however the roles of non-motile or primary cilia in most tissues remain unknown. Several genetic syndromes, called the ciliopathies, are associated with defects in cilia structure or function and have a wide range of clinical presentations. To facilitate the analyses of cilia function we generated a Cilia\textsuperscript{GFP} mouse by targeting cDNA encoding a cilia localized fusion protein (Sstr3::GFP) into the Rosa26 locus. In this system, Sstr3::GFP is expressed from the ubiquitous Rosa26 promoter after Cre mediated deletion of an upstream Neo cassette flanked by loxP sites. Robust and specific fluorescent cilia labeling was observed in multiple live tissues and after fixation. Both cell-type specific and temporally regulated cilia labeling were validated using multiple Cre lines. The analysis of renal cilia in anesthetized mice revealed that their cilia often display a synchronized, repetitive movement. This movement ceased upon death suggesting a relationship with heart beat, blood pressure, and/or glomerular filtration. The ability to visualize cilia in live samples within the Cilia\textsuperscript{GFP} mouse will greatly aid studies of ciliary function. This mouse will be useful for \textit{in vivo} genetic and pharmacological screens to assess pathways regulating cilia motility, assembly, retraction, or length control and to study factors involved in ciliary protein trafficking. Importantly, this resource will also facilitate \textit{in situ} studies into the function of the primary cilium in multiple tissues such as the eye, brain, and kidney, defects in which result in ciliopathy phenotypes.
Introduction:

Cilia are now recognized as critical organelles with essential roles in signaling, development, and are mechanotransduction (For recent comprehensive reviews see [4, 103, 104]). There are two basic types of cilia. The first are primary, non-motile cilia which are solitary organelles composed of a ring of nine microtubule doublets ensheathed by a ciliary membrane. The second type are motile cilia, like those in trachea and ependymal cells of the ventricles in brain, these are similar in structure while having the ability to beat and propel fluid due to additional microtubules and axonemal dynein motors. Cilia are unique sub-domains of the cell comprised of specific proteins and signaling molecules. Several key signaling pathways are now known to be regulated by the cilium, and numerous G-protein coupled receptors, channels, and transcription factors specifically localize to the cilium. As such, a group of syndromes called the ciliopathies are caused by defects in the structure or function of the cilium. Ciliopathy disease states range from mild -where only one organ system is affected, such as in Leber’s Congenital Amaurosis; to more severe syndromes involving nearly all organ systems resulting in perinatal lethality, as seen in Meckel-Grüber Syndrome. Several phenotypes are related specifically to motile cilia defects, such as chronic rhinitis, altered left right body axis specification, and hydrocephalus. Other ciliopathy phenotypes are linked to defects in ciliary signaling such as polydactyly, obesity, and polycystic kidney disease [4, 103, 104].

Model organisms have been used extensively to study ciliary biology. For example, *Caenorhabditis elegans*, a roundworm, has 60 ciliated sensory neurons which allow the animal to find food, mate, and sense its external environment [105-107]. In contrast to mammals, the loss of cilia in *C. elegans* is not lethal and ciliary defects can be easily
assayed using lipophilic dyes. Simple behavioral and visual assays, along with the ease of making transgenic lines in *C. elegans*, has made it possible to rapidly conduct genetic screens, assess protein localization, and elucidate allele function leading to the identification and functional assessment of many ciliary genes. For example, the homolog of the gene PKD1 which is a ciliary gene linked to human autosomal dominant polycystic kidney disease, was initially found in a *C. elegans* mutagenesis screen in search of genes affecting male mating behavior [108].

Another common model system for studying ciliary biology is *Chlamydomonas rheinhardtii*, a green alga with two flagella (homologous structures to motile cilia). A major advantage of *Chlamydomonas* as a model system is that they can shed their flagella *en mass* facilitating large scale proteomic and biochemical studies [109]. Indeed, the major protein components needed to construct cilia, through the process known as intraflagellar transport, were first identified in *Chlamydomonas* [110]. Also, once removed, their flagella can regenerate within an hour, facilitating studies of flagella assembly dynamics [111]. While these organisms have been critical in the development of cilia biology, the confirmation of mammalian relevance and conservation are important for understanding the mechanisms behind ciliopathy associated diseases. Both the *C. elegans* and *Chlamydomonas* models offer the advantage of being able to readily visualize their cilia and flagella in live samples; however, *in vivo* mammalian systems present challenges in this regard. Unfortunately, to date there have been no robust, inducible, *in vivo* models to visualize mammalian primary cilia; limiting studies to those based on fixation and antibody labeling.
To address this limitation, we generated a new mouse model that will allow the
direct visualization of mammalian cilia \textit{in vivo}, the Cilia\textsuperscript{GFP} mouse. Using a known
ciliary membrane protein, the somatostatin type receptor 3 (Sstr3), we generated a fluo-
rescent fusion protein to label cilia \textit{in vivo} \cite{112, 113}. The Sstr3::GFP fusion mouse
cDNA was targeted into the ubiquitously expressed Rosa26 (R26) locus \cite{114}. The allele
contains a floxed Neo cassette upstream of the start site to provide Cre mediated spatial
and temporal control of expression \cite{115}. With this approach we established mouse lines
in which \textit{in vivo} cilia labeling is inducible or constitutively expressed. Our work demon-
strates the utility of the Cilia\textsuperscript{GFP} mouse for \textit{in vivo} and \textit{ex vivo} detection and analysis of
cilia. Importantly, we document ciliary movement within the kidney tubules, a finding
that underscores the utility of this model.

\textbf{Materials and Methods:}

\textbf{Construct Generation and Mouse Engineering:}

To generate the Sstr3::GFP Rosa26 targeting construct, Sstr3::GFP was amplified
from an expression vector (a kind gift of Dr. Pazour, U. of Massachusetts) with primers
adding attB sites to the 3’ and 5’ ends and cloned into the Rosa26 targeting vector using
Gateway technology (Figure 1A, Invitrogen) \cite{115}. Primers forward:
ggggacaagtttgtacaaaaagcaggcttaaccatggccactgttacctatccc, and reverse:
ggggaccacttttgacaagggatcagctttgtacagtctggttcctgccc. The Sstr3::GFP construct was
electroporated into Primo B6 (C57BL/6N-tac) embryonic stem (ES) cells and G418 re-
sistant colonies established as described previously ([81, 116]). The ES cell colonies
were screened by long range PCR using forward primer: aaaagcagcagcatggtagat, and
reverse: cgagggacctataactctgatagc to yield a 2.4kb product (not the same as the \(T^{OFF}\) genotyping product). To test expression and ciliary localization prior to generating the mice, targeted ES cells were differentiated by removing leukemia inhibiting factor and serum from the media. Cells were subsequently transduced with a Cre lentivirus to remove the Neo cassette and activate expression of the ciliary Sstr3::GFP (Figure 1B). Correctly targeted ES cells were injected into blastocysts to generate male chimeras. Germline passage was obtained by crossing chimeric males to albino C57BL/6 females. Genotyping primers are as follows: a common forward within the 5’ homology arm: ctcgtgatctgcaactccag; a reverse in the 3’ homology arm: gctgcataaaccccagatgactcc; a reverse near the 5’ PKG-Neo site: gcgcatgctccagactgccttg; and a reverse at the 5’ end of Sstr3: gcggatgtgttccccagggtgg (Figure 1A, arrows). Together the primers yield a 226bp wild type band, a 317bp product from the targeted allele containing the floxed-stop-sequence\(T^{OFF}\), and a 423bp band that is amplified after excision of the floxed-stop cassette\(T^{ON}\) (Figure 1C)). Systemic and cell type specific deletion of the Neo gene was accomplished using Adenovirus EIIa early promoter Cre ((Tg(EIIa-cre)C5379Lmgd, [117] (hereafter called EIIa Cre)); Proopiomelanocortin Cre ((POMC, Tg(Pomc1-cre)1Gsb), [118] (hereafter called POMC Cre)), and the Pancreatic and Duodenal Homeobox CreER ((Tg(Pdx1-cre/Esr1*)1Mga, [119] (hereafter called Pdx1CreER)). Germline, systemic expression of the \(T^{ON}\) allele was accomplished crossing female EIIa Cre; Sstr3::GFP with wild type C57BL/6 mice.
Ethics

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The protocols were approved and conducted according to the University of Alabama at Birmingham IACUC (APN 121109276).

Tamoxifen Injections

To activate the Cre recombinase in Pdx1CreER animals, mice were injected with 6mg of tamoxifen every other day over a 5 day period and the pancreata were harvested three days after the final injection. Tamoxifen (Sigma T5648) was dissolved in corn oil at 20mg/ml, and bolus IP injections of 300µl were injected intraperitoneally.

Renal tubule isolation:

Mice were anesthetized and then euthanized by cervical dislocation and whole kidneys were isolated and cut into ~2mm pieces. The tissue was incubated at 37°C in three changes of collagenase buffer for 30 minute intervals with gentle agitation every 10 minutes. Collagenase buffer: 10mg/ml Collagenase Type IV (Sigma #C5138), 50U/mL DNase I (Sigma #D5025), and 0.1mg/ml Soybean Trypsin Inhibitor (Invitrogen #17075-029) in DMEM/F12. After each 30 minute incubation, the supernatant was removed and replaced with fresh collagenase buffer. The supernatant, containing the ex vivo tubules, was gently pelleted at 100 RCF for 1 minute, the tubules were washed three times in phosphate buffered saline (PBS) containing 3% bovine serum albumin (BSA). The tu-
bulbs kept on ice and imaged live or were fixed in 4% paraformaldehyde (PFA) for 5 minutes followed by blocking and processing for immunofluorescence analysis.

**Ex vivo Live imaging:**

Brains from Sstr3::GFP; EIIa Cre mice were removed after anesthetization and decapitation. The ex vivo brains were cut sagittally down the midline and placed cut surface down on coverglass in sterile filtered artificial cerebro-spinal fluid (125mM NaCl, 2.5mM KCl, 1.25mM NaH2PO4, 2mM CaCl2, 1mM MgCl2, 25.M NaHCO3, 25mM Glucose, pH 7.3). Cilia were imaged live using a cascade 1K CCD camera (Photometrics Tucson, AZ) on an inverted Nikon TE2000U microscope equipped with a 60X Apochromat oil TIRF NA 1.49 objective, Perkin Elmer ERS 6FE spinning disk confocal module, and Volocity 6.2 software.

**In vivo analysis of renal cilia**

8-16 week old mice were anesthetized by intraperitoneal injection of 2.5% tribromoethanol (Sigma T48402). A dorsal incision was made and the kidney was located and gently teased out through the opening, making sure not to damage the renal artery, vein, or ureter. The mouse was placed, incision side down, inside a heated chamber maintained at 37°C (LiveCell™ stage top incubation system #05-11-0035, Pathology Devices Inc Westminster, MD) with the kidney positioned on a coverslip bathed in PBS. The mice were continuously administered isoflurane at 1-2% (VetOne #13985-030-60 obtained through the UAB ARP veterinary services) throughout the procedure and were euthanized after imaging.
Perfusion:

For immunofluorescence detection of neuronal cilia, animals were anesthetized with a lethal dose of 2.5% tribromoethanol (Avertin, Sigma T48402), and transcardially perfused with PBS, followed by 4% PFA. The brain was isolated and processed for immunofluorescence labeling as described below.

Cryosectioning:

Fresh tissues were isolated and stained as in [113]. Briefly, the tissue was dissected into PBS, oriented in OTC (Fisher #14-373-65), and frozen in a dry ice-ethanol bath. For fixed tissues and immunofluorescence analysis, tissues were dissected into PBS, fixed in 4% PFA overnight at 4°C, penetrated with 30% sucrose in PBS before being embedded in OCT and frozen. Sections 10-15µM thick were cut and mounted onto slides, fixed in 4% PFA for 5 minutes at room temperature (RT), washed in PBS for 5 minutes, treated with 10µg/ml Hoechst (Sigma #33258), and mounted with Immu-Mount (Fisher# 99-904-02).

Immunofluorescence analysis:

For immunofluorescence detection of cilia staining was performed as in [120]. Briefly cryosections were blocked in 1% BSA, 1% Donkey Serum, in PBS + 0.1% Triton-X 100 (PBST) for 30 minutes. Primary and secondary antibodies were diluted in blocking solution and applied to sections for 1 hour at RT or overnight at 4°C, after incubation slides were washed in PBS for 5 minutes before being stained with Hoechst, and mounted with Immu-Mount. The following primary antibodies were used: acetylated \(\alpha\)-
tubulin (Sigma T7451, 1 mg/ml input) pre-conjugated to AlexaFluor 647 (Invitrogen, #A-20186) and used at 1:2000, Adenyly cyclase III (ACIII, Santa Cruz #sc-11617) used at 1:500, and Arl13b (A gift from Dr. Tamara Caspary at Emory University) used at 1:2000. The following secondary antibodies were used: anti-goat and anti-rabbit AlexaFluor-594 each use at 1:1000.

Immunofluorescence on isolated tubules:

Immediately upon isolation the tubules were fixed in 4% PFA for 5 minutes followed by blocking in 1% BSA, 1% Donkey Serum in PBST for 30 minutes. Tubules were incubated with the direct conjugated acetylated α-tubulin antibody for 30 minutes at RT and washed in PBS for 5 minutes. Nuclei were stained with Hoechst (Sigma #33258), and imaged in PBS or 50% glycerol droplets on coverglass.

Cilia movement analysis

The frequency of cilia oscillation was calculated by measuring the fluorescence intensity of a pixel near the moving end of the path of the cilia over the course of the captured video acquisition (26fps, 60s). These intensity timecourses were imported into Microsoft Excel 2010, corrected for background and limited to 1024 samples. Excel’s Fast Fourier Transform function was used to generate frequency power spectrums from which the predominant peak was taken as the frequency of ciliary oscillation. Images of the sweep of the path of cilia were created by taking a single plane of a confocal stack timecourse. All images in the entire timcourse (60s, ~1500 frames) were combined into a
Results and Discussion:

Cilia$^{\text{GFP}}$ mice expressing Sstr3::GFP were engineered to facilitate \textit{in vivo} studies of cilia. Here we demonstrate efficient labeling of motile and primary cilia in live and fixed samples of the Cilia$^{\text{GFP}}$ mouse. Expression of the cilia tag can be controlled by Cre recombinase in an inducible or tissue specific manner if ubiquitous labeling is not desired. To generate the cilia tagged mouse, the Sstr3::GFP mouse cDNA preceded by a floxed Neo cassette was targeted into the ubiquitously expressed Rosa26 locus (Figure 1A) [115]. Targeted embryonic stem cells were tested for Sstr3::GFP expression and cilia localization after Cre transduction \textit{in vitro} (Figure 1B). Germline passage of the conditional (T$^{\text{OFF}}$) and the constitutive (T$^{\text{ON}}$) alleles was confirmed by PCR genotyping (Figure 1C).

The initial analysis of transgene expression was done by mating EIIa Cre males to T$^{\text{OFF}}$/WT females and analyzing the F1 T$^{\text{OFF}}$/WT; EIIa Cre offspring. EIIa Cre is expressed in the early embryo and results in animals with widespread mosaic expression of the recombined Sstr3::GFP allele (T$^{\text{ON}}$) in most tissues (Figures 2, 3, and 4) [117]. In control animals (T$^{\text{OFF}}$/WT Cre-) no GFP labeling was evident (Supplemental Figure 2). The utility of the Cilia$^{\text{GFP}}$ model was validated using tissue/cell type specific Cre lines, as well as inducible Cre mediated activation (Figure 5). Finally using live, \textit{in situ}, time lapse imaging, fluorescent cilia were observed and ciliary movement was noted within single image by taking the average intensity of all the images using ImageJ (NIH, Bethesda, Maryland) and contrast enhanced.
kidney tubules, making this visualization technique and model potentially useful for analyzing cilia mechanosensation and cyst development in vivo.

The Brain:

The clinical importance of cilia in the brain has recently become apparent, despite a general lack of understanding of their function [4, 9]. Motile cilia on the ependymal cells lining the ventricles are important for the movement of CSF, and defects in these cilia or their motility are associated with hydrocephalus [121, 122]. On neurons, disruption of the primary cilium revealed their role in regulating satiation and other behaviors through unknown mechanisms [9, 123, 124]. In addition, cilia are known to be important for sight and smell [9, 125-127]. However, the ubiquitous nature of primary cilia on most neurons in the CNS was unexpected [128]. While the traditional methods of cilia visualization have relied on antibodies to various tubulins, these methods prove especially challenging in the brain where tubulin is highly expressed. This has made visualizing cilia on neurons difficult without special fixation, antibodies, and perfusion conditions. Thus the development of new tools will aid in the assessment of neuronal ciliary function.

To determine whether the Cilia^{GFP} mouse could be utilized for visualizing neuronal cilia in live, ex vivo samples, we analyzed the Cilia^{GFP} mouse in the presence of EIIa with mosaic activation in the brain [117]. Primary cilia were easily detected on cells throughout the brain. For example, primary cilia were found in the hippocampus (Figure 2A) and many motile cilia were detected on the ependymal cells within the ventricles (Figure 2B). Cilia were also observed on the choroid plexus epithelium where there are two populations of ciliated cells, some with multiple cilia and some with only a single
cilium (Figure 2C inset) [121]. Time-lapse live imaging was performed on the ependymal cells to explore the potential utility of the Cilia\textsuperscript{GFP} mouse in cilia motility studies. Images could readily be captured at 15 frames per second with our imaging system (Movie 1). All of these cilia were rapidly visualized with little or no preparation, no counter-staining, on \textit{ex vivo}, live samples (Figure 2A-C, and Movie 1).

In addition to live imaging, the GFP label persisted through fixation and subsequent immunofluorescence staining confirming that Sstr3::GFP labels cilia and is a stable marker in processed tissues. In the lateral ventricle of T\textsuperscript{ON} Cilia\textsuperscript{GFP} mice, Sstr3::GFP and acetylated \(\alpha\)-tubulin colocalize within many of the ependymal cells and in the primary cilia of neighboring cells (Figure 2D). Interestingly the primary cilia labeling is somewhat brighter than on the motile ependymal cilia, this is likely due to the increased membrane surface area in motile cilia and dilution of the protein. This has been observed for the hedgehog pathway mediator, smoothend, in cells induced to form multiple primary cilia [129]. Similar labeling of the primary cilia was noted in the pituitary (Figure 2E); and in the hindbrain region (Figure 2F) where the GFP signal co-localized with the neuronal cilia marker Adenylyl cyclase three ACIII. Together these images, as well as those in the living samples, demonstrate that the expression levels in the brain are sufficient to facilitate studies of neuronal cilia. For example, this model could be used to analyze the prevalence of cilia throughout the brain or the assessment of cilia loss in specific brain regions after Cre deletion of a floxed ciliary allele. Also, this model would easily enhance studies and techniques requiring live tissues such as electrophysiology or pharmacological studies of factors regulating cilia length in the brain.
The Kidney:

The formation of cysts in the kidney is a common pathological feature associated with multiple human ciliopathies [4, 9]. Although the causes are not yet known, significant effort has gone into determining how disrupting cilia function results in cystogenesis. Under normal conditions, the renal cilium is thought to be a mechanosensor wherein deflections of the ciliary axoneme by fluid flow elicit a cytosolic calcium response [130]. This in vivo mechanosensory response is impaired in cilia mutants, as well as in cells lacking polycystin-1 or polycystin-2, two proteins whose function is disrupted in human polycystic kidney disease [131]. These data led to the model that cysts develop through the loss of this mechanosensory signal. However, to date in vivo studies validating this hypothesis have not been readily feasible. To evaluate whether the Cilia GFP mouse may be useful in visualizing the cilia in the kidney and to address clinically important questions such as whether or not flow induces ciliary deflection, we analyzed kidney expression of Sstr3::GFP in EIIa Cre animals (Figure 3). In each of the analyses we conducted, cilia could easily be detected making studies of cilia length, orientation, motility, mechanotransduction, and analysis of whole tubules practical.

In kidney sections, in ex vivo tubules, and in vivo labeled cilia were readily identifiable (Figure 3A-C). Again the label was seen without fixation or staining and the signal persisted throughout handling and imaging. In isolated tubules, many cilia remained attached through isolation, fixation, staining, and imaging (Figure 3D). However, some GFP labeled debris was observed in the isolated tubules and is believed to be ciliary fragments broken off during isolation, a drawback to tubule isolation. Again, the speci-
ficity of ciliary localization was confirmed in tubules and in sections using the cilia marker acetylated α-tubulin (Figure 3D and E).

Next using in situ imaging we evaluated the kidneys of live EIIα Cre mice. Briefly, mice were anesthetized and their left kidney was exposed through a dorsal incision. In a heated chamber, using an inverted microscope, the tubules were imaged in situ (Figure 3C). In several mice, synchronized ciliary movement within multiple tubular lumens was evident using rapid exposures (26fps using 33ms exposures, see Movie 2 and 3, N = 4 of 5). Interestingly, most of the cilia appear to be bending at a regular and specific point along their axoneme above the base. This bending may be due to the rigidity of the tubules within the cilium axoneme, it may be attributed to a particular molecular domain (for example the inversin compartment [132, 133]), or the cilia may be embedded within the ciliary pocket[134].

One might expect with continuous filtration at the glomerulus, and the presumed constant rate of tubular flow that occurs along the length of the proximal tubule that cilia would remain bent in a relatively static position. However, imaging in the CiliaGFP mouse revealed that renal cilia can move rhythmically (Movie 2 and 3). This movement is likely passive and not a result of molecular motors such as dynein for two main reasons. First, the frequency of movement was determined to be 4.58+/−0.20 Hz which is a similar to documented mouse heart rates under anesthesia [135]. This suggests that the movement may be driven by the depth of anesthesia, heart rate, blood pressure, and their impact on glomerular filtration; however we were unable to simultaneously measure the heart rate of mice while imaging. Furthermore, most of the cilia in a whole field move in unison suggesting regulation at the level of the whole kidney not at the level of the indi-
individual cell/tubules/glomeruli. The second reason supporting a passive mechanism is that the movement of the cilium in the tubules stops upon death. After euthanizing the mouse, the cilia stop moving and extend nearly perpendicularly into the nephron lumen (Movie 3, N = 2). It is interesting to note that the cilia have a wide range of movement, creating an arc of >90° in most cases (Supplemental Figure 1). This suggests that tubular flow may not be as continuous as previously believed; however, alterations in the direction of tubular flow or drastic changes in flow rate/pressure must occur to produce this range of ciliary movement, both of which are not known to occur. Alternatively, the “nonmotile” cilia, at least in the proximal tubule images here, are not immotile but actually exhibit motile behavior. It should also be noted that the nodal cilia in the developing mouse embryo have a 9+0 structure and they are indeed motile [136, 137]; however, the nodal cilia also have axonemal dynein motors required for their motility that are not readily apparent in renal primary cilia. Follow-up studies will be necessary to determine whether this luminal movement is active or passive and if these findings will have any impact on our understanding of ciliopathy disorders such as polycystic kidney disease.

The Eye:

Blindness is also associated with ciliopathies such as Senior-Løken syndrome, Leber’s Congenital Amaurosis, and Bardet–Biedl syndrome [125, 138, 139]. This is generally due to dysfunction in the structure or trafficking at the connecting cilium (CC), a highly modified primary cilium in the rod and cone photoreceptors in the retina (Figure 4D diagram) [125]. Defects in trafficking, protein turnover, ciliary assembly, or the distribution of the signaling components required for vision are all associated with retinal
degeneration [4, 125]. Due to the stereotypic anatomy of the retina, and the exaggerated ciliary structure (Figure 4D the CC), the rod cells in the retina are a useful model of ciliary function and trafficking [125, 140, 141], thus endogenous ciliary labeling would be beneficial for longitudinal in vivo studies, and in other studies of the retina where ciliary staining is difficult.

To determine if the CiliaGFP mouse would be sufficient to analyze the photoreceptor CC, we evaluated the retinas of CiliaGFP EIIa Cre mice. Interestingly, the ganglion cells of the retina contained many ciliated cells (Figure 4B arrows) as did many of the cells in the anterior region of the inner nuclear layer (INL, Figure 4C arrows). GFP was concentrated in the CC of the photoreceptors but is detectable in the outer segments (Figure 4D arrows, Supplemental Figure 2H). In addition, rhodopsin staining indicated that the CiliaGFP label does not overtly interrupt trafficking of Rhodopsin nor the health of the rod cell. Finally, in our attempts to co-label the GFP signal within the retina with known ciliary markers, we had difficulty staining the CCs with either Arl13b or acetylated α-tubulin using standard immunofluorescence protocols (Figure 4, acetylated α-tubulin in purple). While labeling of the CC can be done it is unreliable and requires protocol modifications and additional steps such as antigen retrieval. Thus, the CiliaGFP mouse will be useful for identifying the connecting cilia in the retina of live mice over time, and in samples without relying on difficult and lengthy protocols.

Spatio-Temporal Control of Expression:

Having the ability to label cilia on specific cell types in vivo will facilitate studies of the roles cilia have in different tissues. To demonstrate the feasibility of using the
Cilia\textsuperscript{GFP} mouse for this purpose, we crossed the Cilia\textsuperscript{GFP} mouse with the POMC Cre line. The animals from this cross should express Sstr3::GFP in a discrete subset of neurons in the arcuate nucleus (ARC) of the hypothalamus (Figure 5A). These POMC neurons surround the third ventricle, and are important for satiation responses and feeding behavior. Additionally the loss of cilia from these neurons causes hyperphagia and obesity [123, 142, 143]. In these POMC Cre Cilia\textsuperscript{GFP} mice, cilia labeling was detected within the ARC but not in other regions of the brain like the hippocampus (Figure 5B-D and data not shown, N = 2). Staining sections of the hypothalamus with ACIII, a marker found in many but not all neuronal cilia, revealed that the GFP labeling was specific for cilia (Figure 5D).

We also evaluated the temporal responsiveness of the Cilia\textsuperscript{GFP} mouse using the tamoxifen inducible Pdx1CreER line to delete the floxed Neo cassette in the β cells of the islets in the pancreas [119]. Pancreata were harvested three days after a series tamoxifen injections (see methods) and processed for immunofluorescence. As shown in Figure 5E many of the cilia in the islets were labeled with Sstr3::GFP. Primary cilia are present on islet cells and in the ducts of the pancreas as reported previously [144, 145], and in agreement with known PDX1 Cre expression, only the islets were labeled in this model. Specificity was confirmed with acetylated α-tubulin staining (Figure 5E) and the absence of label in the ducts as well as in Cre negative animals was confirmed (Supplemental Figure 2). These studies together demonstrate the spatial and temporal control of cilia labeling offered by the Cilia\textsuperscript{GFP} mouse. This control will aid in ciliary studies of specific cell types, at specific time points, in live, and fixed samples.
Conclusions:

Here we have introduced a new tool for in vivo and ex vivo detection and visualization of mammalian cilia, the Cilia\textsuperscript{GFP} mouse. We have demonstrated that the Cilia\textsuperscript{GFP} mouse is functional, cilia specific, and that spatial and temporal control of expression is possible. In animals where Cre mediated deletion was not initiated no ciliary label was detected (Supplemental Figure 2). In addition, there were a few tissues where ciliary labeling was not found such as the trachea and the motile cilia in the oviduct (Supplemental Figure 2 and data not shown) of T\textsuperscript{ON} mice. The reason that cilia in these regions of the Cilia\textsuperscript{GFP} mouse were not labeled is unknown but it could be that the Rosa26 locus has no or extremely low expression in these tissues, or that these motile cilia fail to localize Sstr3 to their membranes. Despite this, we have generated a mouse to facilitate the study of cilia in many tissues where staining and imaging have been difficult, such as the brain and eye, and for studies that require live or in situ analysis. Importantly, we have not seen any overt phenotypes in T\textsuperscript{ON} animals, despite ubiquitous expression of the somatostatin receptor, although it should be noted that detailed behavioral analyses have not been done.

There are many possible uses for this mouse model. The Cilia\textsuperscript{GFP} mouse line could be used for mammalian mutagenesis and pharmacological screens to identify genes that affect ciliogenesis, or studies of cilia length control and ciliary protein trafficking. One could also assess recovery of ciliary proteins by FRAP analysis, analyze the regulation of cilia motility, or to screen for suppressor mutations using known cilia mutants and assessing the restoration of ciliogenesis. Further, this model could be used as a reporter
of Cre activity and ciliary status in cells that have undergone recombination. For example, the Cilia$^{\text{GFP}}$ mouse could be used for the assessment of cilia loss when used in conjunction with floxed alleles of genes required for ciliogenesis. Lastly, we have shown the utility of this model with \textit{in situ} documentation of ciliary movement in the tubules of the kidney. Indeed this observation indicates the Cilia$^{\text{GFP}}$ mouse will be useful for \textit{in vivo} mechanosensory studies and may provide important insights into how cilia dysfunction contributes to disease. This mouse model will greatly facilitate live imaging studies, and advance the analysis of cilia function in a mammalian context.

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\textbf{References}


Figure Legends

**Figure 1:** Sstr3::GFP Rosa26 targeted alleles. (A) Schematic of the Sstr3::GFP conditional (T<sup>OFF</sup>) and expressing (T<sup>ON</sup>) alleles. Before Cre mediated deletion (T<sup>OFF</sup>), the expression of Sstr3::GFP is inhibited by the Neo cassette (PGK-Neo 3XpA) flanked by LoxP sites (blue triangles). After Cre mediated recombination (T<sup>ON</sup>) the Neo cassette is excised and expression follows. (B) Ciliary localization of Sstr3::GFP was confirmed in differentiated embryonic stem cells transduced with a Cre lentivirus. (C) A four primer mix was used for PCR genotyping of the wild type (WT, 226bp), conditional (T<sup>OFF</sup>, 317bp), and expressing (T<sup>ON</sup>, 423bp) alleles.

**Figure 2:** Sstr3::GFP is expressed and localizes to cilia in the brain. (A-C) *ex vivo*, whole mount brain images. Sstr3::GFP signal (green) in (A) hippocampal primary cilia, (B) ependymal motile cilia (C) and choroid plexus cilia. (D-F) Immunofluorescence labeling with known ciliary markers. (D) The lateral ventricle, (E) pituitary primary cilia, and (F) hind-brain primary cilia. Cilia were immunolabeled with either acetylated α-tubulin (Acet Tub) or adenyly cyclase III (ACIII) in red. Hoechst stained nuclei in blue. Arrows indicate cilia that are double labeled, and arrowheads indicate cilia that are GFP negative in agreement with mosaic EIIa Cre activity.

**Figure 3:** Sstr3::GFP is expressed and localizes to cilia in the kidney. (A-C) Non-stained kidney tissue. Sstr3::GFP signal (green) in (A) Fixed cryosections, (B) Isolated tubules, and (C) *in situ* in the proximal tubule of an anesthetized mouse. (D-E) Immunofluorescence staining of (D) isolated tubules and (E) kidney sections. Acetylated α-
tubulin (red) labeled cilia and nuclei are blue. Arrows indicate cilia that are double labeled.

**Figure 4:** **Sstr3::GFP localization within the retina.** (A-D) Analysis of Sstr3::GFP in the retina. Sstr3::GFP (green) is seen in multiple cell types in the (A) whole retina, (B) in the ganglion layer, (C) in the inner nuclear layer (INL), and (D) in the connecting cilium (CC) of rod cells (arrows). A rod cell is depicted in the schematic on the right. Sections were labeled with acetylated α-tubulin (purple) cilia were not labeled however tubulin rich neural processes were, nuclei are in blue. INL: inner nuclear layer, ONL: outer nuclear layer, CC: Connecting cilium, OS: outer segment. Arrows indicate primary cilia, arrowhead indicates the primary cilium of a glial cell not a CC, and anterior is up.

**Figure 5:** **Cell type specific and inducible expression in the CiliaGFP mouse.** (A) A Nissl stained section of the caudal mouse brain[146], the third ventricle (III) and the arcuate nucleus (ARC) are labeled. In POMC Cre CiliaGFP mice (B-D) Sstr3::GFP cilia (green) are seen in the area of the ARC surrounding the third ventricle (III in B and C). Cilia were labeled with ACIII (red) in (C and D). Double labeled ACIII (red) and Sstr3::GFP (green) cilia (arrows); cilia labeled with ACIII alone (non-POMC, arrowhead), and Sstr3::GFP positive cilia with very low or no apparent ACIII label (asterisk). (E) Sstr3::GFP expression in cilia on a pancreatic islet in a PDX1CreER mouse after tamoxifen induction. Cilia are labeled with (E) acetylated α-tubulin (acetTub) in red. Many cilia were positive for both Sstr3::GFP and acetylated tubulin (arrows) while some acetylated tubulin cilia did not possess Sstr3::GFP (arrowhead).
**Supplement 1:** In situ analysis of ciliary movement in the kidney. (A-B) Confocal images of cilia movement within a (A) whole image or focused on (B) one cilia shown as single plane of a confocal stack. A vector diagram of the cilium arc path (C) shows the maximum arc and the average amount of time in microseconds the cilium was located in a given tenth of the arc. Using the fast Fourier transform (FFT) method the beat frequency of the cilia was calculated (D).

**Supplement 2:** No detectable expression of Sstr3::GFP in Cre negative animals. (A-F) Immunofluorescence analysis of cilia. Sstr3::GFP signal (green) is seen in (A) the islets (C) the ganglion cells (E) and the kidney tubules in Cre positive animals. No GFP signal in detected in the (B, D, F) corresponding tissues from Sstr3::GFP Cre negative animals. Cilia labeled with acetylated α-tubulin (red or purple, except in E). (G) Trachea without Sstr3::GFP label (green) in the motile cilia tufts (labeled with acetylated tubulin, purple). The lamina propria (LP) is autofluorescent, chondrocytes (Ch) are Sstr3::GFP positive (arrow).

**Movie 1:** Ex vivo live imaging of ependymal cilia (same animal represented in Figure 2B). The wave-like movements of the ependymal cilium are easily observed. Images were captured and shown at 11fps.

**Movie 2:** In situ video microscopy of a kidney tubule captured at a high frame rate. Cilia in the tubule can be seen moving back and forth within all of the tubules in the field. The beating movement appears synchronous amongst all of the cilia (shown at 26fps).
Movie 3: *In situ imaging of ciliary movement within a tubule before and after death.*

In this video, the mouse was given a lethal dose of avertin at ~20 seconds into the movie and ciliary movement is nearly complete at 50 sec. Ciliary movement has less range of motion and is more infrequent by the end of the video. Note that the cilia remain synchronous.
Figure 1

A. T^{OFF} allele

B. Image of an experiment result

C. Gel electrophoresis image with lanes labeled T^{OFF}, T^{ON}, WT.
Figure 3

Sstr3::GFP

Kidney Sections

B

Isolated Tubules

C

In Situ Tubules

D

Immunofluorescence

Merge

E

Cilia
Supplement 1

A

B

C

D

4.58 ± 0.20 Hz
n = 37
Movie 1
Live imaging of ependymal cilia beating in the brain
Movie 2
In situ video microscopy of a kidney tubule captured at a high frame rate
Movie 3
In situ imaging of ciliary movement within a tubule before and after death
CHAPTER 5: CONCLUSIONS

Summary:

These studies were initiated to address whether there were different requirements for cilia on the Shh producing, responding, and independent cells of the limb; and to determine which cell populations may contribute to limb defects seen in the ciliopathies. To accomplish this we employed the limb bud as a model system to explore the functional requirement for cilia in the Hedgehog (Hh) signaling, limb patterning, and endochondral bone formation (EBF). Overall, this work provides information regarding the temporal dynamics of cilia loss in relation to signaling events that occur during limb bud morphogenesis. In addition, two important models were developed to advance studies of limb patterning and cilia biology. These studies have offered insights into cilia mediated regulation of limb patterning and bone development.

Chapter Two: Delayed Cilia Loss Results in Skeletal but not Patterning Defects

Review: In chapter two I set out to address what impact regional cilia removal had on limb patterning and bone development. My goal was to assess the relative contribution of the Shh producing and responding cell populations to the polydactyly seen in whole limb cilia mutants. By perturbing cilia on distinct populations of cells during limb development —leaving signaling intact throughout the rest of the limb— we hoped to discern the spatial requirements for primary cilia in limb patterning and EBF. We targeted the
sonic producing and responding cells with two previously characterized Cre lines Shh::GFPCre and Gli1::CreER, respectively. However, there was no Cre line that would affect only the anterior, sonic independent cells in the limb bud. We determined that the structure of the cilia remained for many days beyond Cre induction in vivo, suggesting that the cilia were not sufficiently affected during the patterning phase of limb development. Follow up in vitro studies revealed that despite prompt genetic recombination, ciliary proteins persist in cultured cells for more than 96 hours. This data suggest that the temporal dynamics of protein loss and ciliary ablation were insufficient to address this time-sensitive patterning question. We did note defects in EBF suggesting that Hh signaling was affected by cilia loss later in development.

**Obstacles and Future directions:** Mammalian studies have advantages and disadvantages. In their favor is their breeding, degree of conservation, and application to human disease. On the other hand, being a complex, integrated network of systems, the loss of essential genes is lethal. For example, cilia null mice only develop in utero until embryonic day 9.5 (e9.5)[5]. Due to this, ciliary studies were greatly enhanced by conditional alleles [63]. In chapter two we utilized the conditional Cre/Lox system to delete the Ift88 gene in specific cells of the embryo. Ift88 is a gene required for ciliogenesis, and the floxed allele has been widely used in cilia ablation studies. Using the Shh::GFPCre and the Gli1::CreER mouse lines we set out to delete cilia in the limb bud. We predicted that cilia loss would occur rapidly in the highly proliferative cells of the limb bud and would allow us to assess the importance of the cilium on these distinct cell populations. In contrast, we noted cilia were maintained on many of the cells affected by
the Cre lines for days after the onset of expression/excision and that there was no overt patterning defects.

The Cre/Lox system has been used for over 25 years in mice and has been fully vetted in fate mapping studies, and in gene deletion or activation studies [147]. The fact that cilia remained on Cre affected cells in vivo for over a week using an established floxed allele and previously characterized Cre lines is curious. However, gene activation/expression and reporter activity are rapid processes and may be a misleading measure of gene deletion. For example, the Cre reporter line used in chapter two was the mTmG line, where a floxed membrane tomato (red) cassette is expressed before Cre activity and membrane GFP (green) expression ensues after deletion of the cassette [99]. Thus, the onset of GFP expression is used as a report of Cre activity. Interestingly, very often cells could be identified that were positive for both tomato and GFP, suggesting recent recombination and the persistence of some tomato protein. Consider the temporal dynamics of reporter induction versus gene excision, mRNA degradation, protein turnover, and finally organelle loss. It is quite reasonable, then, to expect that a reporter allele would report Cre activity very promptly, before the effects of deletion have manifest.

We set out to evaluate the impact of cilia loss in limb patterning. Limb patterning is a complex process that occurs rapidly during development, spanning only a few days from embryonic day nine to eleven [90]. Limb outgrowth, skeletal development, musculogenesis, as well as nerve infiltration extend beyond that window, but the autopod is considered to be patterned in those few days early in development. Unfortunately our studies did not reveal any patterning defects when Cre activity was initiated at e9.25-e9.5. Importantly, we did note the presence of ciliary structures on affected, reporter positive,
cells days after the onset of Cre activity. An additional caveat in our studies is that cilia loss was downstream of the initiation of patterning. That is, the genes with the earliest onset of expression in a regional manner happen to be Shh and Gli1, the two promoters we chose to utilize. However, because Cre expression is concomitant with ligand expression and pathway activation, no matter the rate of cilia loss, there would be an initial pulse of Hh signaling. Due to these two considerations; that cilia loss followed the onset of the first patterning signals, and that the loss of cilia was neither complete nor rapid, we now believe that this paradigm was insufficient to answer our initial question.

Endochondral bone formation occurs after limb patterning, when chondrocytes differentiate and condense at about e12.5. After several subsequent developmental steps Ihh secretion and reception drives bone growth and elongation[148]. Just as patterning is affected by the loss of cilia so is this Hh process. Previously, it was determined that cilia loss from chondrocytes results in stunted and malformed bones. It was validating that we did see these changes in our animals, indicating the eventual loss of ciliary function within the regions affected by the Cre lines we used. Our EBF phenotype was similar to those previously published, and differed only in the bones affected [63, 83, 149]. The Shh::GFPCre affected only a part of the autopod, while the Gli1::CreER affected the autopod and ulna causing a more drastic phenotype. We noted the loss of rigidity and improper chondrocyte development in the affected regions, and found the defects to be mainly cell autonomous. Again, these data suggest that cilia loss was sufficient to yield known phenotypes but that the timing or extent of cilia loss early on was insufficient to address patterning.
Moving forward there are very few options available to alleviate these caveats. As noted, the Cre lines we used are those which are expressed as early as possible in a regional manner. Other possible Cre lines would affect the whole limb (CAGGCreER[150], Prx1Cre[151]) or would not reproducibly affect a particular subset of cells (in the case of a mosaic Cre or viral mediated deletion). Alternatively, using the chick model system, it may be possible to electroporate the lateral plate mesoderm to accurately affect regions of the limb. This in conjunction with an siRNA may expedite the knock-down process to affect patterning.

Chapter Three: The Gardener Mouse an Alx4 promoter driven CreERT2 transgenic mouse to study the limb and kidney

Review: When we set out to undertake the studies outlined in chapter two, it became obvious that there were no Cre lines available that were exclusively expressed in the anterior limb bud. Using the Alx4 promoter known to regulate gene expression specifically in the anterior limb [68], we generated the Gardener Mouse. This Cre line can be used to target the independent cells on the anterior limb. Importantly, when used with the Shh::GFPCre and Gli1::CreER lines described above, the Gardener Mouse will allow for regional Cre activity in all cells across the limb bud. We then validated the functionality of the Cre and fate mapped the Alx4 expressing cells in the mouse. In the anterior limb we found that the Alx4 descendents contribute to the anterior limb including digits I-IV, parts of the wrist, and the radius. This data complements that of the Gli1::CreER that affects the ulna [56]. This suggests that the two bones, the ulna and radius, are generated from Shh responding and Shh independent cells respectively, however the Cre expression
in the Gardener Mouse is not exclusive to the Shh independent cells. Interestingly, we found that the kidney also showed cells positive for Cre activity/recombination. While renal expression of Alx4 had been detected in microarrays and via \textit{in situ} hybridization the specific cell types and structures that arise from Alx4 expressing cells have not been defined [68, 152]. This model will have multiple uses, for example monitoring cell fate decisions and changes in limb or kidney development in mutant backgrounds.

\textbf{Obstacles and Future directions:} Limb patterning is a unique event with a lot of asymmetrical gene expression. Due to the regional expression of Shh ligand there is a gradient of pathway across the limb. Subsequently, different genes that are activated at various concentrations of ligand are expressed in discrete regions of the limb [90]. Alx4 was identified and the promoter region required for reliable limb expression was determined. Importantly, other organ systems known to be effected/patterned by Alx4 expression did not show any labeling. For example, skin and hair follicle development, craniofacial patterning, mammary morphogenesis, and tooth development are all Alx4 dependent processes [95]. The promoter element described by Kuijper et al. and used here, however, only reproduces limb and renal expression.

When generating the Gardener Mouse, many steps of the process were simplified because we were able to obtain the $\beta$-gal reporter construct used by Kuijper et al and simply replaced the $\beta$-gal gene with CreER. We confirmed successful and appropriate expression of the allele in the limb, and generated a fate map of the Alx4 descendents throughout limb development. These initial descriptions will be important and informative when analyzed in mutant backgrounds. Many of the details of how the limb is pat-
terned has come from the perturbation of one gene and observing the effects on other genes. It is my hope that the Gardener Mouse will be employed in studies of limb patterning to either monitor cell fate decisions in a mutant context or to ablate genes in the anterior region of the limb.

We were pleasantly surprised to note renal expression of the Alx4::CreER transgene. While this promoter was reported to affect the kidney, there have been no studies of the role or requirement for Alx4 expression in the kidney. We noted expression all along the developing mesonephric tubule/duct suggesting that Alx4 has an early role in the induction or specification in the urogenital system. We noted that the developing nephrons in the kidney were Alx4 positive [152]. Unfortunately, we were not able to differentiate previously labeled, descendents, from current/actively expressing cells. That is, it is unclear whether every new cell born in a nephron expresses Alx4, whether Alx4 expression is sustained or transient, or when the gene is activated during nephrogenesis. In addition, due to the dual fluorescent coloring of our Cre reporter (red before deletion, green after) any fluorescent analysis has to be done in a third color. Lectins are used to identify cell types and tubular segments within the kidney, these lectins are sold pre-conjugated for use in fluorescent microscopy. Regrettably these lectins are only commercially produced in red and green variants. Thus, while this transgene is expressed in the kidney, we have no understanding of the cell types expressing the gene, we do not understand the functional requirement for Alx4 in the kidney, nor do we understand the dynamics of expression. These are simple enough questions that will provide great insight into what impact Alx4 has on renal function and development.
Chapter Four: Development of the Cilia\textsuperscript{GFP} mouse, a model for visualizing cilia in vivo

Review: In chapter four, we generated a mouse expressing a fluorescent ciliary protein for the assessment of primary cilia in vivo, ex vivo, and in processed tissue samples. We call this model the Cilia\textsuperscript{GFP} mouse. In addition to labeling cilia, this mouse will allow for real-time evaluation of cilia regression after Cre mediated recombination. The Cilia\textsuperscript{GFP} mouse, when used in ciliary loss studies like those described in chapter two, will be useful and important for drawing appropriate conclusions about the dynamics of cilia loss and the phenotypes and consequences of cilia loss in vivo. In addition, there are several other applications of in vivo labeled cilia, for example we noted that the cilia in the kidney tubules seemed to move/beat, something that had not been described before.

Obstacles and Future directions: Many models have been used to study primary cilia over the last 25 years. We have learned a lot about ciliogenesis, ciliary beating/motility, ciliary signaling, and the diseases caused by defects in these processes. Owing to their near ubiquity, and their involvement in mammalian signaling and development, ciliary defects in mice and man are often lethal [4, 9]. Thus, to date many studies of cilia are done in organisms that do not require cilia for viability, or whose cilia are easy to observe. Mammalian primary cilia are very difficult to visualize without tissue isolation and processing. To address this limitation to mammalian ciliary studies, we generated a mouse whose cilia can be fluorescently labeled in a ubiquitous or conditional manner.
It has become increasingly common to find certain transcription factors, signaling effectors, channels, and receptors that are specifically and exclusively localized to the primary cilium[14]. Indeed, ciliary targeting motifs and trafficking are both intensively studied today. We exploited one of these known ciliary targeted proteins, the Somatostatin receptor type 3 (Sstr3), and fused its cDNA to that of GFP. This fusion protein has been widely used in in vitro studies of cilia [113]. We decided to target this reporter allele to the Rosa26 locus, which is known for its ubiquitous expression at moderate levels [114, 115]. Our system also had a preceding floxed Neo cassette to inhibit Sstr3::GFP expression until Cre mediated recombination. With this Cilia\textsuperscript{GFP} mouse we were able to see cilia in nearly all tissues examined: the brain, kidney, eye, skin, pancreas, heart, and in fibroblasts derived from the tail. Importantly, these cilia could be seen without any processing or labeling. Interestingly, we noted fluorescent motile cilia on the ependymal cells in the brain, however neither the motile cells in the trachea nor oviduct appeared to localize the fusion protein to their cilia. The Sstr3::GFP seemed to be expressed in these cells, and was localized to the cilia in neighboring cell types, but for unknown reasons these two motile cilia types did not label.

Extending our studies beyond the generation and validation of the Cilia\textsuperscript{GFP} mouse model, we conducted real time imaging of the cilia in the kidneys of live mice through dorsal incisions. Using this paradigm we noted that when imaged at frame rates fast enough to detect movement; that the cilia in the tubules, throughout a field of nephrons were moving synchronously and appeared to be beating. This ciliary movement within the kidney had not been previously noted, and the cilia in the kidney are considered immotile primary cilia. These cilia lack the dynein motors and axonemal structures required
for motility. In addition to this, the beating of the cilia halted after death of the animal, implying that the beating was passive and regulated by the heart. It is still unclear how these cilia are moving there are several possibilities. For example, the synchrony and cadence initially suggested that the movement was caused by the heartbeat. This idea is still the most plausible, however seems physiologically unlikely due to the fact that in the mouse the blood supply to the glomeruli is via capillaries and the hydrostatic pressure is kept nearly constant in the glomerulus (60 mm Hg) by the juxtaglomerular cells. It is possible that in these animals the blood pressure is below 60 mm Hg and thus not restricted/ modulated by the juxtaglomerular cells and the pulsatile pressure of the atrial kick is enough to deflect the cilia. Importantly, we have measured the frequency of the ciliary beat and found it to be 4.58+/−0.20 Hz which converts to ~275 bpm which is quite low for a mouse’s heart rate. However, follow-up studies where we can measure the heart rate and blood pressure will help to determine if this is the case.

**Final Thoughts:** It is unfortunate that our original limb studies did not pan out as expected. While we could have done more to understand why the studies did not yield the expected phenotypes, it would not have changed the fact that our experimental paradigm had flawed. The Gardener Mouse was designed to be included in the limb studies. However, once we were certain that there was no patterning defects in the Shh::GFPCre, Gli1::CreER, nor in the Gardener Mouse (based on preliminary studies), our efforts had to be adjusted. While my studies did not result in powerful mechanistic insights, I have successfully generated two mouse models that will, hopefully, be widely used and result
in awesome scientific advances. Of that, and my whole body of failed, abandoned,
scooped, and tentative works; I am proud.


APPENDIX A

IACUC DOCUMENTATION
MEMORANDUM

DATE: October 15, 2012

TO: BRADLEY K YODER, Ph.D.
MCLM-688 0006
FAX: (205) 934-0950

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on October 15, 2012.

Title of Application: CORE: UAB Recessive Polycystic Kidney Disease Core Center (Dr. Lisa Guay-Woodford); RPKDCC CORE B: The Engineered Mouse Resource

Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).
MEMORANDUM

DATE: November 7, 2012

TO: BRADLEY K YODER, Ph.D.
    MCLM-688 0006
    FAX: (205) 934-0950

FROM:  

Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.

The following application was renewed by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on November 7, 2012.

Title of Application: Intraflagellar Transport Mediated Regulation of Hedgehog Signaling
Fund Source: NIH

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)
# Annual Renewal of Approved Protocols

## Certification Page

### 1. GENERAL INFORMATION
#### A. Principal Investigator:

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<td>Byoder</td>
<td>Mandy Croyle, <a href="mailto:byoder@uab.edu">byoder@uab.edu</a>, 934-0995, 934-0950</td>
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#### B. Approved Project:

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#### C. Will personnel other than the PI (e.g., faculty, staff, students, or fellows) be involved in the animal work being proposed this renewal period?

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<tr>
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<td>Wesley Lewis</td>
<td>Wrlewis</td>
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#### D. Will there be any changes in animal procedures from that described in the previous year’s approval?

If the response is YES, describe the changes. See instructions for changes not permitted on this form.

#### E. Certification:
APPENDIX B
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