Supplemental Figure 1

***Ret KO* (*Ret* $^{EGFP/EGFP}$) mutant mice exhibit similar Wolffian duct (WD) defects as in *Ret Y1062F* mice.**

A. (top) The illustrations depict the diagram of *Ret KO* and *Ret EGFP* (*Ret*$^{EGFP/+}$) control mice. (bottom) E11.5 whole-mount immunofluorescence of urinary tract with E-cad (white, WD, CND and cloaca) antibody shows *Ret KO* WD fails to reach cloaca. The images were acquired by confocal imaging with z-stacks. Red arrowhead in *Ret EGFP* denotes successful contact between the WD and cloaca and in the *Ret KO* mutant image shows the gap between WD and cloaca . See also Figure 1.
B. Whole-mount immunofluorescence images with GFP (green, WD & UB) and Pax2 (red, WD, UB & MetM) antibodies in E11.5 urinary tract shows normally developed MetM in *Ret KO* mutant urinary tract, same phenotype as seen in *Ret Y1062F* mutantWDs. WD; wolffian duct, UB; ureteric bud, CND; common nephric duct, MetM; metanephric mesenchyme, scale bar=100μm.

Supplemental Figure 2

pERK and proliferation defects in *RetY10162F* mutant Wolffian ducts..

A. The anterior (proximal) *RetY1062F* mutant WDs do not show loss of pERK staining.

Whole-mount immunofluorescence images with EGFP (green, Ret positive) and pERK (magenta) antibodies of E9.5 lower body of embryos of *RetEGFP* control (left) and *RetY1062F* mutant (right) mice reveal that ERK activity in the rostral part of WD (dashed line) shows no significant difference. The caudal aspect of the mutant WDs

show decreased ERK activity as discussed in the main text. The inset shows low power view with green GFP signal in the WD. The decrease of pERK outside the WDs in *RetY1062F* mutants is likely due to two reasons. First, developmental defects in neural crest cells where Ret signaling is important (Jain et al, JCI, 2010). Second, potential effect of RET-mediated abnormal signaling in WD on mesenchymal cells (Hoshi et al, Development, 2012); here increased ERK activity was shown to promote mesenchymal survival. More studies would be needed to determine Ret-WD autonomous and nonautonomous mechanisms on neighboring cell populations.

B. Reduced proliferation in distal WDs of *RetY1062F* mutant mice at E9.5. Whole-mount immunofluorescence images with EGFP (green, Ret positive WD) and pHH3 (blue, proliferating cells) show significant reduction of proliferation in the distal end of mutant WD. See also Fig. 2B. WD; wolffian duct, scale bar=100μm.

Supplemental Figure 3

AKT phosphorylation has no significant difference in WDs at E9.5 between *RetEGFP* control and *RetY1062F* mutant mice.

A. Whole-mount immunofluorescence images with EGFP (green, Ret positive WD) and pAKT (red) antibodies of lower body of E 9.5embryos of *RetEGFP* control (top) and *RetY1062F* mutant (bottom) mice show no significant phosphorylation of AKT in WD (dashed lines). At this stage, pAKT staining is weak in general in the WDs compared to the surrounding sympathetic chain region in both control and mutant (right, magnified images).

B. At E10.5 pAKT has more prominent staining in WD in both *RetEGFP* control and *Ret KO* mutant in contrast to pERK defects in the WD in these mice. WD; wolffian duct, scale bar=200μm.

C. No primary antibody control showed no significant staining in the specimen (right). Scale bar=50μm.

Supplemental Figure 4

Loss of ERK activity causes failure of the connection of WD with cloaca.

E10.0 control urinary tracts were grown *ex vivo* with MEK inhibitor U0126 (10μM) or vehicle (DMSO). Whole-mount immunofluorescence images with Pax2 (green, WD and MetM) and E-cad (red, WD and cloaca) after 24h of culture reveal that U0126-treated WDs failed to reach cloaca, suggesting ERK-MAPK activity is crucial for proper WD-cloaca connection. WD; wolffian duct, Scale bar=200μm.

Supplemental Figure 5

***RetY1062F* mutant WDs fail to contact the cloacal epithelia and show runted cellular protrusions at the leading edge of WD .**

A. Whole-mount 3D maximal intensity projection confocal GFP immunofluorescence images with 100x objective clearly show marked reduction in cellular protrusions in *RetY1062F* mutant ($Ret^{EGFP/Y1062F}$) WD tip. Note the long protrusions in the control *RetEGFP* ($Ret^{EGFP/+}$) WDs. Scale bar=20μm.

B. Cross sectional and 3D X-ray nano CT images of *RetEGFP* control ($Ret^{EGFP/+}$) and *RetY1062F* mutant ($Ret^{EGFPY1062F}$) WDs confirm WD fusion and cellular protrusion

defects to contact cloaca in *Ret Y1062F* mutant mice. Whole E9.75d embryos were stained with Ret antibody (Cell Signaling Technology, #C31B4) and processed for 3D X-Ray nano CT scanning capable of submicron resolution (see methods). Green dashed lines depict WDs in the cross sectional images on the left. Yellow arrowheads show the contacts of cellular protrusions of WD with cloaca in *RetEGFP* control embryo in the right 3D rendered images. Note that fine protrusions from the control WDs (pseudocolored red) contacts cloaca (cyan), almost piercing it (also see Mov 4). In contrast, the mutant WDs viewed from various angles fail to contact cloaca or extend cellular protrusions to it (also see Mov5). WD, wolffian duct, Scale bar=50μm.

Supplemental Movie 1.

This movie shows whole-mount urinary tract culture of E11.5 *Ret EGFP/+* and *Ret Y1062F/EGFP* mutant embryos for 48 hours. Both movies are merged into one beginning with control *Ret EGFP/+* embryos. Note that the control urinary tract culture shows normal UB branching (green) and contact with urogenital sinus. Ret mutant urinary tract shows no obvious UB budding or branching during the course of the culture.

Supplemental Movie 2.

The movie is of sequential z-stack confocal images processed from whole-mount immunofluorescence microscopy of *RetEGFP* WD (*Ret EGFP/+*) stained with GFP (green, WD with protrusions) and E-cad (red, cloaca and body wall) at E9.5 (28 somites). Note that many sharp cellular protrusions (like neurites or filopodia) are growing from the control WD tip region especially a “spike” contacting cloaca.

Supplemental Movie 3.

The movie of sequential z-stack confocal images processed from whole-mount immunofluorescence microscopy of *RetY1062F* mutant WD (*Ret Y1062F/EGFP*) stained with GFP (green, WD with protrusions) and E-cad (red, cloaca and body wall) at E9.5 (28 somites). Compared to the control images (Supplemental movie 2), this mutant WD does not reach cloacal epithelia and has no prominent cellular protrusions.

Supplemental Movie 4.

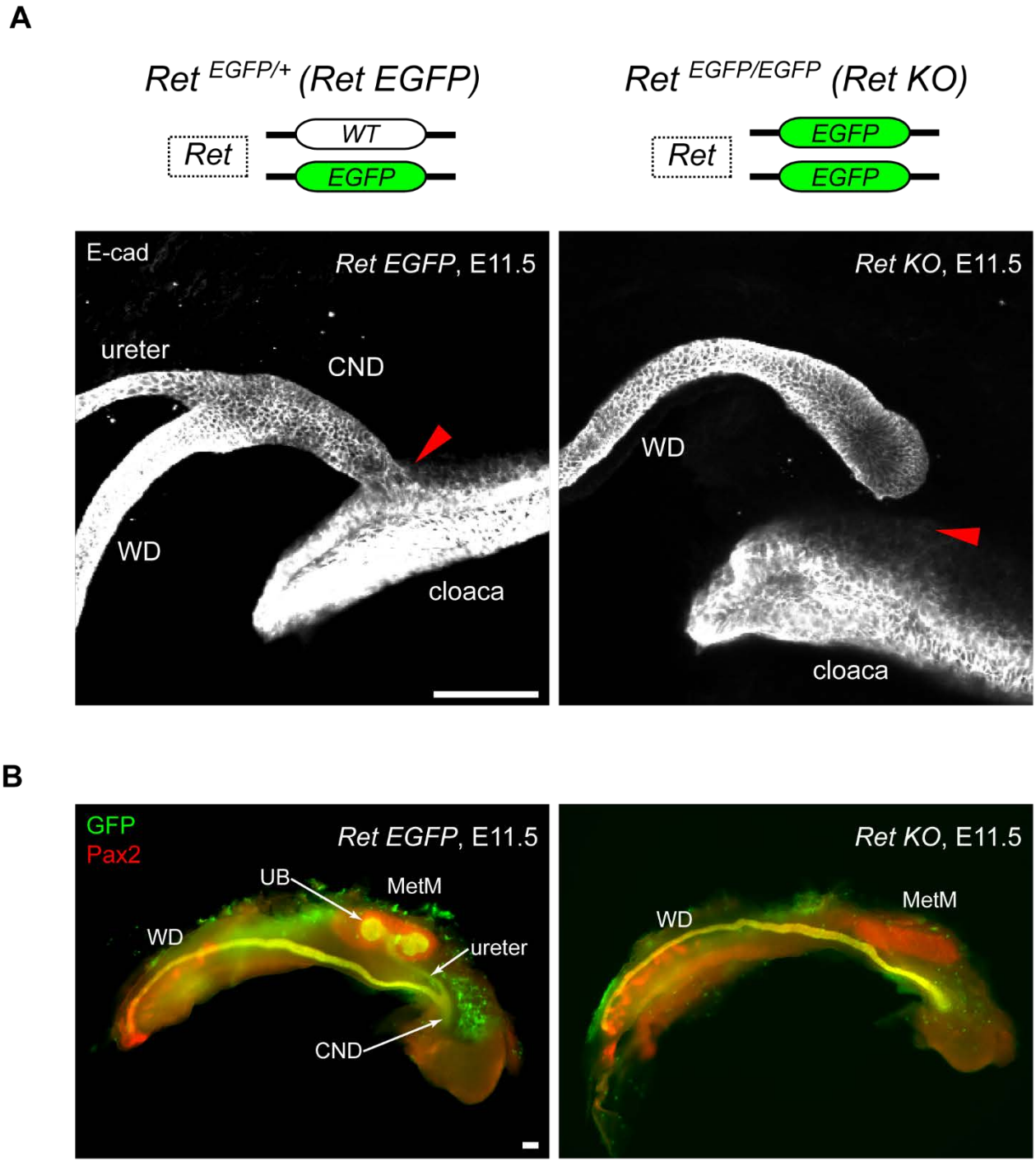
The movie is generated form 3D X-ray nano CT of whole E9.75d RetEGFP control embryos. The movie begins with cross-sectional images in the plane of cloaca and show WD1 at the cloaca appearing in the bottom left at about 3s, and the WD2 at about

4s at bottom right (bright intensities, see Supp Fig 5B). The movie transitions in to 3D rendering with pseudo coloring of the cloaca with cyan and the WD with bright red (about 12s), shows a panoramic view of the lower part of the embryo and then goes into details of the anatomic relationship of the WD with the cloaca (about 30s). At about 35s, it shows both WD contacting the cloaca and then zooms into closer view showing clear contact of the WD1 with the cloaca through cellular protrusions (about 39s) and then gives a panoramic view of these contacts and the other WD (about 44s onwards).

Supplemental Movie 5.

The movie is generated form 3D X-ray nano CT of whole E9.75d RetY1062F mutant embryos (*Ret Y1062F/EGFP*). The movie begins with cross-sectional images in the plane of cloaca and show WDs appearing in the bottom and top right at about 2s (see Supp Fig 5B). The movie transitions in to 3D rendering with pseudo coloring of the cloaca with cyan and the WD with bright red (about 8s), shows a panoramic view of the lower part of the embryo (18s onwards) and then goes into closer details of the anatomic relationship of the WD with the cloaca (about 30s). At about 35s, it shows both mutant WDs are unable to reach the cloaca and then zooms into closer view showing failure of WD1 to contact cloaca through cellular protrusions (about 40s) and then gives a panoramic view of WD-cloaca fusion defect including the other WD (about 42s).

Supplemental Figure 1



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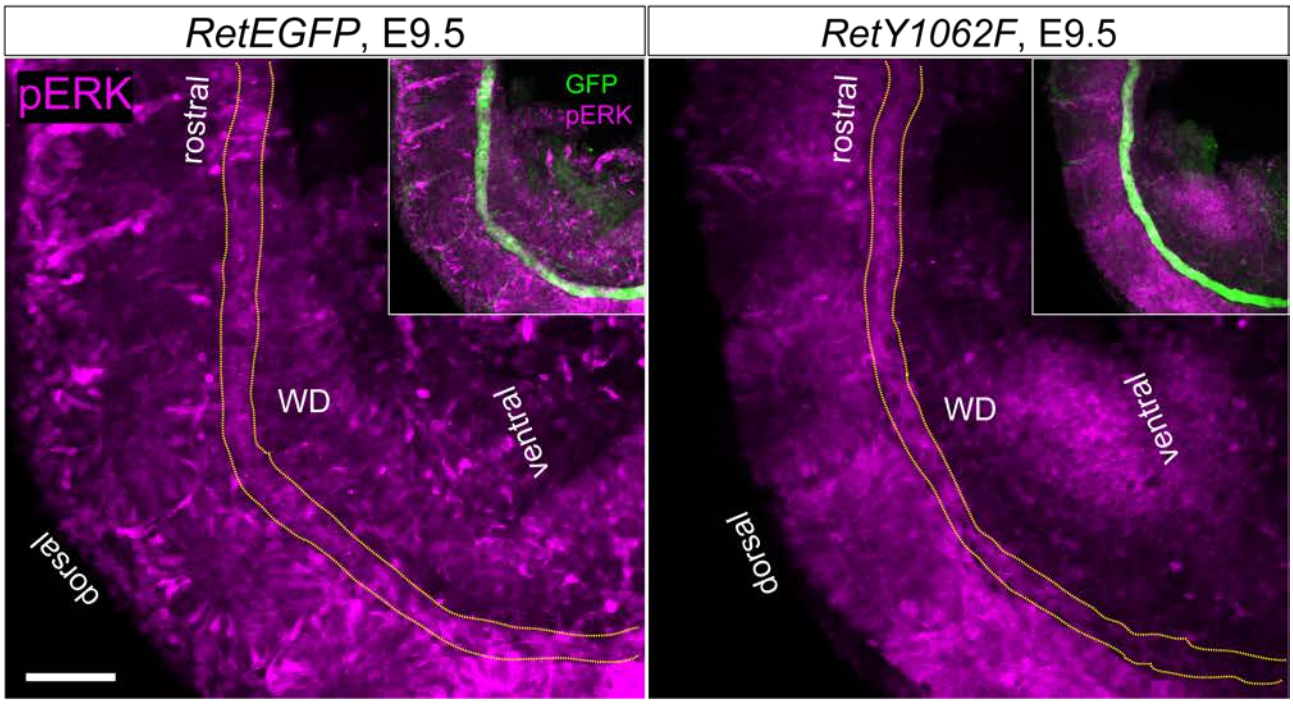
Supplemental Figure 2

A



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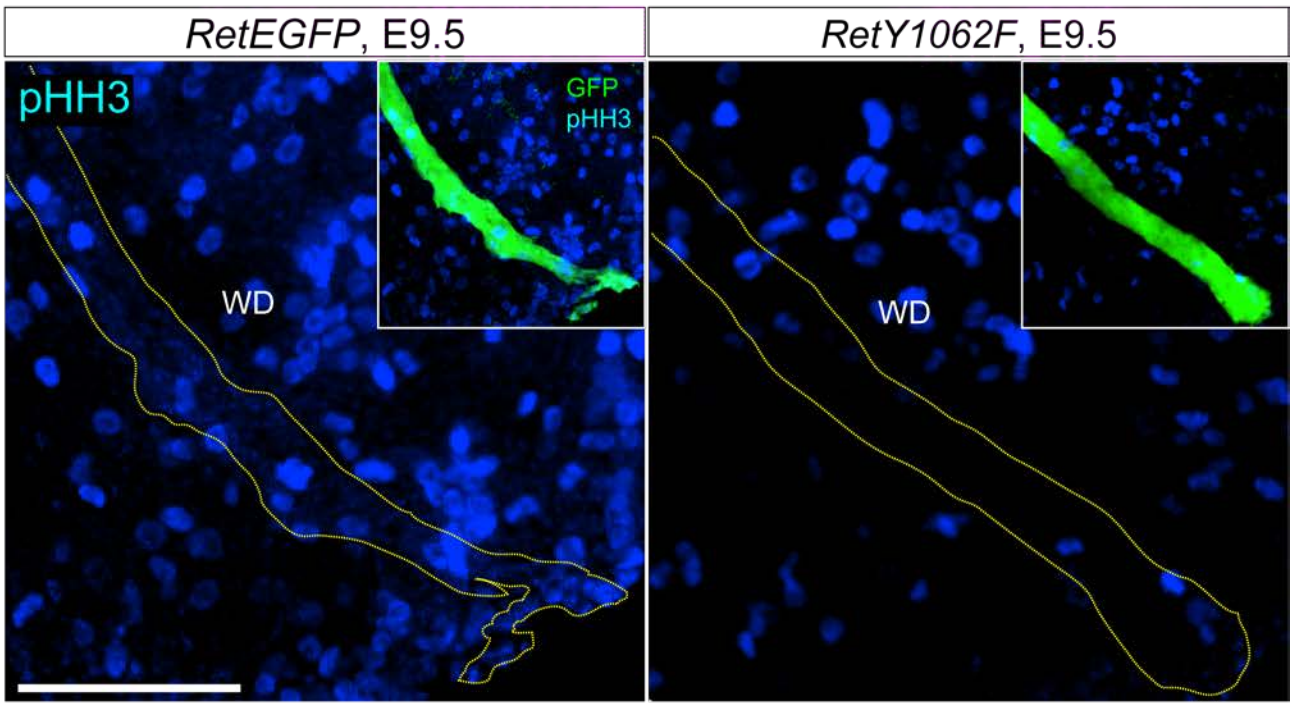

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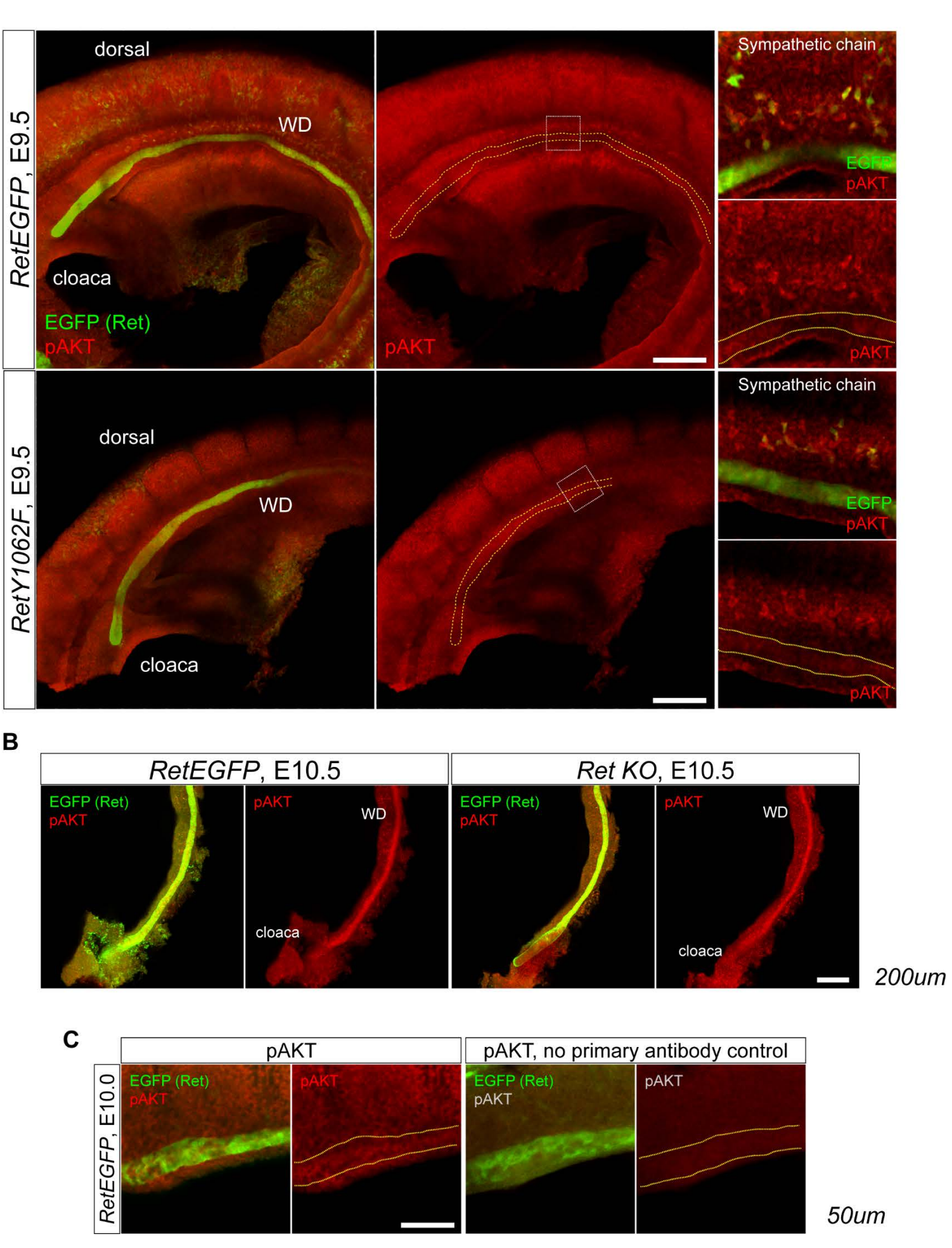
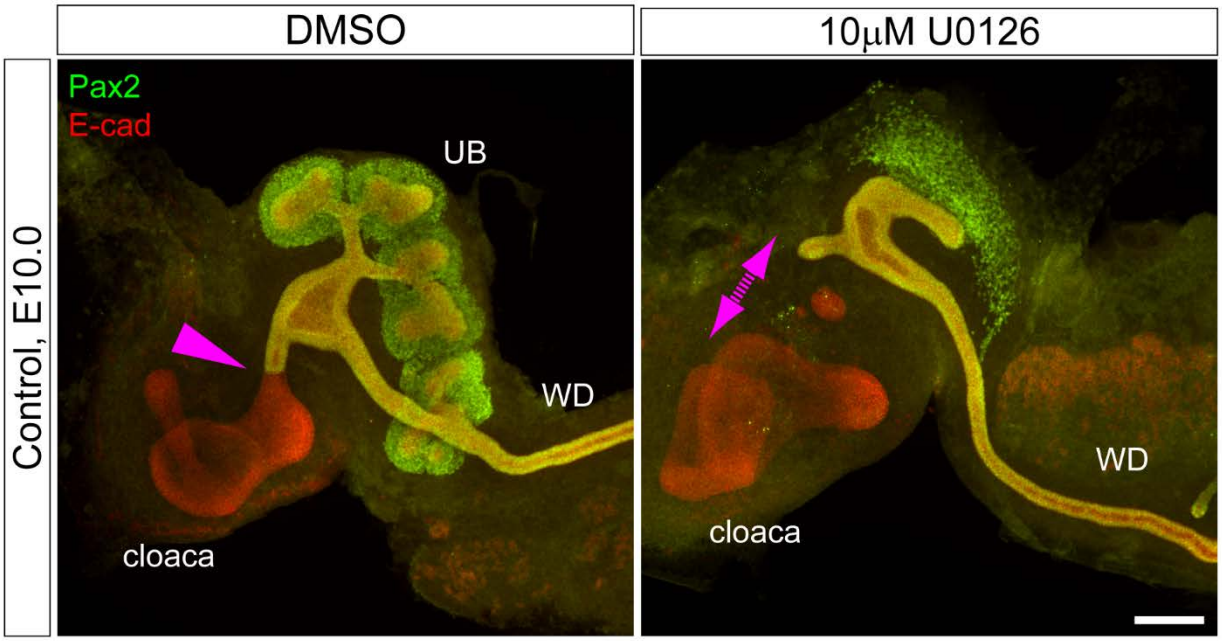
Supplemental Figure 3



Supplemental Figure 4



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Supplemental Figure 5