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Figures and figure supplements

Integrated β-catenin, BMP, PTEN, and Notch signalling patterns the nephron

Nils O Lindström, et al.

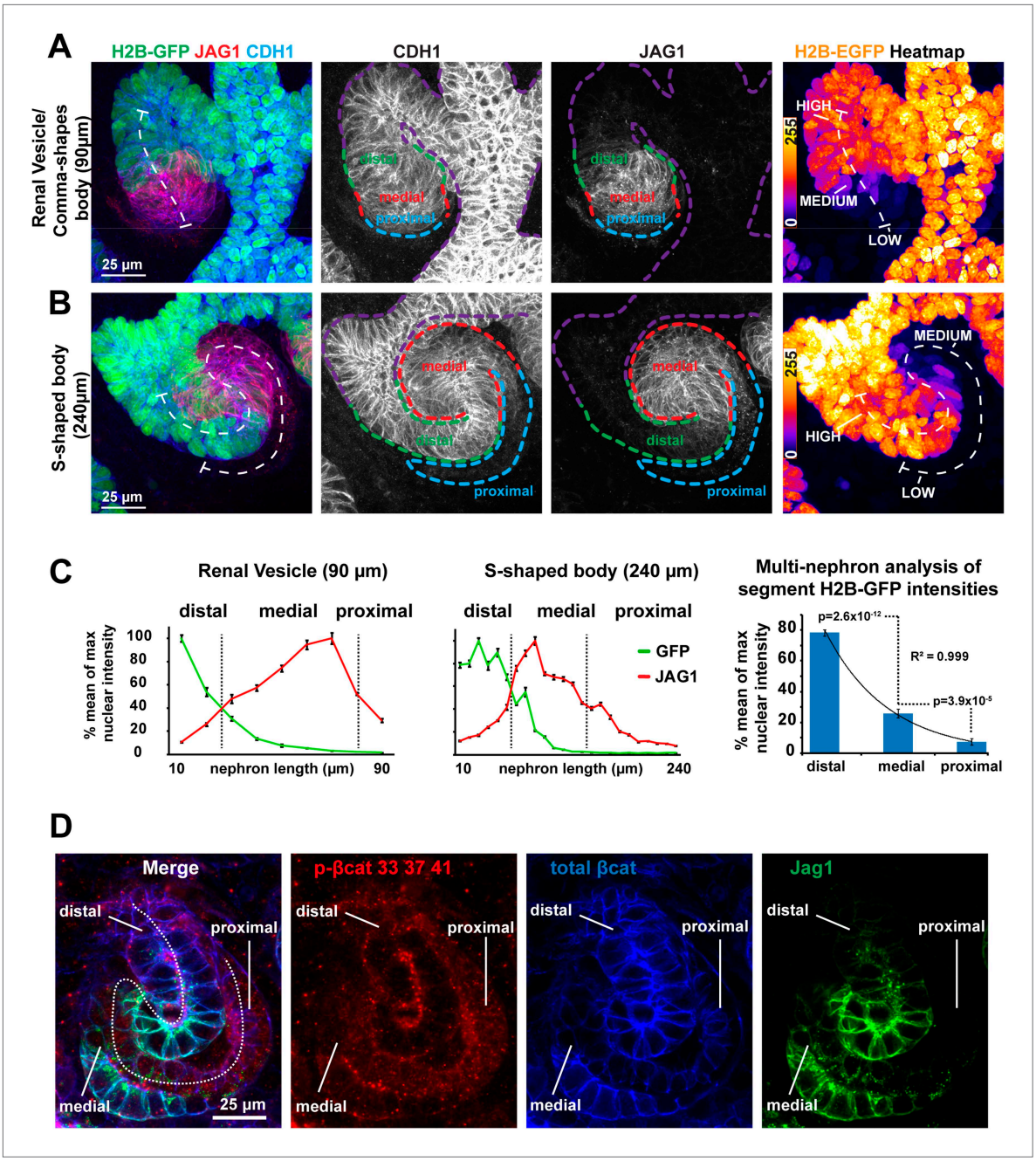

Figure 1. β-catenin activity levels form a reversed gradient along the axis of the nephron. (**A**–**B**) *TCF/Lef::H2B-GFP* expression in nephrons: (**A**) late renal vesicle/early comma-shaped body nephron, (**B**) S-shaped body nephron, lines: white–nephron axis, purple–ureteric bud, green–distal nephron, red–medial nephron, blue–proximal nephron/glomerular precursors. Heat-maps display signal intensity in different nephron segments. (**C**) Quantification of nuclear H2B-GFP and cell-membrane Jag1 antibody stain signal-intensity along the proximal–distal axis. Error bars represent SEM of pixels representative of 10 µm segments. Right-hand side graph shows mean values for segments, as identified by H2B-GFP and Jag1 profiles (n = 11 nephrons), error bars indicate SEM. p-values derived from t-tests. (**D**) Antibody stains against total β-catenin and phosphorylated β-catenin in S-shaped body nephron—Jag1 marking the medial segment. White dashed line indicating nephron axis.

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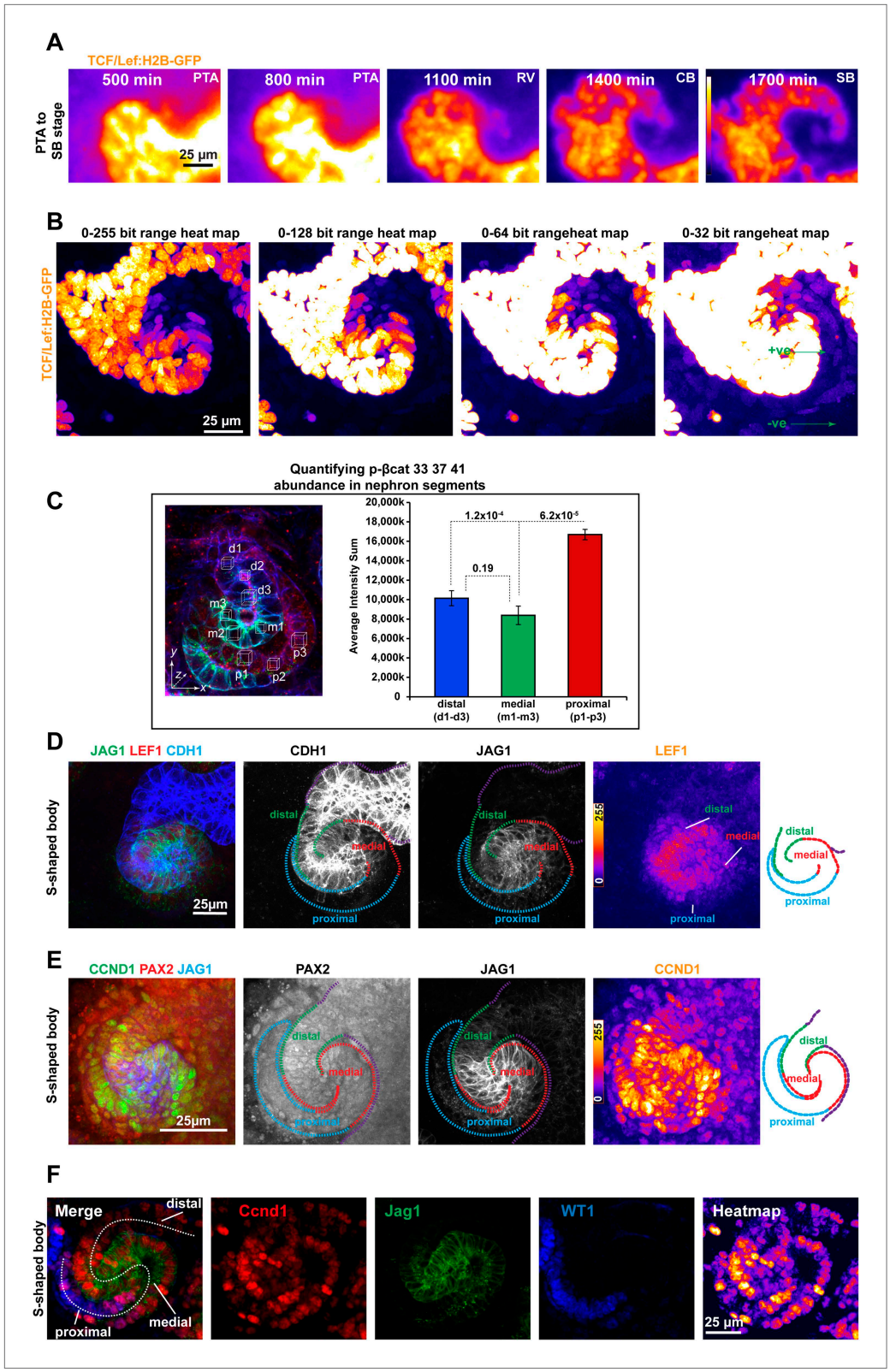

Figure 1—figure supplement 1. β-catenin reporter and antibody data show different β-catenin activity levels along the axis of the nephron. (**A**) *TCF/Lef:H2B-GFP* time-lapse data of a nephron developing through post-MET Pretubular Aggregate (PTA), Renal Vesicle (RV), Comma-shaped (CB), and S-shaped (SB) stages. These data are shown as time-lapse in ***Video 2***. (**B**) S-shaped body nephron from ***Figure 1B*** shown at four different brightness

Figure 1—figure supplement 1. Continued on next page

Figure 1—figure supplement 1. Continued

settings that represent a doubling in brightness for each field going left to right. This clearly shows that all segments of the nephron are positive for the *TCF/Lef:H2B-GFP* reporter but follow a visual gradient. Green arrows point to positive cells in the dimmest portion of the nephron and an area of the kidney that even in at the brightest settings show up as negative. (**C**) Quantification of antibody stain for β-catenin phosphorylated at Ser33/Ser33/Thr41 in the distal, medial, and proximal segments (nine measurements per nephron in five nephrons). Error bars represent SEM, p-values derived from t-tests. (**D**–**F**) Lef1 and Ccnd1 (CycD1) antibody stains in S-shaped body nephrons; Pax2 is used as a structural marker against all nuclei within the nephron, Jag1 is used to detect the medial segment, Wt1 is used to detect the developing podocytes in the proximal segment.

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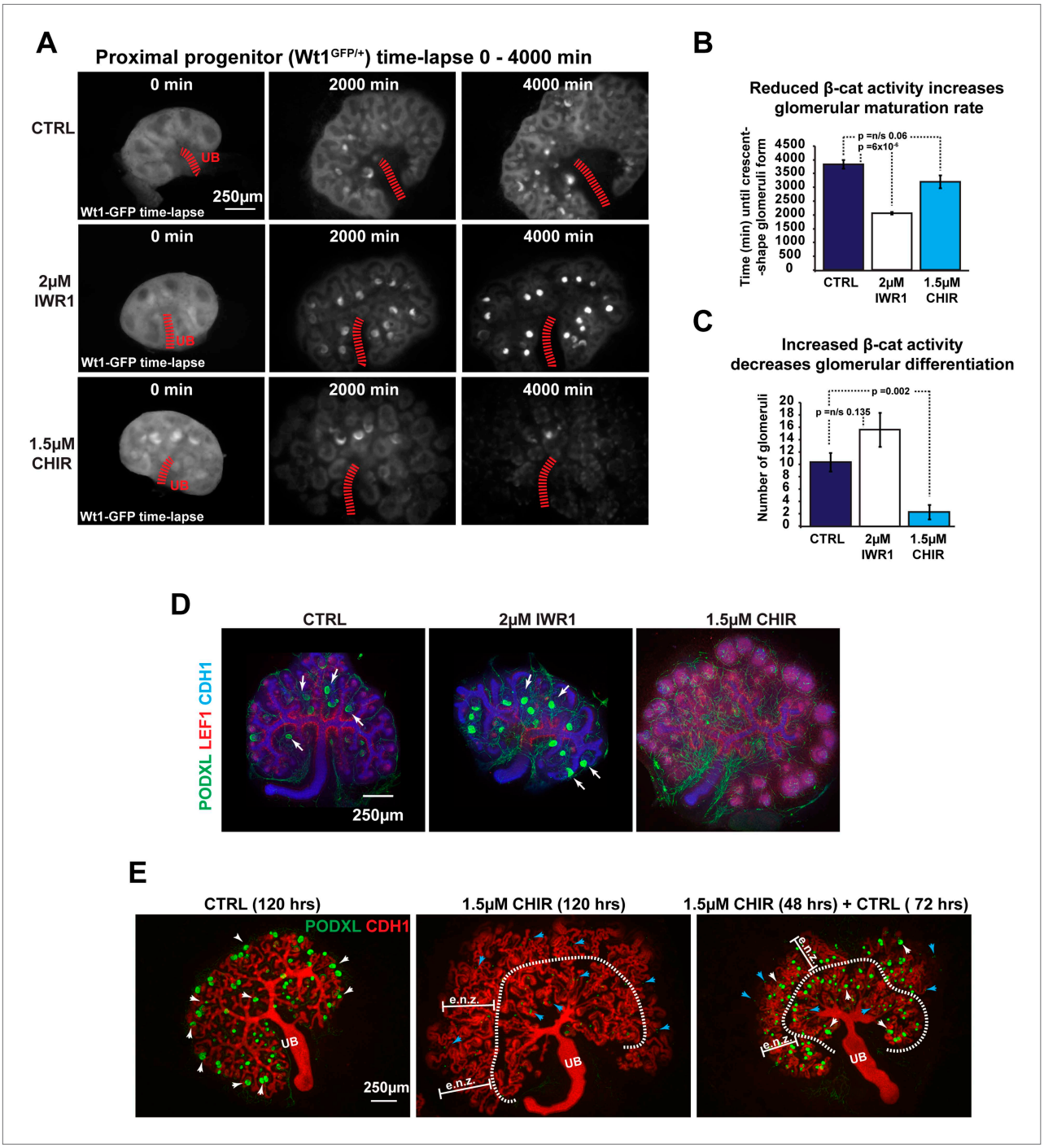

Figure 2. Pharmacological modulation of β-catenin signalling alters proximal segment development. (**A**) Time-lapse analysis of treated *Wt1*$^{+/GFP}$ kidneys—same as shown in ***Video 5***. (**B**) Quantification of mean time taken for first glomeruli to mature to crescent-shaped stage where glomeruli are tightly packed and exhibit a bright signal. (**C**) Mean number of mature glomeruli after 3800 min of culture. (**D**) Kidneys stained for podocyte marker Podxl, β-catenin target Lef1, and epithelial marker Cdh1. Arrowheads indicating structures positive for Podxl. (**E**) The proximal identity resumed its formation when CHIR was removed after 48 hr—white arrowheads indicate larger Podxl positive structures, blue arrowheads indicate very small Podxl positive structures, dashed line separates ectopic nephron zone (e.n.z.) nephrons from those inside the ureteric tree (UB).

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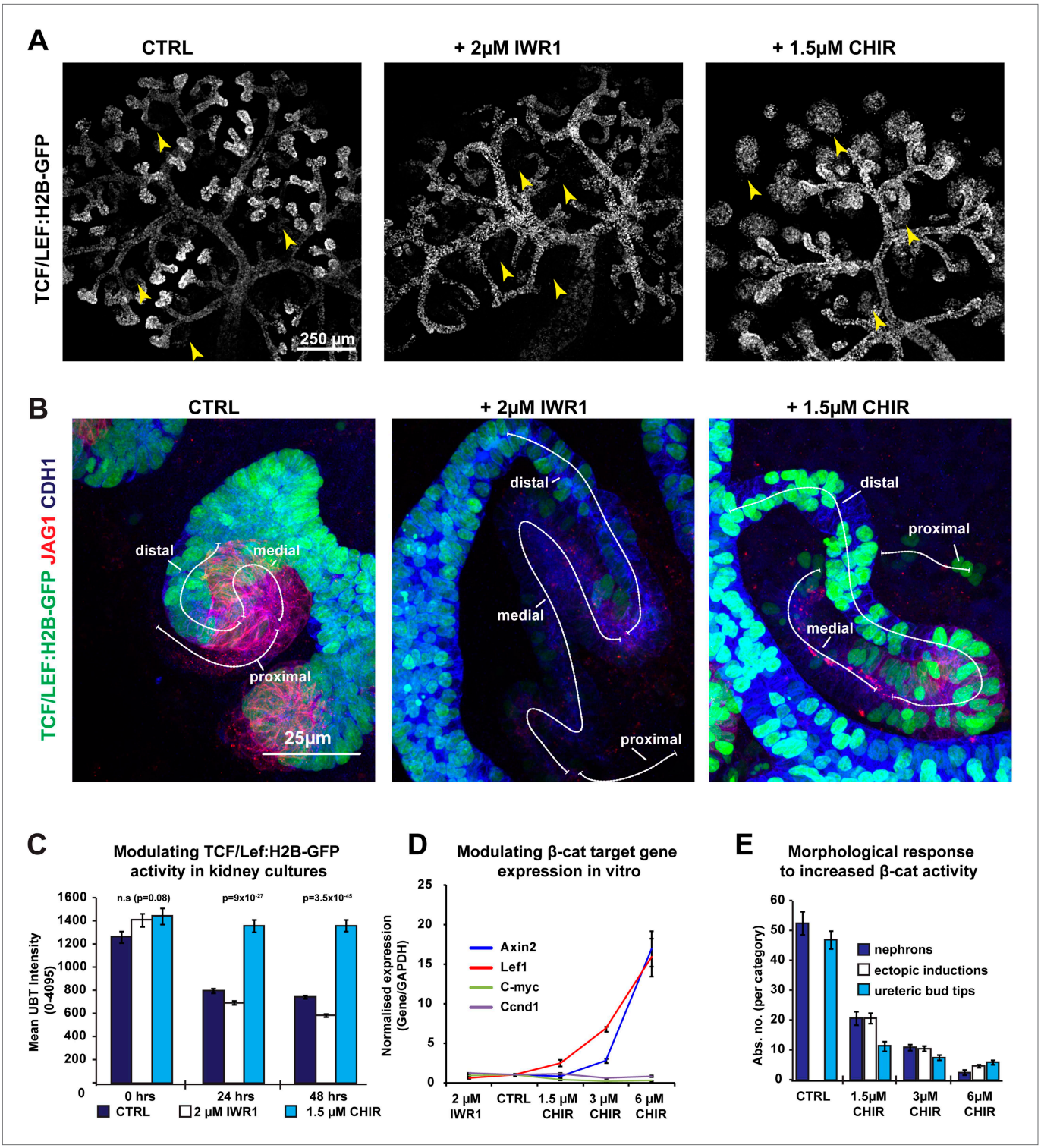

Figure 2—figure supplement 1. β-catenin signalling is altered in response to pharmacological inhibitors. (**A**–**B**) *TCF/Lef:H2B-GFP* kidneys displaying low levels of β-catenin signalling in IWR1 conditions whereas strong activation of β-catenin signalling is detected in CHIR conditions. Typical nephrons within respective conditions are indicated with yellow arrowheads and nephrons captured at 60× and stained Jag1 and Cdh1 are shown in (**B**). (**C**) Signal intensity of *TCF/Lef:H2B-GFP* β-catenin reporter is increased and decreased in the tips of the ureteric bud in response to CHIR and IWR1, respectively. (**D**) qRT-PCR of known β-catenin target genes in response to IWR1 and CHIR treatment. Some β-catenin target genes responded as predicted (*Axin2*, *Lef1*), others did not (*c-Myc* and *Ccnd1*) as is expected from cell type-specific β-catenin targets (***Sansom et al., 2005***; ***Sansom et al., 2007***)—*Axin2* and *Lef1* data is also shown in ***Figure 4D*** to put them into the context of other expression changes. (**E**) Effects of different β-catenin activity levels on kidneys morphogenesis and nephron-formation.

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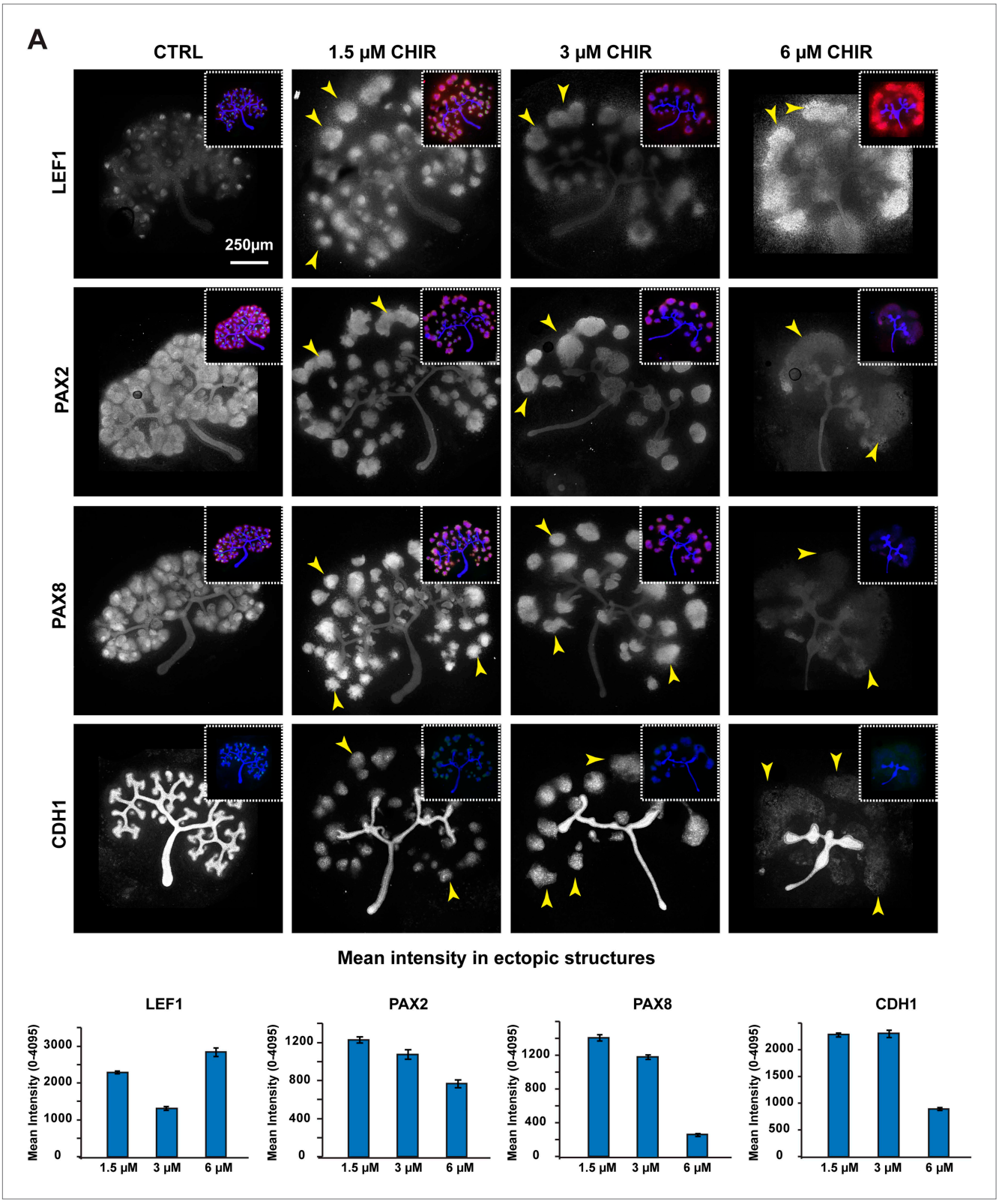

Figure 2—figure supplement 2. 'Just-right' β-catenin signalling levels drive MET. (**A**) Response of β-catenin target gene (Lef1), induction markers (Pax2 and Pax8), and epithelialisation marker (Cdh1) to different CHIR concentrations. Inserts showing all channels for kidneys stained for Jag1, Cdh1, and indicated marker. As in previous studies, strong activation of β-catenin disrupted the normal MET. Arrowheads indicate ectopic nephrons. All error bars indicate SEM.

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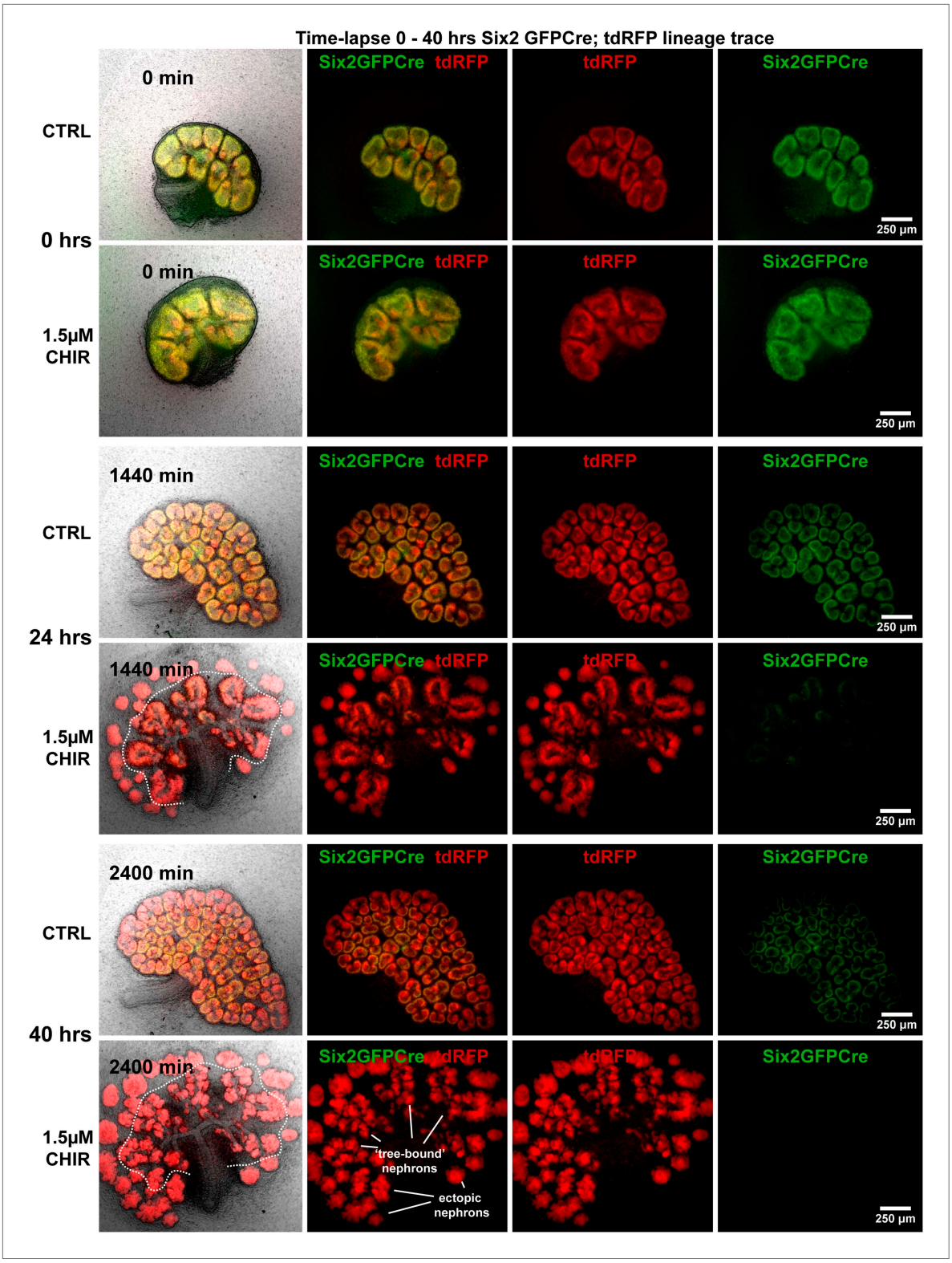

Figure 2—figure supplement 3. Ectopic nephrons form from Six2 expressing progenitors. *Six2*^{GFPCre} with conditional RFP lineage tracing highlighting all nephron progenitors cell (GFP^{+}) and all nephron lineages (RFP^{+}). Kidneys cultured in CHIR show ectopic RFP^{+} structures forming from previous GFP^{+} nephron progenitors. Dashed line indicates separation of ectopic nephrons from the ureteric bud tree and endogenous 'tree-bound' nephrons. Data also presented in ***Video 3***.

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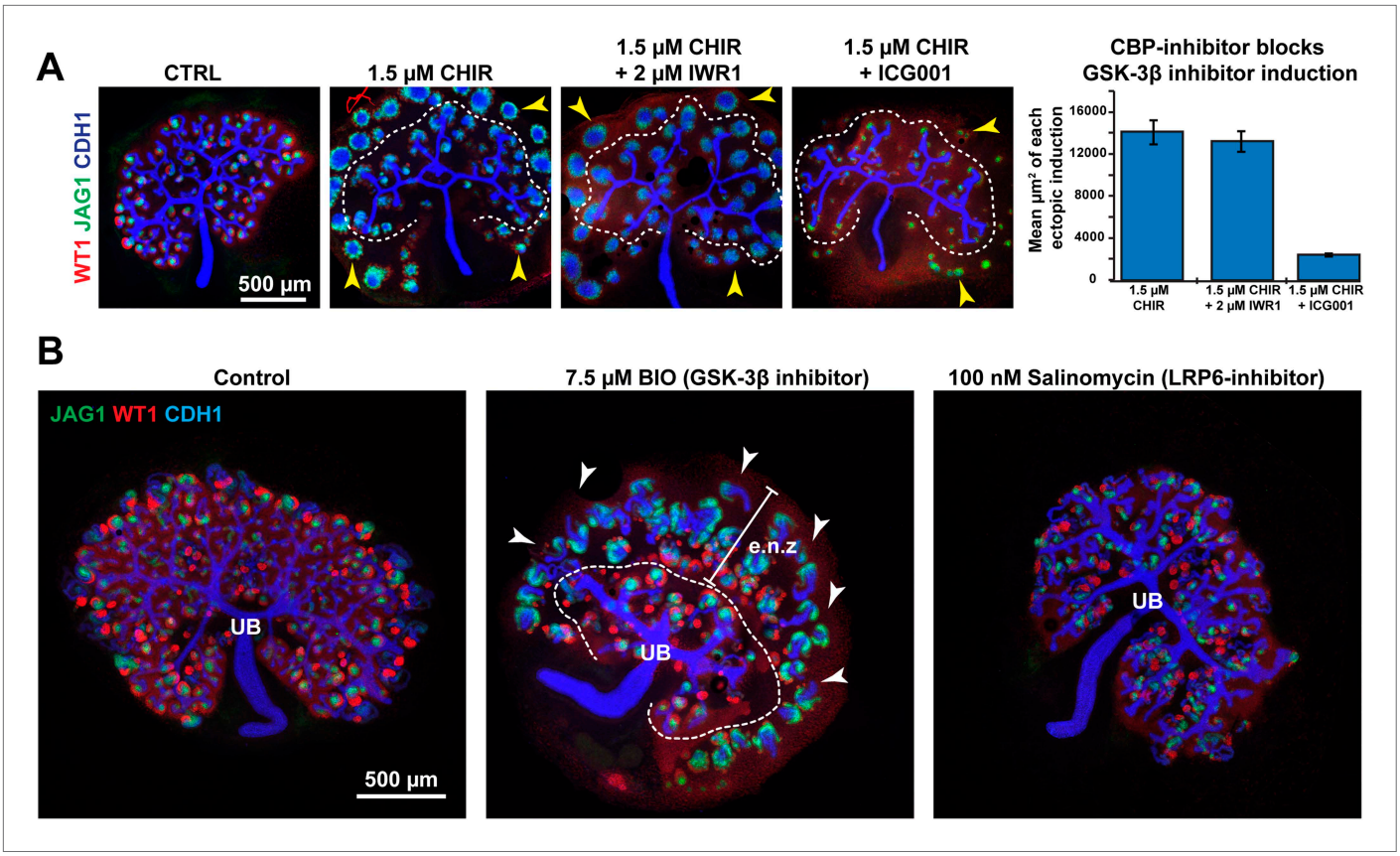

Figure 2—figure supplement 4. Co-inhibition experiments confirm specificity of pharmacological inhibitors. (**A**) Co-inhibition of GSK-3β (CHIR) and Tankyrase (IWR1) or CBP (ICG001). ICG001 blocks TCF interacting with the CBP co-activator (***Emami et al., 2004***) downstream of (CHIR). CHIR effects are not blocked by IWR1 inhibiting Tankyrase upstream of GSK-3β but are blocked by ICG001. Dashed line separates ectopic nephrons from those adjacent to the ureteric bud. Error bars indicate SEM. Arrowheads indicate ectopic nephrons. (**B**) 7.5 μM BIO (GSK-3β inhibitor) mimics 1.5 μM CHIR and ectopic nephrons form and the UB (ureteric tree) branches is as in (**A**). Arrowheads indicate ectopic nephrons, dashed line separates ectopic nephron zone (e.n.z.) nephrons from those inside the ureteric tree (UB). (**B**) 100 nM salinomycin (LRP6 inhibitor) treated samples show UB branching similar to that caused by IWR1. The effect on nephrons is also similar. Antibody stains and scale bars as indicated.

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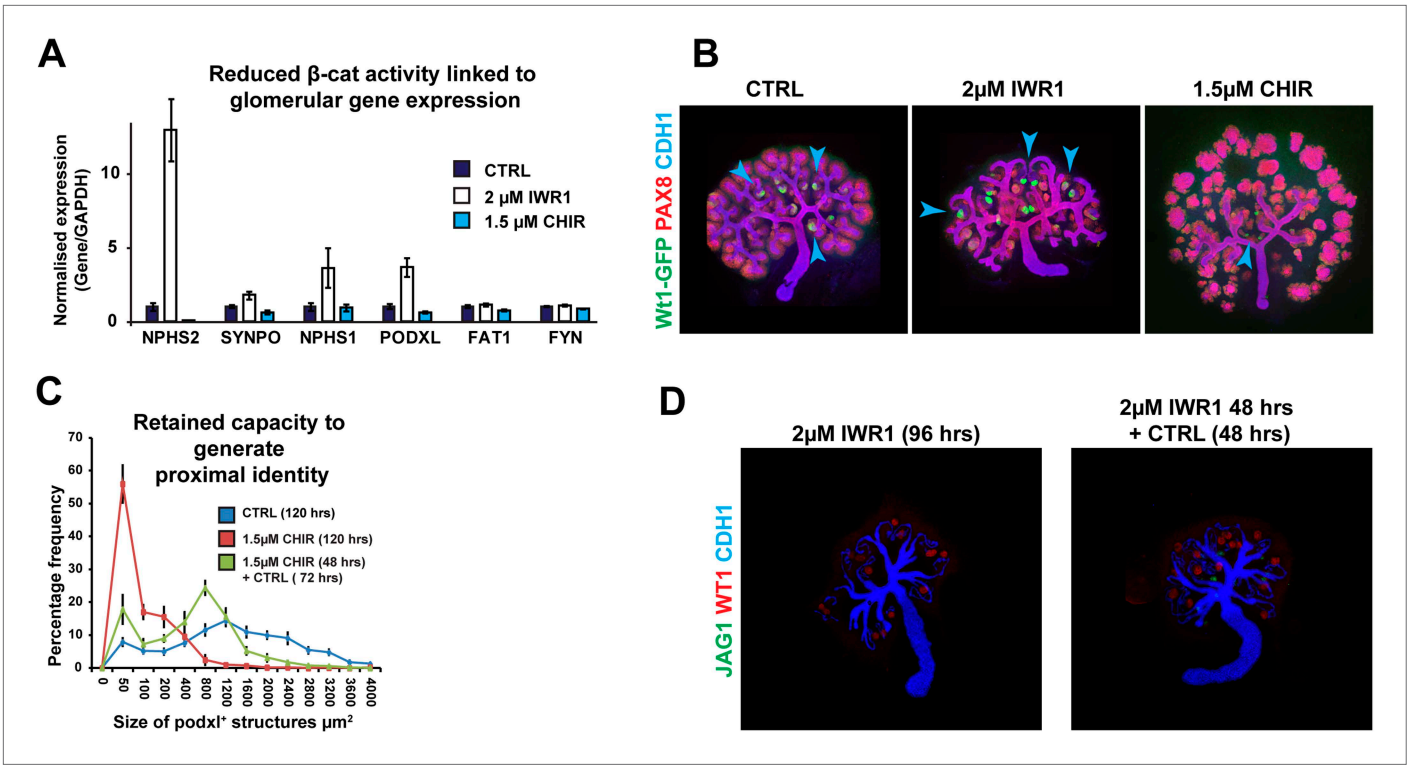

Figure 2—figure supplement 5. The proximal cell-identity is promoted by decreased β-catenin signalling. (**A**) qRT-PCR data for genes indicative of terminally differentiated glomerular cells. (**B**) *Wt1*$^{+/GFP}$ kidneys stained for Pax8 and Cdh1–arrowheads indicating structures positive for GFP. (**C**) Size of glomerular structures in rescued nephrons as shown in ***Figure 2E***. All error bars indicate SEM. (**D**) Kidney development in kidneys cultured for a full 96 hr in IWR1 or 48 hr in IWR1 followed by 48 hr in control medium. Antibody stains as indicated.

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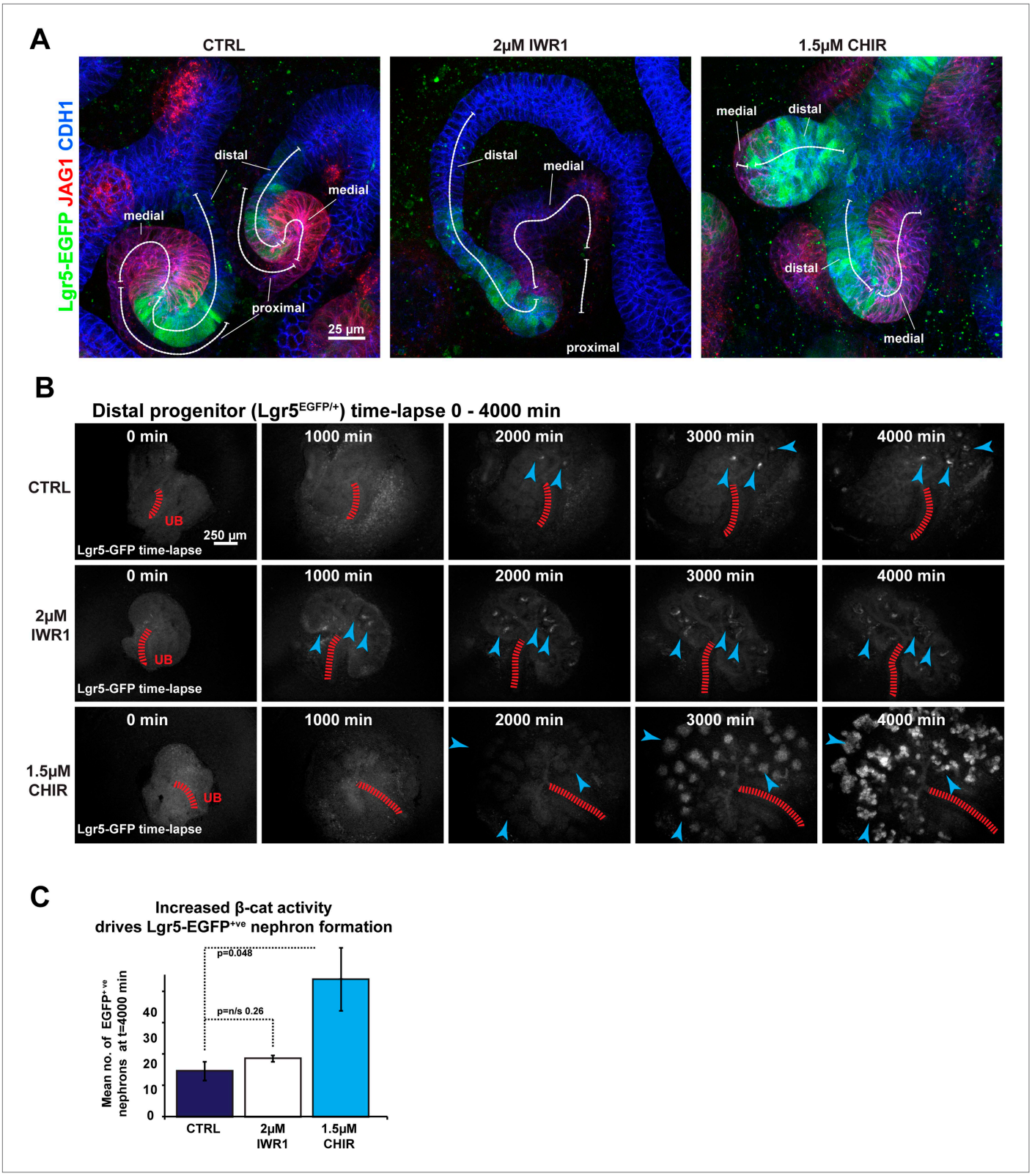

Figure 3. Pharmacological modulation of β-catenin signalling alters distal segment development. (**A**) *Lgr5-EGFP* expression in treated nephrons with segmentation markers. (**B**) Time-lapse analysis of treated *Lgr5*$^{+/EGFP\text{-}IRES\text{-}CreERT2}$ kidneys–arrowheads indicate developing nephrons, red-dashed line indicates ureteric bud (UB). (**C**) Mean number of *Lgr5-EGFP* positive nephrons per kidney.

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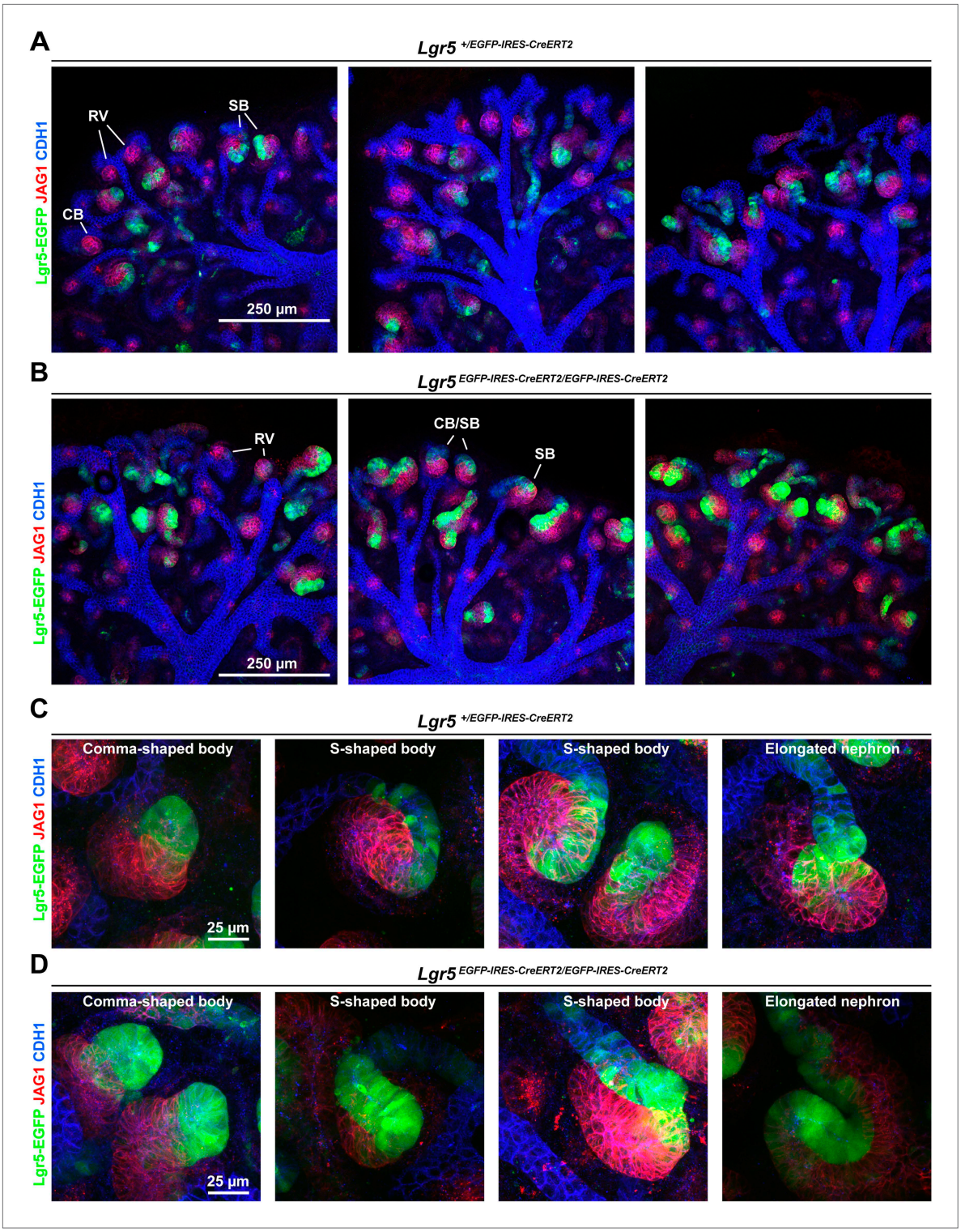

Figure 3—figure supplement 1. Lgr5 expression domain in heterozygous and homozygous Lgr5$^{+/EGFP\text{-}IRES\text{-}CreERT2}$ kidneys. Cultured Lgr5$^{+/EGFP\text{-}IRES\text{-}CreERT2}$ kidneys stained for segmentation marker Jag1 and epithelial marker Cdh1. (**A** and **C**) Lgr5$^{+/EGFP\text{-}IRES\text{-}CreERT2}$ heterozygous kidneys and kidneys and (**B** and **D**) Lgr5 $^{EGFP\text{-}IRES\text{-}CreERT2/EGFP\text{-}IRES\text{-}CreERT2}$ homozygous kidneys and nephrons display normal segmentation. Homozygous tissue displays stronger EGFP expression as expected but nephrons appear morphologically normal.

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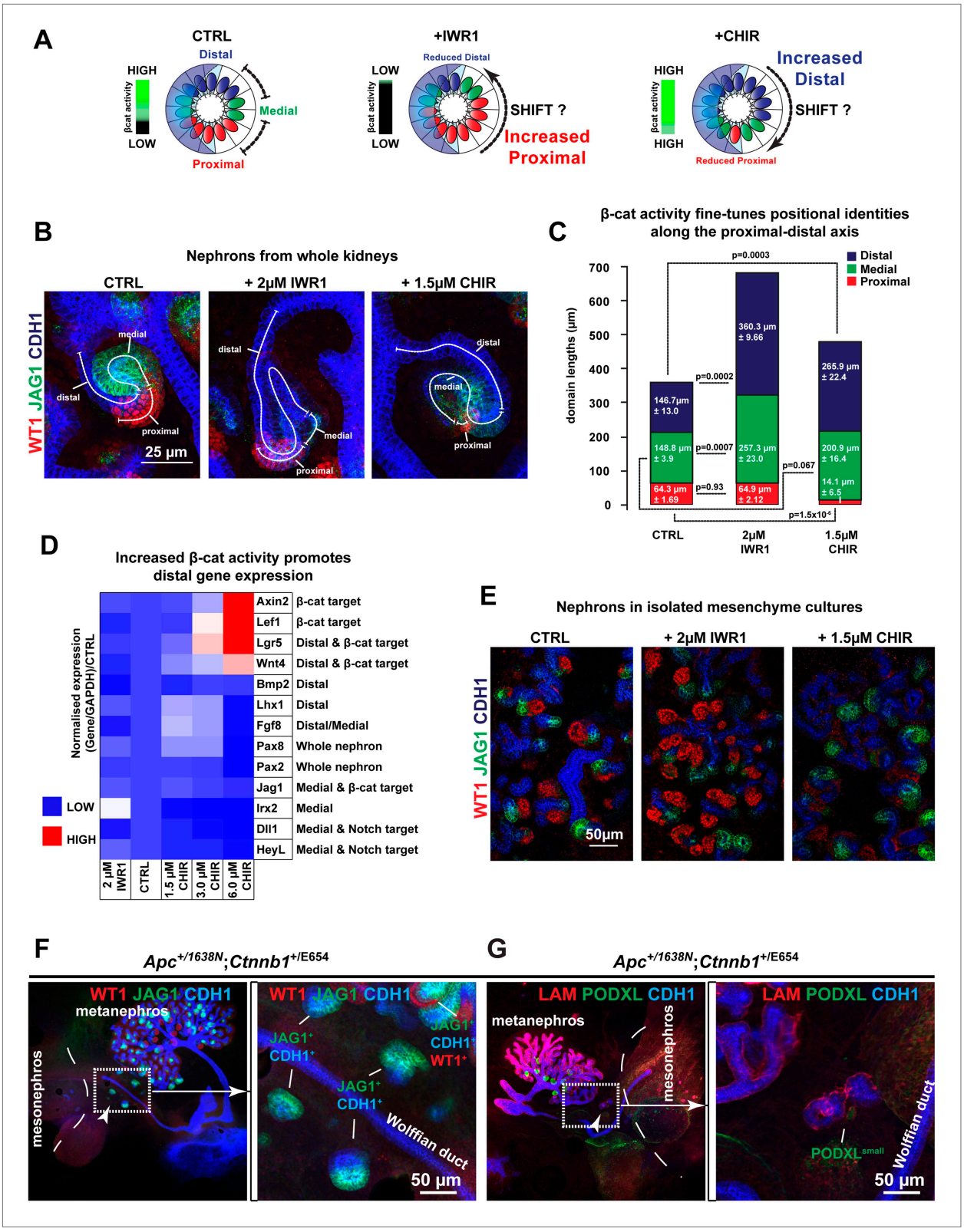

Figure 4. Shifts in positional identity by altered β-catenin activity. (**A**) Model of predicted changes in segmentation if the gradient of β-catenin activity specifies positional identities in the nephron. Nephrons depicted as spheres representing renal vesicle stage. Dashed line indicates nephron segments. Gradient bar indicates β-catenin activity. (**B**) Antibody stains against segment specific markers in nephrons with different β-catenin signalling conditions. (**C**) Proximal, medial, and distal nephron domain-sizes in Control, CHIR, and IWR1 treated kidneys. Mean values and SEMs indicated within bars on

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graph. (**D**) qRT-PCR analysis of markers for nephron induction displayed as a heat-map with information displayed in figure. The RNA was isolated after 48 hr of culture from whole kidneys. (**E**) Antibody stains on nephrons developed in isolated mesenchyme. (**F** and **G**) *Apc*$^{+/1638N}$ *Ctnnb1*$^{Y654/E654}$ kidneys where *Ctnnb1*^{Y654} is the wild-type allele. Kidneys characterised using anti-Wt1, Jag1, Podxl, Lam, and Cdh1. Arrowheads and boxed area indicate ectopic nephrons in **E**–**F**. Dashed lines separate metanephric and mesonephric regions in **E**–**F**.

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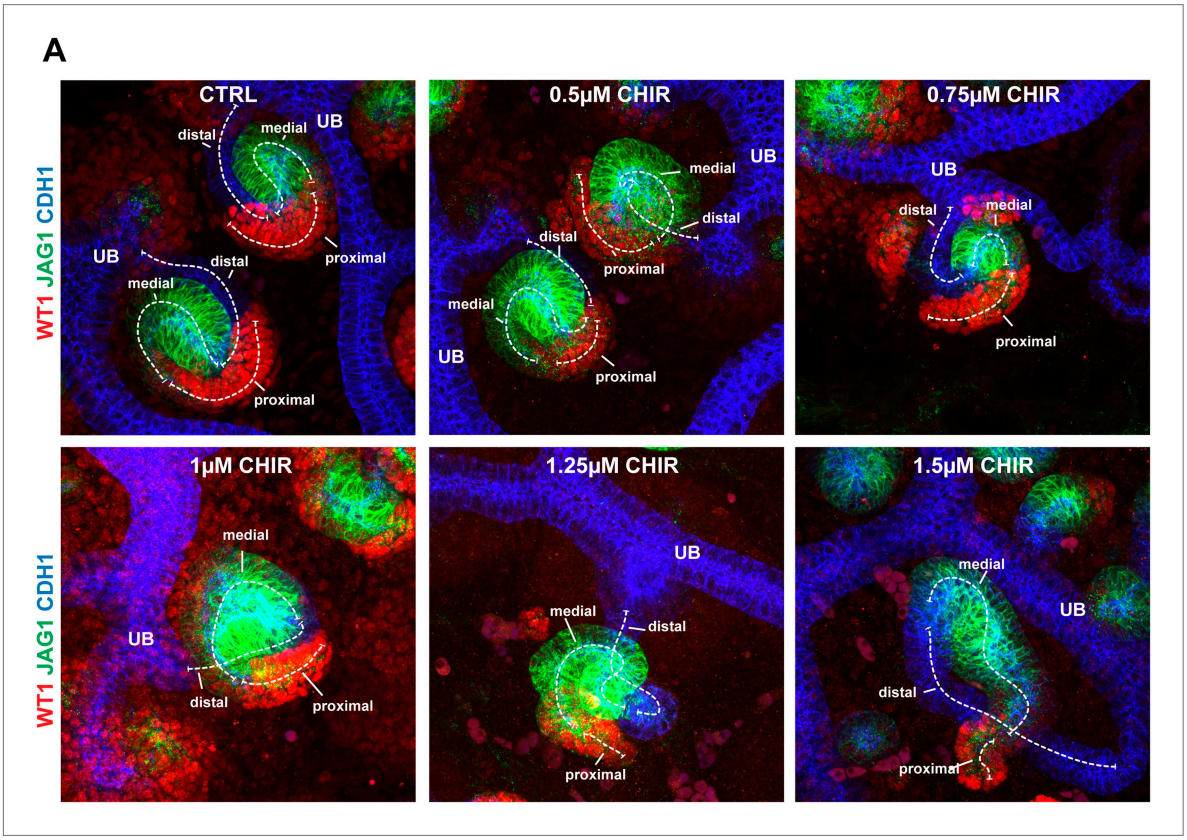

Figure 4—figure supplement 1. Gradual shifts in positional identity by gentle changes in β-catenin activity. (**A**) Typical nephrons for their conditions as cultured at incremental CHIR dosages (0 μM, 0.50 μM, 0.75 μM, 1 μM, 1.25 μM, and 1.5 μM). Dashed lines indicate the axis and lengths of nephron segments. Kidneys stained for Wt1, Jag1, and Cdh1. UB–ureteric bud.

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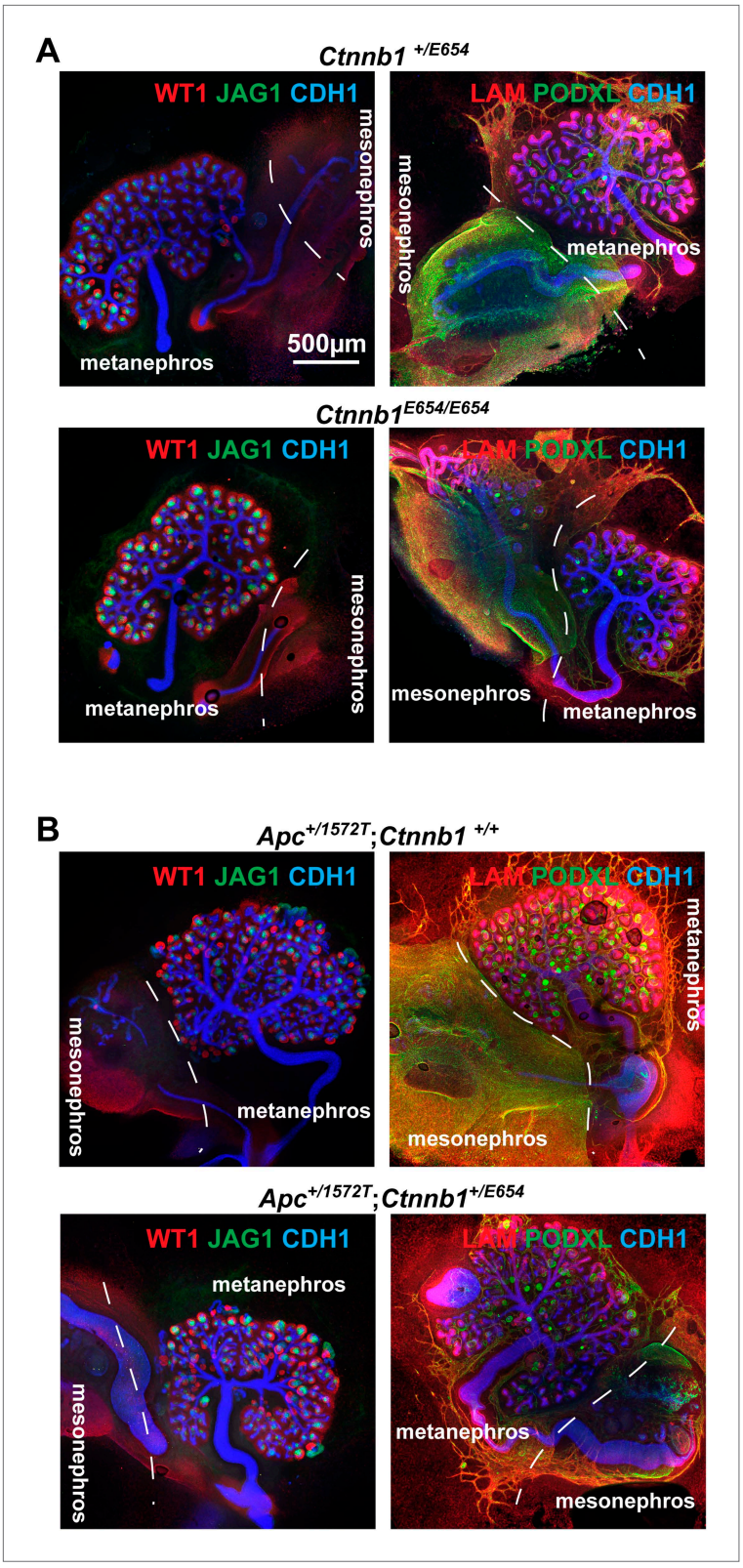

Figure 4—figure supplement 2. β-catenin activity dosage-dependent phenotypes in series of *Apc* and *Ctnnb1* models. Kidneys characterised using anti-Wt1, Jag1, Podxl, Lam, and Cdh1. (**A**) *Ctnnb1*$^{Y654/E654}$ and *Ctnnb1*$^{E654/E654}$, (**B**) *Apc*$^{+/1572T}$ *Ctnnb1*$^{Y654/Y654}$ and *Apc* $^{+/1572T}$ *Ctnnb1*$^{Y654/E654}$. *Ctnnb1*^{Y654} is the wild-type allele. Dashed lines separate metanephric and mesonephric regions.

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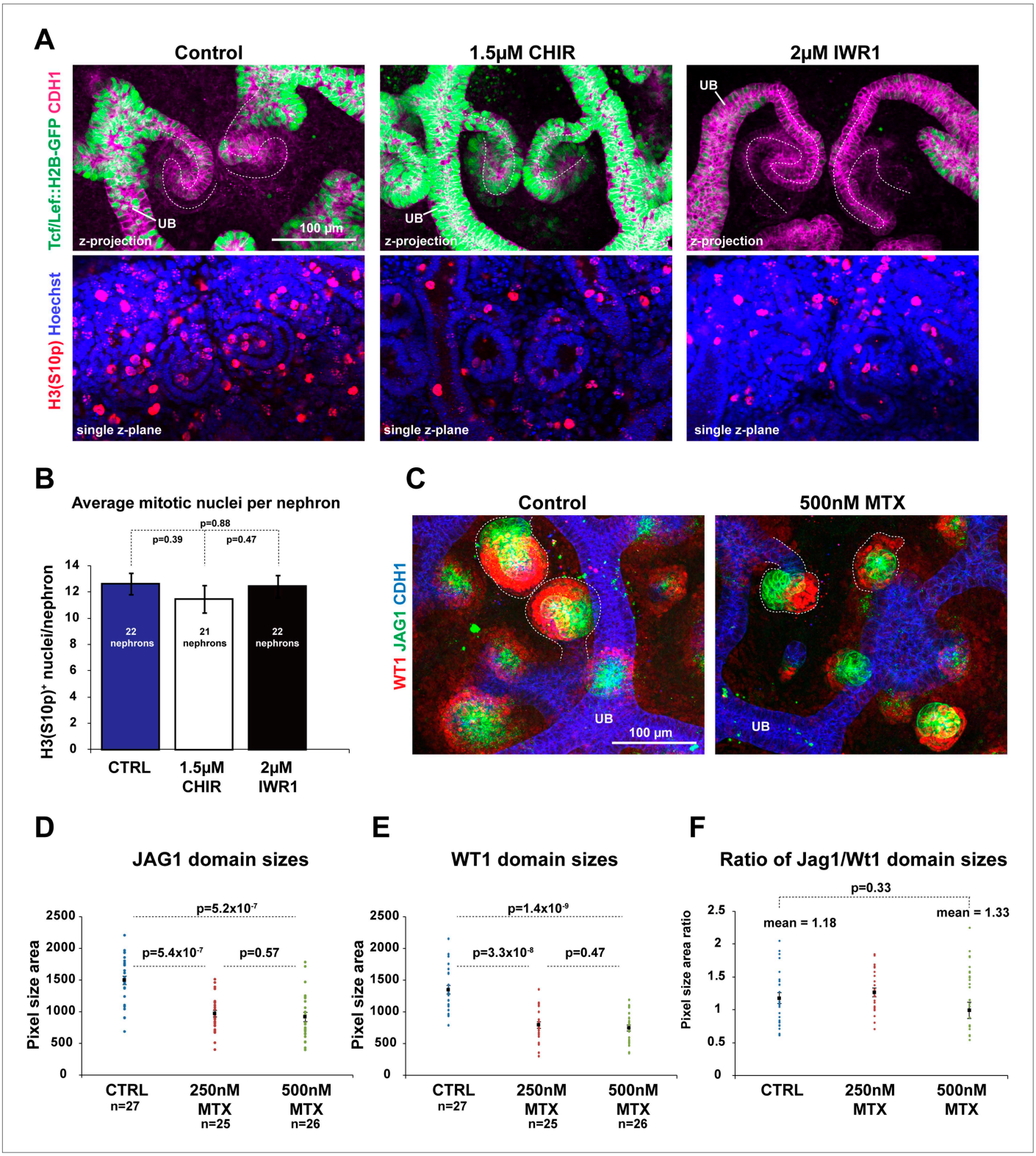

Figure 5. Modulating β-catenin activity shifts positional identities along the nephron without altering proliferation. (**A**) Proliferation in *TCF/Lef::H2B-GFP* expressing nephrons treated with CHIR and IWR1. Nephron axis–dashed white line. Phosphorylated Histone 3 used as a marker for mitotic cells. (**B**) Quantification of mitotic nuclei per nephron. (**C**) Effect of Methotrexate (MTX) on nephron development and patterning. Nephrons outlined with white dashed line. (**D**–**F**) Measurement of Jag1$^+$ and Wt1$^+$ segment sizes of nephrons in control, 250 nM Methotrexate (MTX), and 500 nM MTX conditions. All error bars indicate SEM. p-values generated using Student's *t* test. Scale bars and antibodies as indicated on fields. UB–ureteric bud.

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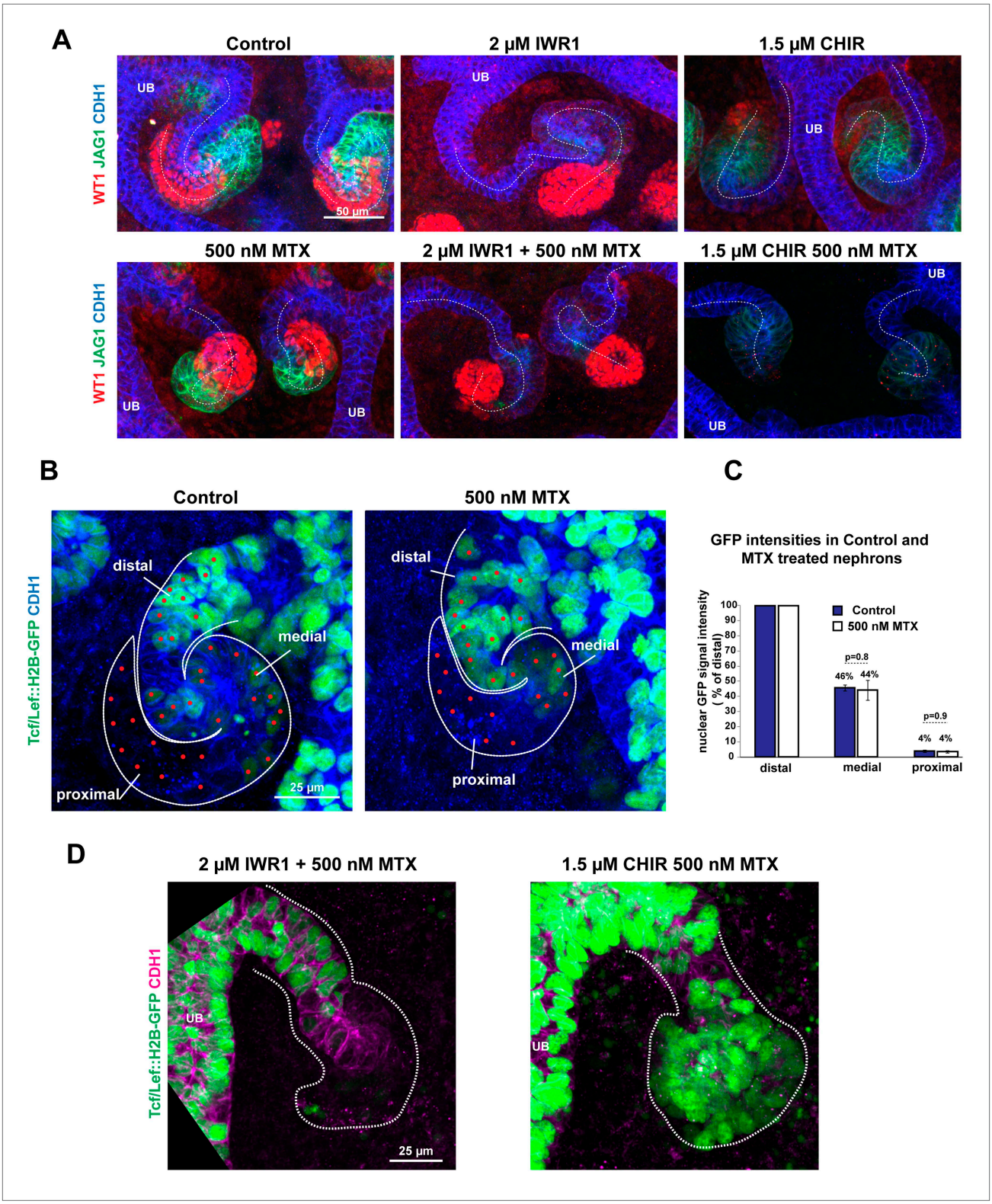

Figure 5—figure supplement 1. Modulating β-catenin activity shifts positional identities along the nephron regardless of proliferation levels. (**A**) CHIR and IWR1 alter segmentation similarly with or without MTX. Dashed-white line indicates nephron axis. (**B**–**C**) Inhibiting proliferation does not block the formation of a GFP-gradient in *TCF/Lef:H2B-GFP* expressing nephrons. Nephrons outlined with white dashed line. Segment labelled on images. Red dots indicate examples of nuclei that were quantified for graph (**C**). Error bars indicate SEM. p-values were calculated using t-tests. (**D**) Signal intensity of *TCF/Lef:H2B-GFP* β-catenin reporter is decreased and increased with IWR1 and CHIR also, when MTX is added. Nephrons outlined with white dashed line. Scale bars and antibodies as indicated on fields. UB–ureteric bud.

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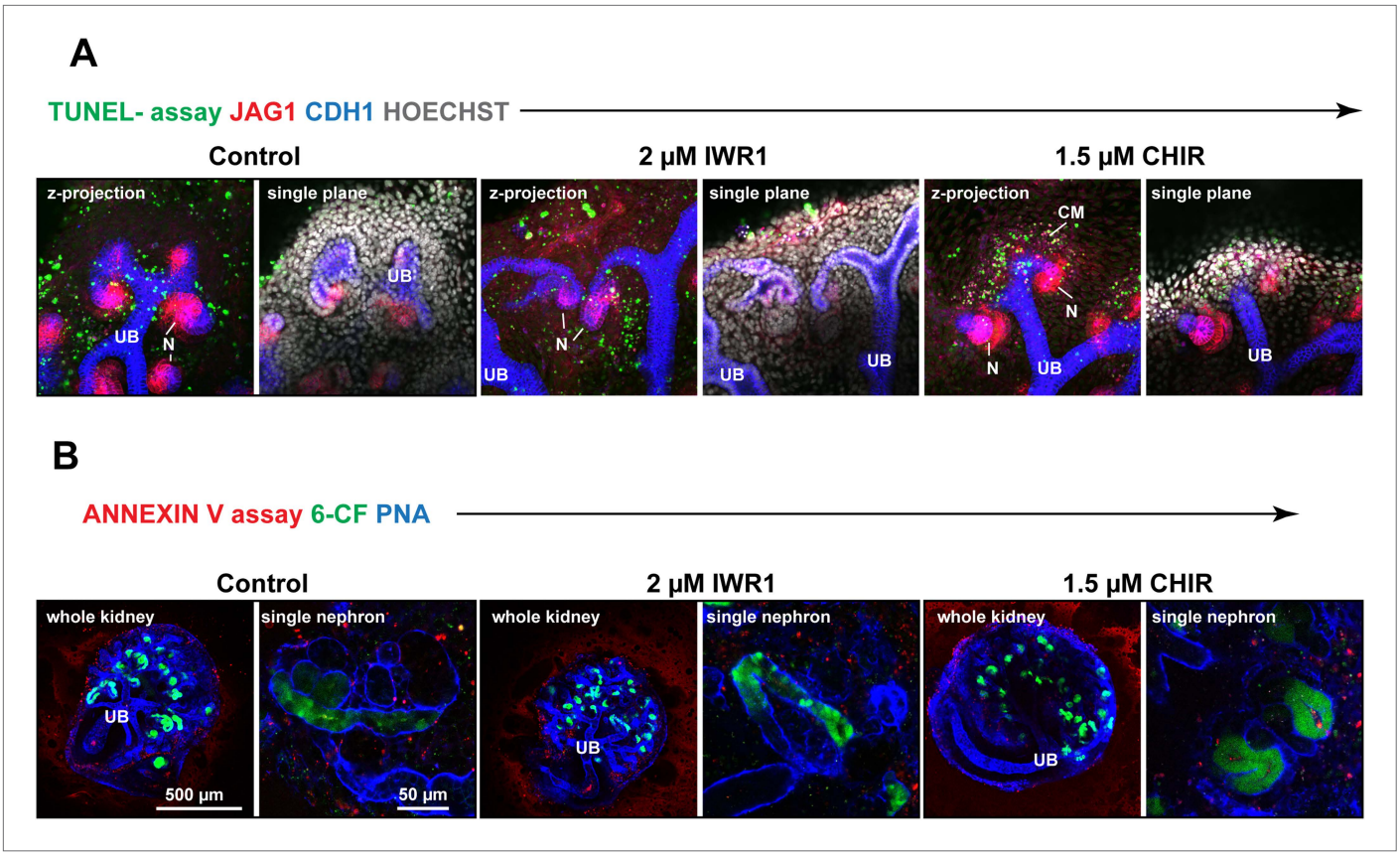

Figure 5—figure supplement 2. Modulating β-catenin activity shifts positional identities along the nephron regardless of apoptosis levels. (**A**) Treated kidneys stained with TUNEL-assay to detect apoptotic cells. (**B**) Treated kidneys (live) stained with Annexin V assay to detect apoptotic cells. 6-CF marks proximal tubules and PT and as specified. Scale bars and antibodies as indicated on fields. UB–ureteric bud, N–Nephron.

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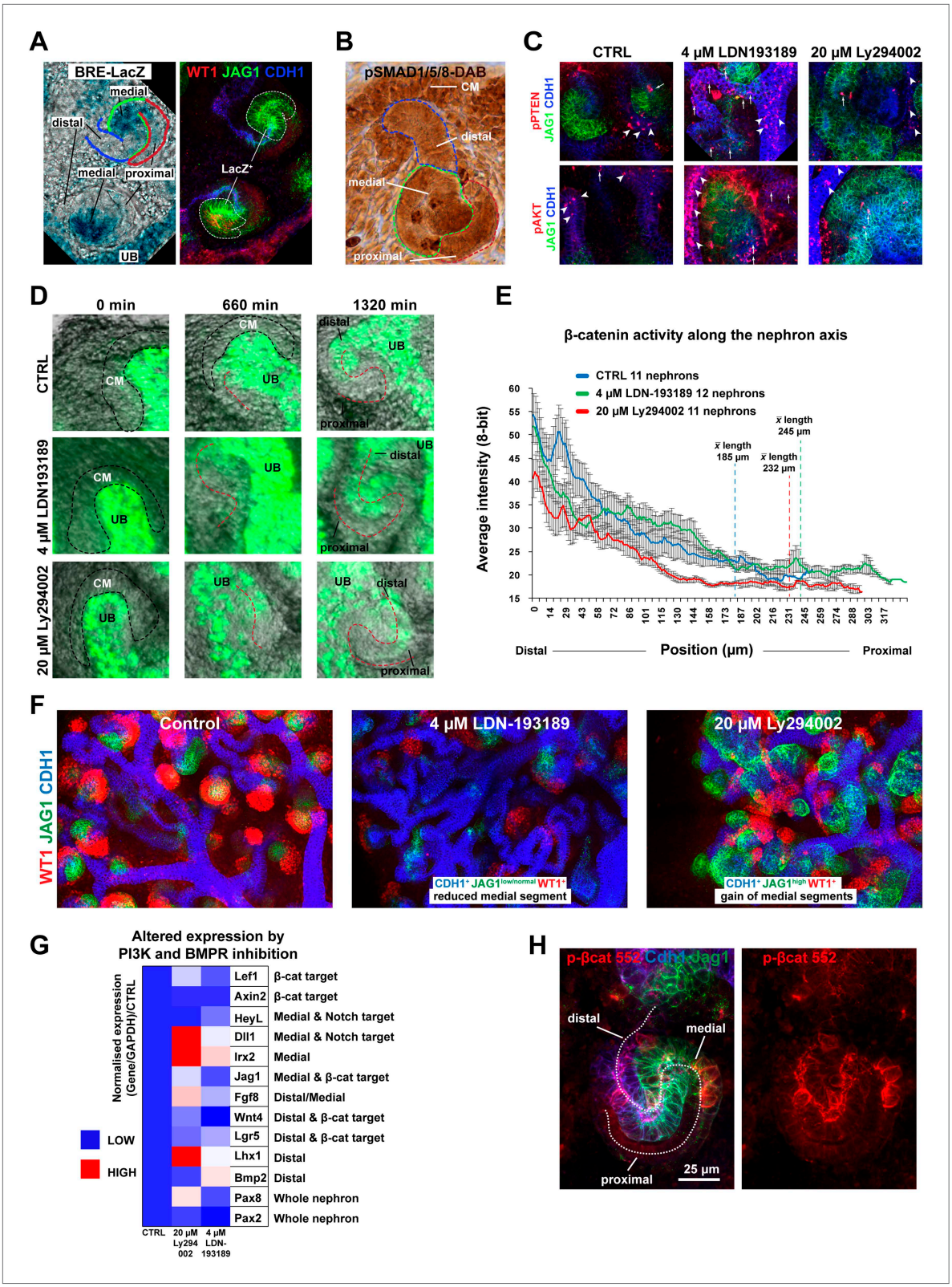

Figure 6. The β-catenin activity gradient is modified by changes to BMP and PI3K signalling. (**A**) BRE-LacZ pSMAD reporter shows strong labelling in medial segment; co-stained for Wt1, Jag1, Cdh1. (**B**) pSMAD1/5/8 specific antibody stain. Lines and labelling in **A**–**B** indicate different segments. (**C**) pPTEN and pAKT levels in the nephron after inhibition of BMPR with 4 μM LDN-193189 or PI3K with 20 μM Ly294002. White arrowheads and arrows

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indicate staining in ureteric bud and nephrons, respectively. (**D**) Time-lapse of single *TCF/Lef::H2B-GFP* positive nephrons developing from induction stage through S-shaped body stage. Nephron axis in red. Nephrons treated as specified. CM -cap mesenchyme, UB–ureteric bud. (**E**) Quantification of *TCF/Lef::H2B-GFP* intensities at different positions along the proximal–distal axis at S-shaped body stage/22 hr. Multiple nephrons used as indicated on graph. Error bars indicate SEM. Average lengths of nephrons at 22 hr for each condition are indicated on the graph. (**F**) Inhibition of BMPR or PI3K alters the medial segment negatively and positively, respectively. (**G**) qRT-PCR data of segment-specific markers and β-catenin target genes displayed as a heat-map with gene information displayed in figure. (**H**) p-β-catenin Ser552 localisation in S-shaped nephron. Antibody stains and scale bars as indicated.

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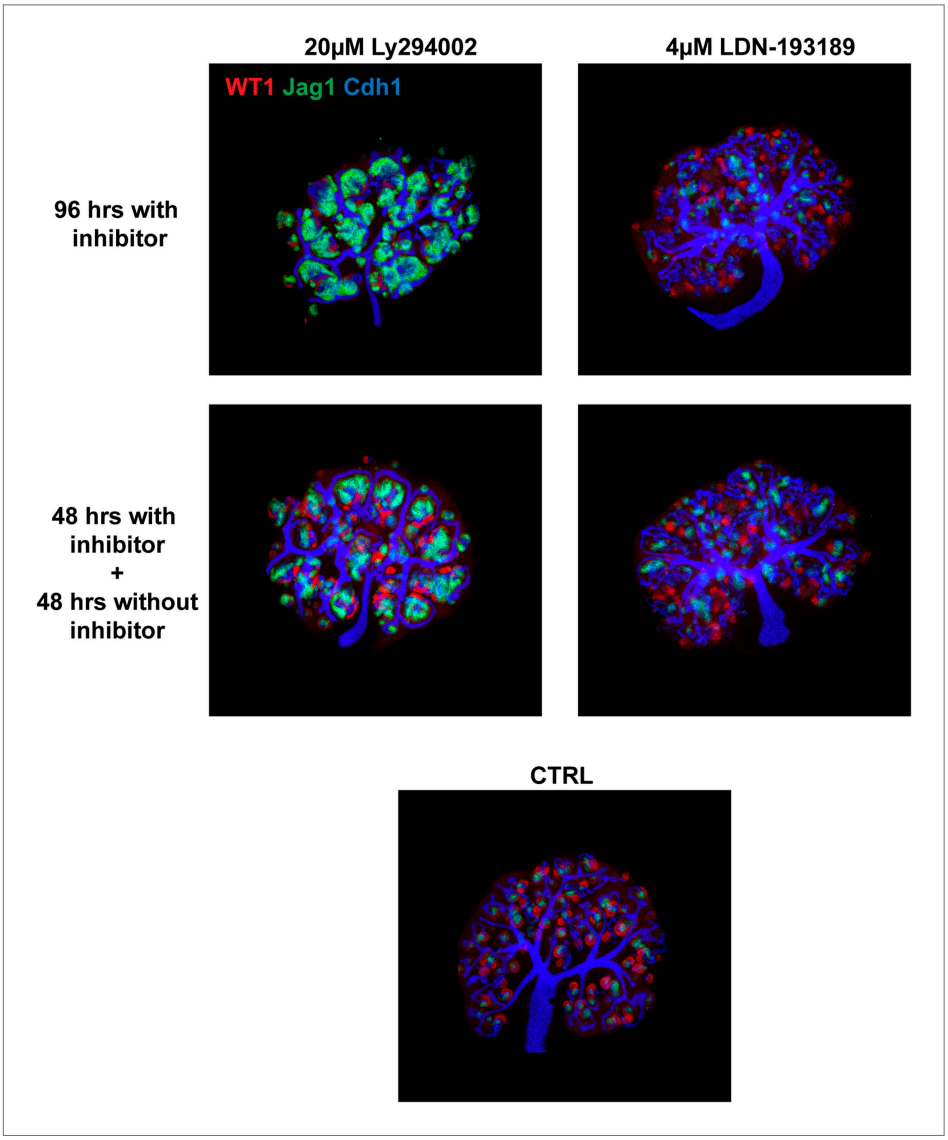

Figure 6—figure supplement 1. Rescue/reversals experiments for kidneys treated with 20 μM Ly294002 or 4 μM LDN193189. Kidneys were cultured for 96 hr in the inhibitors or for 48 hr in inhibitor followed by another 48 hr in control medium. Kidneys were stained for Wt1, Jag1, and Cdh1 to display nephron formation and overall morphology.

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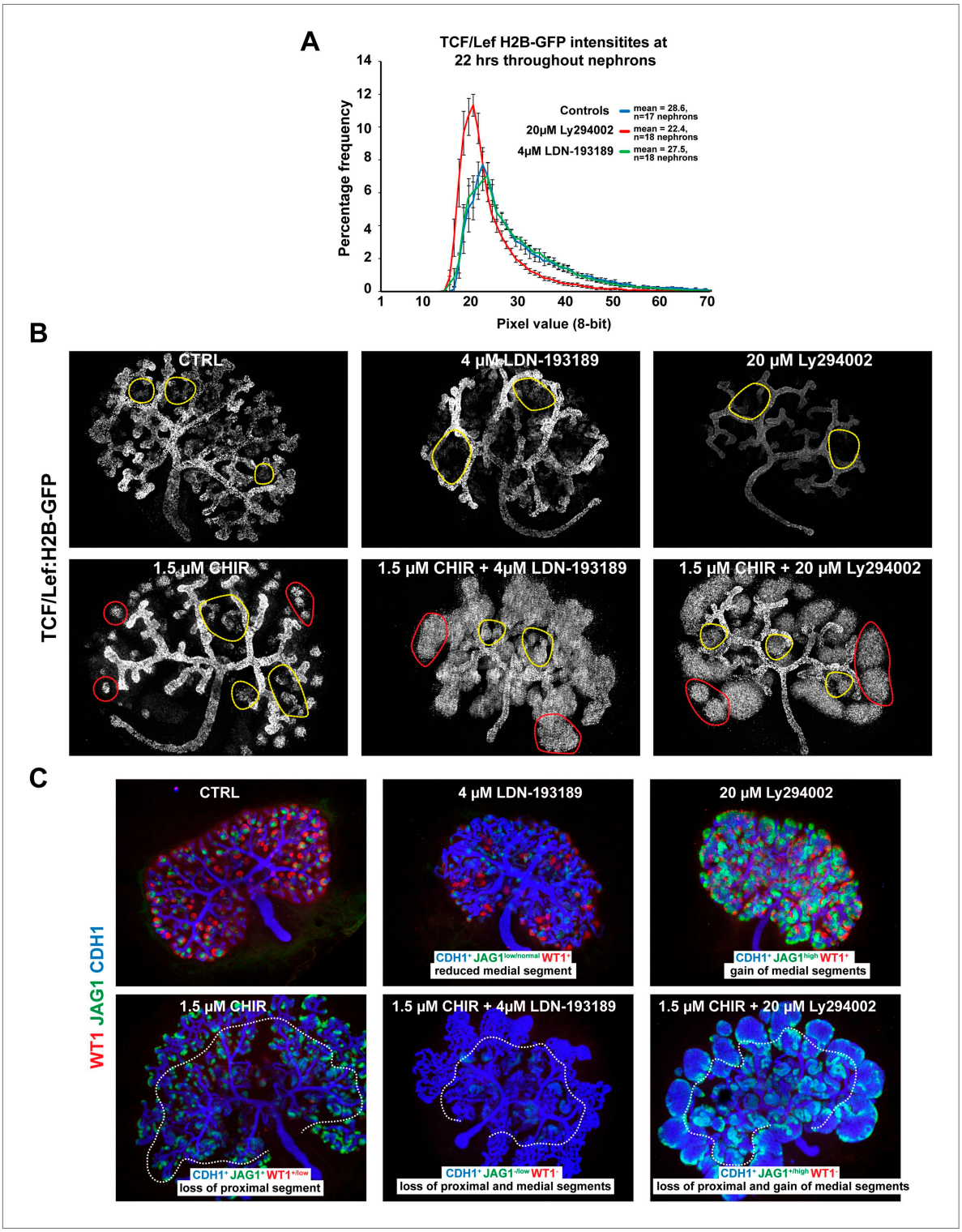

Figure 6—figure supplement 2. Changes to BMP and PI3K signalling alters nephron segmentation and β-catenin signalling. (**A**) Data from time-lapse captured *TCF/Lef:H2B-GFP* nephrons treated with LDN-193189 or Ly294002 or as controls. The percentage frequency is plotted against pixel intensity values. The GFP intensity was measured for all pixels throughout the nephron at 22 hr of culture. Ly294002 treated nephrons had fewer bright nuclei whilst LDN-193189 and control nephrons were not distinguishable at this stage with this method. Error bars indicate SEM. (**B**) Whole *TCF/Lef:H2B-GFP* kidneys treated with LDN-193189, Ly294002, CHIR, and combinations thereof. Yellow and red dashed circles indicate ureteric bud tree-bound endogenous and ectopic nephrons, respectively. (**C**) Inhibition of BMPR, PI3K, and activation of β-catenin predictably alters the medial and proximal segments. Dashed line indicates separation between ectopic and endogenous ureteric bud tree-bound nephron structures.

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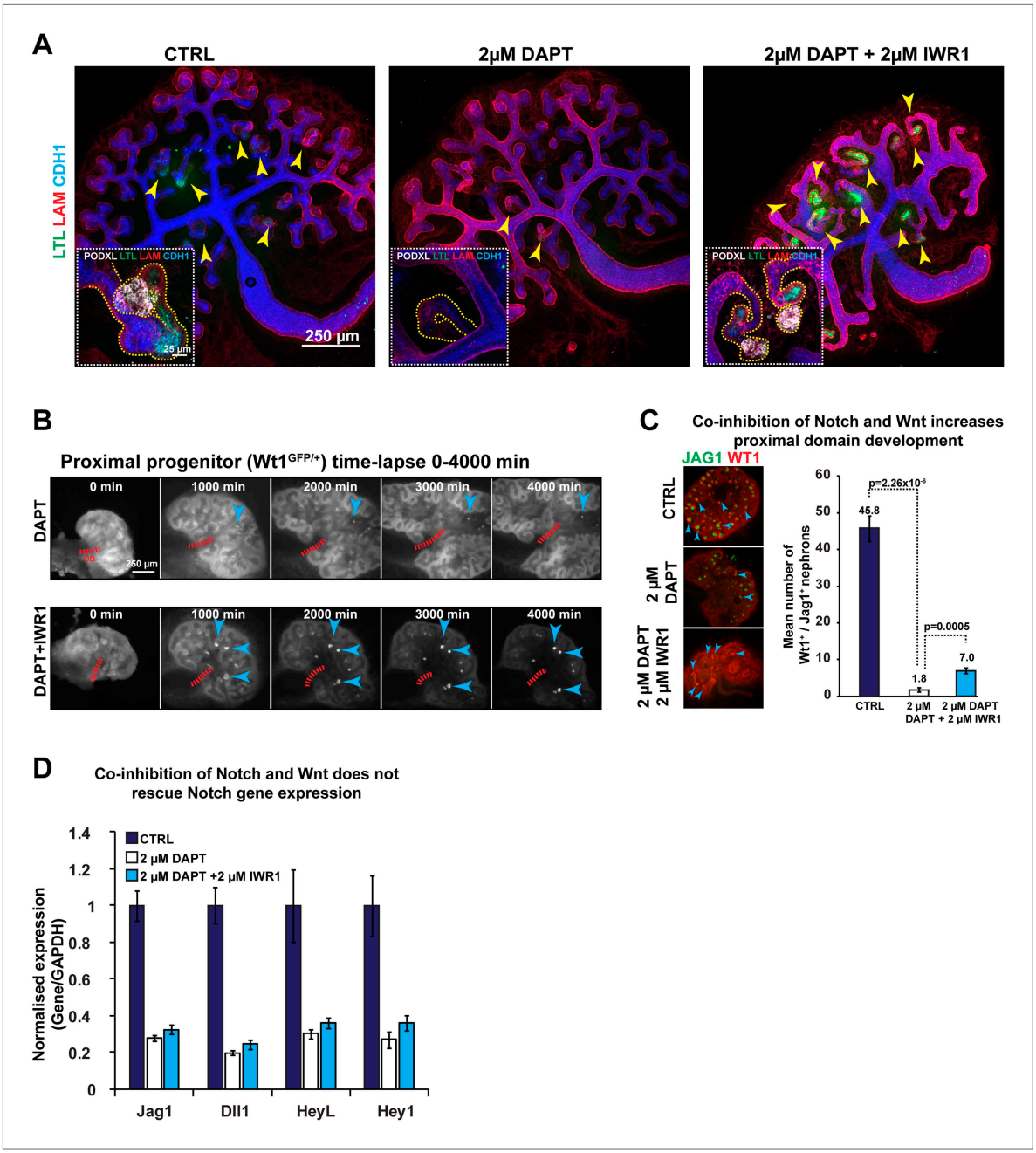

Figure 7. Altered β-catenin activity rescues the loss of Notch. (**A**) Kidneys treated with DAPT and DAPT/IWR1 and stained for LTL, β-laminin, Cdh1, and Podxl–arrowheads indicate LTL-positive nephrons, inserts show magnified nephrons with Podxl staining for podocytes, yellow line outlines nephron tubules. (**B**) Time-lapse analysis of $Wt1^{+/GFP}$ kidneys treated with DAPT and DAPT + IWR1–arrowheads show GFP^{HIGH} structures in developing proximal segments, red dashed line indicates ureteric bud positions (UB). (**C**) Structures positive for Jag1 and Wt1 in treated kidneys–arrowheads indicating double-positive structures. (**D**) qRT-PCR data for Notch target genes (*Jag1*, *Dll1*, *Heyl*, *Hey1*). All error bars indicate SEM.

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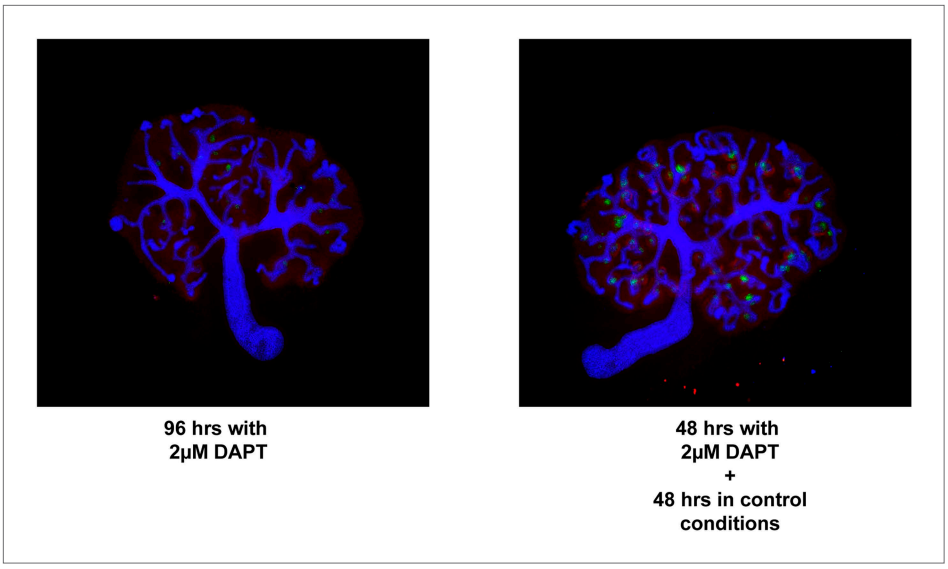

Figure 7—figure supplement 1. Rescue/reversals experiments for kidneys treated with 2 μM DAPT. Kidneys were cultured for 96 hr in DAPT or for 48 hr in DAPT followed by another 48 hr in control medium. Kidneys were stained for Wt1, Jag1, and Cdh1 to display nephron formation and overall morphology. Antibody stains indicated.
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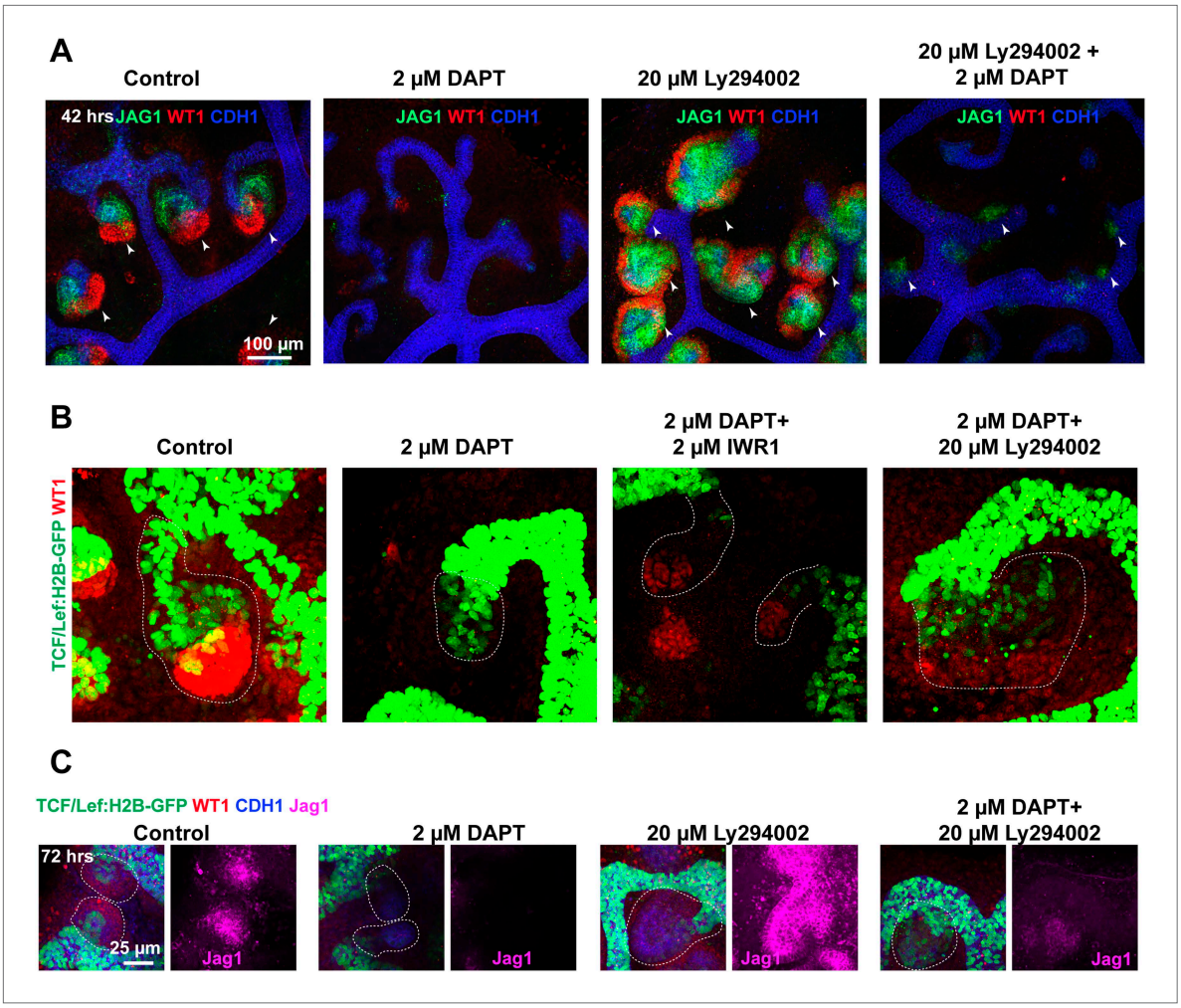

Figure 7—figure supplement 2. Altered β-catenin activity or PI3K signalling rescues the loss of Notch. Data from kidneys treated with 2 μM DAPT and in combination with 20 μM Ly294002 or 2 μM IWR1. (**A**–**B**) *TCF/Lef:H2B-GFP* nephrons stained for WT1, JAG1, and CDH1. WT1 and JAG1 stains are not optimally compatible with the PFA fixation required to preserve the GFP signal. Thus, wild-type kidneys were also treated and stained for WT1 JAG1 and CDH1—shown in (**C**). Jag1^{+} structures indicated with arrowheads. Stains, scales, and treatments as specified.

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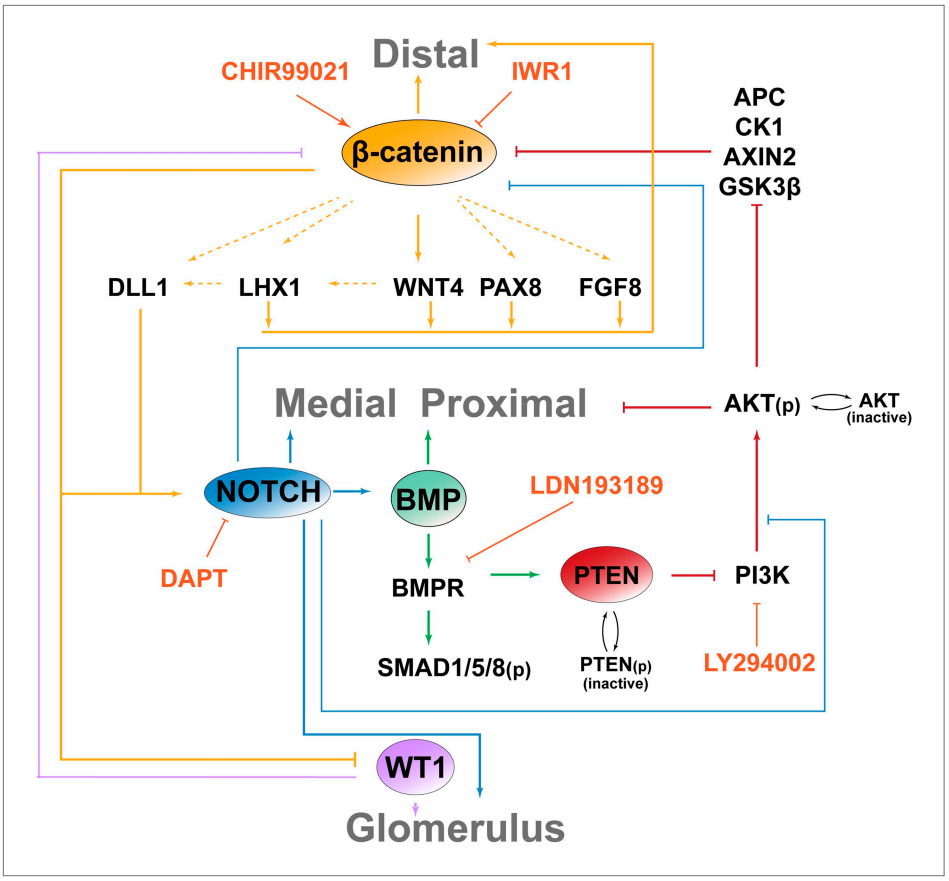

Figure 8. Model for molecular pathways interacting to control the patterning of the nephron. β-catenin activity is necessary to determine a distal cell identity but must be excluded from the proximal nephron. BMP/PTEN/PI3K antagonises β-catenin activity in the medial segment and positively promotes a medial fate and Notch ligand expression. Notch is essential for medial and proximal development. Glomerular progenitor cells strongly inhibit β-catenin function, possibly via a WT1-dependent mechanism as in Sertoli cells (***Chang et al., 2008***).

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