Figure S1. Identification of embryonic structures with whole embryo immunofluorescence. (A) Whole embryo maximum intensity projections from Fig. 1 with PAX8-positive (red) and SF1:eGFP-positive (green) structures labeled. (B) Digital slices of E11.5 XY embryos demonstrating somite and dorsal root ganglia identification through TUJ1 (gray) and αSMA (blue). Arrows point to the third dorsal root ganglia. Arrowheads indicate adjacent somite boundaries. (C) Comparison of total somites with tail somites in SF1:eGFP samples from E10.5 to E12.5. The lower x-axis displays the embryonic stages typically correlated with the tail somite counts in the upper x-axis, as established by Hacker et al., 1995. Note that there are two XY E10.5 samples with identical total somite and tail somite counts.

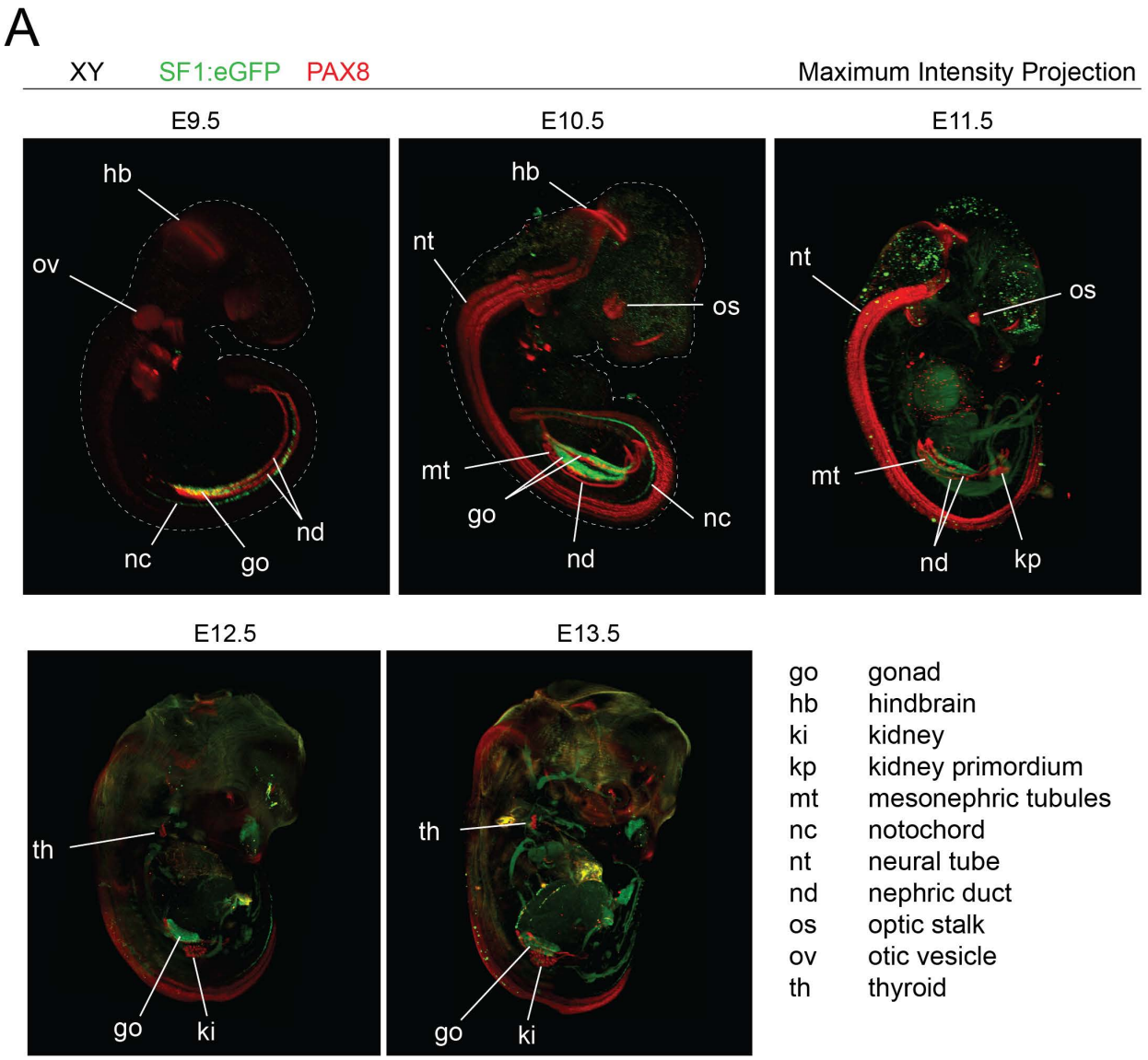


Figure S2. Digital slices of XY whole embryos showing organs adjacent to the genital ridges from E10.5 to E12.5. Transverse (50μm thick) and sagittal (150μm thick) digital slices. Transverse slices are centered at 300μm along the AP axis of the gonads. Gray values are a merge of background immunofluorescence signals from multiple antibodies. Organs are false colored for identification.

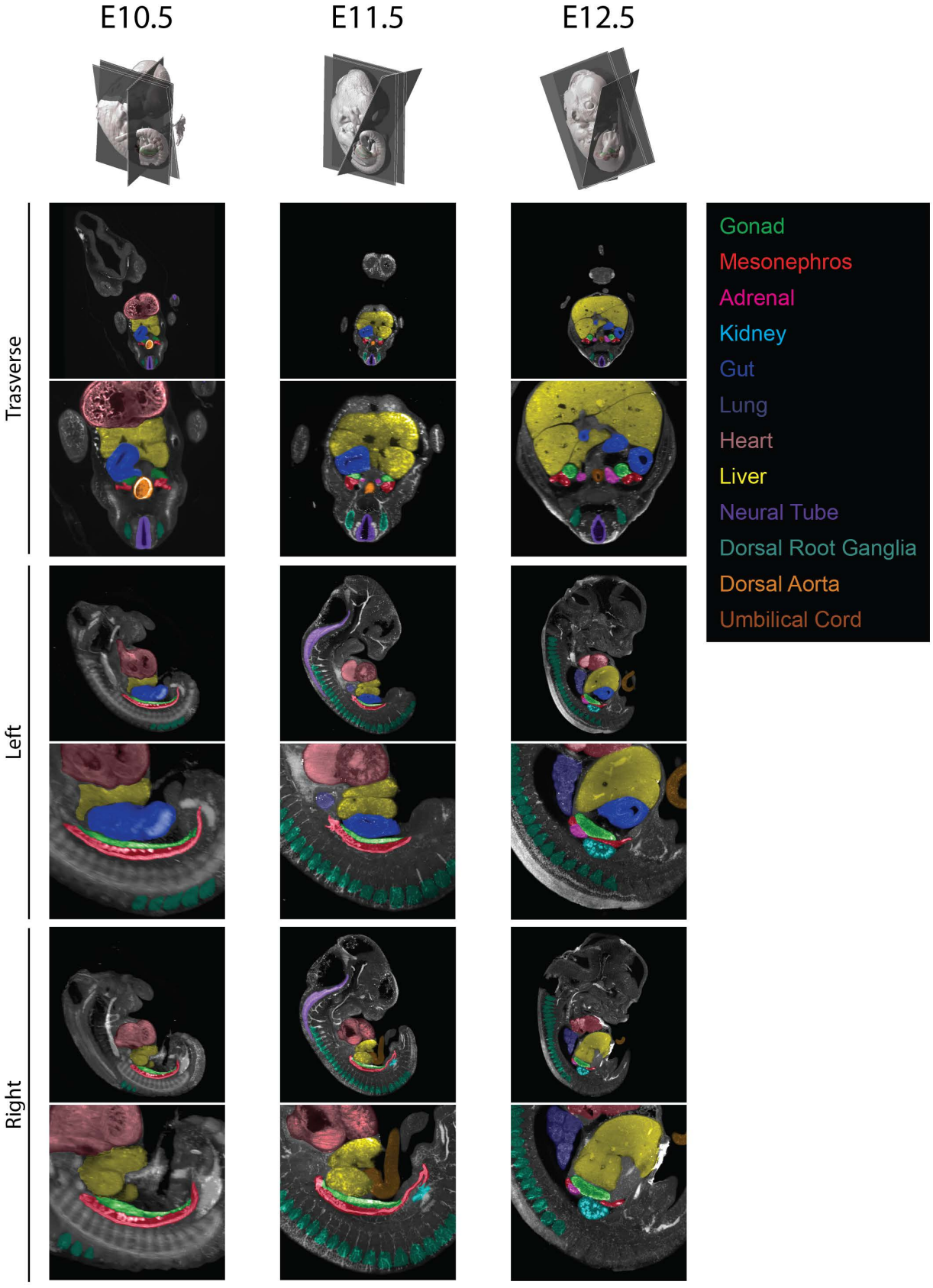


Figure S3. Somite alignment of gonad, mesonephric duct, and limbs for individual Tg(*Nr5a1-GFP*) samples. Graph showing somite alignment of genital ridge (green), mesonephric ducts (red), and limb buds (cyan) relative to somites for all fourteen embryos in the Tg(*Nr5a1-GFP*) time course. Arranged by total somites. Showing right (R) and left (L) sides.

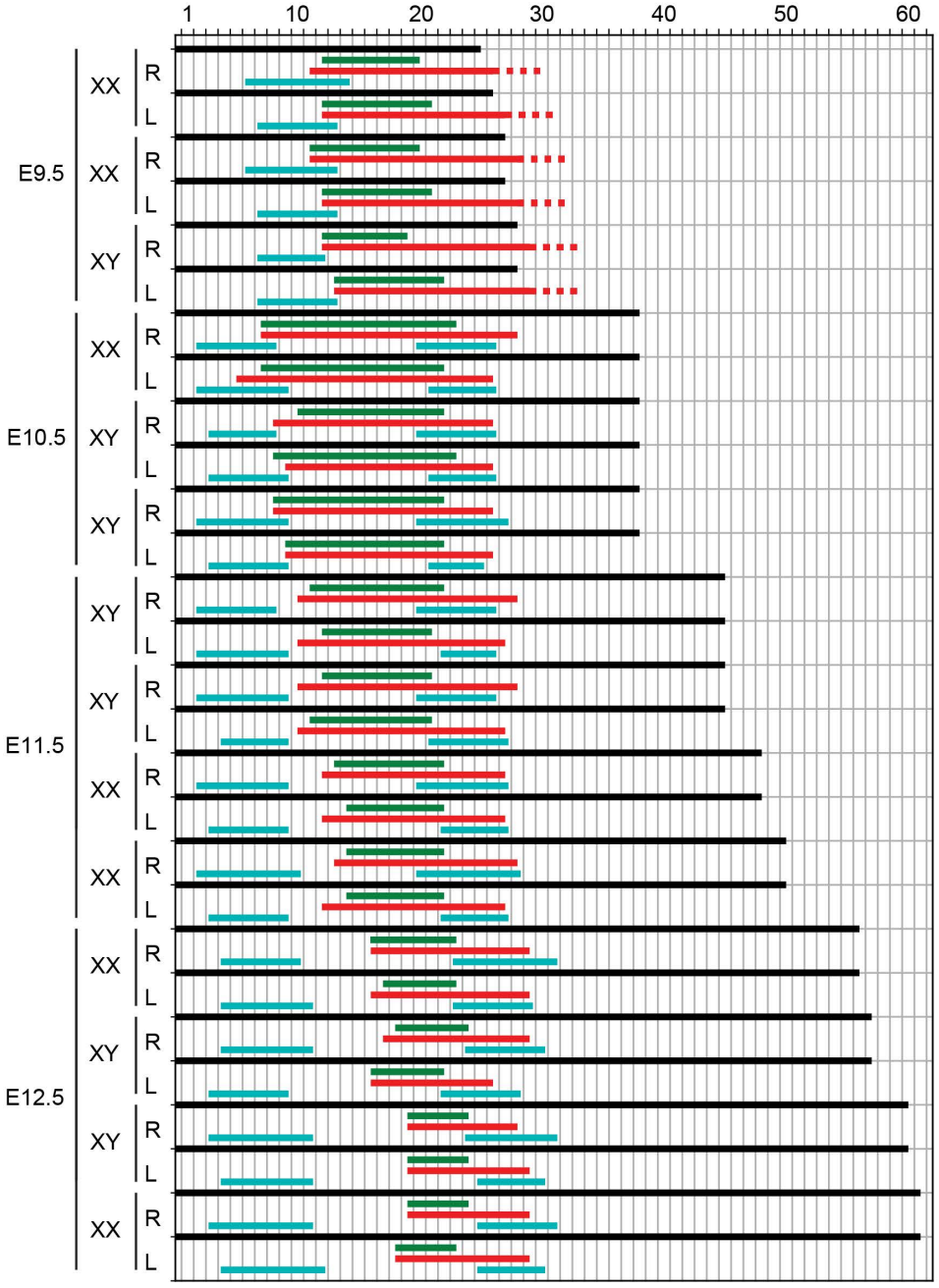


Figure S4. Comparison of NR5A1 and SF1:eGFP in Tg(*Nr5a1-GFP*) urogenital complexes. Each stage includes a maximum intensity projection of the full urogenital complex as well as two single z-slices (2.5 µm thick, region indicated by white box). Slices were taken from ventral (v, upper panel) and dorsal (d, lower panel) planes. The adrenal can be seen developing dorsomedially to the gonad beginning at E11.5. Organs are indicated by dotted lines (white, gonad; magenta, adrenal). Scale bar: 100µm.

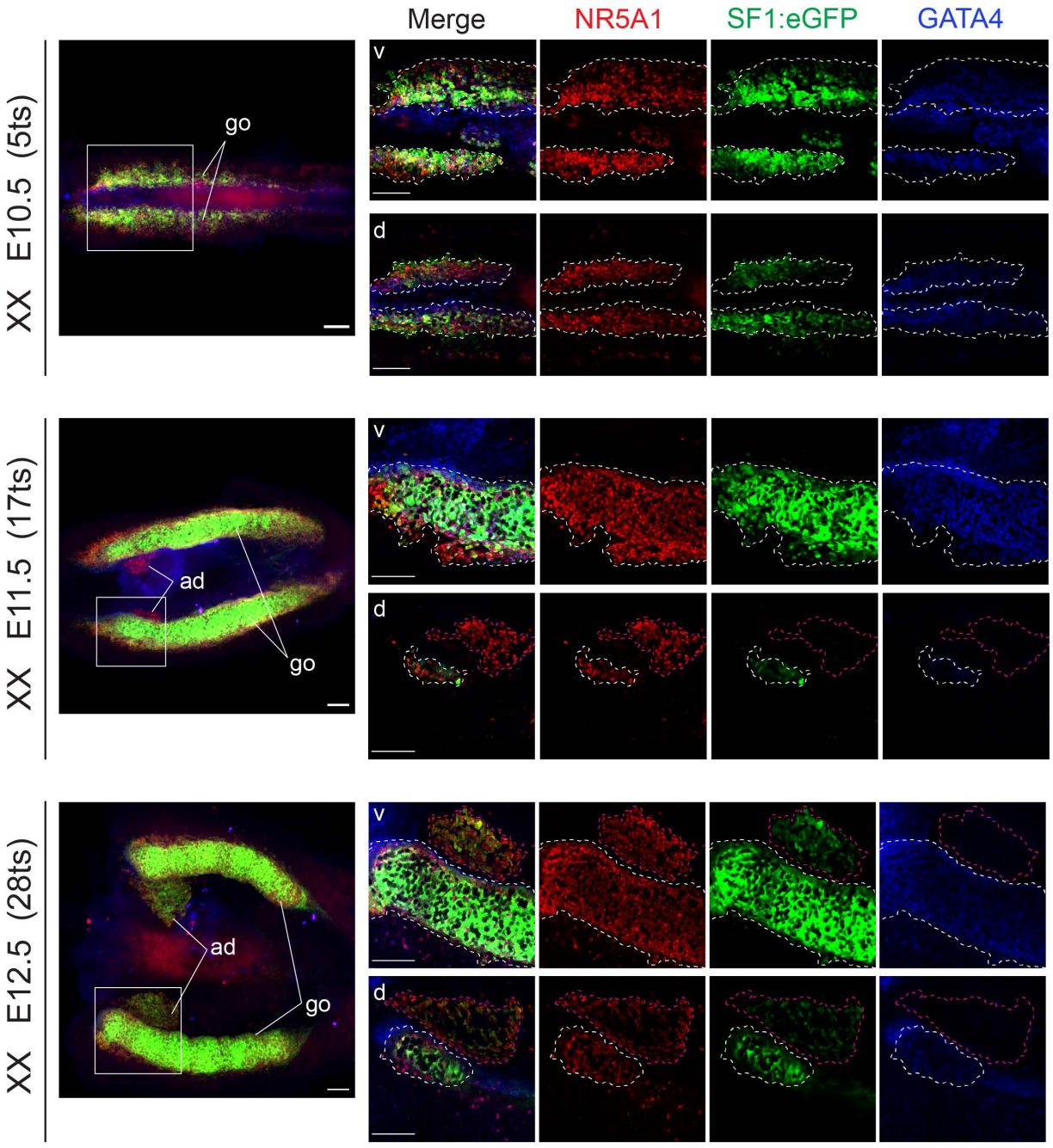


Figure S5. Multidimensional shifts of urogenital organs relative to each other in XX embryos. Data depicting shifts in position of the ducts, gonads, adrenals and kidneys from XX embryos at E11.5, E12.5, and E13.5. Images in the first column contain SF1:eGFP and PAX8 immunofluorescence displayed with 'normal shading', using urogenital segmentations (shown to the right) to mask values outside the urogenital system to 0, with semi-transparent whole embryo isosurfaces to show the position of the complex in the embryo. Isosurface segmentations include gonads (green, based on SF1:eGFP), adrenals (magenta, based on SF1:eGFP), mesonephric ducts (M. DUCT) and kidneys (red, based on PAX8). Black arrowheads indicate the kidney primordium, white arrowheads indicate the growing kidney. R, right side. See Fig. 4 for XY embryos. Measurements are given for the distance from the center point between the left and right gonads to the surface of the embryo between the aligned somites (*) and the distance between the center points of the left and right gonads (**).

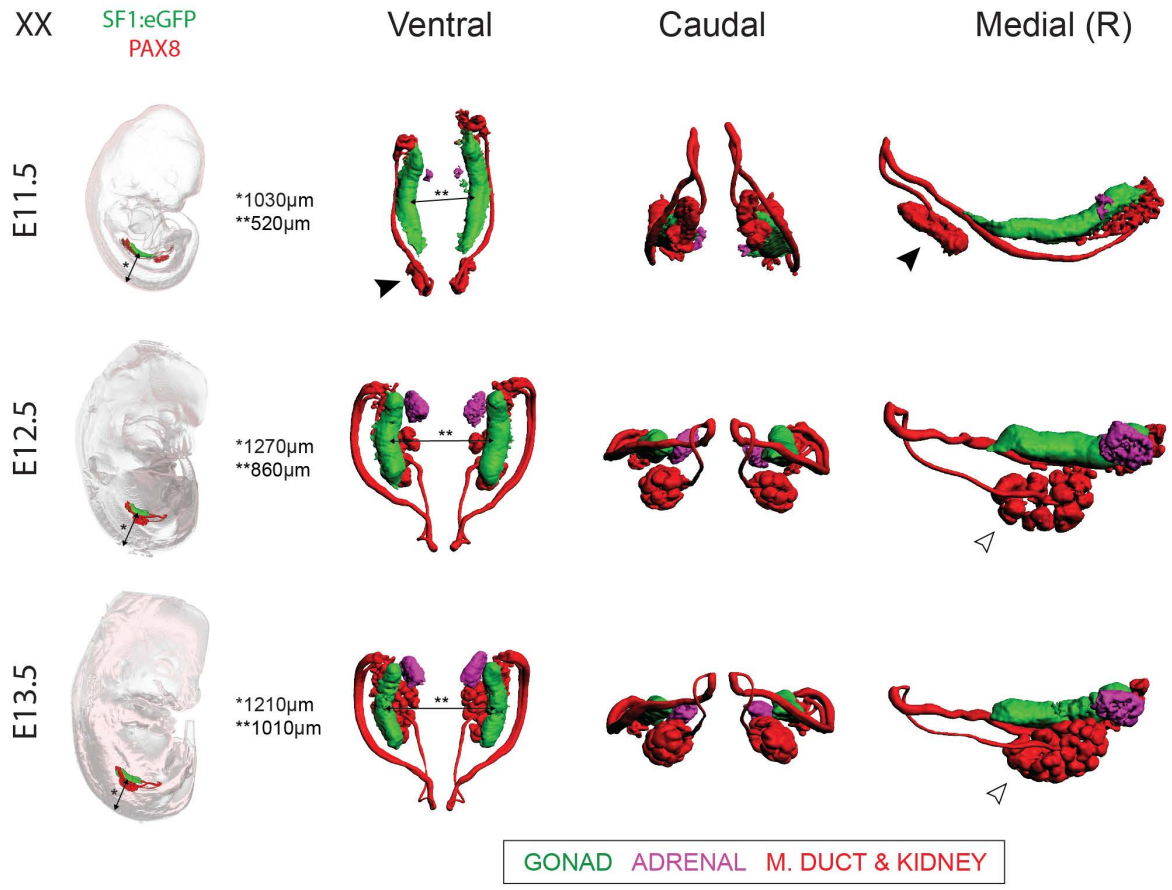


Figure S6. Genital ridge morphological analysis pipeline. (A) Maximum intensity projections from an E11.5 XY sample showing the whole embryo, the genital ridges, and the gonads with a measurement scaffold overlaid for analysis with Imaris software. The scaffold includes a centerline over each gonad, cross lines every 100μm starting from the anterior pole, and a line below the right gonad composed of points used to orient the digital slices. SF1:eGFP, green; PAX8, red; αSMA, blue. (B) At every cross line in the scaffold, a digital slice (10μm thick) was created perpendicular to the AP axis of the gonad around that point. In each slice, the Imaris "measurement" tool (in white) was used to measure morphological features as indicated by the schematics based on the gonad (SF1eGFP, green), the nephric duct (PAX8, red), and the dorsal aorta (αSMA, blue).

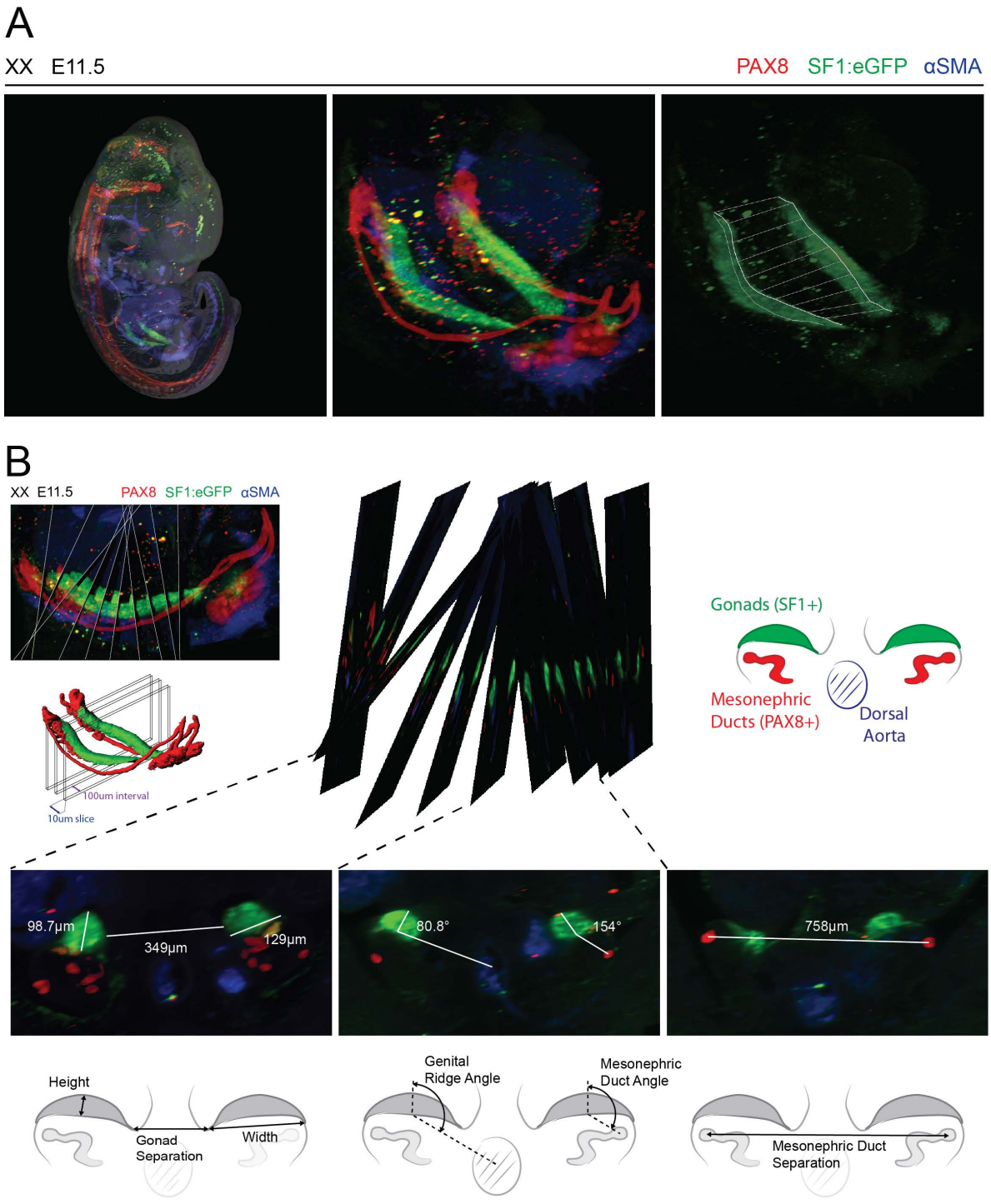


Figure S7. Mesonephric tubule isosurfaces comparing embryo sides and sexes. (A) Isosurface segmentations of nephric ducts and kidneys (based on PAX8) from XY embryos at E10.5, E11.5, and E12.5. Arrowheads indicate pronephric regions (white), connections between individual mesonephric tubules and the main mesonephric duct (black), and initial separation of the Müllerian duct from the mesonephric duct at E12.5. Note the variation in mesonephric tubule morphology between the left and right sides. No consistent differences were detected. (B) Isosurface segmentations of nephric ducts and kidneys (based on PAX8) form XY and XX embryos as E13.5 and E14.5. At these stages, sex-specific duct development is not yet apparent.

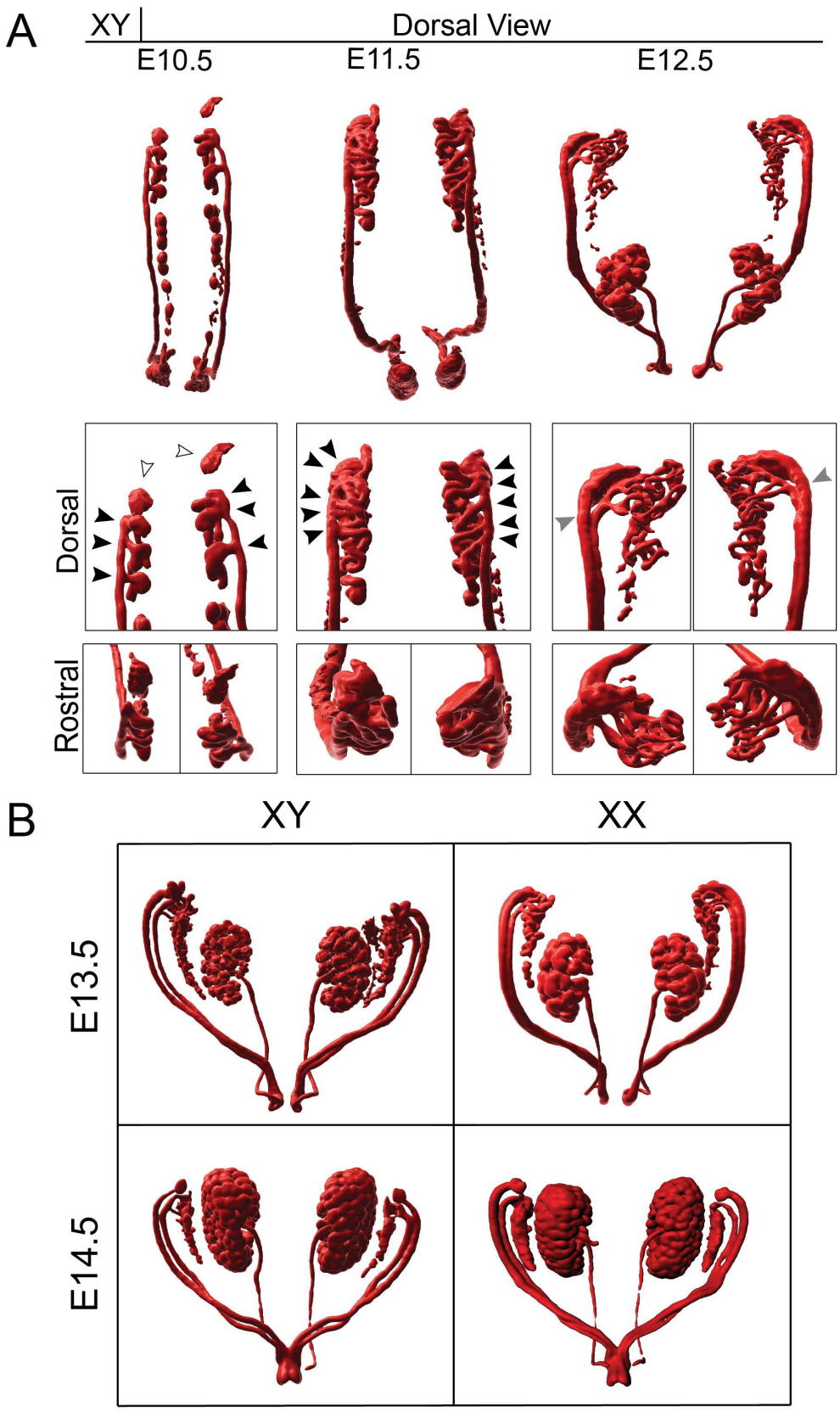
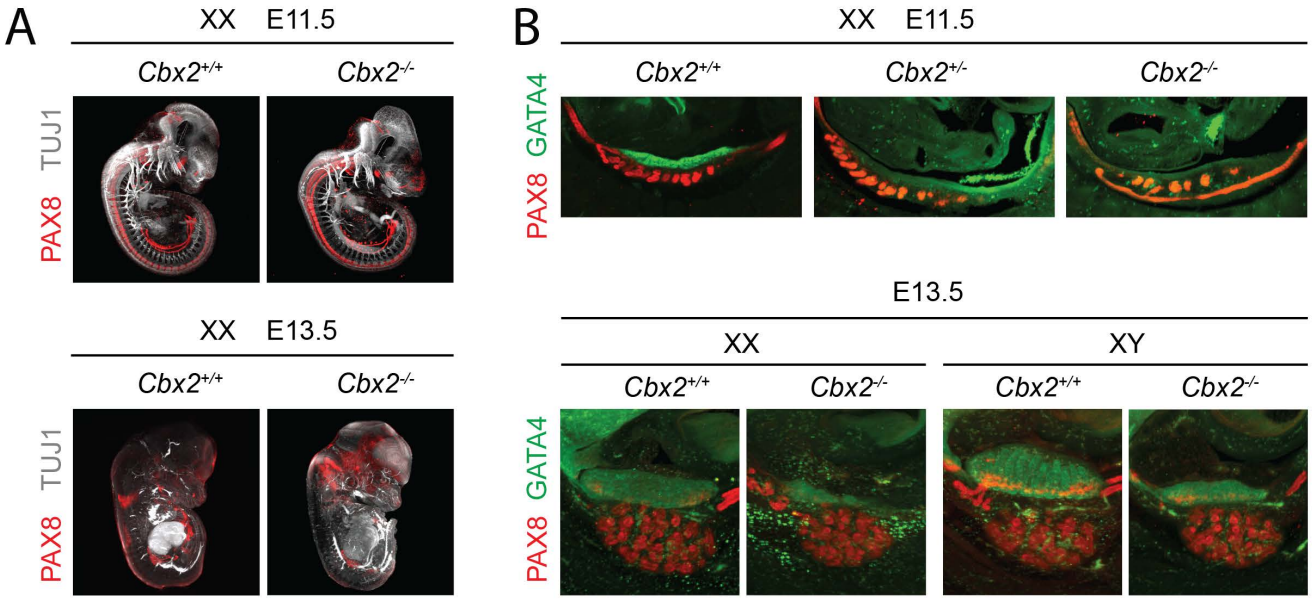


Figure S8. *Cbx2* mutants display reduced nephric duct development at E11.5. (A) Maximum intensity projections of XX wild type and *Cbx2*-knockout embryos at E11.5 and E13.5. PAX8, red; TUJ1, gray. (B) Sagittal slices (20μm thick) of right genital ridges and nephric ducts from E11.5 and E13.5 wild type and *Cbx2* mutant embryos. Note the reduced genital ridge thickness in XX *Cbx2* mutants at E11.5 and apparent XY sex reversal at E13.5 (absence of testis cords). PAX8, red; GATA4, green. (C) Comparison of nephric ducts between *Cbx2* wild type, heterozygous, and knockout embryos at E11.5. Whole embryo views show PAX8 (red) and TUJ1 (gray) immunofluorescence displayed with 'normal shading'. Nephric duct isosurfaces are colored by side (left, cyan; right, red). White arrowheads indicate pronephric regions. Black arrowheads indicate connections between individual mesonephric tubules and the main mesonephric duct. Mesonephric tubule orientation is indicated with dotted lines.



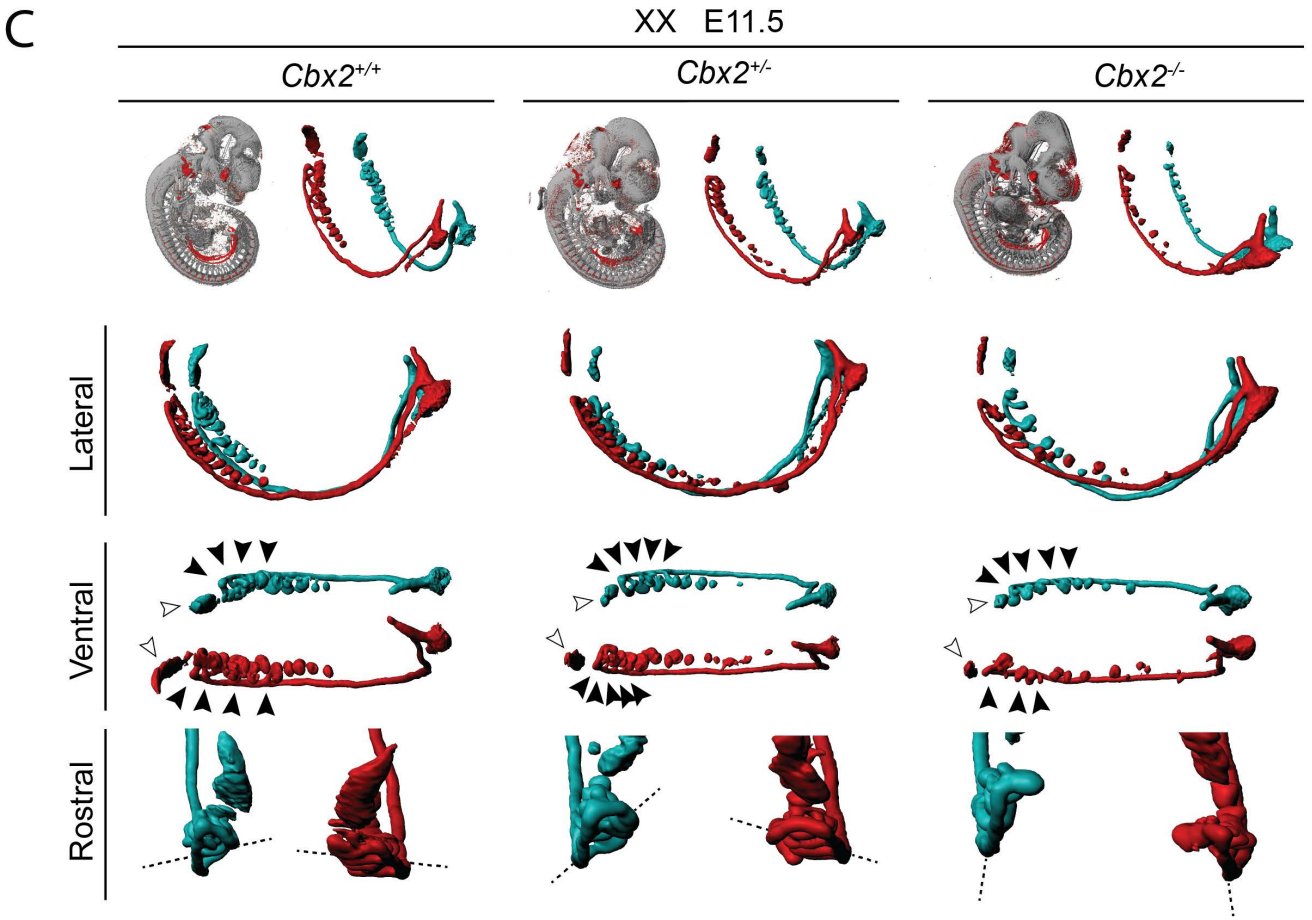
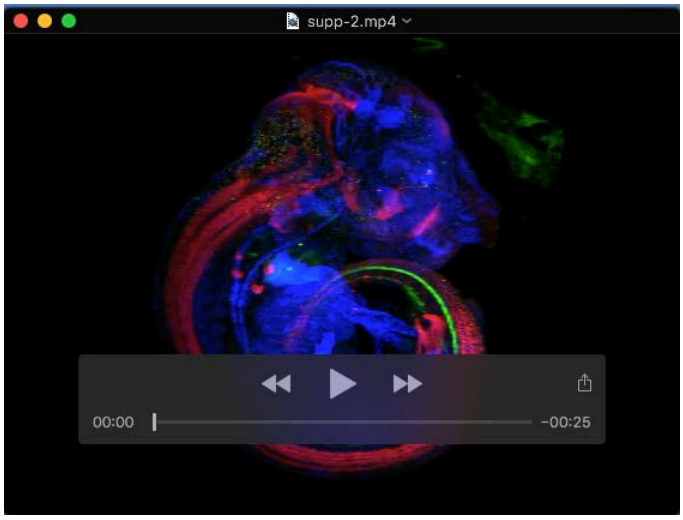


Table S1. Primary and secondary antibodies used in this study.

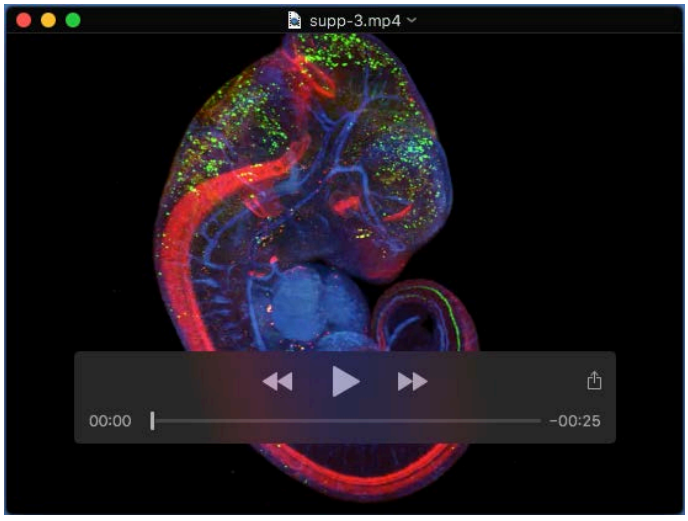
Antibody	Host	Dilution	Source	Product number
GATA4	Goat	1:500	Santa Cruz Biotechnology	Sc-1237 (discontinued)
GFP	Chicken	1:1000	Abcam	Ab13970
NR5A1	Rabbit	1:500	TransGenic Inc.	KO611
PAX8	Rabbit	1:500	Proteintech	10336-1-AP
αSMA (FITC-conjugated)	Mouse	1:500	Sigma	F3777
TUJ1 (AF488-conjugated)	Mouse	1:500	BioLegend	801203
AF647-anti-Rabbit	Donkey	1:500	Jackson ImmunoResearch	711-165-152
Cy3-anti-Goat	Donkey	1:500	Jackson ImmunoResearch	705-165-147
AF546-anti-Chicken	Donkey	1:500	Invitrogen	A-11040

Table S2. Genotyping primers used in this study.

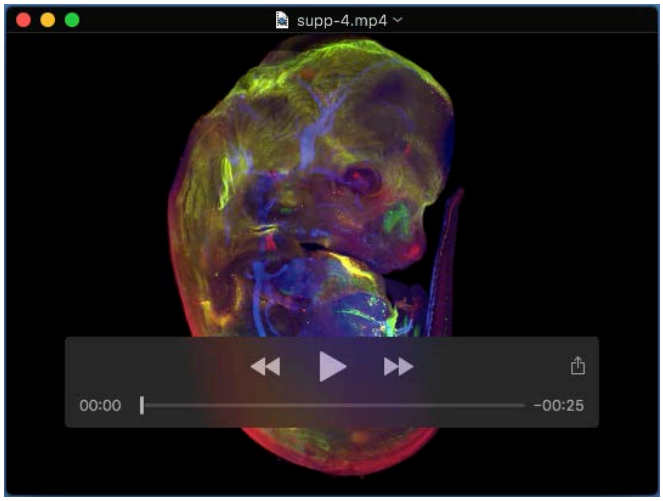
Allele	Forward primer	Reverse primer	Allele-specific primer
Cbx2 null	GTAGCCAAGCCAGAGCTGAA	ACCACAGGCCTCTTTGGTGT	CCGCTTCCATTGCTCAGCGGT
Tg(*Nr5a1-GFP*)	CACCATCTTCTTCAAGGACGAC	GAATGACACCTACTCAGACAATGC	N/A
Sry	GTGTCTCAAAGCCTGCTCTTC	CATGTACTGCTAGCAGCTATC	N/A
Myogenin (internal control)	TTACGTCCATCGTGGACAGCAT	TGGGCTGGGTGTTAGTCTTAT	N/A



Movie 1. 3D imaging of urogenital morphology at E10.5. 3D reconstruction of an E10.5 XY mouse embryo displayed as a maximum intensity projection with SF1:eGFP (green), PAX8 (red), and aSMA (blue) immunofluorescence and isosurface segmentations of embryo surface (gray), gonads (green), adrenals (magenta), and mesonephric ducts and kidneys (red).



Movie 2. 3D imaging of urogenital morphology at E11.5. 3D reconstruction of an E11.5 XY mouse embryo displayed as a maximum intensity projection with SF1:eGFP (green), PAX8 (red), and aSMA (blue) immunofluorescence and isosurface segmentations of embryo surface (gray), gonads (green), adrenals (magenta), and mesonephric ducts and kidneys (red).



Movie 3. 3D imaging of urogenital morphology at E12.5. 3D reconstruction of an E12.5 XY mouse embryo displayed as a maximum intensity projection with SF1:eGFP (green), PAX8 (red), and aSMA (blue) immunofluorescence and isosurface segmentations of embryo surface (gray), gonads (green), adrenals (magenta), and mesonephric ducts and kidneys (red).