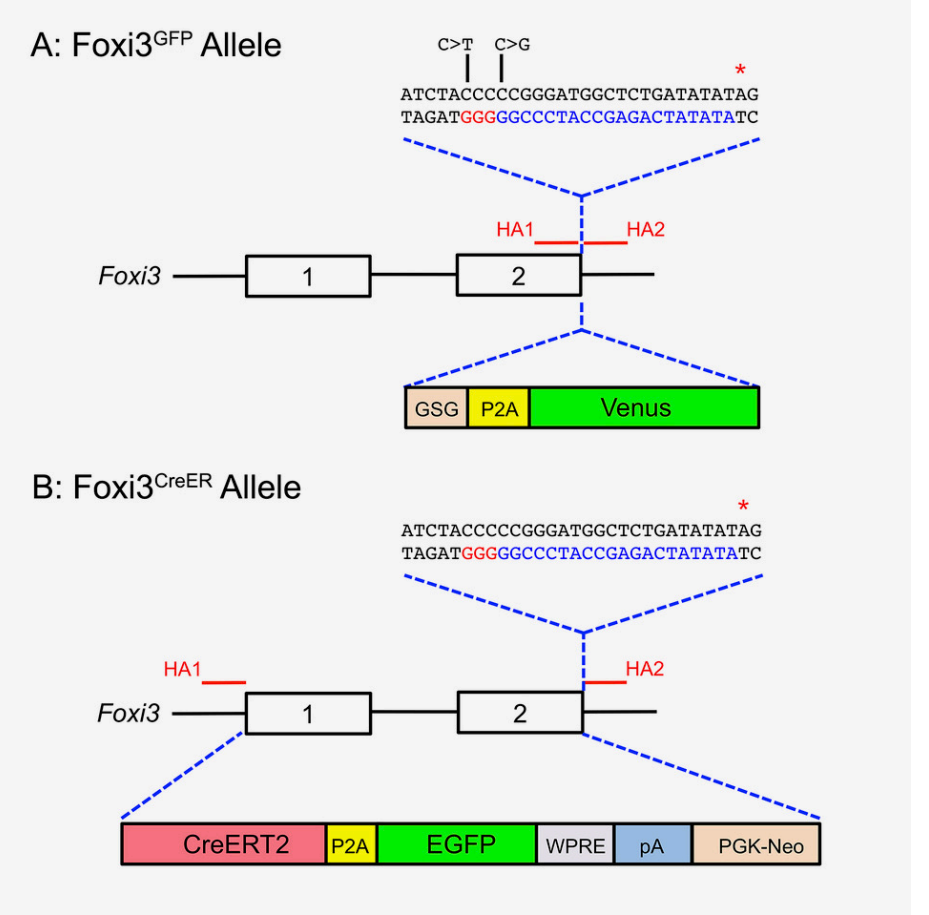


Fig. S1. Novel transgenic mouse lines. [Image from Ankamreddy et al. (2023)] **(A)** Foxi3-GFP used for recording the gene expression. **(B)** Foxi3-CreER line for fate mapping the Foxi3 positive cells at the desired stage of development.

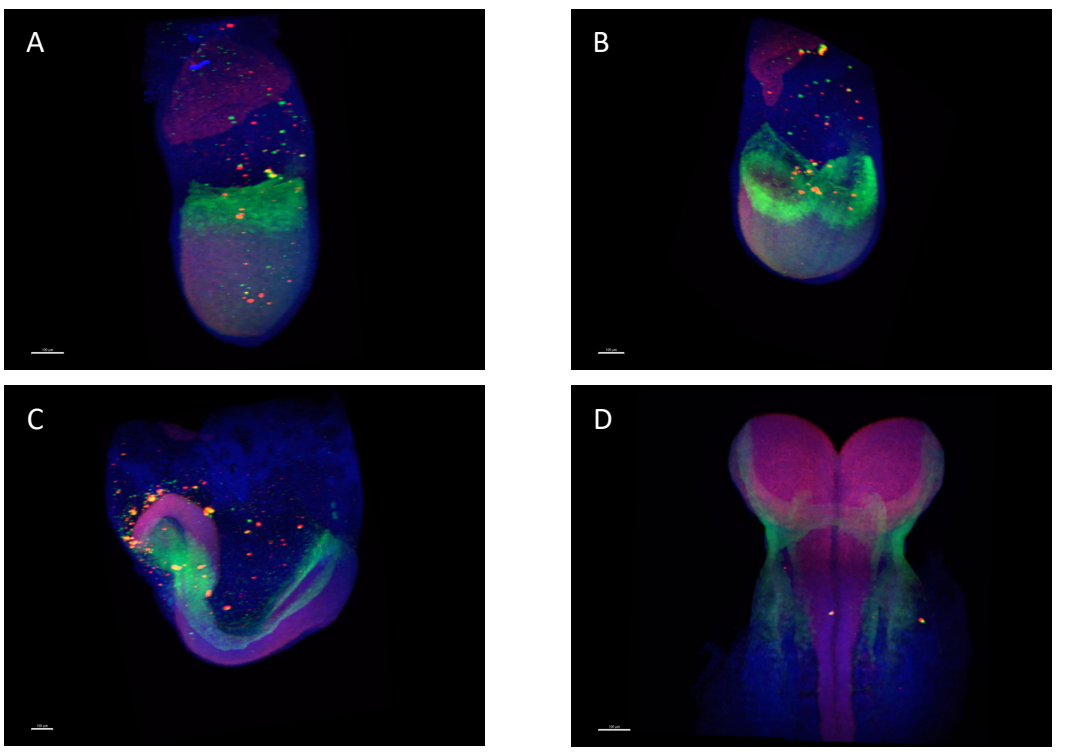


Fig. S2. Whole embryos showing Foxi3-GFP expression (see Supplemental Movies S1-8 for high resolution). **(A)** E7.0 embryo stained for Foxi3-GFP (green), SOX2 (red), DAPI (blue). The top half of the egg shape is extraembryonic tissue. See **Movie S1** for 3D rotation and **Movie S2** for optical sections from anterior to posterior side of the embryo. **(B)** E7.5 embryo stained for Foxi3-GFP (green), SOX2 (red), DAPI (blue). The top half of the egg shape is extraembryonic tissue. See **Movie S3** for 3D rotation and **Movie S4** for optical sections from anterior to posterior side of the embryo. **(C)** E8.5 embryo stained for Foxi3-GFP (green), SOX2 (red), DAPI (blue). The top half of the egg shape is extraembryonic tissue. See **Movie S5** for 3D rotation and **Movie S6** for optical sections from anterior to posterior side of the embryo. **(D)** E8.75 embryo stained for Foxi3-GFP (green), SOX2 (red), DAPI (blue). The extraembryonic tissue has been removed. See **Movie S7** for 3D rotation and **Movie S8** for optical sections from dorsal to ventral side of the embryo. Scale bars indicated in the videos.

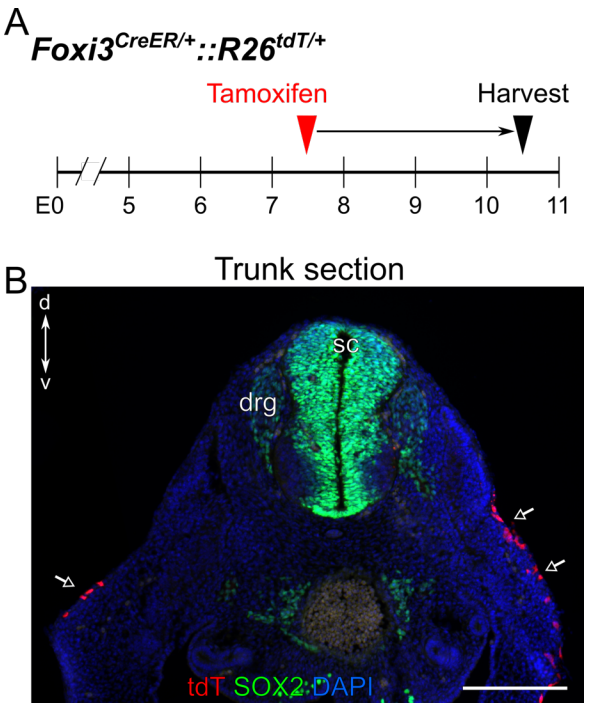


Fig. S3. Foxi3 lineage contributes to only epidermis in the trunk. (A) Experimental timelines for Foxi3-CreER induction at E7.5 followed by E10.5 harvests. Cryosections for immunostaining were collected along the anterior-posterior axis. **(B)** Sections through the trunk immunostained for tdTomato (red), SOX2 (green), DAPI (blue). tdTomato+ cells were only found in the epidermis (open arrows; *n*=5). d, dorsal; drg, dorsal root ganglion; sc, spinal cord; v, ventral. Scale bar = 200μm.

Placode \ Gavage age	E5.5	E6.5	E7.5	E8.5	E9.5
Lens	1/5	7/7	6/8	0/5	0/6
Olfactory	1/5	7/7	8/8	2/5	0/6
Adenohypophysis	0/5	4/7	6/8	0/5	0/6
V ganglion	1/5	7/7	7/8	1/5	0/6
VII ganglion	1/5	7/7	8/8	4/5	0/6
VIII ganglion	1/5	7/7	8/8	2/5	0/6
Inner ear	1/5	7/7	8/8	4/5	0/6
IX&X ganglia	2/5	7/7	8/8	5/5	5/6

Legend
Labeling in 50% or more embryos
Labeling in less than 50% of embryos

Fig. S4. Summary of spatiotemporal analysis of lineage labeling found in Foxi3-CreER::Rosa-tdTomato embryos. Embryos were gavaged between E5.5 and E9.5, followed by harvest at E11.5 (n=5 to 8). Number of embryos with significant tdTomato signal in a placodal derivative is indicated as a fraction of the total number of embryos analyzed for each experimental condition. Placodes that showed significant tdTomato labeling in 50% or more embryos were considered a derivative of Foxi3- CreER lineage and are highlighted in red. See Figure 3 for representative images, and methods section for data analysis criteria.

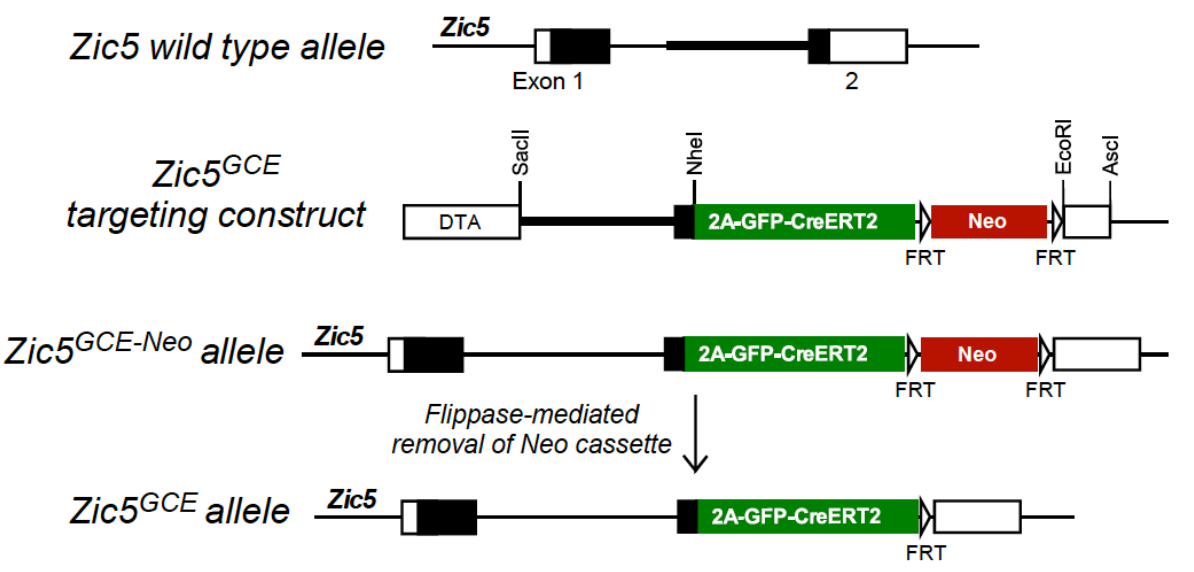


Fig. S5. Zic5-CreER lineage tracing mouse construct. GFP-CreERT2 fusion gene was inserted in exon 2, interrupting the Zic5 coding region [MGI: 1929518], leaving the 3'UTR intact. Neomycin selection cassette was removed by crossing the mouse line with Rosa26-Flippase (Jackson Laboratory Strain #:003946; 129S4/SvJaeSor-*Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$/J).

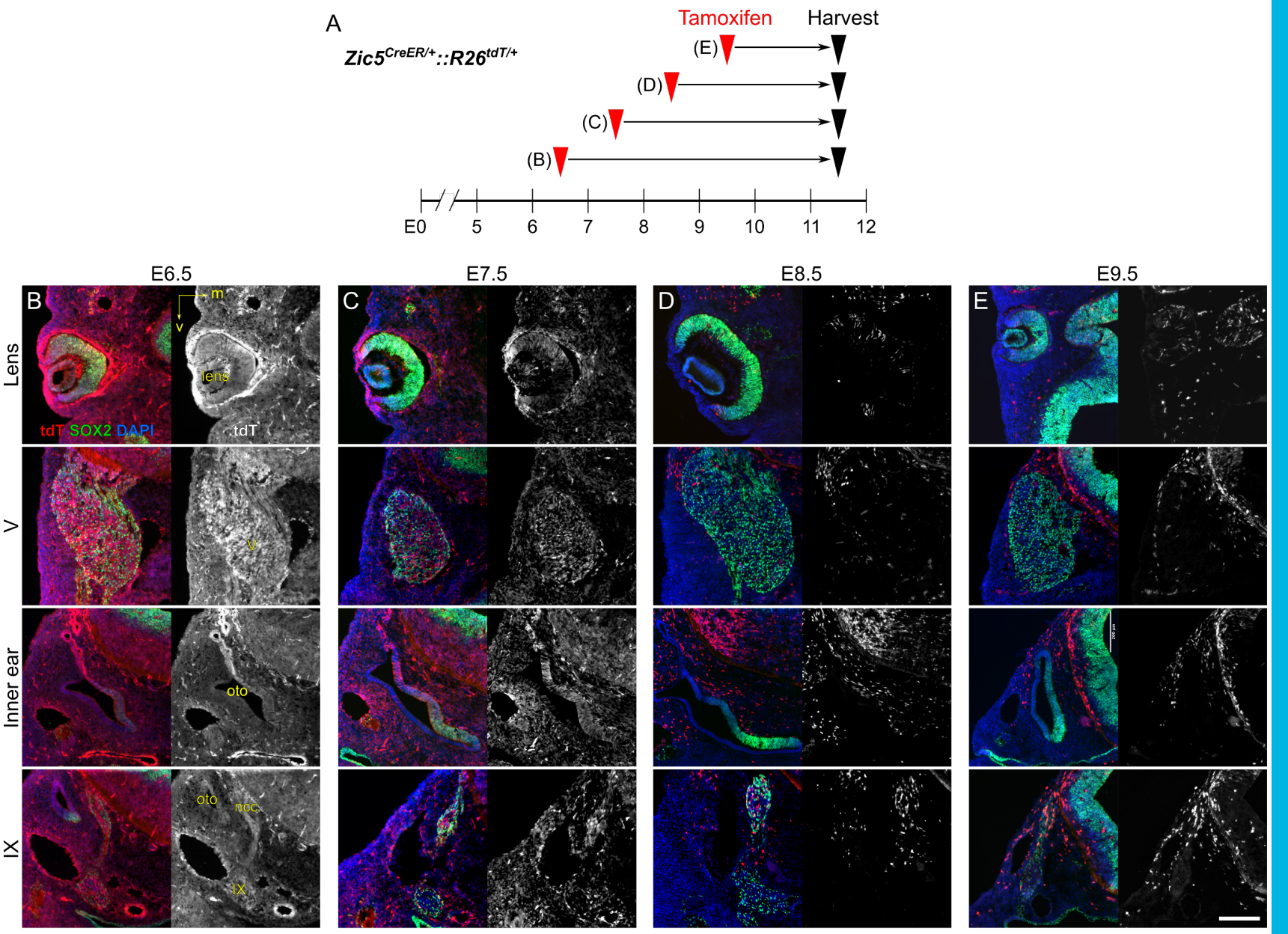


Fig. S6. Zic5 lineage contribution to cranial placodes diminishes after E7.5. (A) Experimental timelines for Zic5-CreER induction between E6.5 and E9.5 followed by E10.5 to 11.5 harvest. Cryosections for immunostaining were collected along the anterior-posterior axis, as in Figures 2 and 3. **(B – E)** Sections through the lens, trigeminal ganglion (V), inner ear, and epibranchial (petrosal) ganglion (IX) immunostained for tdTomato (red), SOX2 (green), DAPI (blue) (*n*=3-8). There is no contribution of the Zic5 lineage to placodal derivatives following Cre induction at E8.5 or E9.5. Note that the dorsal neural crest-derived ganglion in the figure panels for IX shows Zic5 lineage derivatives at all ages but the ventral placodal derived petrosal ganglion (IX) does not show any tdTomato+ + cells after E7.5. m, medial; ncc, neural crest derived ganglion; oto, otocyst/inner ear; pit, pituitary placode; v, ventral. Scale bar = 200 µm.

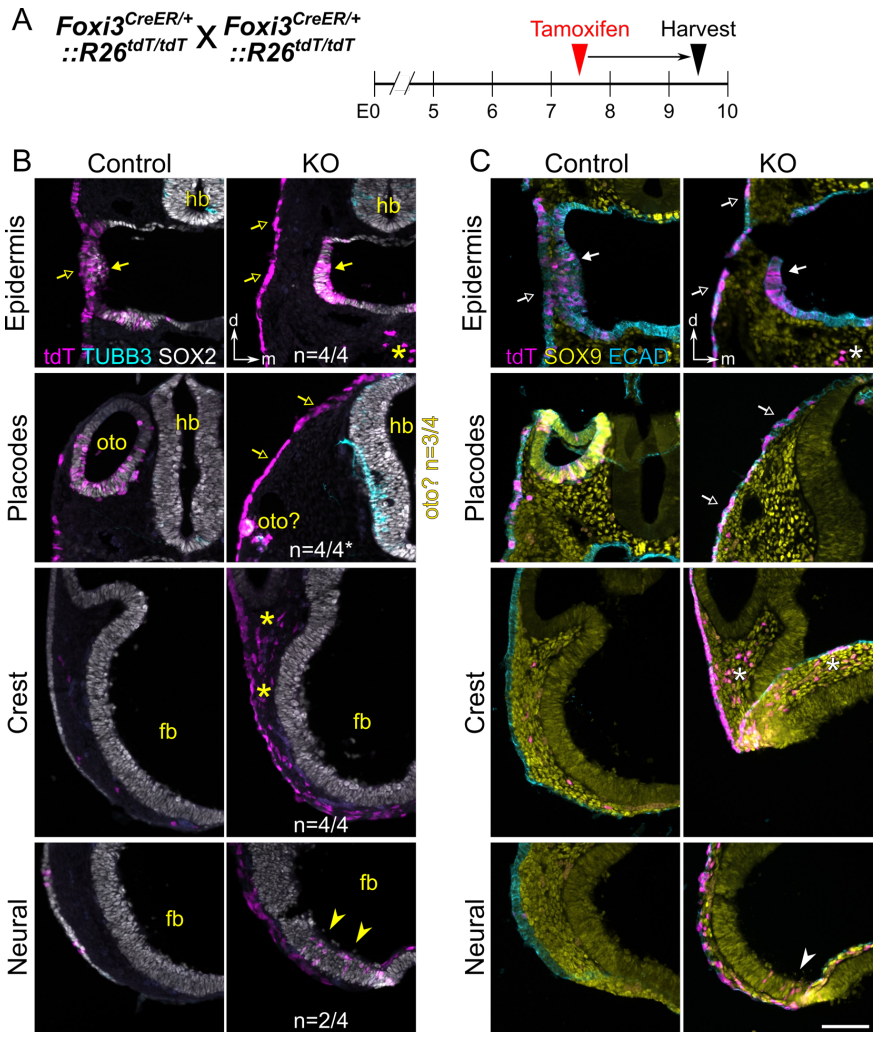


Fig. S7. Fate change of the neural plate border in Foxi3-knockout mutants appears by E9.5. (A) Experimental timeline for *Foxi3-CreER::Rosa-tdTomato* sibmating. Tamoxifen was delivered at E7.5 and embryos harvested at E9.5. **(B)** Representative images of tdTomato labeling (magenta) with SOX2 (gray) and TUBB3 (cyan). See Figure 6 for genotype of the controls and KO. Increased tdTomato labeling in epidermis is evident in KO mutants at E9.5 compared with controls (*n*=4 controls; *n*=4 KO); open arrows point to ectoderm and solid arrows to endoderm. Note the absence of fusion of ectoderm and endoderm in the mutants. Arrowheads indicate neuroepithelial tdTomato labeling. Sample sizes (n) for how many embryos showed fate change to each ectodermal derivative is indicated. 3 out of 4 embryos show a small rudimentary vesicle where otocyst should have formed in control embryos. **(C)** Representative images of tdTomato labeling (magenta) with SOX9 (yellow) and ECAD (light blue). Asterisks in panels B and C indicate neural crest cells. tdTomato+ neural crest cells are positive for SOX9. d, dorsal; fb, forebrain; hb, hindbrain; m, medial; oto, otocyst/inner ear. Scale bar = 100μm.

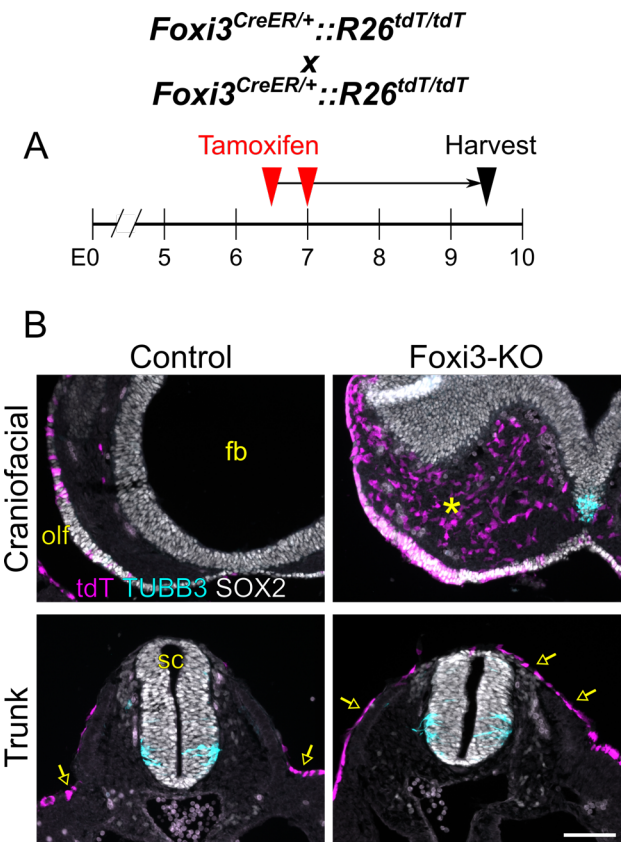


Fig. S8. No ectodermal lineage fate change observed in the trunk. (A) Experimental timeline for *Foxi3-CreER::Rosa-tdTomato* sibmating. Tamoxifen was delivered at E6.5 and E7.0 and embryos harvested at E9.5. **(B)** Representative images of tdTomato labeling (magenta) with SOX2 (gray) and TUBB3 (cyan). See Figure 6 for genotype of the controls and KO. Increased tdTomato labeling in epidermis is evident in KO mutants at E9.5 in both cranial and trunk sections compared with controls (*n*=3 controls; *n*=2 KO); open arrows point to ectoderm. However, the mesenchymal (presumed neural crest) labeling was only observed in the craniofacial region and not the trunk region. Scale bar = 100μm.

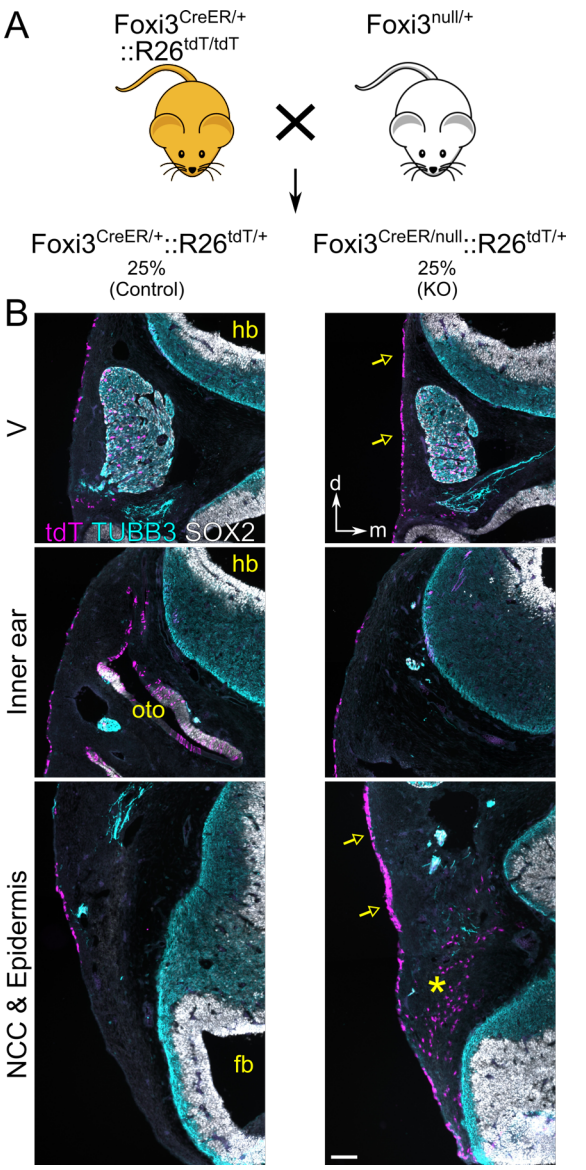
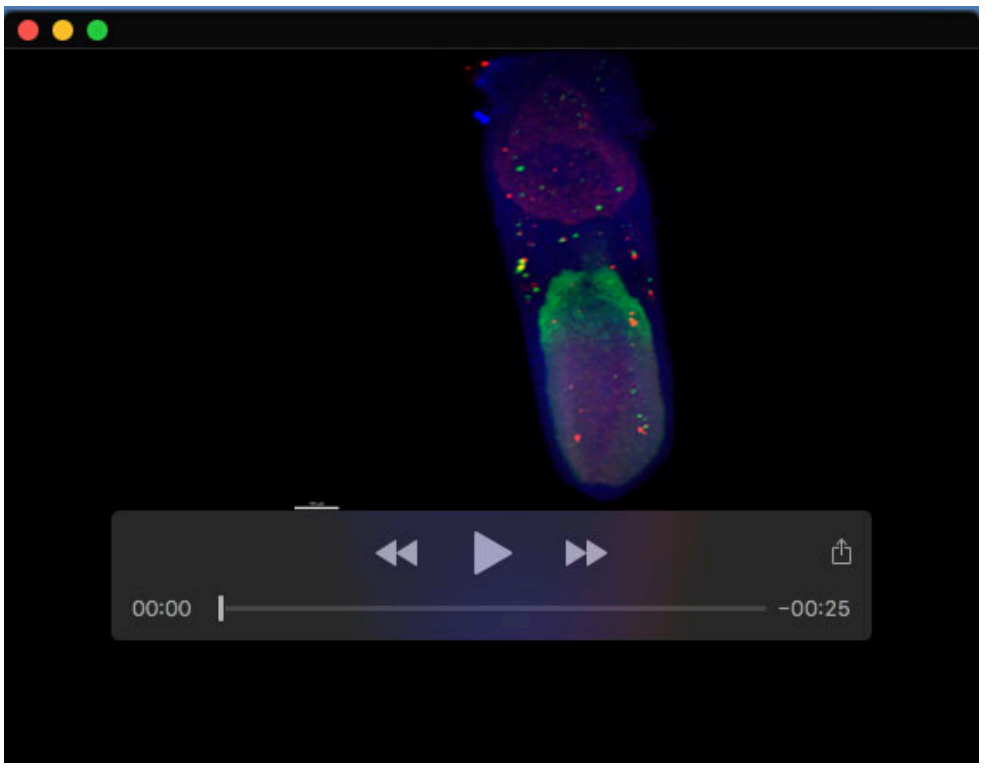
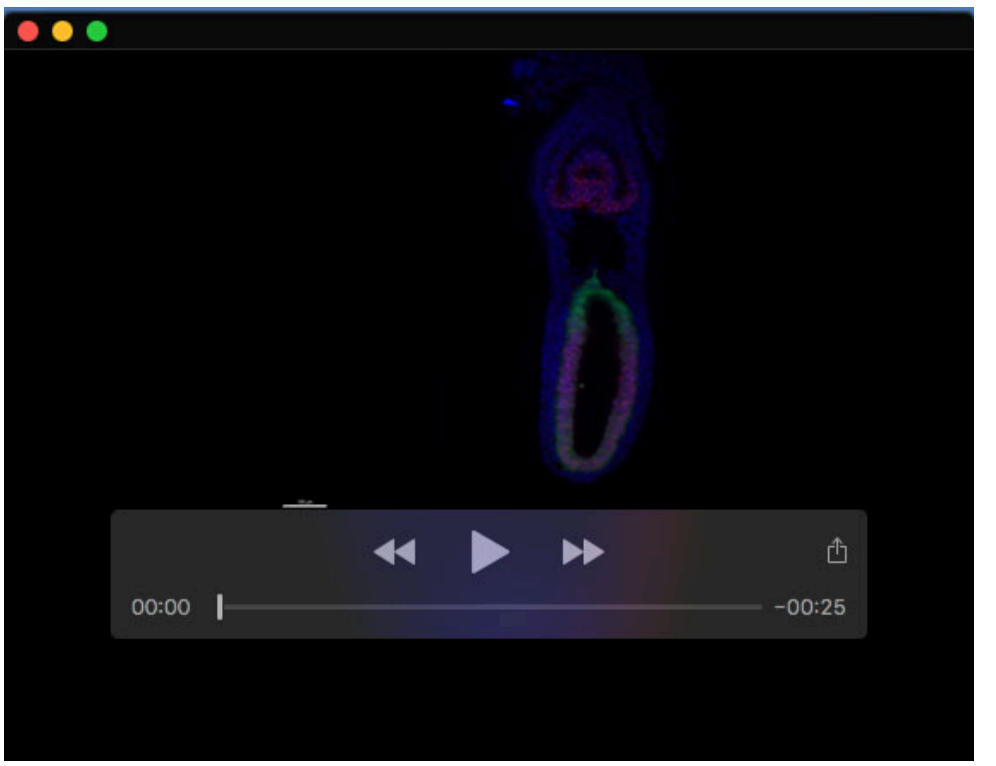


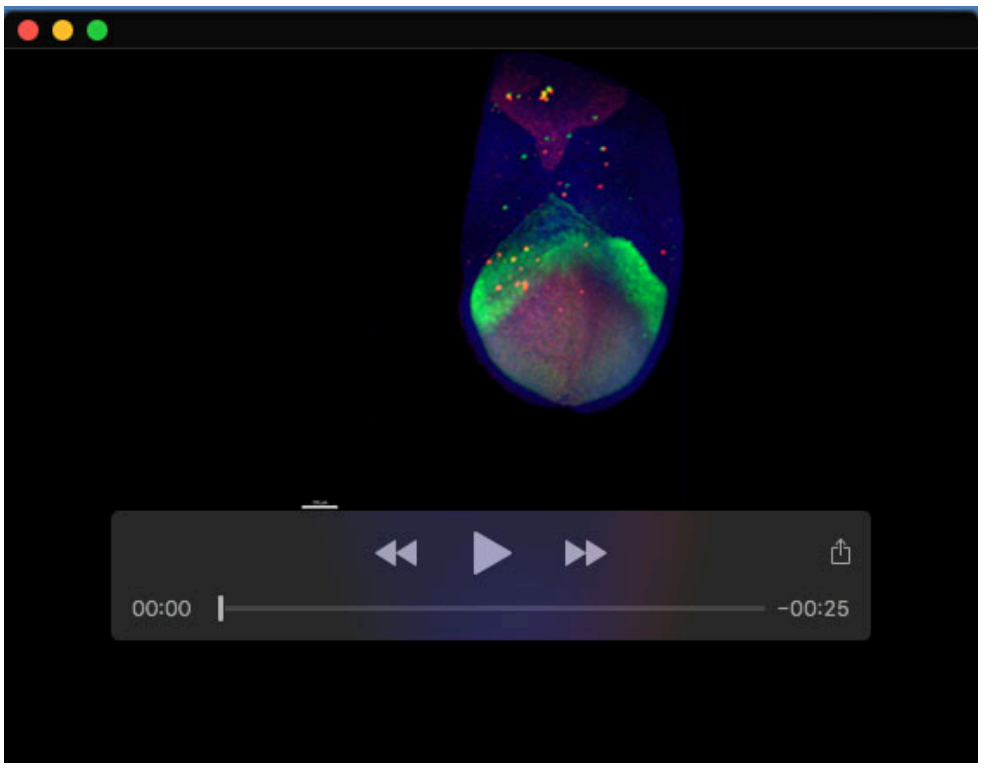
Fig. S9. One copy of CreER in Foxi3-knockout embryos also shows fate change of the neural plate border similar to the knockout mutants with two copies. (A) Mating scheme for generating knockout embryos with one copy of CreER compared to control littermates. Tamoxifen gavage was performed at E7.5 and embryos harvested at E11.5. **(B)** Examples of images showing similar tdTomato staining pattern at knockouts in Figure 6. Cryosections stained for tdTomato (magenta) with SOX2 (gray) and TUBB3 (cyan). Note the increased tdTomato signal in the epidermis in knockout mutants (open arrows) and neural crest derivatives (asterisks) compared with controls (*n*=3 controls; *n*=5 KO). None of the embryos showed significant tdTomato labeling in the neural epithelium. d, dorsal; fb, forebrain; hb, hindbrain; m, medial; oto, otocyst/inner ear. Scale bar = 100μm.



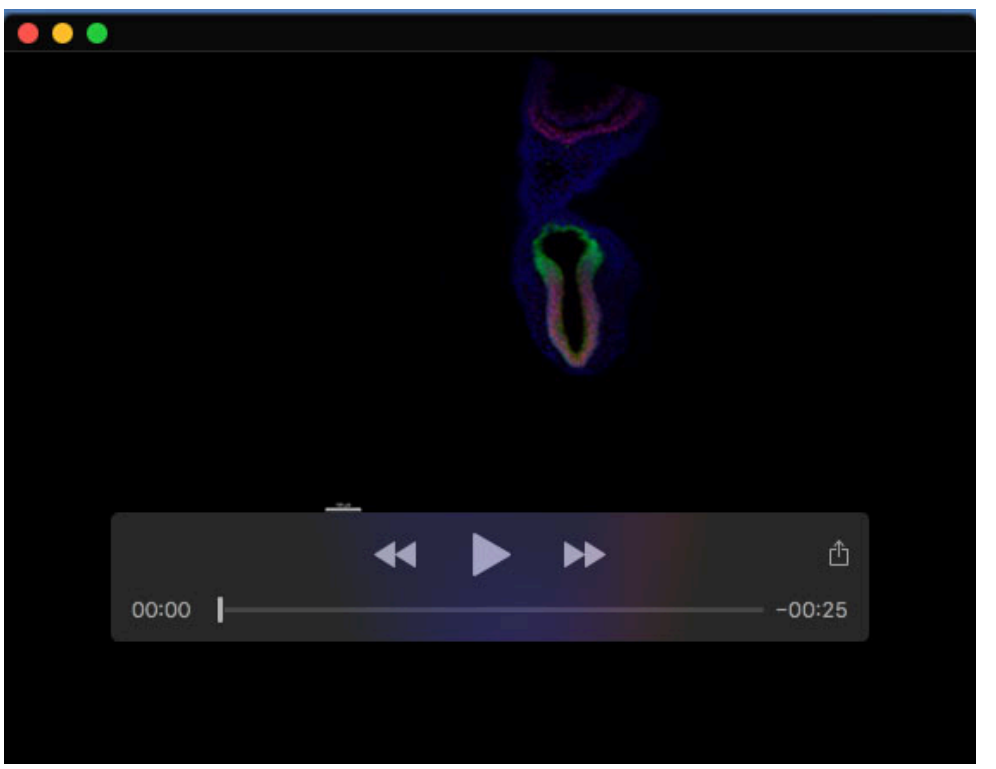
Movie 1. Lightsheet movie of a whole E7.0 Foxi3-GFP embryo stained for Foxi3-GFP (green), SOX2 (red) and DAPI (blue). The top half of the egg shape is extraembryonic tissue, bottom half is embryonic.



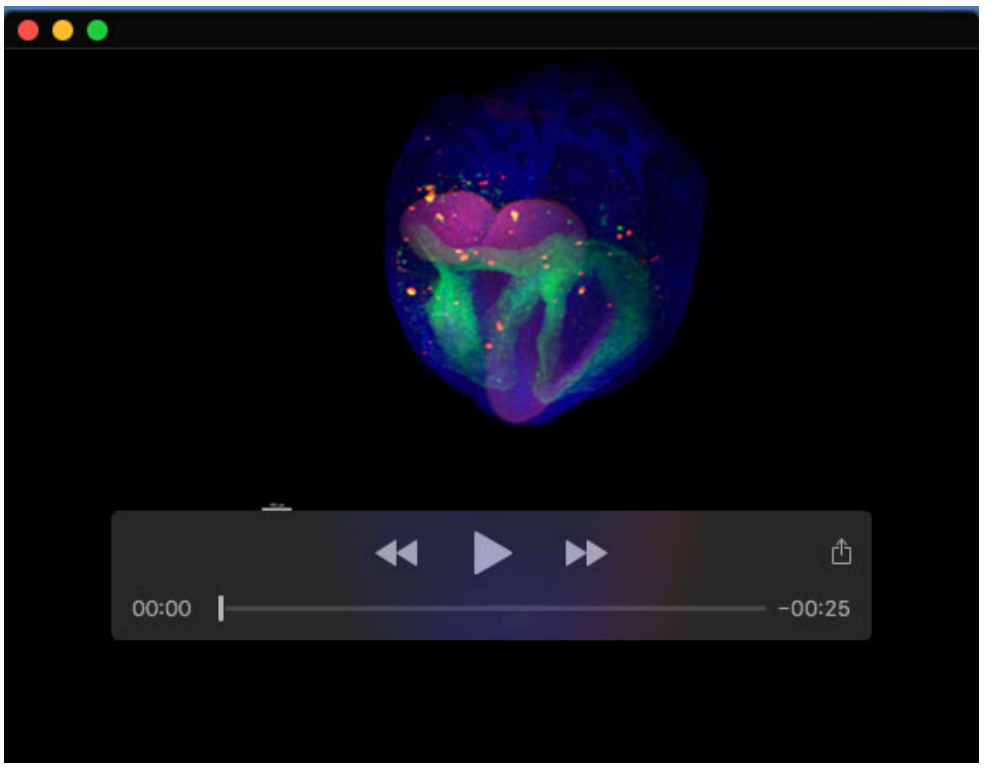
Movie 2. Optical sections through an E7.0 Foxi3-GFP embryo stained for Foxi3-GFP (green), SOX2 (red) and DAPI (blue). The optical sections along the anterior-posterior axis of the embryo were rendered from the light sheet data in Movie 1.



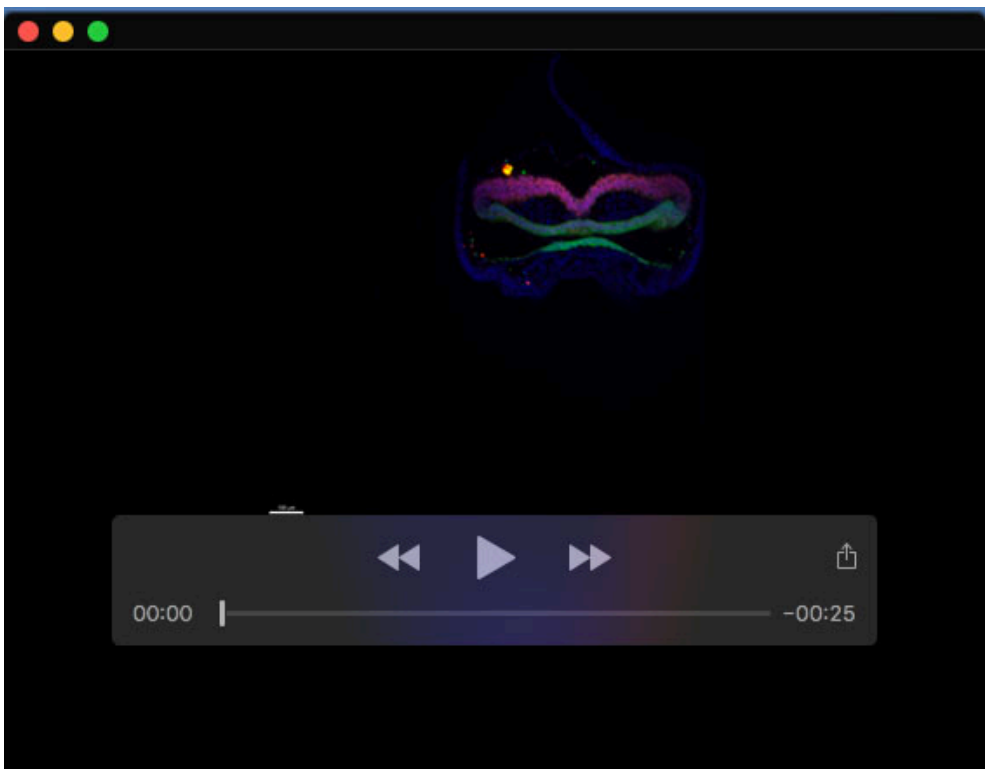
Movie 3. Lightsheet movie of a whole E7.5 Foxi3-GFP embryo stained for Foxi3-GFP (green), SOX2 (red) and DAPI (blue). The top half of the egg shape is extraembryonic tissue, bottom half is embryonic.



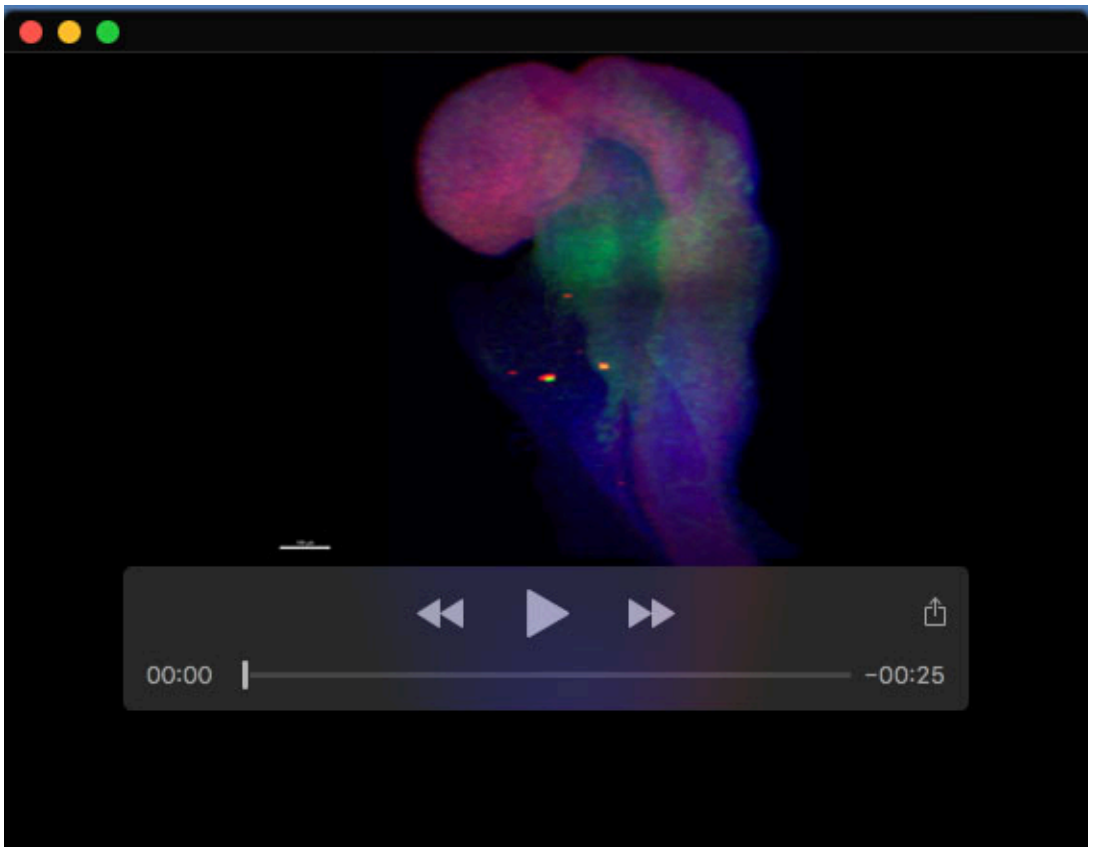
Movie 4. Optical sections through an E7.5 Foxi3-GFP embryo stained for Foxi3-GFP (green), SOX2 (red) and DAPI (blue). The optical sections along the anterior-posterior axis of the embryo were rendered from the light sheet data in Movie 3.



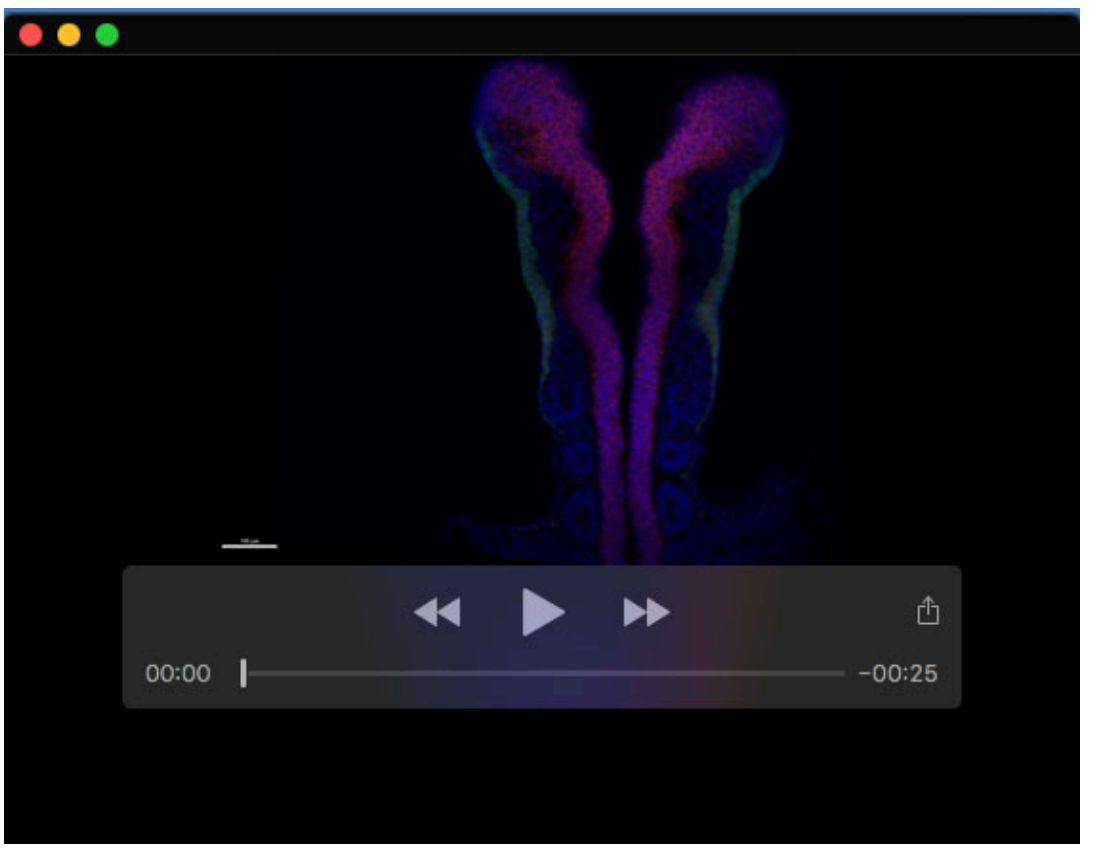
Movie 5. Lightsheet movie of a whole E8.5 Foxi3-GFP embryo stained for Foxi3-GFP (green), SOX2 (red) and DAPI (blue). The top half of the egg shape is extraembryonic tissue, bottom half is embryonic.



Movie 6. Optical sections through an E8.5 Foxi3-GFP embryo stained for Foxi3-GFP (green), SOX2 (red) and DAPI (blue). The optical sections along the anterior-posterior axis of the embryo were rendered from the light sheet data in Movie 5.



Movie 7. Lightsheet movie of the anterior half of an E8.75 Foxi3-GFP embryo stained for Foxi3-GFP (green), SOX2 (red) and DAPI (blue). the extraembryonic tissue has been removed.



Movie 8. Optical sections through an E8.75 Foxi3-GFP embryo stained for Foxi3-GFP (green), SOX2 (red) and DAPI (blue). The optical sections along the dorsal-ventral axis of the embryo were rendered from the light sheet data in Movie 7.