Published in final edited form as:

Genesis. 2019 October ; 57(10): e23328. doi:10.1002/dvg.23328.

Generation and characterization of *Lhx4^{tdT}* reporter knock-in and *Lhx4^{loxP}* conditional knockout mice

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Summary

LHX4 is a LIM-homeodomain transcription factor essential for the development of spinal cord and pituitary gland. Mice with homozygous *Lhx4-null* mutation suffer early postnatal death from lung defect. In this study, to facilitate the research on *Lhx4* function, we designed a targeting construct to generate two novel *Lhx4* mouse lines: *Lhx4^{loxP}* conditional knockout and *Lhx4^{tdT}* reporter knock-in mice. *Lhx4^{tdT/+}*, *Lhx4^{loxP/+}*, and *Lhx4^{loxP/loxP}* were viable, fertile, and did not display any gross abnormalities. By breeding *Lhx4^{loxP}* line with Cre-expressing mice, the Exon 3 of *Lhx4* was efficiently removed, resulting in a shift in the reading frame and the inactivation of *Lhx4*. The expression of tdTomato knock-in reporter recapitulated the endogenous LHX4 expression and was detected in the retina, spinal cord, pituitary gland, and hindbrain of *Lhx4^{tdT}* mice. Thus, *Lhx4^{tdT}* and *Lhx4^{loxP}* mouse lines provide valuable tools for unraveling the tissue-specific role of *Lhx4* at postnatal stages in mice.

Keywords

homeobox; LIM-homeodomain; pituitary gland; retina; spinal cord

1 | INTRODUCTION

LHX4 is one of the LIM-homeodomain (LIM-HD) transcription factors that are characterized by two LIM domains for protein–protein interactions and a homeodomain for DNA binding to regulate the expression of target genes. LHX4 shows significant similarity to LHX3 in the amino acid sequence and belongs to the LHX3/4 subgroup of LIM-HD proteins (Yamashita, Moriyama, Sheng, & Westphal, 1997). Previous studies have shown that *Lhx4* is expressed in the spinal cord, pituitary gland, hindbrain, and retina (Balasubramanian, Bui, Ding, & Gan, 2014; Elshatory et al., 2007; Li et al., 1994; Sharma et

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al., 1998; Sheng et al., 1997). Mice with homozygous *Lhx4* null mutation die shortly after birth with lung defect, and heterozygous mice are apparently normal (Li et al., 1994). In mice lacking both *Lhx3* and *Lhx4*, the pouch rudiment develops but the formation of Rathke's pouch fails (Sheng et al., 1997). In *Lhx3* and *Lhx4* double knockout mice, the ventrally projecting spinal cord motor neurons switch their subtype identity to the motor neurons with axons exiting dorsally from the neural tube (Sharma et al., 1998).

In human, heterozygous mutations of *LHX4* are associated with congenital pituitary hormone deficiency (Castinetti et al., 2008; Machinis et al., 2001; Tajima et al., 2007). In addition, patients display abnormalities in the sella turcica of the central skull base, severe respiratory disease, and hypoglycemia. A homozygous mutation of *LHX4* is associated with a lethal form of congenital hypopituitarism (Gregory et al., 2015). The patients are born small for gestational age with a small phallus, undescended testes, and mid-facial hypoplasia, and died within the first week of life.

In this study, we generated *Lhx4* conditional knockout and tdTomato reporter knock-in mouse lines and characterized these for further study of the role of *Lhx4*. This study demonstrated the faithful tdTomato expression in *Lhx4*-tdTomato reporter knock-in mice and the effective knockout of *Lhx4* through Cre recombinase-mediated removal of loxP-flanked *Lhx4* Exon 3 in *Lhx4^{loxP}* conditional knockout mice. The efficient ablation of *Lhx4* by Cre recombination could surmount the problem that *Lhx4* conventional knockout mice die within 24 hr after birth. Therefore, these two novel mouse lines offer useful tools for studying the role of *Lhx4*.

2 | MATERIALS AND METHODS

2.1 | Animals

To generate the *Lhx4* conditional knockout and tdTomato knock-in alleles, the *Lhx4^{ckotdT}* targeting vector was constructed containing a floxed Exon 3, an FRT-flanked En2 splicing acceptor-IRES-tdTomato-pGK-Neo (tdT-Neo) cassette for negative selection, 5.6 kb 5' arm, 4.5 kb 3' arm, and pGK-diphtheria toxin fragment A (DTA) cassette for negative selection (Figure 1a). The floxed region contained 99 bp of Intron 2–3, Exon 3, and 224 bp of Intron 3–4. The targeting construct was linearized and electroporated into C57BL/6J-129S6 hybrid ESCs. Correctly targeted ESC clones were confirmed by Southern blotting using a 3'-probe, which detects the 7.8 kb wild type and 5.8 kb *Lhx4^{ckotdT}* DNA fragments from BamHI-digested genomic DNA, respectively (Figure 1b). Positive ESCs were then injected into C57BL/6J blastocysts to generate mouse chimeras. The primers used to amplify a 655 bp Southern blotting probe are 5'-GCTGACTGCATTACGGTGGTTTTTCGCTACAGA-3' and 5'-CTGCTCACCTCGGAAGCTCAGCTCACTGTCAC-3'. Then, targeted ESCs were injected into C57BL/6J blastocysts to generate chimeric mice. *Lhx4^{ckotdT}* mice were obtained by crossing chimeras with C57BL/6J mice and were then crossed with ROSA26-FLPe mice (B6.129S4-*Gt(ROSA)26Sor^{tm1(FLP1)}Dym*/RainJ, The Jackson Laboratory Stock Number: 009086) to generate *Lhx4^{loxP/+}* mice by removing the FRT-flanked tdT-Neo cassette. The *Lhx4^{loxP}* conditional knockout and wild type alleles were identified by PCR primer set P1: 5'-TGAAGCTATCAGGAGGCCTAGAGT-3' and 5'-AGCATGGCCAGCTCTGCTTACCGT-3' (Figure 1c) or primer set P3: 5'-

TGAAGCTATCAGGAGGCCTAGAGT-3' and 5'-CAGCTGTGCTCCAGGCCAGGAGA-3' (Figure 1e). *Lhx4^{ckotdT}* mice were also crossed with CMV-Cre mice (B6.C-Tg[CMV-cre]1Cgn/J, The Jackson Laboratory Stock Number: 006054) to generate *Lhx4^{tdT/+}* mice by removing the Exon 3 of *Lhx4* and the Neo cassette. The PCR primer set P2 used to identify the *Lhx4^{tdT}* knock-in allele was 5'-CACGCTGATCTACAAGGTGAAGA-3' and 5'-ACCTTGAAGCGCATGAACTCT-3' (Figure 1c). University Committee of Animal Resources at the University of Rochester and Hangzhou Normal University approved all animal procedures described here. The corresponding authors will make these mouse strains available to the research community upon acceptance of this manuscript.

2.2 | Immunohistochemistry

Immunohistochemistry was performed as previously described (Huang et al., 2014). In brief, tissues dissected from mice were fixed in 4% paraformaldehyde (PFA) in PBS for 1–2 hr (eyes) or overnight (heads) at 4°C. Tissues were then rinsed with cold PBS three times for 15 min each, cryoprotected in 30% sucrose, embedded and frozen in OCT compound (TissueTek). Cryosections were cut at 18 µm thickness. After blocking with 10% horse serum in PBST (0.3% Triton-X100 in the PBS, pH 7.3), sections were incubated with primary antibodies overnight at 4°C. Then, sections were rinsed four times with PBST and incubated with Alexa-conjugated secondary antibodies (Invitrogen) at room temperature for 90 min. After sections were washed with PBST and PBS, they were nuclear counter-stained with DAPI and mounted with coverslips. Images were obtained on a Zeiss LSM 510 META confocal microscope. The following primary antibodies were used: mouse anti-ISL1/2 (1:200, Developmental Studies Hybridoma Bank, #39.4D5), mouse anti-GNAO1 (1:500, Millipore, # MAB3073), rabbit anti-RFP (1:1,000, Rockland, #600-401-379), guinea pig anti-LHX4 (1:250, gift of T.M. Jessell, Columbia University), mouse anti-LHX4 (1:50, Santa Cruz Biotechnology, #sc-374562), and sheep anti-VSX2 (1:1,000, Exalpha Biological, # X1180P).

3 | RESULTS AND DISCUSSION

In order to generate the *Lhx4* conditional knockout and tdTomato (tdT) knock-in mice, we used promoter-driven Neo selection cassette for the generation of *Lhx4* knockout-first and conditional-ready (*Lhx4^{ckotdT}*) allele (Figure 1a,b). The *Lhx4^{tdT}* reporter mouse line was obtained by breeding the *Lhx4^{ckotdT}* mice with CMV-Cre mice to remove the Exon 3 and Neo, while crossing the *Lhx4^{ckotdT}* mice with *FLPeR* mice to remove the tdT-Neo cassette generated the *Lhx4^{loxP}* allele. PCR genotyping primer sets P1 and P2 were used to identify *Lhx4* wild type and the floxed allele, and the tdT knock-in allele, respectively (Figure 1c). *Lhx4^{loxP/+}*, *Lhx4^{loxP/loxP}*, *Lhx4^{tdT/+}*, *Lhx4^{tdT/loxP}* mice were viable, fertile, and did not display any gross abnormalities. The native fluorescence of tdTomato reporter was detectable in the inner nuclear layer (INL) of the *Lhx4^{tdT/+}* retina (Figure 1d).

Removal of Exon 3 was expected to result in the deletion of the second LIM domain and a frameshift starting in Exon 4, which truncated the coding sequence C-terminal to Exon 2 including the DNA-binding homeodomain. To determine whether loxP-flanked Exon 3 in

the *Lhx4^{loxP}* conditional knockout allele could be efficiently excised by Cre recombinase to generate the *Lhx4*-null mutation as designed, we first crossed the *Lhx4^{loxP}* conditional knockout mice with CMV-Cre mice, which causes germline deletion of loxP-flanked genes (Schwenk, Baron, & Rajewsky, 1995). We performed PCR genotyping with primer set P3 to identify *Lhx4* wild type, *Lhx4^{loxP}* and null alleles, and found that global *Lhx4^{-/-}* null mice died within 24 hr after birth (Figure 1e), consistent with the previous report (Li et al., 1994). The *Lhx4^{loxP}* conditional knockout mice were then crossed with retina-specific *Six3-Cre* mice (Furuta, Lagutin, Hogan, & Oliver, 2000), to remove Exon 3 in the retina. *Lhx4^{loxP/loxP};Six3-Cre* or *Lhx4^{tdT/loxP};Six3-Cre* (*Lhx4*-null) mice were viable but immunostaining using both guinea pig anti-LHX4 and mouse anti-LHX4 antibody showed that the expression of LHX4 was ablated in the retina (Figure 1f). Thus, the *Lhx4^{loxP}* allele allows the efficient inactivation of *Lhx4* by tissue specific Cre recombination in vivo, overcoming the problem that *Lhx4* conventional knockout mice die within 24 hr after birth.

To determine whether the expression of knock-in tdTomato reporter recapitulates the expression of endogenous *Lhx4*, we compared the expression pattern of tdTomato and LHX4 in *Lhx4^{tdT/+}* retinas by co-immunolabeling with anti-LHX4 and anti-RFP that detects tdTomato. The expression of LHX4 was first observed in the inner nuclear layer (INL) at P5 while tdTomato expression was undetectable (Figure 2a). At P6, the cytosolic expression of tdTomato was seen in the INL and colocalized with the cells expressing LHX4 in the nuclei (Figure 2b). The co-expression of tdTomato and LHX4 in the INL was similarly observed in the P8 retina (Figure 2c). At P14, while the expression of tdTomato largely overlapped that of LHX4 in the INL (Figure 2d), the expression level of LHX4 was reduced in the cells located at the outermost INL while the expression of tdTomato was obviously present there (Figure 2d,e). Overall, our results showed that the expression of tdTomato largely recapitulates that of endogenous LHX4. The slight delay in the onset of tdTomato expression could be due to a lower detection sensitivity of cytosolic tdTomato than nuclear LHX4, and prolonged expression of tdTomato might reflect the longer half-life of tdTomato than that of LHX4 in this cell population.

Previous studies have shown that LHX4 is expressed in retinal bipolar cells (BCs) (Balasubramanian et al., 2014; Elshatory et al., 2007), we thus determined the identity of tdTomato-expressing cells by co-labeling with anti-RFP and bipolar cell markers in the *Lhx4^{tdT/+}* retina. Co-immunolabeling of tdTomato and VSX2, a pan-BC marker (Elshatory et al., 2007; Jung et al., 2015), confirmed that tdTomato was exclusively expressed in the VSX2⁺ BCs (Figure 3a). GNAO1 and ISL1 are ON-BC markers (Bramblett, Pennesi, Wu, & Tsai, 2004; Elshatory et al., 2007; Haverkamp & Wässle, 2000). We found that some tdTomato⁺ cells colocalized with the GNAO1⁺ and ISL1⁺ cells in the INL (Figure 3b,c), suggesting their subtype identity as ON-BCs. These results are consistent with the previously described expression of LHX4 specifically in BCs (Balasubramanian et al., 2014; Elshatory et al., 2007).

To examine the expression pattern of tdTomato in other structures where *Lhx4* expression has been documented (Li et al., 1994; Sharma et al., 1998), sections of mouse spinal cord, pituitary gland, and hindbrain were immunolabeled with anti-RFP. In the spinal cord, the expression of tdTomato was detected in neuronal cell bodies and axons at E10.5 and E11.5

(Figure 4a,b), consistent with the previous study that LHX4 is expressed in motor neurons whose axons emerge ventrally from the neural tube (Sharma et al., 1998). We also found the tdTomato staining in the hindbrain and pituitary gland (Figure 4c, d), in agreement with previous studies (Li et al., 1994; Sheng et al., 1997). In summary, tdTomato expression generally recapitulated the expression pattern of the endogenous LHX4. Therefore, *Lhx4^{tdT}* and *Lhx4^{loxP}* mouse lines serve as useful tools for exploring novel roles of *Lhx4* in mice during development and postnatally.

ACKNOWLEDGMENTS

We thank Drs. Amy Kiernan, Richard Libby, Patricia White, and the members of Gan laboratory for their insightful and helpful discussions, and University of Rochester Mouse Genome Editing Resource for the assistance with the generation of the *Lhx4^{tdT}* and *Lhx4^{loxP}* mouse models.

Funding information

Department of Ophthalmology at the University of Rochester, Grant/Award Number: RO1EY026614

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FIGURE 1.

Generation of *Lhx4^{loxP}* and *Lhx4^{tdT}* mice. (a) The *Lhx4^{ckotdT}* knockout-first and conditional-ready allele was generated by flanking Exon 3 with loxP sites and inserting an FRT-flanked *tdT*-*Neo* cassette in Intron 2–3. The *Lhx4^{loxP}* mouse line was obtained by crossing *Lhx4^{ckotdT}* mice with the *FLPe* deleter strain to remove the *tdT*-*Neo*, while crossing *Lhx4^{ckotdT}* mice with *CMV-Cre* mice to remove Exon 3 and *Neo* generated *Lhx4^{tdT}* mouse line. Black boxes represent exons and thick bars are homologous arms. The 3' southern probe is marked as the hatched box. P1, the PCR primer set used to identify the *Lhx4^{loxP}* conditional knockout allele. P2, the PCR primer set used to identify the *Lhx4^{tdT}* knock-in

allele. P3, the PCR primer set used to identify the *Lhx4^{loxP}* conditional knockout and the *Lhx4* null alleles. (b) Representative southern blotting for identification of targeted ESCs using a 3'-probe, which identifies 7.8 kb wild type and 5.8 kb *Lhx4^{ckotdT}* DNA fragments from BamHI digested genomic DNA. (c) Representative PCR amplification identified a 350 bp wild type fragment, a 460 bp *Lhx4^{loxP}* fragment, and a 400 bp *Lhx4^{tdT}* fragment using primer set P1 and P2, respectively. (d) The tdTomato reporter was detected in the INL of *Lhx4^{tdT/+}* retina at P14. (e and f) Effective inactivation of Lhx4 by Cre recombinase-mediated removal of the loxP flanked exon 3 in *Lhx4^{loxP}* mice. *Lhx4^{loxP/loxP}* mice were crossed with *Lhx4^{+/-}*; *CMV-Cre* mice, which were produced by breeding *Lhx4^{loxP/loxP}* with *CMV-Cre* mice, to obtain *Lhx4^{-/-}* mice. P3 primer set was used to amplify the *Lhx4^{loxP}*, the wild type, and the *Lhx4*-null alleles. We found that *Lhx4^{-/-}* mice with germline inactivation of *Lhx4* died within 24 hr after birth, while *Lhx4^{+/-}* mice did not display any gross abnormalities (e). Immunolabeling with guinea pig and mouse anti-LHX4 showed that the expression of LHX4 was ablated in the *Lhx4*-null retina at P7 and P8 (f). Scale bars equal 50 μ m

FIGURE 2.

Overlapping expression of LHX4 and tdTomato in the *Lhx4*^{tdT/+} retina. (a) LHX4 expression was first detected in the INL at P5 while the expression of tdTomato was not observed. (b) The expression of tdTomato was seen in the INL at P6 and colocalized with LHX4-expressing cells in the INL. (c) The expression of tdTomato and LHX4 was largely increased in the retina at P8, and the co-expression of LHX4 and tdTomato was found in the INL. (d) LHX4 expression largely overlapped that of tdTomato in the INL at P14. However, the expression of LHX4 was downregulated in the cells located at the outer edge of INL at P14, while the strong expression of tdTomato was present there. (e) LHX4 colocalized with a majority of tdTomato⁺ cells in the INL of adult retinas. However, the expression of LHX4 was absent in the cells located at the outer edge of the INL where the expression of tdTomato persisted. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Scale bars equal 50 μ m

FIGURE 3.

The expression of tdTomato in adult retinal BCs. (a) Anti-VSX2 labeled the nuclei of all BCs in the INL. All of tdTomato⁺ cells expressed VSX2 in the INL. (b) GNAO1 was expressed in the cell bodies of all ON-BCs, and tdTomato colocalized with GNAO1⁺ cells in the adult retina. (c) The nuclear marker ISL1 stained ON-BCs and cholinergic amacrine cells, and tdTomato was co-expressed with ISL1 in BCs. Scale bars equal 50 μ m

FIGURE 4.

The expression of tdTomato in the spinal cord, hindbrain, and pituitary gland. (a and b) The expression of tdTomato was observed in the ventral horn of developing spinal cord at E10.5 and E11.5. The tdTomato⁺ motor neurons extended axons ventrally from the neural tube. (c) The intense expression of tdTomato was detected in the neuronal cell bodies and axons in the developing hindbrain at E14.5. (d) tdTomato was expressed in the future anterior lobe of the pituitary gland at E13.5. The dashed line outlined the pituitary gland. Scale bars equal 100 μ m