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Generation and Characterization of Tamoxifen-Inducible *Pax9-CreER* Knock-In Mice using CrispR/Cas9

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SUMMARY

Pax9 encodes a paired-box homeodomain transcription factor and is critical for the development of multiple organs. Using CrispR/Cas9-mediated homologous directed repair (HDR), we generated a new *Pax9-CreER* knock-in mouse line in which the CreER^{T2} fusion protein is produced after synthesis of endogenous Pax9 protein. We found that tdTomato reporter expression in *Pax9-CreER;tdTomato* reporter mice is detectable in a similar pattern to the endogenous *Pax9* expression, faithfully recapitulating the *Pax9* expression domains throughout the embryo and in the adult mouse. At early embryonic stages, the tdTomato reporter is expressed first in the pharyngeal pouch region and later in the craniofacial mesenchyme, somites, limbs, and lingual papillae in the adult tongue. These results demonstrate that this new *Pax9-CreER* knock-in mouse line can be used for lineage tracing and genetic targeting of *Pax9*-expressing cells and their progeny in a temporally and spatially controlled manner during development and organogenesis.

Keywords

Pax9; *Pax9-CreER*; genetic labeling; mouse development

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INTRODUCTION

Pax9 belongs to a family of highly conserved genes that encode paired-box homeodomain (Pax) transcription factors and is expressed in a wide variety of mouse embryonic tissues during development. Expression of *Pax9* mRNA is first detectable in the pharyngeal pouch around embryonic day E9.0 (Peters *et al.*, 1998) and, subsequently, in the derivatives of the pharyngeal pouch, sclerotome, and limb buds (Neubüser *et al.*, 1995; Peters *et al.*, 1998). In addition, Pax9 is also expressed in the craniofacial mesenchyme, including the nasal processes and mandibular and maxillary arch (Peters *et al.*, 1998). Pax9 expression also marks sites of prospective tooth development before morphological signs of odontogenesis appear, and is maintained in the developing embryonic tooth mesenchyme (Neubüser *et al.*, 1997; Peters *et al.*, 1998).

In Pax9 null mutant mice ($Pax9^{LacZ/LacZ}$), not all the structures expressing Pax9 are affected, suggesting that Pax9 is not indispensible for development of all *Pax9+* tissues. These Pax9 knock-out mice showed defects in derivatives of the third and fourth pharyngeal pouches, such as the thymus and parathyroid glands (Peters *et al.*, 1998). Although the elbows, knees and tails of $Pax9^{LacZ/LacZ}$ knock-out mice appeared unaffected, they did exhibit abnormal digit formation and preaxial digit duplication of the fore- and hindlimbs (Peters *et al.*, 1998). Pax9 is also critical for the development of craniofacial mesenchymal tissues, particularly those that are derived from the cranial neural crest. Both Pax9 null and Pax9 conditional knock-out ($Wnt1\text{-}Cre;Pax9^{fl/fl}$) mice, in which Pax9 is only deleted in neural crest derivatives, exhibit cleft secondary palate with absent palatal processes of the premaxilla. In the mandible region, alveolar bone and coronoid process formation was also impaired. Furthermore, tooth development is arrested at the bud stage (Kist *et al.*, 2007; Peters *et al.*, 1998).

Interestingly, although Pax9 is not expressed in the skin, it is expressed in the epithelium of the tongue at late embryonic stages and in adults (Jonker *et al.*, 2004). Moreover, this Pax9 expression is required for maintaining the morphology and permeability of tongue epithelial cells and may also prevent these cells from adopting a skin-specific epithelial fate (Jonker *et al.*, 2004). In adults, Pax9 expression is also detectable in epithelial tissues of the digestive system, such as the esophagus (Jonker *et al.*, 2004; Peters *et al.*, 1997). Although the function of Pax9 in later stages remains unclear, previous studies have suggested a role in malignancy and cell survival of cancerous epithelium (Gerber *et al.*, 2002; Lee *et al.*, 2008).

Despite the critical requirement for Pax9 during embryonic development, the specific contribution of the *Pax9+* subpopulation remains unclear. Moreover, the molecular regulation associated with maintaining distinct cellular identities in *Pax9+* cells remains unknown. CrispR/Cas9-mediated gene knock-out/knock-in methods have recently emerged as useful tools for the efficient generation of transgenic mouse models (Wang *et al.*, 2013; Yang *et al.*, 2013). Using the CrispR/Cas9-mediated homologous directed repair (HDR) mechanism, we generated a new *Pax9-CreER* knock-in allele allowing the production of the tamoxifen-inducible CreERT2 recombinase (Feil *et al.*, 1997) in cells normally expressing Pax9. Therefore, Pax9-driven Cre-mediated recombination can be used to leave indelible genetic markers that identify *Pax9+* cell lineages during embryonic developmental stages as

well as in adults. These mice will help identify the molecular mechanisms regulating *Pax9+* cells. Here, we describe this new allele and its utility for lineage tracing and genetic targeting of Pax9 expressing cells and their progeny during embryonic and postnatal stages.

RESULTS AND DISCUSSION

The mouse *Pax9* gene is located on Chromosome 12:56,691,767-56,712,822. It consists of 5 exons interrupted by 4 introns (Ensemble, ENSMUSG00000001497). In order to create an allele in which CreER^{T2} expression recapitulates endogenous Pax9 expression without interfering with Pax9 function, we integrated the 2A-CreER^{T2} coding sequence into the last exon of *Pax9*, immediately before the stop codon (Fig. 1a). Following sgRNA-mediated double strand DNA breakage by Cas9 enzyme, the homology arms guide the 2A-CreER to insert in-frame with the *Pax9* open-reading frame by homologous directed repair (HDR). As a result, CreER expression is regulated by the *Pax9* promoter in tandem with endogenous *Pax9* expression.

To detect the insertion of *CreER^{T2}* into the *Pax9* gene, we designed polymerase chain reaction (PCR) primers to target the altered DNA fragments spanning the *Pax9* gene outside the left homology arm and the CreER cassette inside the donor construct (Fig. 1b). We identified one mouse from 24 littermates that contained mutant PCR products consistent with the presence of the CreER cassette in the 3' end of the *Pax9* gene. To examine the insertion site in greater detail, we performed whole-genome sequencing and aligned the sequence reads to a mouse reference genome, in which Chromosome 12 was replaced with an artificial Chromosome 12 sequence containing the *Pax9* donor construct (left homology arm+2A-CreER+right homology arm). We confirmed that the *Pax9* donor construct is inserted in the 3'end of the *Pax9* gene (Fig. 2a). Moreover, analysis of the genome sequencing map from a wider region of Chromosome 12 indicates that the *Pax9* donor construct read is comparable to that of other regions (Fig. 2b), consistent with insertion of only one copy of the *Pax9* donor construct. Taken together, our results show that the *Pax9* donor construct was only inserted at the predicted site, not in an ectopic site.

To analyze the CreER expression pattern *in vivo*, we generated *Pax9-CreER;tdTomato* reporter mice to detect Cre activity driven by dynamic Pax9 expression at various stages. Throughout this study, mice were analyzed 48 hours post-tamoxifen induction, in order to allow 12–24 hours for the tamoxifen to take effect and another 12–24 hours for the tdTomato protein to be synthesized. To confirm that the CreER recombination reflects the expression of endogenous Pax9, we compared the pattern of tdTomato expression to that previously reported for Pax9. Previously, Pax9 expression was first reported in the pharyngeal pouch region around E9.0. Consistent with this, after Cre activity was induced with tamoxifen at E8.5, tdTomoto reporter expression was detectable in a similar region 48 hours later (Fig. 3).

Pax9 is a critical regulator of the craniofacial region during development (Kist *et al.*, 2007; Peters *et al.*, 1998), so we examined Pax9 expression at various stages of craniofacial development. Two days after tamoxifen induction of E10.5 and E14.5 *Pax9-CreER;tdTomato* reporter mice, tdTomato expression was detectable in the craniofacial region. In sections, expression was detectable in the nasal mesenchyme (Fig. 4a,b).

Furthermore, we found that tdTomato was expressed in the palatal shelf mesenchyme (Fig. 4c,d). In the mandible, we detected tdTomato-expressing cells in the mesenchymal region closely associated with the tooth bud at E12.5 (Fig. 4e). At the bell stage, tdTomato expression was more restricted to the dental mesenchyme, dental follicle and mesenchymal region immediately adjacent to the dental epithelium (Fig. 4f). Overall, this pattern of tdTomato expression is consistent with that of Pax9 in previously published studies.

Next, we examined tdTomato expression in other tissues of the body during development. Two days after tamoxifen induction of E10.5 and E12.5 *Pax9-CreER;tdTomato* reporter mice, tdTomato expression was detectable in the vertebral column (Fig. 5a,b). Specifically, we detected tdTomato expression in the condensation of the intervertebral disk primordia at both stages. In addition, we detected tdTomato expression in the limb region (Fig. 5c,d). At E12.5, strong expression of tdTomato was detectable in the anterior proximal region of the forelimb mesenchyme, with weaker expression more posteriorly (Fig. 5c,c'). At E14.5, tdTomato expression was detectable ventral to the primordia of the phalangeal bones and in the pawpad (Fig. 5d,d'). At later stages, we also observed Pax9 expression in other regions where Pax9 was previously reported to be expressed, such as the tongue (Fig. 6a–d). Two days after tamoxifen induction of E14.5 *Pax9-CreER;tdTomato* reporter mice, tdTomato expression was detectable in the tongue epithelium and the mesenchyme of the lateral region of the tongue (Fig. 6a,b). In adults, Pax9 expression was previously reported in the filiform papillae of the tongue. Consistently, in adult *Pax9-CreER;tdTomato* reporter mice, we detected tdTomoto reporter expression throughout the lingual papillae, including the filiform papillae (Fig. 6c,d).

Taken together, the expression of tdTomato in *Pax9-CreER;tdTomato* reporter mice recapitulated the previously reported expression pattern of endogenous Pax9. Therefore, our *Pax9-CreER* knock-in mice can serve as a useful model for lineage tracing and genetic targeting of the craniofacial and limb mesenchyme, tongue epithelium and pharyngeal pouch derivatives during embryonic development and in adult life, and will be available to the research community after appropriate arrangements are made.

METHODS

Single guided RNA (sgRNA) and donor vector construct

Pax9 sgRNA GTGCAGAAGCGGTCACAGAATGG was designed to target the 3' end of *Pax9*, optimized for T7E1 assay efficiency and against off-targets (PNABio). pCAG-CreERT2 plasmids were obtained from Addgene (Plasmid #14797) for donor vector construction (Matsuda and Cepko, 2007). An E2A peptide cleavage site was inserted in front of the CreERT2 coding sequence, which was flanked by a left homology arm containing 500bp of the last intron and exon of *Pax9* and right homology arm containing 500bp of 3' UTR of *Pax9* (PNABio).

One-cell stage embryo injection

All animal procedures were performed according to NIH guidelines and approved by the University of Southern California Institutional Animal Care and Use Committee (IACUC).

B6D2F1 (C57BL/6×DBA2) and CD-1 mouse strains were used as embryo donors and foster mothers, respectively. Super-ovulated female B6D2F1 mice (8 weeks old) were mated to B6D2F1 stud males, and fertilized embryos were collected from oviducts. After 100–200 ng/μl Cas9 mRNA (L-6125, TriLink Biotechnologies) and 50–100 ng/μl sgRNA were mixed on ice for 10 minutes, 5–10 ng/μl purified *Pax9* donor DNA was added. The mixture was then injected into the cytoplasm and pronucleus of fertilized eggs with well-recognized pronuclei in M2 medium (M2112, Cytospreen). Injected eggs were cultured in M16 medium (M6111, Cytospreen) at 37 °C under 5% CO2 overnight. Approximately 20–25 two-cell stage embryos were transferred into oviducts of the pseudopregnant CD-1 females at 0.5dpc.

Genotyping of the *Pax9-CreER* founder mouse

The mutant allele of the *Pax9-CreER* knock-in mouse was confirmed by genotyping PCR products that span from the upstream region of the left homology arm of the *Pax9* gene to the CreER insert in the donor construct. We used two sets of primers to detect the mutant allele independently. Primer set 1 (forward TCTGCCTTTCTCTCCTGGAA and reverse ATTCTCCCACCGTCAGTACG) generates a 1373bp PCR product for the mutant allele, and primer set 2 (forward CAGTGCTGAGCAGAATTCCA and reverse AGGCAAATTTTGGTGTACGG) generates PCR products of 722bp. Both PCR primer pairs identified the same one founder mouse containing the mutant allele from 24 littermates.

Whole-genome sequencing of the *Pax9-CreER* founder mouse

We performed whole-genome sequencing using genomic DNA from the founder *Pax9-CreER* knock-in mouse (Fulgent Diagnostics). The sequenced reads were aligned to the mouse reference genome *mm9* downloaded from the UCSC database (http://hgdownload.cse.ucsc.edu/goldenPath/mm9/chromosomes/). The reference sequence reads in Chromosome 12 of *mm9* were replaced *in silico* with an artificial Chromosome 12 sequence containing the Pax9 donor construct. The modified *mm9* genome was indexed using the Burrows-Wheeler Aligner (BWA) *BWA Index* command to prepare it for use with the BWA aligner. Alignment was performed using the BWA software using the *BWA-Mem* algorithm. The fastq file containing the sequenced reads was aligned against the modified *mm9* genome and a .bam file was generated. Duplicate reads in the .bam file were marked using *Samblaster* software and then sorted and indexed using *Samtools* software. To view the aligned .bam file in the Integrative Genomics Viewer (IGV), a genome file was created using the modified *mm9* reference genome fasta file with the artificial Chromosome 12 within the IGV software using the *Create.genome File* tool. The genome was loaded into IGV followed by the aligned .bam file and the reads aligning to the *Pax9* donor construct were analyzed.

Genetic labeling of *Pax9-CreER* knock-in mice

The *Pax9-CreER* founder mouse (F0) was mated with Bl6 females to generate F1 *Pax9-CreER* mice. *ROSA26LoxP−STOP−LoxP−tdTomato* (tdTomato reporter) mice were obtained from the Jackson Laboratory (JAX no. 007905) (Madisen *et al.*, 2010). tdTomato reporter female mice were mated with *Pax9-CreER* F1 male mice and received 1.5mg/10g bodyweight tamoxifen (Sigma T5648) at E8.5 through E14.5. The female mice were euthanized 48 hours after injection to collect *Pax9-CreER;tdTomato* embryos. Adult *Pax9-CreER;tdTomato* mice received 5 daily injections of 1.5 mg/10 g bodyweight tamoxifen and

were euthanized 48 hours after the last injection. Whole samples were imaged using a Leica MZ10F. For cryosections, samples at various stages were fixed in 4% paraformaldehyde, passed through a sucrose series, then embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Sakura) and frozen onto a dry ice block to solidify. Embedded samples were cryosectioned at 7μm thickness using a cryostat (Leica CM1850). Sections were counterstained with DAPI (Sigma, D9542). Images were captured using a fluorescence microscope (Leica DMI 3000B) with filter settings for DAPI/FITC/TRITC.

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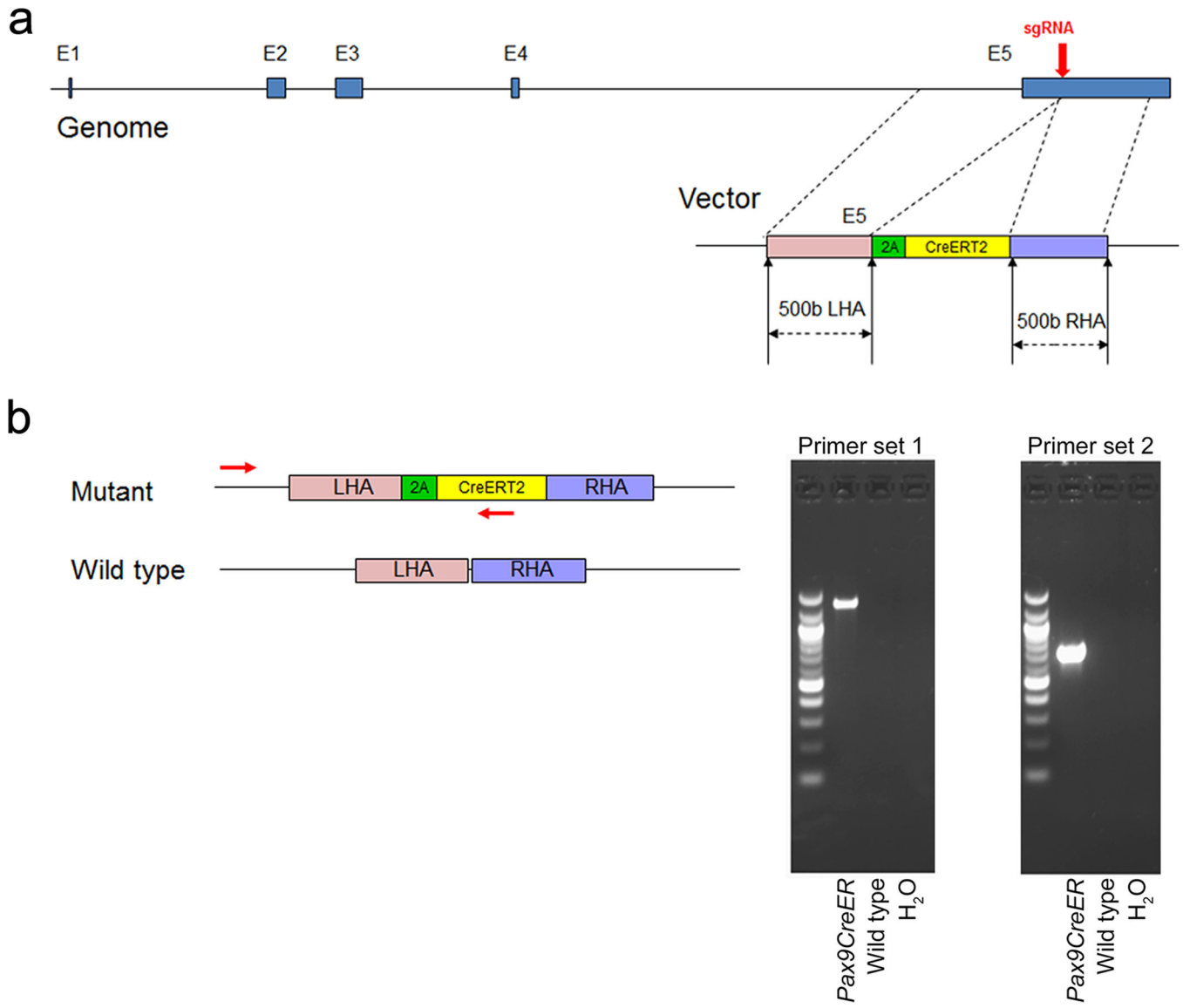

FIG. 1.

Generation of *Pax9-CreER* knock-in mice. (a) Schematic diagram of the knock-in strategy showing the insertion site of the *Pax9* donor construct into the *Pax9* gene. (b) Primers and PCR products for the identification of *Pax9-CreER* mice. Schematic diagram depicts the location of the PCR primers detecting the mutant allele.

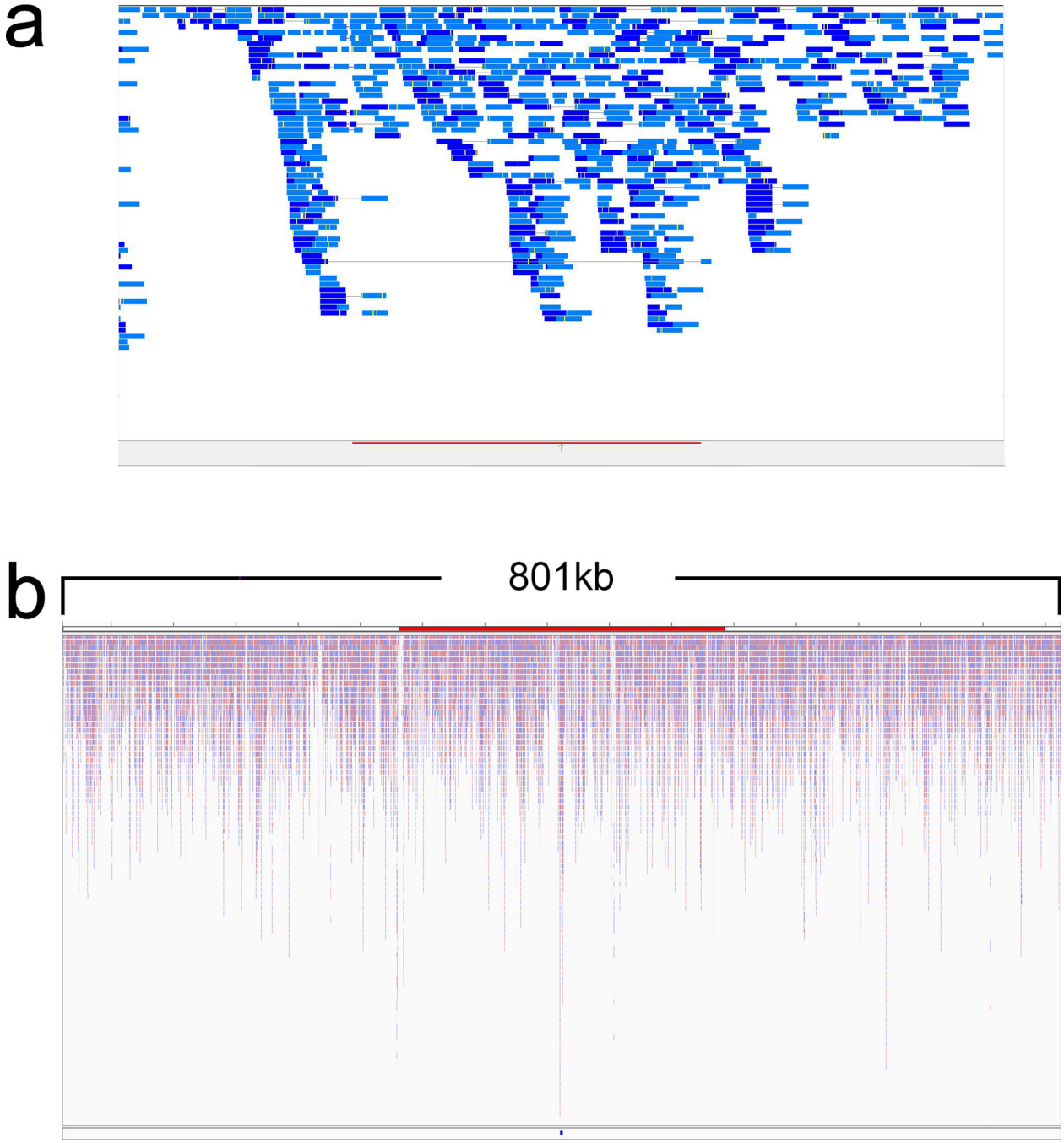

FIG. 2.

Whole-genome sequencing analysis of *Pax9-CreER* knock-in mice. (a) Focused snapshot image of whole-genome sequencing reads that aligned to the 800kb region of the *Pax9* donor construct (orange bar at the bottom of the figure) within mouse Chromosome 12. Light and dark blue horizontal bars reflect the paired-end read direction with a minimum read depth of 20× with no sequencing gaps located within the insert. (b) Zoomed out view of the *Pax9* donor construct (small blue rectangle at the bottom of the figure) and surrounding genomic region demonstrating no significant deviation of the average coverage and typical read

depth. The figures were produced using the Genetrix Viewer (Epicenter Software) and the Integrative Genomics Viewer (IGV) from the Broad Institute.

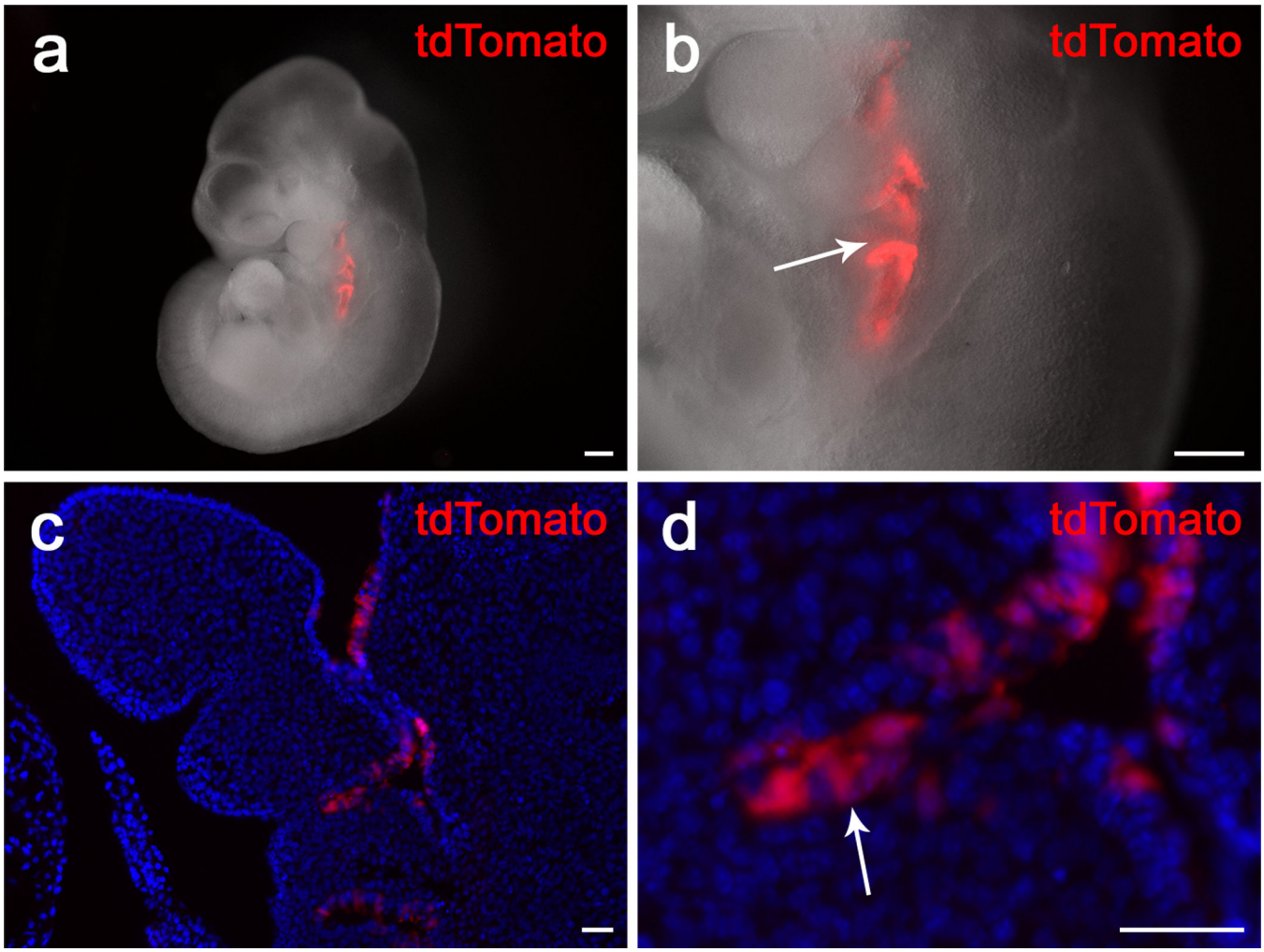

FIG. 3.

tdTomato expression in the pharyngeal pouch region of *Pax9-CreER;tdTomato* mice induced at E8.5. (a, b) Visualization of whole-mount *Pax9-CreER;tdTomato* mice 48 hours after tamoxifen induction at E8.5. Arrow indicates tdTomato staining in the pharyngeal pouch regions (c, d) Visualization of sections of *Pax9-CreER;tdTomato* mice 48 hours after tamoxifen induction at E8.5. Note that tdTomato signaling is restricted to the epithelium of the pharyngeal pouches (arrow). Scale bars, (a) and (b), 200μm; (c) and (d), 25μm.

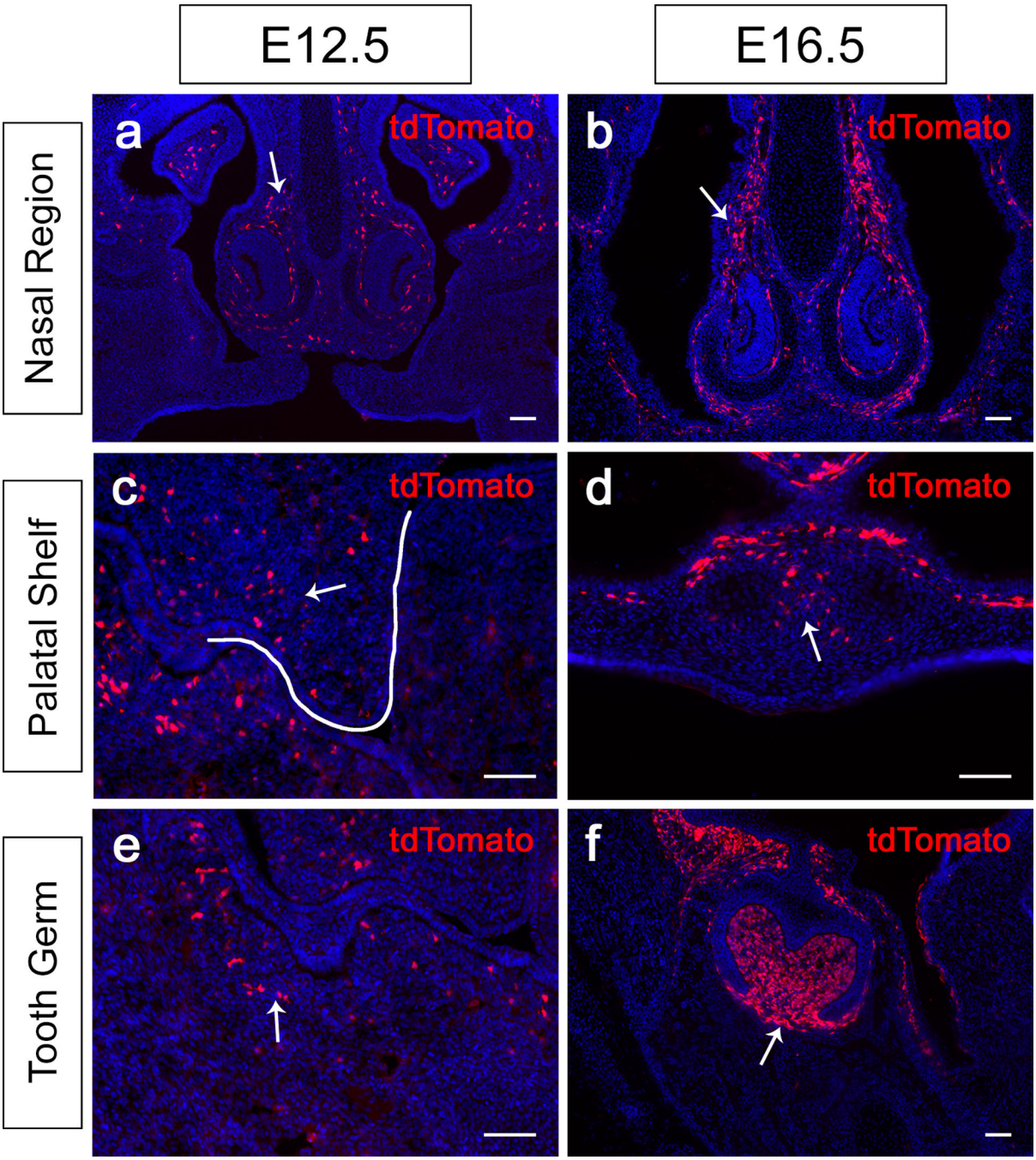

FIG. 4.

tdTomato expression in the craniofacial region of *Pax9-CreER;tdTomato* mice induced at E10.5 and E14.5. (a–i) Visualization of coronal sections of the nasal region (a–b), the palatal shelves (c–d), and the tooth germs (e–f) of *Pax9-CreER;tdTomato* mice 48 hours after tamoxifen induction at E10.5 and E14.5. White line indicates the palatal shelf prior to elevation and fusion (c). Arrows indicate tdTomato staining in the mesenchyme of these regions. Scale bars, 50μm.

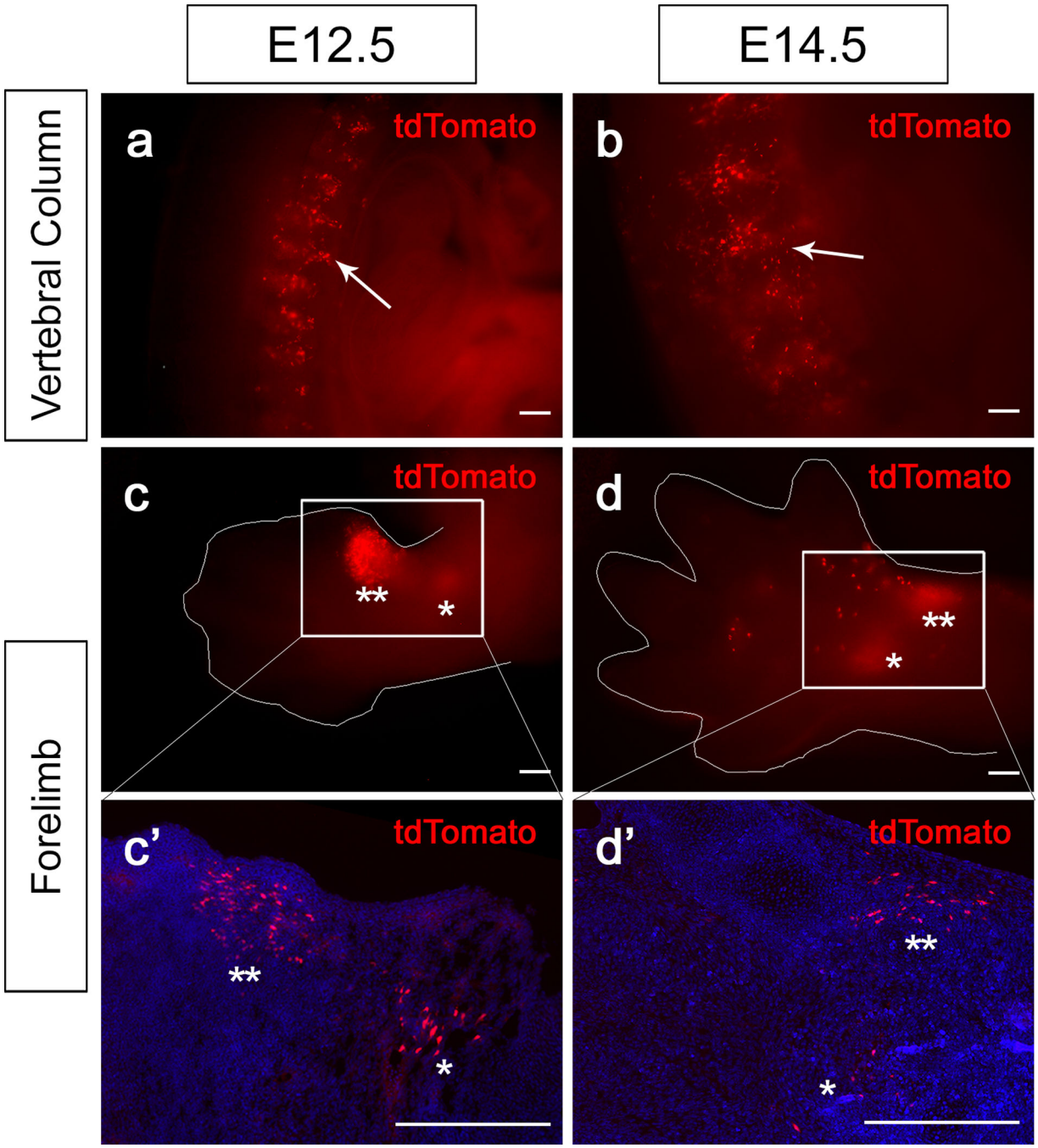

FIG. 5.

tdTomato expression in the vertebral column and forelimbs of *Pax9-CreER;tdTomato* mice induced at E10.5 and E12.5. (a, b) Visualization of whole mount transverse section view of the vertebral column of E12.5 and E14.5 *Pax9-CreER;tdTomato* mice 48 hours after tamoxifen induction. Arrows indicates tdTomato expression in the intervertebral disk primodia. (c, d) Ventral views of forelimbs from E12.5 and E14.5 *Pax9-CreER;tdTomato* mice 48 hours after tamoxifen induction in whole mounts (c, d) and sections (c', d'). ** and * in (c) and (c') indicate strong and weaker expression region of tdTomato in the limb bud.

** and * in (d) and (d') indicate expression close to the phalangeal bone primodia and in the pawpad region. Boxed areas in (c) and (d) are shown enlarged in (c') and (d'), respectively. Thin white line outlines the forelimb. Scale bars, 200μm.

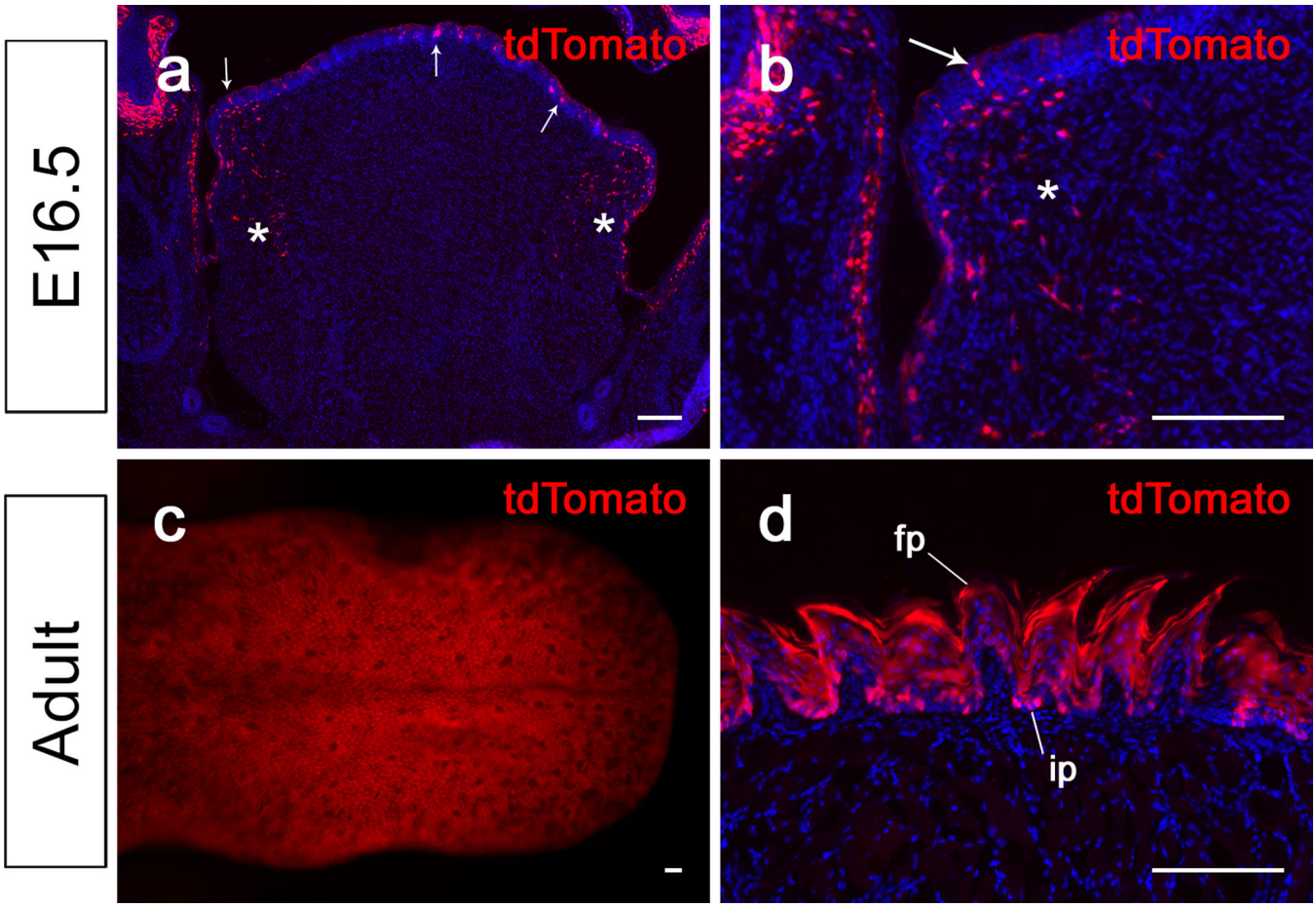

FIG. 6.

tdTomato expression in the tongue of *Pax9-CreER;tdTomato* mice induced in E14.5 embryos and 4-week old adults. (a, b) Visualization of coronal sections of tongues of *Pax9-CreER;tdTomato* mice 48 hours after tamoxifen induction at E14.5. Arrows indicate tdTomato expression in the tongue epithelium. Asterisks indicate tdTomato expressing mesenchymal cells on the lateral side of the tongue. Panel (b) is a magnified view from (a). (c,d) Visualization of whole mount (c) and coronal sections (d) of tongues from adult *Pax9-CreER;tdTomato* mice 48 hours after tamoxifen induction. fp, filiform papilla; ip, interpapillary region. Scale bars,100μm.