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A transgenic Alx4-CreER mouse to analyze anterior limb and nephric duct development

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Abstract

Background: Genetic tools to study gene function and the fate of cells in the anterior limb bud are very limited.

Results: We describe a transgenic mouse line expressing CreER^{T2} from the Aristaless-like 4 (*Alx4*) promoter that induces recombination in the anterior limb. Cre induction at embryonic day 8.5 revealed that Alx4-CreER^{T2} labeled cells using the mTmG Cre reporter contributed to anterior digits I to III as well as the radius of the forelimb. Cre activity is expanded further along the AP axis in the hindlimb than in the forelimb resulting in some Cre reporter cells contributing to digit IV. Induction at later time points labeled cells that become progressively restricted to more anterior digits and proximal structures. Comparison of Cre expression from the Alx4 promoter transgene with endogenous Alx4 expression reveals Cre expression is slightly expanded posteriorly relative to the endogenous Alx4 expression. Using Alx4-CreER^{T2} to induce loss of intraflagellar transport 88 (*Ift88*), a gene required for ciliogenesis, hedgehog signaling, and limb patterning, did not cause overt skeletal malformations. However, the efficiency of deletion, time needed for Ift88 protein turnover, and for cilia to regress may hinder using this approach to analyze cilia in the limb.

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AUTHOR CONTRIBUTIONS

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Alx4-CreER^{T2} is also active in the mesonephros and nephric duct that contribute to the collecting tubules and ducts of the adult nephron. Embryonic activation of the Alx4-CreER^{T2} in the Ift88 conditional line results in cyst formation in the collecting tubules/ducts.

Conclusion: Overall, the Alx4-CreER^{T2} line will be a new tool to assess cell fates and analyze gene function in the anterior limb, mesonephros, and nephric duct.

Keywords

Aristaless-like 4; Cre^{ERT2}; fate mapping; limb bud development; mesonephros; ureteric bud

1 | INTRODUCTION

Morphogenesis of the limb bud has long been a model to understand the interactions of signaling pathways in three-dimensional space in vivo. The limb bud has proven useful since it is easily accessible, and perturbations in the developmental process result in readily identifiable phenotypes such as polydactyly, syndactyly, loss of digits, or short/malformed limbs. The limb is patterned by signals emanating from distinct organizing regions along multiple axes.[1,2] For example, the dorsal-ventral axis is established by Wnt signals from the dorsal ectoderm.[3] The proximal-distal axis is regulated by Fgf signals produced by the apical ectodermal ridge, a specialized group of cells that runs along the most apical boundary of the limb bud spanning from anterior to posterior.[4] Finally, the anterior-posterior (AP) axis of the limb bud is specified by sonic hedgehog (Shh) signaling. The morphogen Shh originates from a population of mesenchymal cells referred to as the zone of polarizing activity (ZPA) in the posterior side of the limb bud.[5] In mammals, precise spatial and temporal coordination of these signals give rise to the fully formed limb consisting of a stylopod (humerus/femur), zeugopod (radius-ulna/tibiafibula), and autopod with five digits numbered I to V from the most anterior (thumb) to posterior.[6]

Shh patterns the limb by establishing a gradient across the AP axis of the limb bud with the cells requiring Shh for their patterning in the posterior, and cells never receiving a Shh signal in the anterior.[7,8] Shh mediates these effects by regulating the expression and activity of the Gli transcription factors. Briefly, hedgehog (Hh) ligands bind to their receptor, patched, located in the primary cilium; this subsequently leads to the activation of the Gli1 and Gli2 transcription factors as well as inhibition of the Gli3 repressor in the posterior region of the limb.[9,10] The cilium is an important coordinator of hedgehog signal regulation, as the pathway components transiently localize to the cilia depending on Hh signaling activity. Central to their role as Hh pathway coordinators, both activation and repression of the pathway is disrupted when cilia are perturbed.[11–17]

The connection between cilia and limb morphogenesis was demonstrated in mice with mutations affecting intraflagellar transport (IFT). IFT mediates the bidirectional movement of large protein complexes along the ciliumaxoneme, and disruption of this process impairs cilia formation. A hypomorphic mutation in *Ift88* (*Ift88*^{Tg737Rpw}) with stunted cilia results in the duplication of the anterior most digit, while congenital null *Ift88* (*Ift88*$^{tm1.1Bky}$) mutants, lacking cilia, exhibit up to nine digits and typically do not survive past embryonic day 12.5 (E12.5).[18–20] To better evaluate Ift88 and cilia function in the limb, a conditional

Ift88 allele (*Ift88*^{tm1Bky}) was crossed with Prx1Cre.[14,21] Prx1Cre is active throughout the limb bud mesenchyme beginning at E9.0. The resulting mice had significantly shortened limbs, along with eight digits across the limb bud field.[14] The limb malformations in the *Ift88* mutants have been attributed to altered regulation of the Hh pathway. However, it remains uncertain as to whether cells on the anterior or posterior side of the limb contribute differentially to the polydactyly in these conditional mutants, and how that relates to Hh pathway activity.

Manipulation of the hedgehog pathway components, such as Shh and Gli1, specifically in the posterior portion of the limb bud has led to the generation of multiple important tools to study development and patterning of the posterior region of the limb bud. For example, mouse lines that express Cre from the Shh or Gli1 promoters have been used to analyze the fate of the descendants of the Shh expressing cells in the ZPA, or Gli1 expressing/Shh responsive cells, respectively.[7,8] The utilization of mouse Cre lines and Cre reporters such as the mTmG mouse ((Cg)-Gt(ROSA)26Sortm4$^{(ACTB-tdTomato,-EGFP)Luo}$/J) have proven to be useful approaches to study cell fate, to analyze how genetic mutations affect cell fates, and for assessing the consequence of disrupting genes involved in limb patterning. However, similar studies have not been conducted for cells in the anterior region of the limb, in large part due to the lack of appropriate genetic tools to alter gene expression in this region.

Here, we describe the generation of a transgenic mouse line expressing CreER^{T2} under the control of the mouse Aristaless-like 4 (*Alx4*) promoter. We analyze the activity of this inducible Cre line and show Alx4-CreER^{T2} is expressed in the anterior region of the forelimb (FL) bud and anterior ~2/3 of the hindlimb (HL) bud as well as in the nephric duct and mesonephros. In situ hybridization analysis indicates that endogenous Alx4 expression overlaps that of Cre expression from the transgene; however, Cre expression was frequently extended more posteriorly. This was particularly evident in the HL. Using this Cre line and the fluorescence-based mTmG Cre reporter,[22] we indelibly labeled Alx4-CreER^{T2} expressing cells in the limb, and determined their fates as well as the fate of the nephric duct derived *Alx4CreER^{T2}* expressing cells. We evaluated the new Cre line by deleting *Ift88* in *CreER^{T2}* expressing cells. While we observed no overt skeletal defects in the limb of *Alx4CreER^{T2}* conditional *Ift88* mutants, deletion of cilia from the *Alx4CreER^{T2}* expressing cells of the nephric duct leads to cyst development in the collecting duct. Importantly, this new Cre mouse line will be useful for analyzing the effects of temporal deletion of conditional alleles in these regions.

2 | RESULTS

The *Alx4* gene encodes a homeodomain containing transcription factor expressed in the mesenchyme of several developing tissues including skin appendages (hair, whiskers, teeth), craniofacial bones, mammary stromal cells, the nephric duct, and the anterior region of the limb bud.[23–26] While there are numerous studies analyzing signaling coordination from the developing posterior region of the limb bud, the role of the cells in the anterior region of the limb in this process is less understood. This is in part due to limited availability of genetic tools to evaluate gene function and to conduct fate mapping studies that are specific to the anterior region of the limb bud. To address this limitation, we developed a

transgenic mouse line that uses the mouse *Alx4* promoter to drive expression of a tamoxifen (TM)-inducible Cre recombinase (hereafter referred to as Alx4-CreER^{T2}) in the anterior region of the developing limb bud.

To characterize the expression pattern and Cre activity of the Alx4-CreER^{T2} transgene, we utilized a Cre reporter line that expresses membrane associated GFP (mGFP) after Cre mediated deletion of a ubiquitously expressed membrane associated Tomato fluorescent protein (mTomato). This reporter is hereafter referred to as mTmG.[22] The temporal-spatial activity of Alx4-CreER^{T2} was analyzed by injection of TM into pregnant mice at different gestational stages and the embryos were isolated 24 hours after injection (Figure 1A, 24 hours post TI, first two columns).

Cre reporter-associated mGFP activity was not observed in embryos isolated 24 hours after an E8.5 TM induction (Figure 1A, E8.5, 24 hours post TI). This is consistent with the known onset of *Alx4* expression between E9.0 and E9.5. Induction of Cre at E9.5 showed mGFP expression in the anterior region of both the FL and HL (Figure 1A, E9.5, second column) within 24 hours of TM injection. At subsequent Cre induction time points (E10.5, E11.5), larger domains of mGFP reporter were observed throughout the anterior portion of the limbs (Figure 1A second column, FL and HL). The HL has a broader domain of expression, with mGFP found across nearly half to 2/3^{rds} of the HL bud at E10.5 (Figure 1A, E10.5, second column). Analysis of embryos induced at E12.5 and analyzed 24 hours later resulted in sporadic mGFP reporter expression located mainly in the proximal anterior side of limb, consistent with a reduction in *Alx4* expression in the autopod at this time point (Figure 1A, E12.5, second column). Few labeled cells were evident in the limbs when CreER^{T2} was induced at E13.5 or later (data not shown).

Using Alx4-CreER^{T2}, we also analyzed the fate of anterior cells labeled in the limb using the mTmG reporter. Based on the in situ hybridization data, it must be noted that we are not assessing the fate of Alx4 cell progeny, but rather cells of the anterior limb bud that express the Alx4CreER^{T2} transgene. Cre was induced at the same developmental time points as above but embryos were not analyzed until E16.5. Although the E8.5 Cre induced embryos did not show CreER^{T2} activity when analyzed 24 hours post TM induction, the cell fate analysis at E16.5 revealed mGFP positive cells in the limb (Figure 1A, E16.5, columns 3–6). The pharmacokinetics of TM in the early embryo is not certain, but data from Hayashi et al indicate it can remain active for at least 24 hours.[27] Thus, when the Alx4-CreER^{T2} transgene was initially expressed at around E9.0, Cre was activated. Early injection of TM at E8.5 to 10.5 resulted in Cre reporter-labeled cells in the most anterior parts of the limb at E16.5, digits I to III, and to some regions of digit IV (Figure 1A, E16.5) in the hind limb. This likely reflects the broader domain of CreER^{T2} expression observed in the HL at the early time points relative to the FL. Analysis of the limbs where Cre was induced at the later time points showed a range of mGFP contribution encompassing digit I and II and portions of digit III, but this became progressively more anteriorly restricted at later time points. Further, the cells labeled at this late time point are located more proximally than at the other time points. The mGFP label was present in many cell types in the limb including muscle and connective tissues, chondrocytes, and the perichondrium (Figure 1B,C).

We compared endogenous Alx4 expression with CreER^{T2} RNA expression from the transgene using whole mount in situ hybridization (Figure 1D) and RNA scope (Figure 1E). These data indicate significant overlap in the domains of expression in cells on the anterior limb buds in both FL and HL. In agreement with the mTmG data (Figure 1A), expression of Cre typically extended more posteriorly on the limb buds than did the endogenous Alx4 expression.

To further evaluate the Alx4-CreER^{T2} mouse, we crossed it with the *Ift88*$^{tm1Bky/J}$ conditional mutant line. Based on PCR genotyping, we can detect deletion in cells dissected from the anterior but not from the posterior limb bud cells or in the abdomen where Alx4CreER^{T2} is not expressed (Figure 2A). However, deletion in the anterior limb bud of *Ift88* was not complete using the Alx4-CreER^{T2} line or alternatively there is contamination with posterior derived cells during the dissection process. We further evaluated deletion efficiency by PCR genotyping of flow cytometry isolated GFP+ cells from E8.5 induced Ift88 conditional Alx4CreER^{T2} embryonic limb buds at E13.5 (Figure 2B). We note significant variability between embryos with several (eg, embryos 1 and 4) having near complete deletion, while others have less than half deletion (embryo 3) based on the intensity of the PCR products. The reason for the variability with Ift88 is uncertain but could reflect strength of the Cre in combination with the Ift88 floxed allele being more difficult to delete relative to the mTmG Cre reporter allele.

Since IFT88 is required for cilia assembly and maintenance,[28,29] we evaluated the impact of Cre induction on the presence of cilia as identified by acetylated α-tubulin immunofluorescence microscopy in Alx4-CreER^{T2}; *Ift88* samples compared to controls induced at E8.5 and isolated E16.5 (Figure 2D). This was done on cells in the anterior vs posterior side of the limb bud on an mTmG reporter background. Qualitative assessment revealed there was a reduction, but not absence, of cilia on mGFP+ cells from the anterior of the limb in *Ift88* floxed mice compared to mGFP + cells isolated from the normal control. There were no overt differences in presence of cilia on the posterior cells from the mutant or control where Cre is not expressed.

Unexpectedly, while we detect deletion of the *ift88* floxed allele in the Alx4-CreER^{T2};*Ift88* embryos, we did not observe any overt skeletal or limb patterning defects in any of the mutant embryos we analyzed (Figure 2C). Based on the variability in Ift88 deletion, cilia loss, and the presence of no overt limb defects, it is recommended that studies focused on analyzing gene function or phenotypes using the Alx4-CRER^{T2} mouse be evaluated using a mouse line where the floxed allele is balanced over a null allele such that only a single recombination event is required.

Consistent with the observations made by Kuijper et al using an 17 kb region of the *Alx4* promoter to drive expression of LacZ expression in the mouse, we also observed Cre reporter mGFP signal in the developing nephric duct and mesonephric tubules upon TM induction at E8.5 and analysis at E11.5 (Figure 3A, left panel).[26] Sectioning of the embryos confirmed mGFP expression in the epithelial cells of the nephric duct (Figure 3A, right panel). To analyze the contribution of these Alx4-CreER^{T2} expressing descendants to the kidney, we evaluated E16.5 kidneys from embryos that were TM induced at

E11.5 (Figure 3B). Both whole mount and sections revealed mGFP positive cells in the epithelium of renal tubules (Figure 3B). To determine the nephron segments to which the Alx4-CreER^{T2} positive cells contributed, we assessed their fate using the proximal tubule marker Aquaporin-1 (AQP1) and collecting duct marker Aquaporin-2 (AQP2) staining. Cre activity was induced at E11.5 and embryonic kidneys were assessed on E17.5. Consistent with the Cre expression observed in the nephric duct, we observed AQP2 and mGFP double positive tubules, but were unable to find AQP1 and mGFP double positive proximal tubule cells (Figure 3C). These data indicate that the Alx4-CreER^{T2} expressing cells from the nephric duct contribute to the collecting ducts/tubules that are derived from the nephric duct.

In the kidney, *Ift88* deletion and cilia loss results in cyst formation.[30] Cell fate mapping indicated that Alx4-CreER^{T2} is active in the nephric duct and that these cells contribute to the collecting duct/tubules of the kidney when Alx4-CreER^{T2} was induced at E8.5. Thus, we evaluated whether deletion of *Ift88* in embryos using Alx4-CreER^{T2} was able to cause renal phenotypes in postnatal mice. Large cysts were evident in the kidneys at postnatal day 35 (P35). As observed with the collecting duct fate mapping with an E11.5 induction, the cysts formed specifically in the AQP2 positive collecting tubules; however, there are mGFP tubules that do not become cystic (Figure 4A,B). The cysts lacked cilia as determined by co-staining with an acetylated α-tubulin antibody. In contrast, noncystic but mGFP+ tubules maintain their cilia indicating that while the Cre reporter was activated, Ift88 was likely not deleted in those cells (Figure 4C), again indicating that phenotype analysis with this Cre line would benefit from balancing the floxed allele over a germline null mutation.

3 | DISCUSSION

The use of genetic tools such as Cre lines to analyze gene function in limb morphogenesis or to perform fate mapping has provided the foundation for understanding not just limb patterning but broader themes in development and signal transduction. Several Cre lines are currently available for studies of the posterior side of the limb bud.[7,8] This includes cell fate analysis of Shh expressing (Shh-CreER^{T2}) or hedgehog responding (Gli1-CreER^{T2}) cells useful to define their respective contributions to digits of the autopod or to evaluate how these fates change in context of mutant backgrounds. This type of analysis is currently not possible for the anterior side of the limb as the necessary Cre lines are not yet available. To our knowledge, the Alx4-CreER^{T2} line described here is the first such resource to specifically study the anterior side of the limb bud.

We demonstrate that Alx4-CreER^{T2} can induce expression of a Cre reporter in cells of the developing anterior limb bud from ~E9.5 to E12.5. We used Alx4-CreER^{T2} to conduct fate mapping of the anterior cells at progressive gestational time points. These data reveal that tagged cells can contribute to digits I to III of the FL and I to IV of the HL. These fates become increasingly more restricted to anterior proximal structures as gestation progresses. Caution must be taken in interpretation of these data to indicate the fate of Alx4 expressing cells as in situ hybridization data indicate that Cre expression from the transgene extends more posteriorly than does endogenous Alx4 expression. Our data do agree with previous analysis of the Alx4 promoter driving LacZ expression.[26]

Although induction of Cre at E8.5 did not label any cells by 24 hours (E9.5) when Alx4 expression initiates, labeled cells were present when induction occurred at E8.5 and embryos were isolated and analyzed at E16.5. These data may demonstrate one limitation of temporal control of Cre induction during development. The half-life of TM with regards to Cre activation is not well defined. Once administered, TM may continue to activate Cre for at least 24 hours afterward.[27] This temporal factor of induction must be considered in interpreting the fate mapping data. It would be beneficial to include a fluorescent tag directly on Cre to distinguish between cells that are actively expressing Cre vs cells in which Cre activity has subsided but are still expressing the Cre reporter.

The fates of posterior cells in the limb bud and the impact of genetic mutations on these fates have been evaluated using tools such as the Gli1CreER^{T2} and ShhCreER^{T2}. For example, Ahn and Joyner utilize Gli1CreER^{T2} to ascertain changes in the fate/distribution of hedgehog responding cells resulting from the loss of Shh, Gli2, or Gli3.[8] These mutants disrupt induction of the hedgehog pathway from the ZPA and transcriptional activator and repressor of the pathway, respectively, and alter the distribution and fate of these cells within the forming limb. The Alx4-CreER^{T2} line now opens the opportunity for such studies targeting the anterior cell populations.

Primary cilia have critical roles in regulating the hedgehog pathway. Disruption of genes required for ciliogenesis, such as *Ift88*, throughout the limb bud cause extensive polydactyly similar to Gli3 mutants.[18–20] In *Ift88* mutants, the proteolytic processing of Gli3 to its repressor does not occur efficiently nor can the Gli2 transcription factor be activated.[15,31] Thus, the hedgehog pathway can neither be turned fully on or fully off. Our goal was to ascertain how much of the polydactyly phenotype associated with cilia dysfunction was derived from cells in the anterior limb bud. Surprisingly, there were no overt limb phenotypes in *Ift88* conditional mutants using the Alx4-Cre^{ERT2} lines when induced at E8.5. One possible interpretation of this outcome is that the polydactyly observed in *Ift88* mutants, which lack cilia throughout the limb bud, arises from signaling defects in the posterior cells in agreement with where Hh signaling is known to be important.[14,18] However, based on both PCR genotyping of specific regions of the limb bud and cilia staining, deletion of *Ift88* was not complete and the possibility exists that it is not enough to induce polydactyly. Another possibility is that the Ift88 protein and the subsequent regression of the cilium occur too slowly once the floxed allele is deleted in this inducible model, therefore failing to produce the skeletal defects observed in other mice with *Ift88* deletions. Studies using a flox/null approach where only a single deletion is required may help to address this issue and is highly recommended when using the Alx4CreER^{T2} line.

During characterization of the Alx4CreER^{T2} line, we also noted Cre reporter activity in the nephric duct. Fate mapping revealed that these cells contribute to the collecting duct and tubules that are derived from the ureteric bud. When analyzed in the context of the *Ift88* conditional allele, these tubules frequently lack cilia and become cystic. Thus, in the kidney the Alx4CreER^{T2} line does cause the predicted phenotype resulting from the loss of cilia function.

Alx4 expression has also been reported in the hair follicle[32]; however, this Alx4-CreER^{T2} allele did not show any pilosebaceous expression in whiskers where endogenous gene expression has been found (data not shown). This is also consistent with the work from Kuijper et al that utilized the same promoter region to drive LacZ expression in a mouse line.[26] This lack of expression suggests that the promoter elements required for follicle expression are not within the 17 kb promoter region used in our transgene allele.

In conclusion, the new Alx4-CreER^{T2} mouse line described here results in Cre reporter activity in the developing anterior limb bud, mesonephros, and nephric duct. We demonstrate that Alx4-CreER^{T2} labels cells that become progressively more restricted to the anterior side of the limb contributing to the anterior most digits. In the kidney, Alx4-CreER^{T2} labels cells that are restricted to the collecting duct and tubules. Thus, this new Cre line will be useful for assessing the consequence of conditional gene deletion within these cells as well as to analyze changes in cell fates, location, and migrations in mutant backgrounds.

4 | EXPERIMENTAL PROCEDURES

4.1 | Generation of the Alx4-CreER^{T2} transgenic line

To generate the Alx4-CreER^{T2} construct, we utilized a 17 kb genomic fragment previously characterized by Kuijper et al and shown to recapitulate many of the expression domains of the endogenous Alx4 gene.[26] We replaced the LacZ reporter with a CreER^{T2} cDNA fusing Cre at codon 13 located within exon 2 of the Alx4 gene. The final construct was sequence verified and the vector backbone was excised by an XhoI/BfuI digest. The gel purified fragment was used for pronuclear injection into C57BL/6J zygotes (UAB Transgenic and Genetically Engineered Models Core). Genomic PCR was used to identify founder lines carrying the transgene (Alx4-CreER^{T2} primers: forward 5′-ctcaaggccagctgtaggga-3′ in the *Alx4* promoter and reverse 5′-cgcgcggctccgacacgggcactg-3′ within the Cre open reading frame).

All animals were maintained in an AAALAC accredited facility in accordance with IACUC regulations at the University of Alabama at Birmingham (animal protocol #09276). In accordance with our NIH resource sharing plan, these transgenic mice will be made available to the research community upon request.

4.2 | Inducible Cre TM Induction

Alx4-CreER^{T2} mice were crossed to the Gt(ROSA) 26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$ (mTmG) Cre reporter line (stock number 007676, Jackson Laboratories, Bar Harbor, Maine) for analysis of Cre activity, and to the *Ift88*^{tm1Bky} (*Ift88*^{F}, *Ift88tm1*$^{Bky/J}$, stock number 022409, Jackson Laboratories) mice to assess the efficacy in deletion of conditional alleles as well as analyses of cilia loss in the early kidney and in the anterior limb bud. Mice for 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 Cre induction, TM (cat # T5648 Sigma-Aldrich, St. Louis, Missouri) was dissolved in corn oil at 20 mg/mL, and a single 6 mg dose was given to pregnant females via IP injection at the times indicated.

4.3 | Whole mount in situ hybridization

Embryos were isolated at E9.5 to E11.5 and processed for in situ hybridization using standard protocols.[33] Antisense probes for Cre and Alx4 were generated by addition of a T7 promoter sequence included in the reverse primer. Sequences were amplified by standard PCR and transcribed using T7 RNA polymerase and the DIG RNA Labeling kit according to manufacturer's instructions (Millipore Sigma, St. Louis, Missouri). Primer sequences: Alx4 forward 5′-cgcggggatcctttaacaagttcc-3′; Alx4 reverse (with T7 promoter sequence) 5′-ggatccTAATACGACTCACTATAggg aga tgatgccagctgctccaaacag-3′; Cre forward 5′-ccgtttgccggtcgtgggcggcatgg-3′; Cre reverse (with T7 promoter sequence) 5′-ggatccTAATACGACTCACTATAgggaga cgc gcggctccgacacgggcactg-3′.

4.4 | RNA scope analysis

Limb buds from embryos at E9.5 to E11.5 were fixed and sectioned. Slides were post-fixed overnight with 4% para-formaldehyde at 4°C. Detection of transcripts in limb bud sections was performed using the RNA scope Fluorescent Multiplex Reagent Kit (Cat. No. 320850, ACD, Newark, California). Tissues were pretreated according to technical note 320535-TN. Probe hybridization, counterstaining, and mounting of the limb bud slides were performed following user manual number320850-USM. Slides were assayed using probes for Cre (Cat. No. 474001-C3) and Alx4 (Cat. No. 574271-C2) prepared in probe diluent (Cat. No. 300041). Positive (PPIB-C2/UBC-C3, Cat. No. 320881) and negative (dapB; Cat. No. 320871) control probes were run with each experiment. Color module option Amp4 Alt A-FL (Atto 647 for C2 and Alexa 488 for C3) was chosen for probe identification. Slides were counterstained with DAPI and then mounted using Prolong Gold AntifadeMountant (Cat. No. P36930, Thermo Fisher Scientific, Waltham, Massachusetts).

4.5 | Flow sorting of GFP+ cells and genotyping

Embryos were isolated at E13.5 after induction at E8.5. Both left and right FL and HL were isolated and pooled together for each embryo for processing. Limbs were minced and placed in 0.25% Trypsin, rocked at 37°C for 5 minutes and mixed via pipetting with DMEMF-12 containing 10% FBS. Dissociated limbs were put through a 70 μm strainer, spun at 220*g* for 5 minutes at 4°C, and were resuspended in autoMACS buffer (cat# 130-091-222 Miltenyi Biotec, Auburn, California) with 1% bovine serum albumin (BSA). FACS was done on a BD FACSMelody Cell Sorter using an RFP only control to set the gates to separate GFP+ cells from the remaining cells. Genomic DNA was isolated, and PCR was performed to check for deletion of IFT88.

4.6 | Tissue and embryo preparation

For analysis in sections for immunofluorescence and in situ, embryos and tissues were fixed in 4% PFA overnight at 4°C, rinsed in PBS, and cryoprotected in 30% sucrose/PBS at 4°C until samples sank. Samples were embedded in OCT (#14-373-65 Thermo-Fisher Scientific), frozen in a dry ice ethanol bath, and stored at −80°C until processed. Cryosections 12 μm thick were generated, fixed for 10 minutes in 4% PFA at room temperature, and washed for 5 minutes in PBS at room temperature to remove OCT. Cover

slips were mounted with Immumount (9990402 Thermo-Fisher Scientific) or DABCO (cat# D2522 Sigma Aldrich).

4.7 | Microscopy

Imaging of fluorescent mTmG Cre reporter in embryos was performed immediately after isolation or within 24 hours of isolation after having been stored in 4% PFA at 4°C. Wholemount limbs and sections were imaged on a Ziess Lumar v12 fluorescence stereomicroscope equipped with an Axiocam MRm camera using Axio-Vision software or a Leica MZ16FA microscope equipped with a Retiga 1300 camera using MetaMorph software. Kidney and limb confocal images were captured using a PerkinElmer ERS 6FE spinning disk microscope utilizing Volocity version 6.1.1 software for image analysis (PerkinElmer, Shelton, Connecticut). RNA Scope images were acquired using a Leica SP8 confocal using ×60 objective (NA = 1.4).

4.8 | Immunofluorescence

Kidneys and embryos were fixed in 4% PFA overnight at 4°C and cryoprotected in 30% sucrose/PBS until tissue sank. Samples were embedded in OCT, frozen in a dry ice ethanol bath, and stored at −80°C until processed. Cryosections 12 μm thick were generated, fixed for 10 minutes in 4% PFA, permeabilized in 0.2% Triton X-100 for 8 minutes at room temperature, and blocked in 0.1% Triton X-100, 1% donkey serum, 0.01% sodium azide, and 1% BSA in PBS for 30 minutes at room temperature. Primary antibody incubations were performed overnight at 4°C and secondary antibody incubations performed for 1 hour at room temperature. Primary antibodies were rabbit anti-aquaporin-1 (AQP1) (1:100, cat# AQP11-A, Alpha Diagnostic International), goat anti-aquaporin-2 (AQP2) (1:100, cat# sc-9882, Santa Cruz Biotechnology Inc., Santa Cruz, California), or Alex Fluor 647 direct conjugated to (cat# A20186 Thermo-Fisher Scientific) anti-α-acetylated tubulin (1:3000, cat# T7451, Sigma-Aldrich). Secondary antibodies were Alexa Fluor 647 conjugated donkey anti-rabbit and anti-goat IgG (cat# A21244 and cat# A21447 Thermo-Fisher Scientific). Nuclei were visualized by Hoechst nuclear stain (cat#33342 Sigma Aldrich). Sections were cover slipped and mounted with DABCO.

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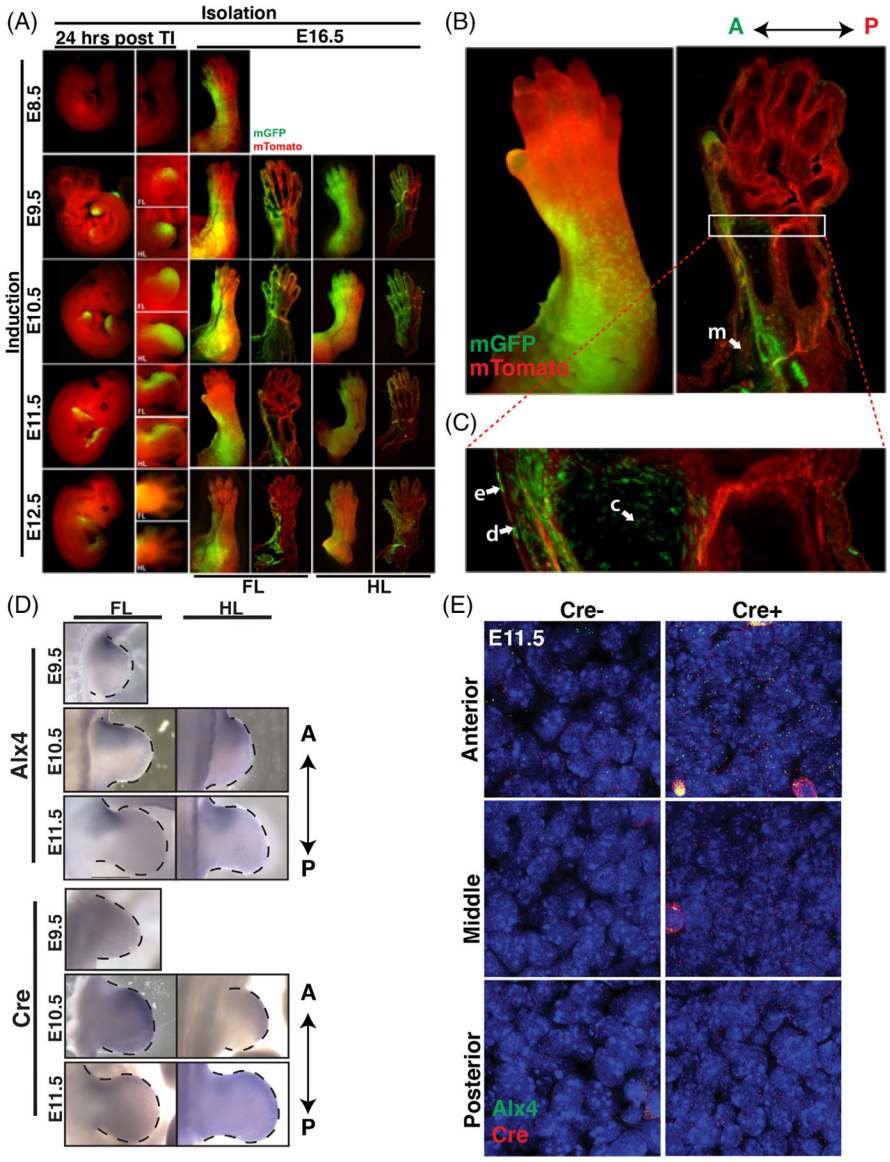

FIGURE 1.

Alx4-CreER^{T2} activity and expression in the developing limb. A, Alx4-CreER^{T2} fluorescent mTmG Cre reporter analysis of wholemount forelimb (FL) and hindlimb (HL) 24 hours post-tamoxifen induction (TI), and in whole mount and sections of the limbs at E16.5. Tamoxifen induction occurred at E8.5, E9.5, E10.5, E11.5, and E12.5. Cre reporter is membrane GFP (mGFP, green) while the nonrecombined (Cre-) cells remain membrane tomato (red). Anterior is up in whole mount embryos, and to the left in the isolated limbs and sections. B, mTmG Cre reporter analysis of limbs of E16.5 embryos induced at E11.5, whole mount (left) and section of forelimbs (right). C, Inset of the boxed region in panel B. Arrows indicate Alx4-CreER^{T2} descendants in the (m) muscle, (e) epidermis, (d) dermis, and (c) chondrocytes. Arrow in top right corner indicates the anterior (A) and posterior (P) orientation of limbs throughout the figure. D, Whole mount RNA in situ hybridization analysis of Alx4 and CreERT2 expression was performed on FL and HL buds isolated from

wild type and Alx4-CreERT2 (Cre) transgenic embryos isolated at E9.5 through E11.5. Data indicate overlapping domains of expression on the anterior side of the limb, but Cre expression typically extended further posteriorly and distally than observed for endogenous Alx4. Limb buds are oriented such that the anterior side of the limb is located on top. E, RNA scope was conducted on sections of the forelimb from E11.5 embryos using probes generated for Alx4 (green) and Cre (red). As observed for whole mount in situ hybridization, Alx4 and Cre is greatly enriched on the anterior side of the limb bud, but Cre probe is detected in some cells in the medial region of the limb bud

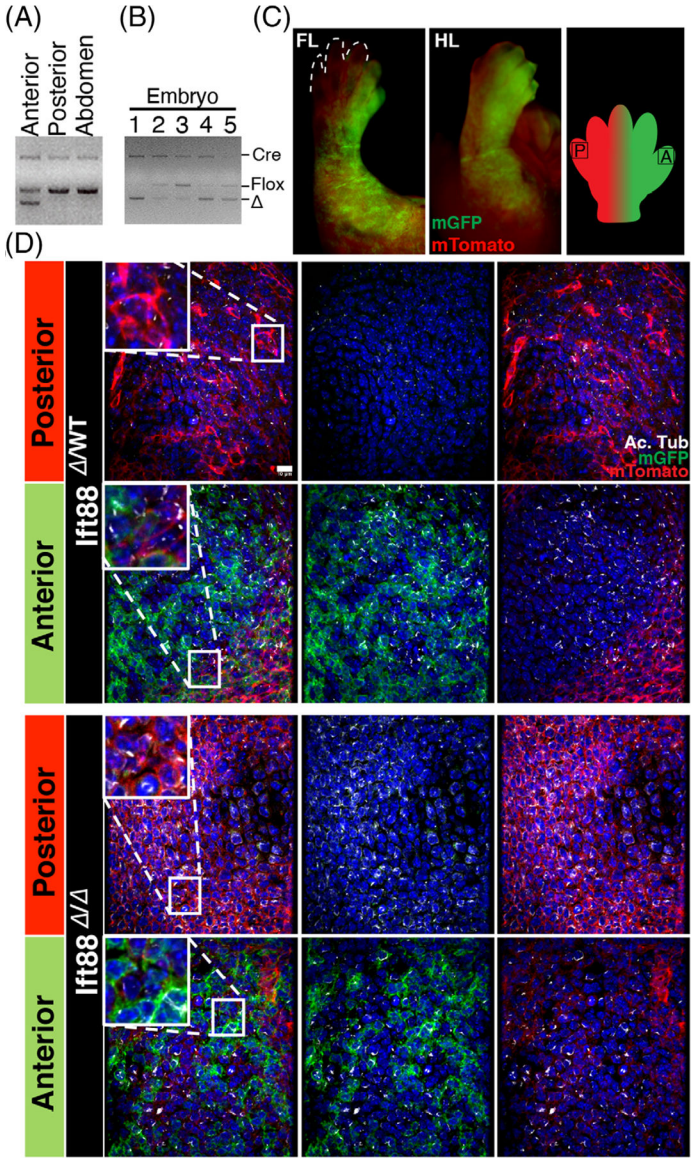

FIGURE 2.

Analysis of IFT88 deletion in the limb bud using Alx4-CreER^{T2}. PCR genotype analysis of tissue isolated from Alx4-CreER^{T2};*Ift88*$^{F/F}$ E13.5 embryos following tamoxifen injection at E8.5. A, Mechanically separated anterior limb bud (Anterior), posterior limb bud (posterior) and a control region in the abdomen (Abdomen), and, B, GFP positive cells isolated from five separate embryos via FACS. The PCR bands for Cre, *Ift88* conditional (Flox), wild-type (WT), and induced mutant (Δ) bands are indicated. C, Analysis of whole mount limbs from Alx4-CreER^{T2};*Ift88*$^{F/F}$ embryos induced at E8.5, isolated at E16.5, showed no overt phenotypes. A depiction of the anterior (A) and posterior (P) regions of the limb bud is shown with black boxes indicating the location of images shown in panel d. D, Immunofluorescence staining of cilia using acetylated α-tubulin (Ac. Tub, white) in the anterior and posterior limb buds of *Ift88*$^{\Delta/WT}$ and Ift88$^{F/F}$ (*Ift88*$^{\Delta/\Delta}$ after Cre excision)

embryos induced at E8.5 and isolated at E16.5. The mTmG Cre reporter shown with mGFP reporter (green) and mTomato (red). Scale bar 10 μm. Hoechst stained nuclei blue

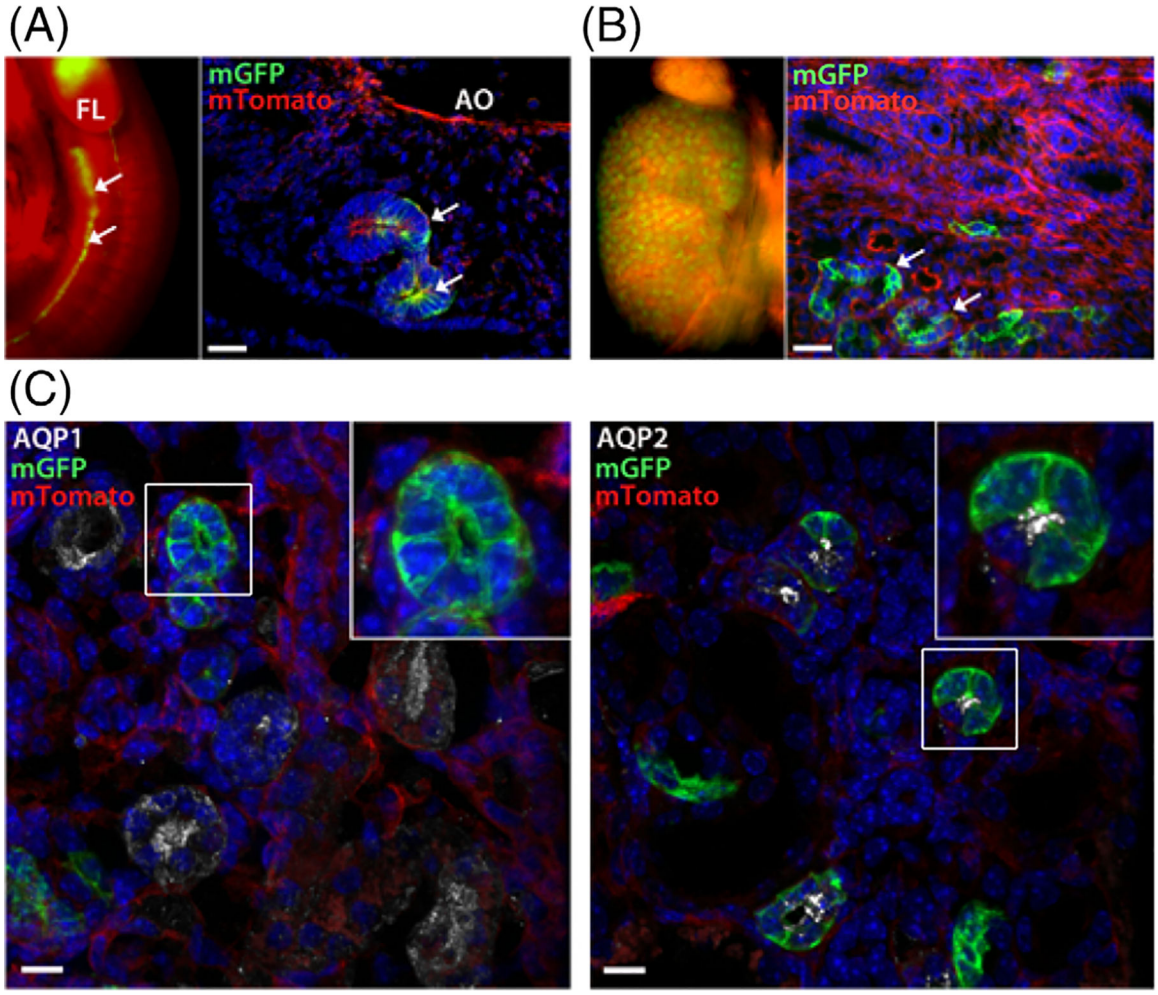

FIGURE 3.

Alx4-CreER^{T2} activity in the embryonic kidney. (A, Left panel) mTmG Cre reporter expression in an E11.5 whole mount embryo after tamoxifen induction at E8.5. Cre activity was evident in the mesonephric tubules and nephric duct (arrows) and in the forelimb (FL). (A, Right panel) A transverse section below the forelimb shows Cre reporter (membrane associated GFP [mGFP], green) labeled epithelial cells in the mesonephric duct next to the aorta (AO). Scale bar 30 μM. B, Cre reporter analysis at E16.5 following tamoxifen induction at E11.5 in a (left) whole mount kidney and (right) a section showing Cre reporter membrane GFP (mGFP, green) in tubule epithelium (arrows). Scale bar 30 μm. C, Kidney sections from E17.5 embryos after tamoxifen induction at E8.5 show tubules expressing Cre reporter (mGFP, green) that are not positive for the proximal tubule marker Aquaporin-1 (AQP1, white, left) but are positive for the collecting duct marker Aquaporin-2 (AQP2, white, right). Membrane tomato (mTomato, red) indicates Cre negative cells. Scale bars 10 μm. All Hoechst stained nuclei (blue)

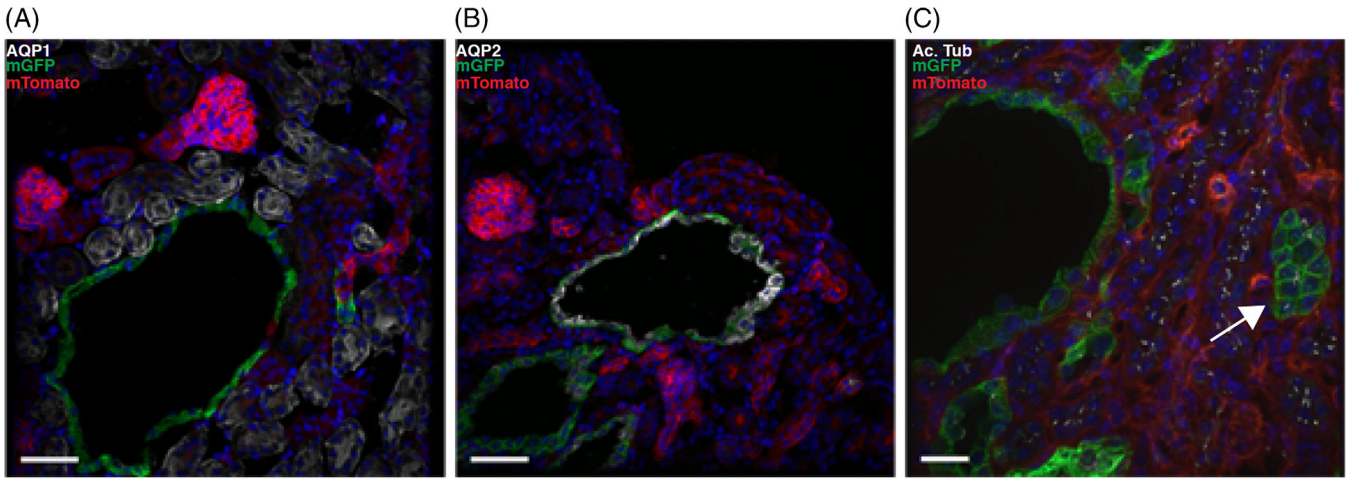

FIGURE 4.

Effect of Alx4-CreER^{T2} mediated cilia loss during development on the adult kidney. Kidney analysis at postnatal day 35 after Cre induction at E8.5. All renal cysts were Cre reporter (membrane associated GFP [mGFP], green) positive. A, Cystic epithelia were not labeled with a proximal tubule marker (AQP1, white) but were labeled with the, B, collecting duct marker (AQP2, white). Scale bars 50 μm. C, Acetylated α-tubulin stained positive cilia (Ac. Tub., white) were present throughout the section but absent specifically from the Cre positive epithelial cells lining cysts (mGFP, green). Scale bar 20 μm. All Hoechst stained nuclei blue. Arrow indicates noncystic but mGFP+ tubule where the Cre reporter was activated but *Ift88* was likely not deleted as cilia are detected on these cells using acetylated α-tubulin antibody