RESEARCH ARTICLE

Figures and figure supplements

Canonical Wnt signaling and the regulation of divergent mesenchymal Fgf8 expression in axolotl limb development and regeneration

Giacomo L Glotzer *et al*

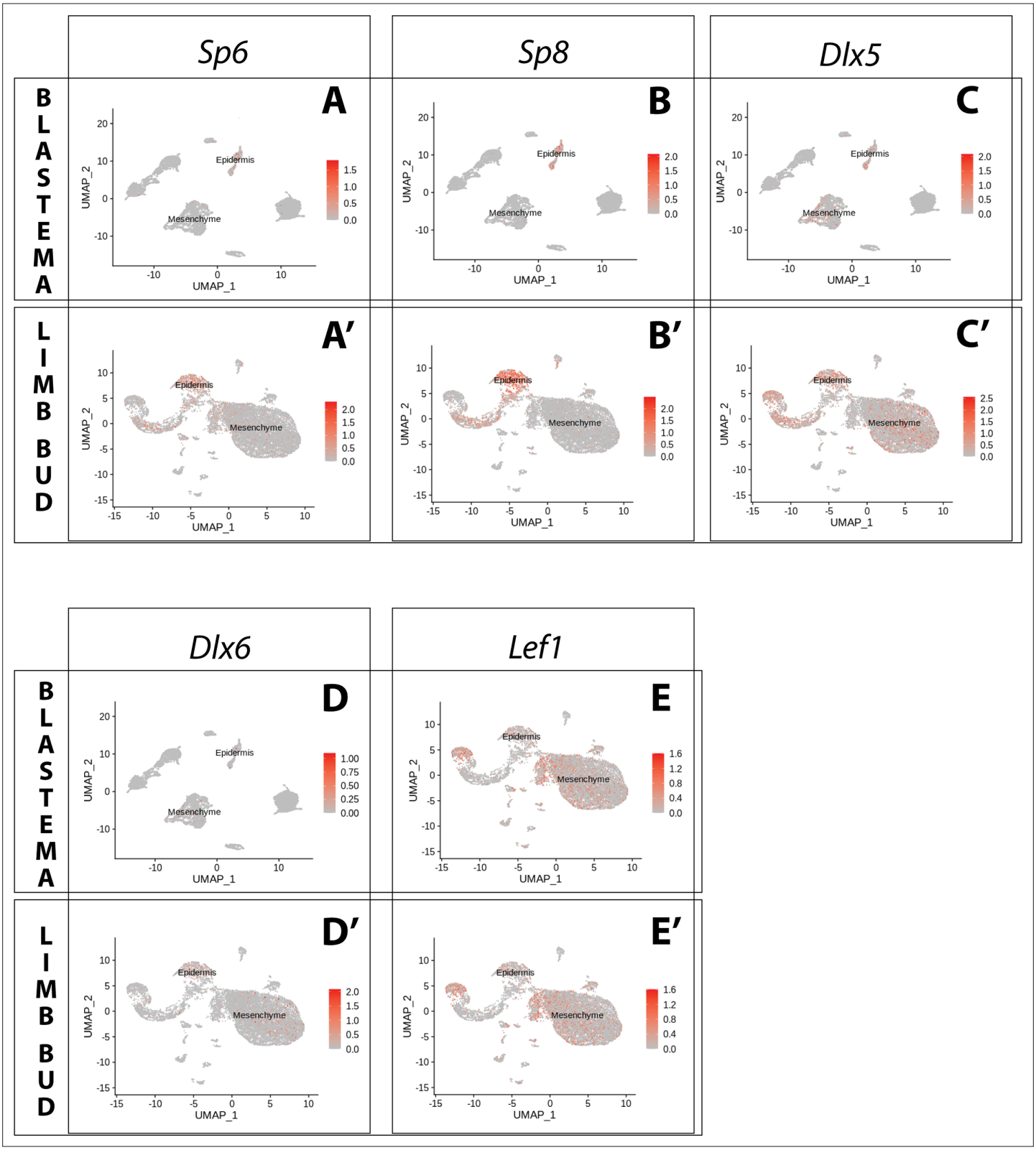

Figure 1. Expression of AER transcription factors in the axolotl limb bud and limb blastema assessed by reanalysis of Axolotl scRNA seq datasets. Reanalysis of scRNA seq data from blastema (**A–E**, data from ***Li et al., 2021***) or limb bud (**A′–E′**, data from ***Lin et al., 2021***). (**A, A′**) UMAPs of Axolotl scRNA seq expression data for *Sp6*. Expression is detected in the epidermis of the axolotl blastema and limb bud. (**B, B′**) UMAPs of Axolotl scRNA seq expression data for *Sp8*. Expression is detected in the epidermis of the axolotl blastema and limb bud. (**C, C′**) UMAPs of Axolotl scRNA seq expression

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data for *Dlx5*. Expression is detected in the epidermis and mesenchyme of the axolotl blastema and limb bud. (**D, D'**) UMAPs of Axolotl scRNA seq expression data for *Dlx6*. Expression is detected in the epidermis and mesenchyme of the axolotl limb bud. Expression is detected only in few blastema cells. (**E, E'**) UMAPs of Axolotl scRNA seq expression data for *Lef1*. Expression is detected in the epidermis and mesenchyme of the axolotl blastema and limb bud.

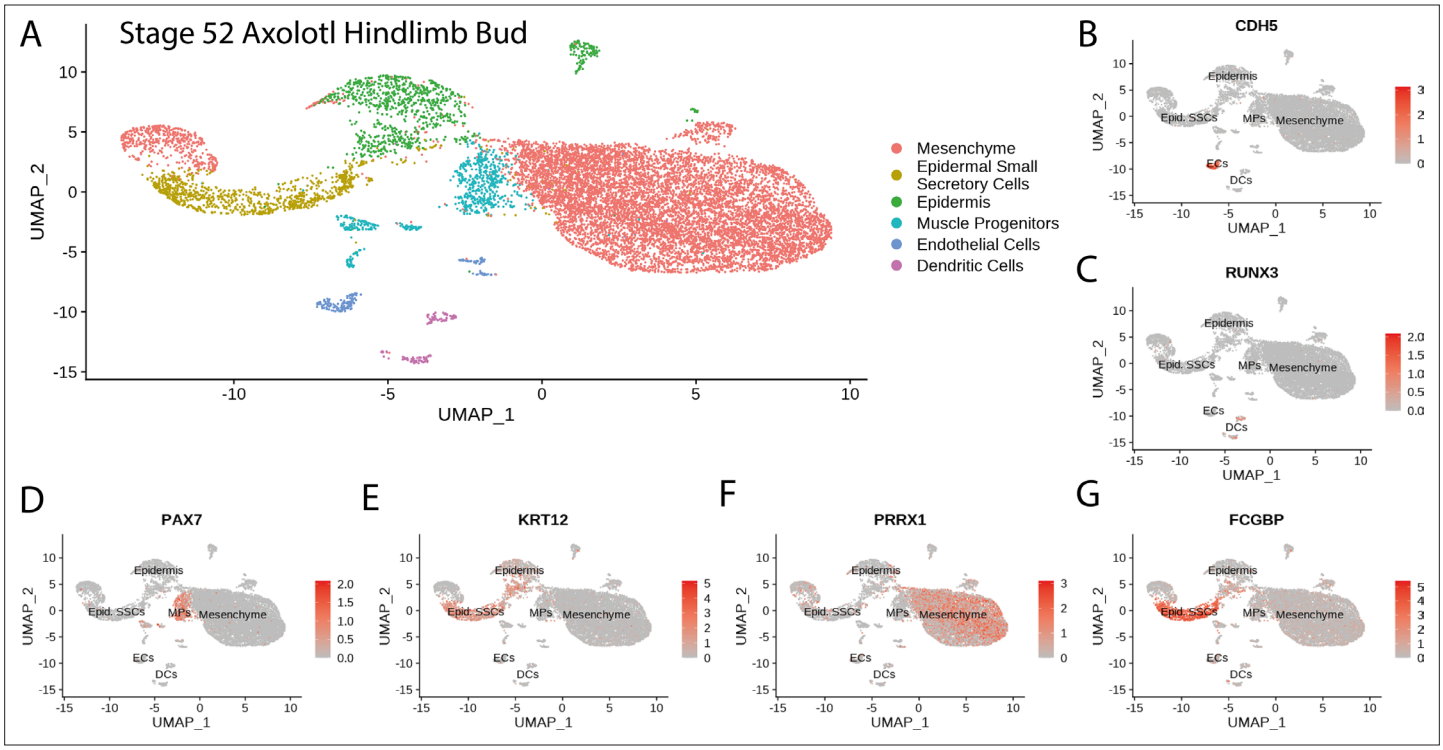

Figure 1—figure supplement 1. Cluster identification in the reanalysis of axolotl limb bud scRNA seq dataset from ***Lin et al., 2021***. (**A**) Clustering of axolotl limb bud scRNA seq expression data reveals six clusters that are readily identifiable by the expression of key markers. (**B**) *Cdh5* labels endothelial cells in the axolotl limb bud scRNA seq dataset. (**C**) *Runx3* labels dendritic cells in the axolotl limb bud scRNA seq dataset. (**D**) *Pax7* labels muscle progenitors in the axolotl limb bud scRNA seq dataset. (**E**) *Krt12* labels epidermal cells (including SSCs) in the axolotl limb bud scRNA seq dataset. (**F**) *Prrx1* labels mesenchymal cells in the axolotl limb bud scRNA seq dataset. (**G**) *Fcgbp* labels epidermal small secretory cells in the axolotl limb bud scRNA seq dataset.

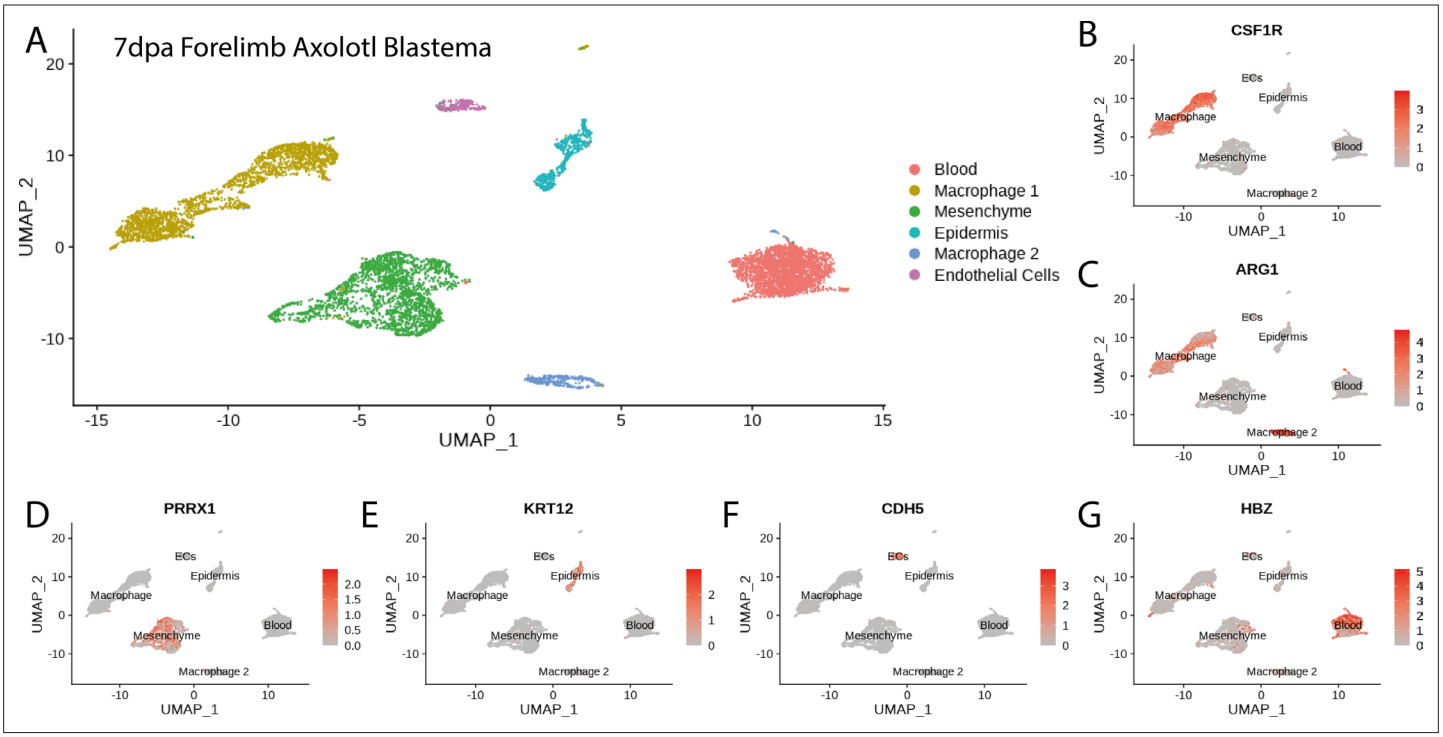

Figure 1—figure supplement 2. Cluster identification in the reanalysis of axolotl blastema scRNA seq dataset from ***Li et al., 2021***. (**A**) Clustering of axolotl limb bud scRNA seq expression data reveals six clusters that are readily identifiable by the expression of key markers. (**B, C**) *Csf1r* and *Arg1* label two macrophage clusters in the axolotl blastema scRNA seq dataset. (**D**) *Prrx1* labels mesenchymal cells in the axolotl blastema scRNA seq dataset. (**E**) *Krt12* labels epidermal cells in the axolotl blastema scRNA seq dataset. (**F**) *Cdh5* labels endothelial cells in the axolotl blastema scRNA seq dataset. (**G**) *Hbz* labels blood cells in the axolotl blastema scRNA seq dataset.

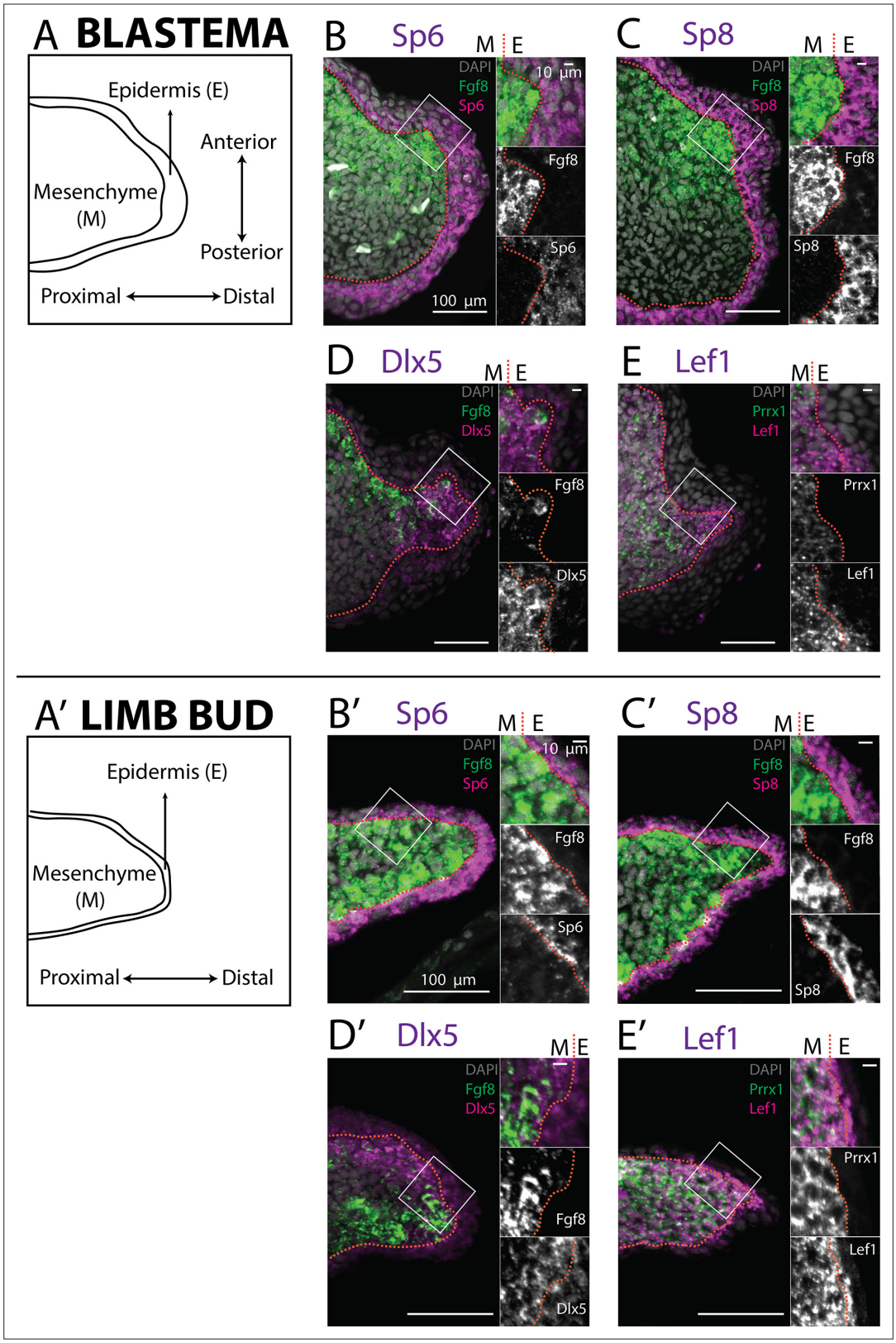

Figure 2. Expression of AER transcription factors in the axolotl limb blastema and limb bud assessed by HCR in situ hybridization. (**A, A′**) Schematic outlining the mesenchymal and epidermal compartments in a longitudinal section of an axolotl blastema and limb bud. (**B, B′**) Expression of *Sp6* in the epidermis and of *Fgf8* in the mesenchyme of the axolotl blastema and limb bud revealed by HCR (single planes from whole-mount images, *n* =

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4). (**C, C'**) Expression of *Sp8* in the epidermis and of *Fgf8* in the mesenchyme of the axolotl blastema and limb bud revealed by HCR (single planes from whole-mount images, *n* = 4). (**D, D'**) Expression of *Fgf8* in the mesenchyme as well as of *Dlx5* in the mesenchyme and basal epidermis of the axolotl limb bud and blastema (single planes from whole-mount images, *n* = 4). (**E, E'**) Expression of the mesenchymal marker *Prrx1*, and of *Lef1* in the mesenchyme and basal epidermis of the axolotl blastema and limb bud (single planes from whole-mount images, *n* = 4). For microscopy images right panels represent magnifications of the outlined boxes, M = mesenchyme, E = epidermis. Dashed lines demarcate epidermal–mesenchymal boundaries.

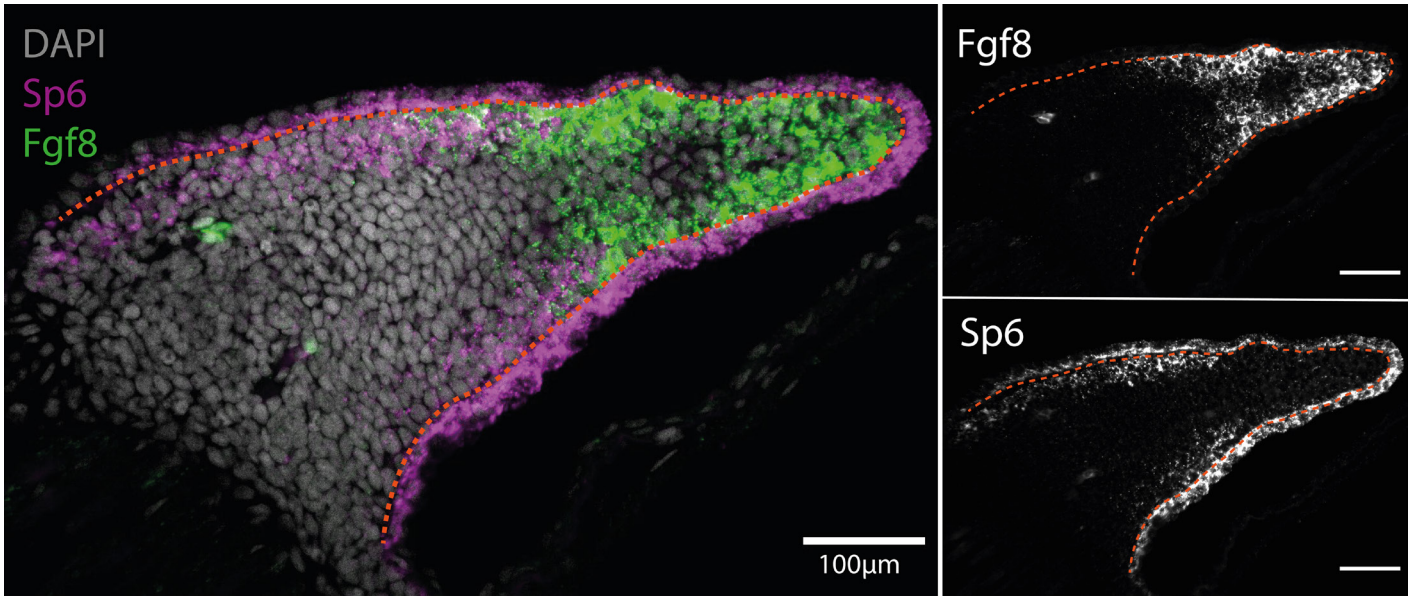

Figure 2—figure supplement 1. *Sp6* is expressed in the basal epidermis and proximal mesenchyme in the axolotl limb bud (n = 4). (**A**) Single plane image from a whole-mount limb bud HCR staining of *Sp6* and *Fgf8* transcripts. (**B**) *Fgf8* expression is localized to the distal mesenchyme of the axolotl limb bud. (**C**) *Sp6* is ubiquitously expressed in the epidermis but only expressed in the proximal mesenchyme of the axolotl limb bud. Dashed lines demarcate epidermal–mesenchymal boundaries.

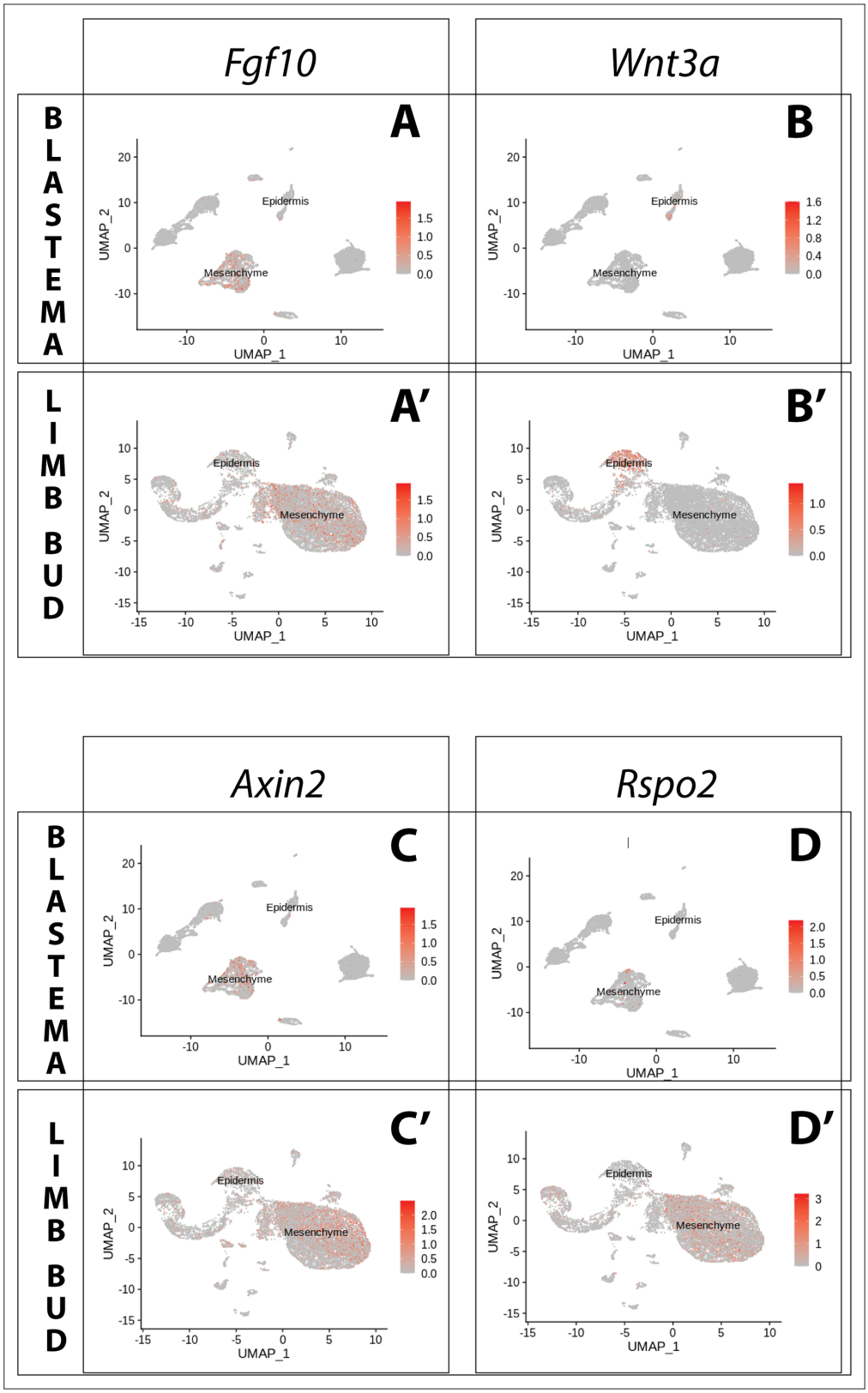

Figure 3. Expression of *Fgf10* and of the canonical Wnt pathway components *Wnt3a*, *Axin2*, *Rspo2* in the axolotl limb bud and limb blastema assessed by reanalysis of Axolotl scRNA seq datasets. Reanalysis of scRNA seq data from blastema (**A–D**, data from ***Li et al., 2021***) or limb bud (**A′–D′**, data from ***Lin et al., 2021***). (**A, A′**) UMAPs of Axolotl scRNA seq expression data for *Fgf10*. Expression is detected in the mesenchyme of the axolotl blastema

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and limb bud. (**B, B'**) UMAPs of Axolotl scRNA seq expression data for *Wnt3a*. Expression is detected in the epidermis of the axolotl blastema and limb bud. (**C, C'**) UMAPs of Axolotl scRNA seq expression data for *Axin2*. Expression is detected in the prevalently in the mesenchyme of the axolotl blastema and limb bud. (**D, D'**) UMAPs of Axolotl scRNA seq expression data for *Rspo2*. Expression is detected in the mesenchyme of the axolotl limb bud. Expression is detected only in few blastema cells.

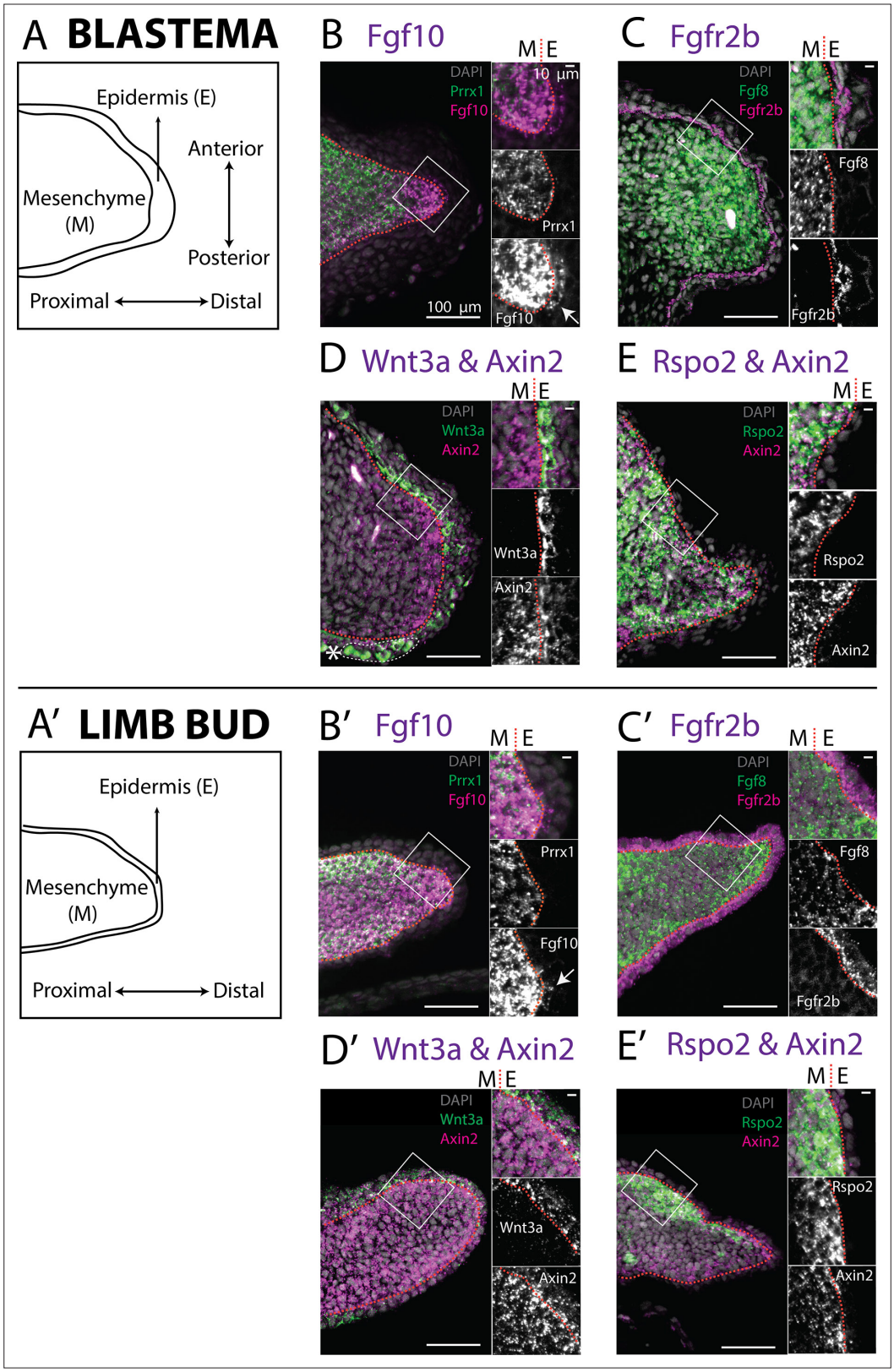

Figure 4. Expression of *Fgf10*, *Fgfr2b* and of the canonical Wnt pathway components *Wnt3a*, *Axin2*, *Rspo2* in the axolotl limb bud and limb blastema evaluated by HCR in situ hybridization. (**A, A′**) Schematic outlining the mesenchymal and epidermal compartments in a longitudinal section of an axolotl blastema and limb bud. (**B, B′**) Expression of *Prrx1* and *Fgf10* in the mesenchyme of the axolotl blastema and limb bud revealed by HCR

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(single planes from whole-mount images, *n* = 4). Arrows point to weak *Fgf10* expression present in the distal basal epidermis of the blastema and limb bud. (**C, C'**) Expression of *Fgf8* in the mesenchyme and expression of *Fgfr2b* in the basal epidermis of the axolotl blastema and limb bud revealed by HCR (single planes from whole-mount images, *n* = 4). (**D, D'**) Expression of *Wnt3a* in the basal epidermis and *Axin2* in the mesenchyme and basal epidermis of the axolotl blastema and limb bud (single planes from whole-mount images, *n* = 4). *Axin2* expression is stronger in the mesenchyme and weaker in the epidermis. (**E, E'**) Expression of *Rspo2* in the mesenchyme and *Axin2* in the mesenchyme and basal epidermis of the axolotl blastema and limb bud (single plane from whole-mount image, *n* = 4). Bright green structures (*) in the blastema outer epidermis are autofluorescent signal. For microscopy images right panels represent magnifications of the outlined boxes, M = mesenchyme, E = epidermis. Dashed lines demarcate epidermal–mesenchymal boundaries.

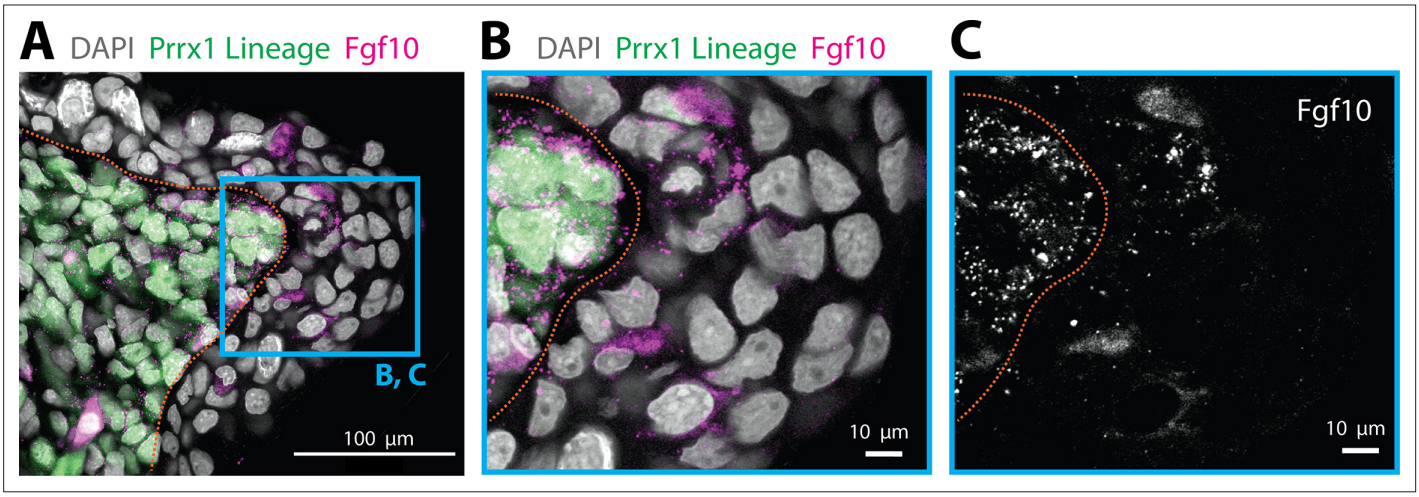

Figure 4—figure supplement 1. Epidermal cells expressing *Fgf10* are not of mesenchymal origin. (**A**) *Fgf10* transcripts are present in epidermal cells that are not *Prrx1* lineage labeled in the axolotl blastema. *Fgf10* transcripts are present in epidermal cells that are not *Prrx1* lineage labeled in the axolotl blastema (n = 4 blastemas). HCR in situ hybridization performed in a tissue section of a *Prrx1:Cre-ER;CAGGs:lp-Cherry* animal that was tamoxifen converted during limb development. Green cells are cells of mesenchymal lineage origin. (**B**, **C**) Magnifications of the box outlined in A. Dashed lines demarcate epidermal–mesenchymal boundaries.

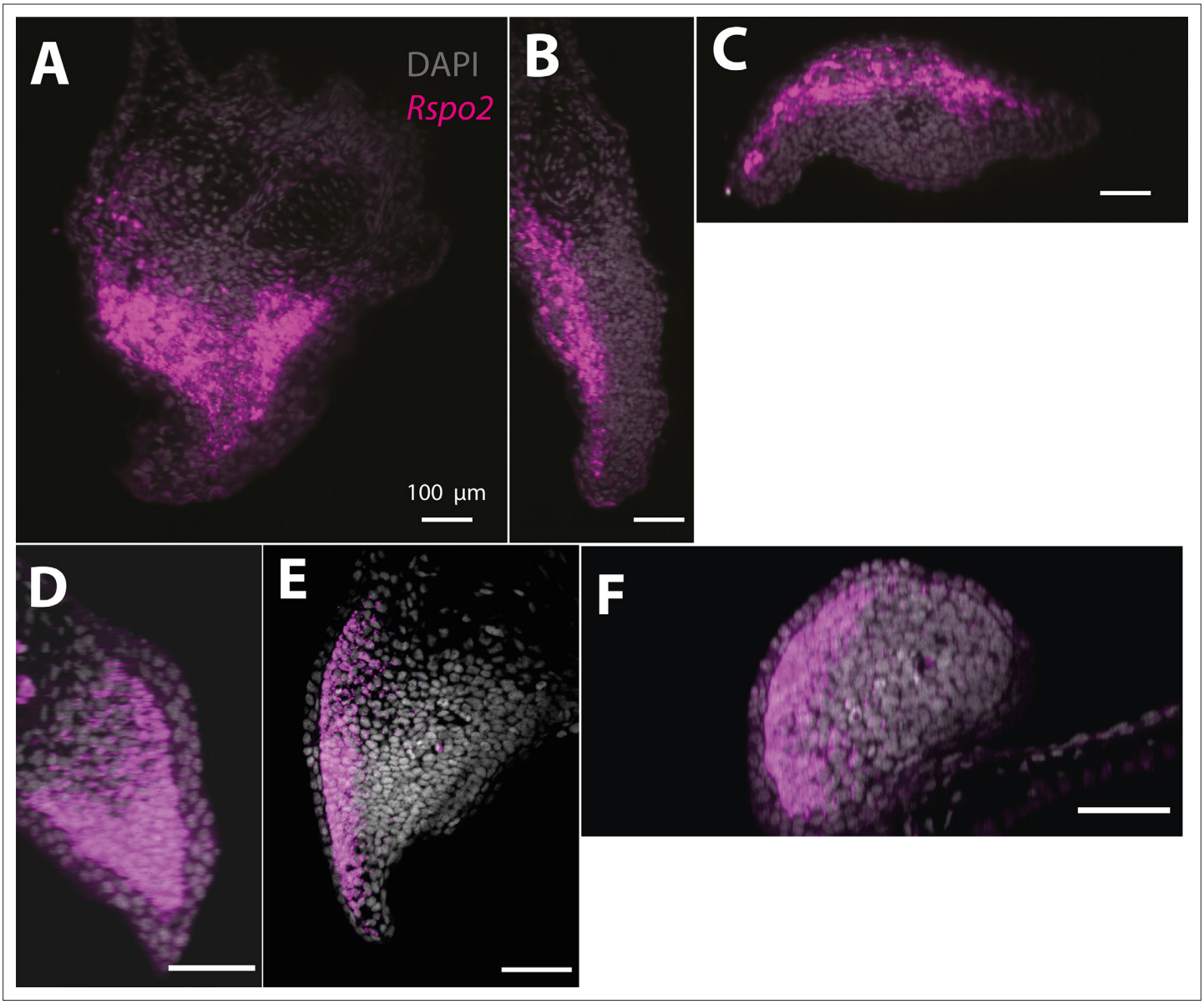

Figure 4—figure supplement 2. *Rspo2* expression is biased along the dorsoventral axis in the axolotl blastema (**A–C**) and limb bud (**D–F**) (*n* = 4). (**A**) Single plane image from a whole-mount blastema HCR staining of Rspo2 transcripts. (**B**) Cross-section of the blastema proximodistal axis showing biased expression along the dorsoventral axis. (**C**) Cross-section of the blastema anterior–posterior axis showing biased expression along the dorsoventral axis. (**D**) Single plane image from a whole-mount limb bud HCR staining of Rspo2 transcripts. (**E**) Cross-section of the limb bud proximodistal axis showing biased expression along the dorsoventral axis. (**F**) Cross-section of the limb bud anterior–posterior axis showing biased expression along the dorsoventral axis.

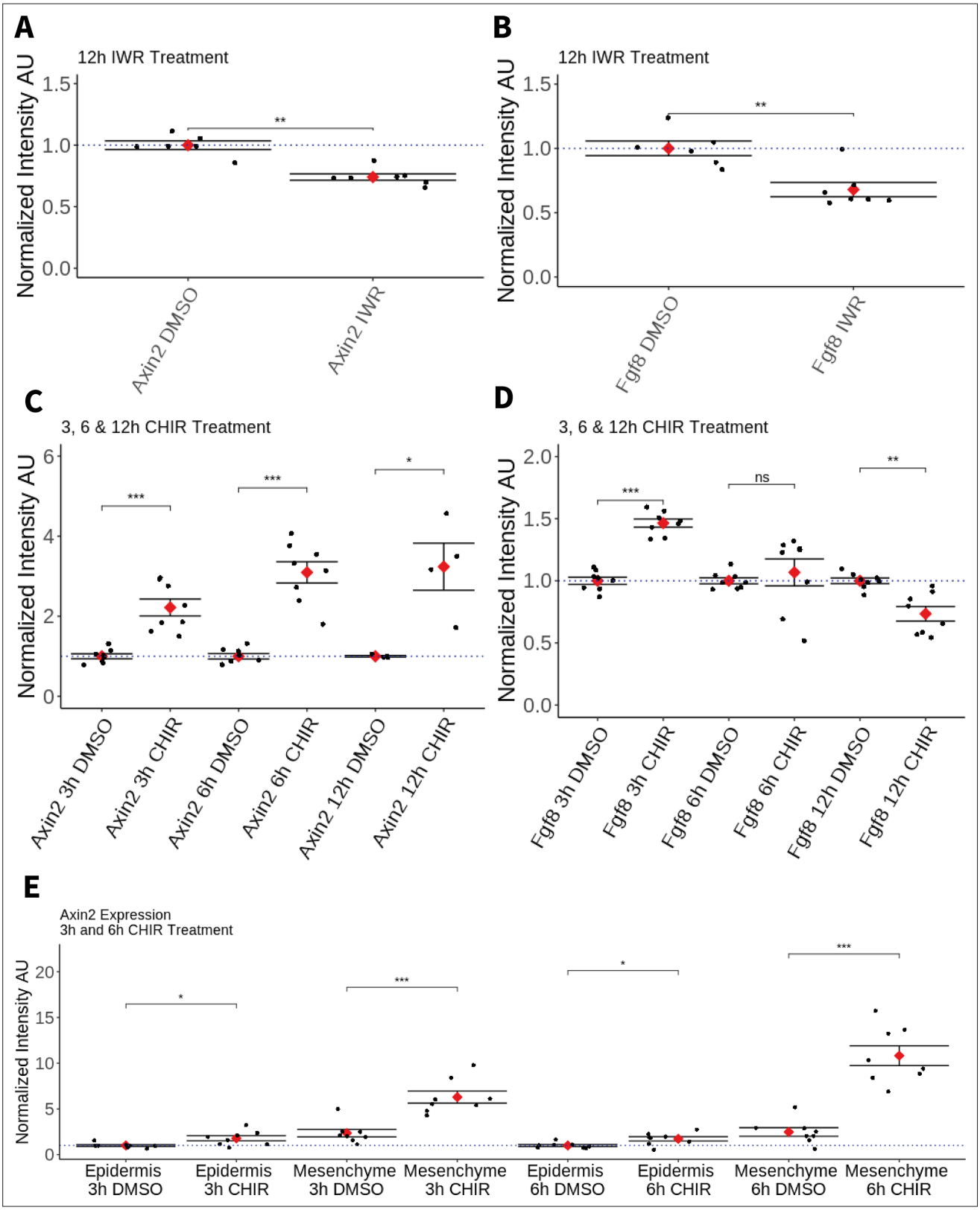

Figure 5. Effect of pharmacological perturbation of Wnt signaling on *Fgf8* expression in the axolotl blastema. Plots depicting expression levels of *Fgf8* or *Axin2* as assessed by mean fluorescent intensity of HCR signal inside the corresponding gene expression domains. Each black dot represents the mean signal quantified from one blastema, each red dot represents mean values for each condition. Whiskers indicate the standard error of the mean. * indicates statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ns = nonsignificant = p > 05). Statistics were performed using the nonparametric

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Wilcoxon rank sum test. (**A, B**) Pharmacological inhibition of Wnt signaling using IWR1-endo downregulates *Axin2* and *Fgf8* expression in the axolotl limb blastema. (**C**) CHIR treatment activates canonical Wnt signaling in the axolotl blastema as shown by upregulation of *Axin2* expression after 3, 6, and 12 hr of treatment. (**D**) Pharmacological activation of Wnt signaling using CHIR upregulates *Fgf8* in the axolotl blastema after 3 hr of treatment. *Fgf8* expression comparable to DMSO control levels after 6 hr of treatment and downregulated after 12 hr of CHIR treatment. (**E**) Mesenchymal and epidermal *Axin2* expression are both upregulated after 3 and 6 hr of CHIR treatment. Mesenchymal *Axin2* increases 2.6-fold after 3 hr and 4.4-fold after 6 hr of treatment. Epidermal *Axin2* increases 1.8-fold after 3 hr and 1.7-fold after 6 hrs of treatment.

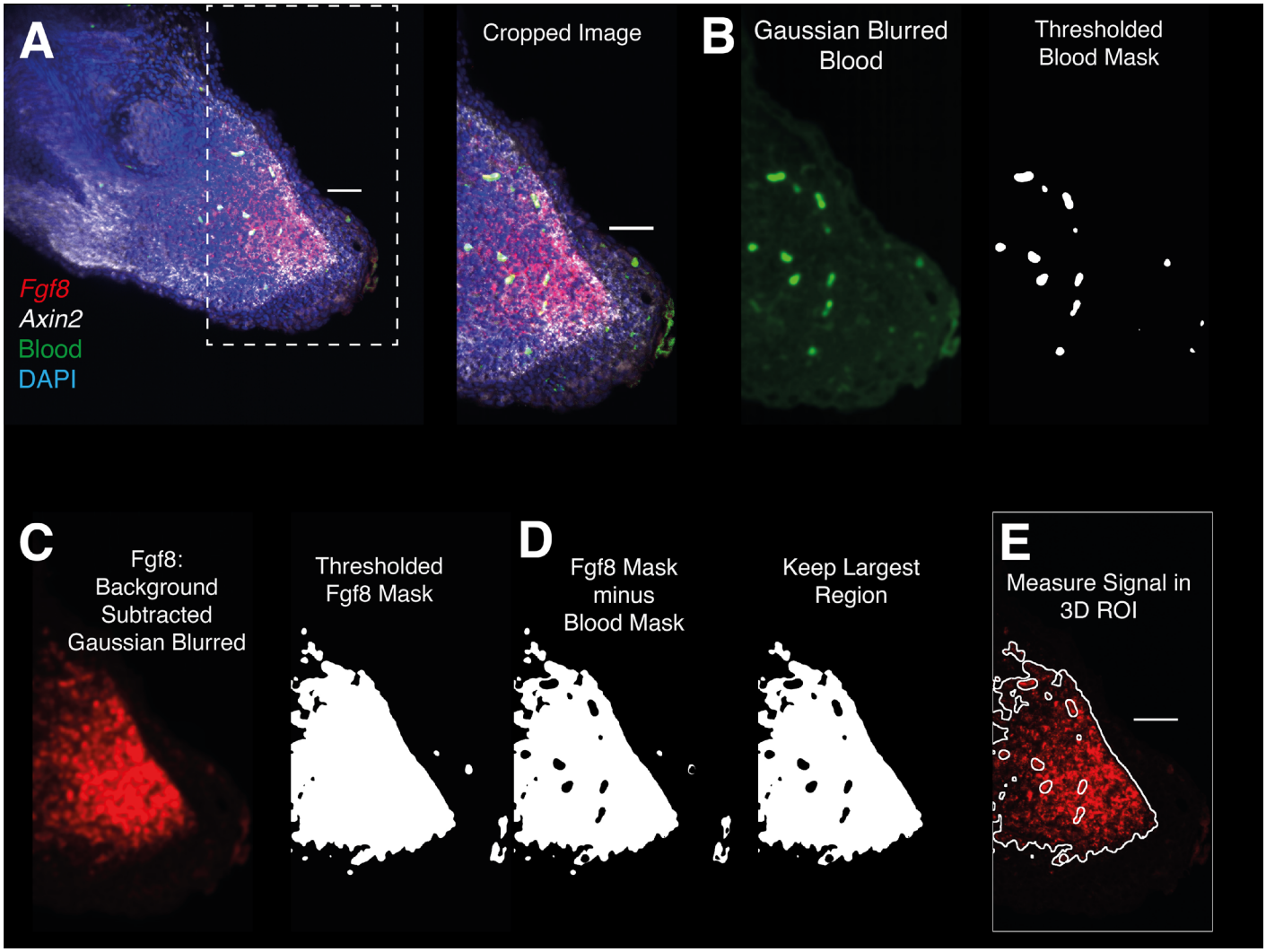

Figure 5—figure supplement 1. Image analysis workflow for whole-mount HCR signal quantification inside gene expression domains. (**A**) 3D images are cropped distal to the amputated bone. (**B**) The Image acquired using 488 illumination (no HCR staining) is used to generate a 3D mask of the autofluorescent blood cells. The mask is generated by thresholding after denoising (Gaussian Blur). (**C**) HCR images are background subtracted, denoised, and thresholded to generate a mask containing HCR signal + autofluorescence. (**D**) HCR signal is isolated subtracting the previously obtained autofluorescence mask from the gene expression mask. (**E**) The resulting mask is refined and used for 3D signal quantification.

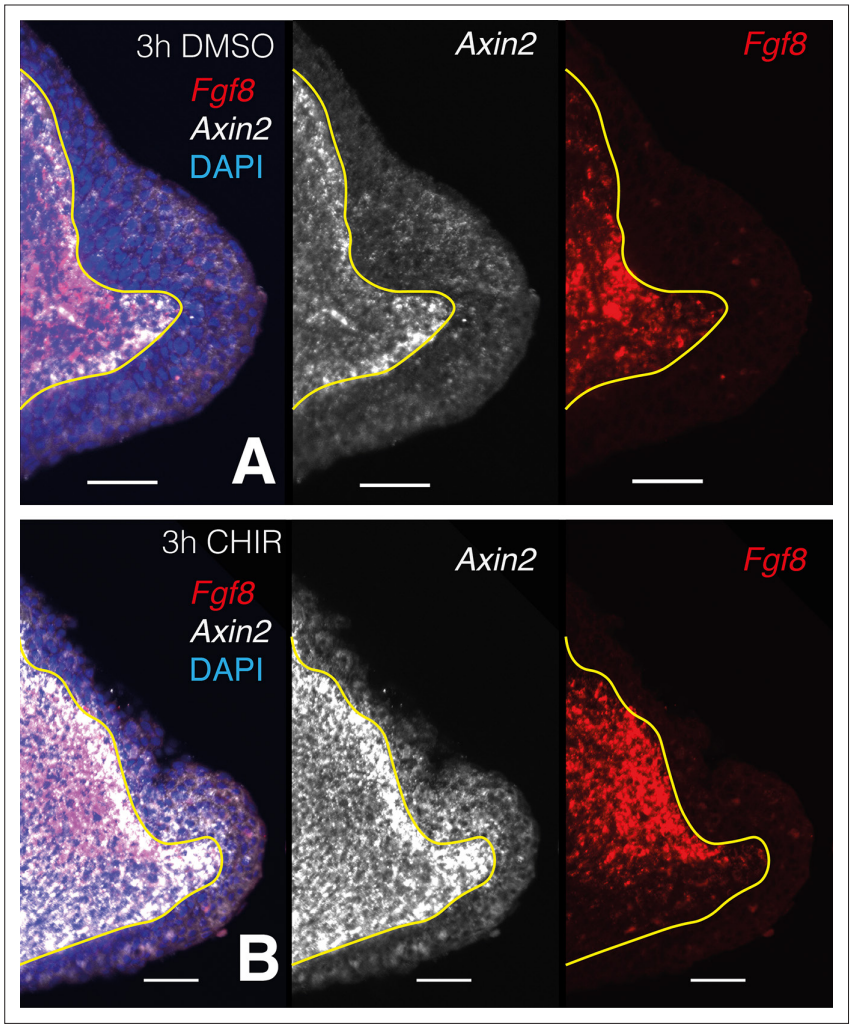

Figure 5—figure supplement 2. Single plane images from whole-mount stacks of 3-hr CHIR-treated and DMSO control samples. (**A**) Three hours DMSO control blastema. From left to right: composite multichannel image, *Axin2* alone, Fgf8 alone. (**B**) Three hours CHIR-treated blastema. From left to right: composite multichannel image, *Axin2* alone, *Fgf8* alone. (**A, B**) All pairs from treated and control are displayed with the same settings. Yellow lines demarcate epidermal–mesenchymal boundaries.

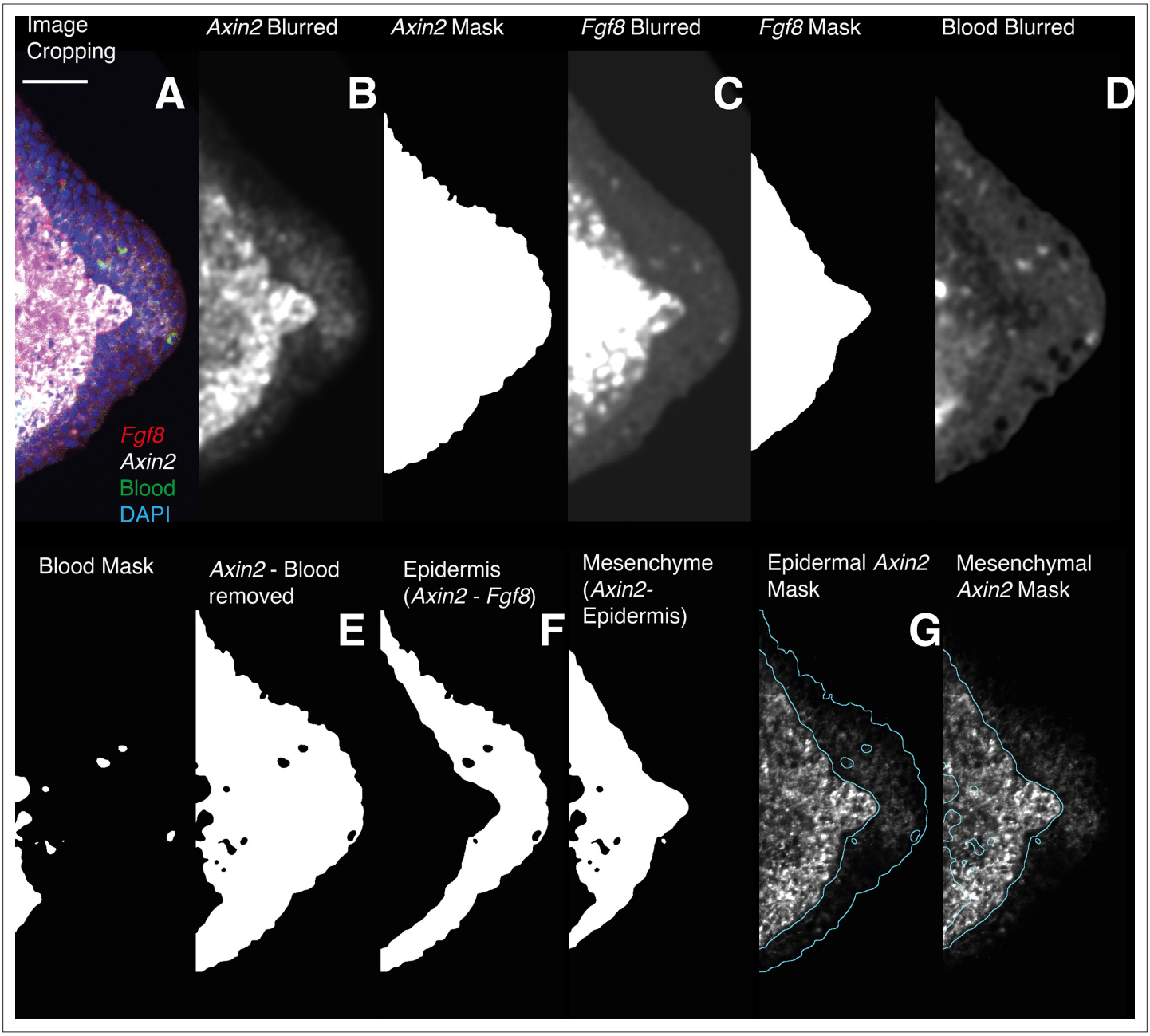

Figure 5—figure supplement 3. Image analysis workflow for the quantification of *Axin2* HCR signal quantification in the epidermal and mesenchymal domains. (**A**) 3D images are cropped, in the cropped images the entire mesenchyme contains *Fgf8* transcripts. (**B, C**) HCR images for *Axin2* and *Fgf8* are denoised and thresholded to generate a mask containing HCR signal + autofluorescence. (**D**) The Image acquired using 488 nm illumination (no HCR staining) is used to generate a 3D mask of the autofluorescent blood cells. (**E**) The *Axin2* gene expression domain is isolated by subtracting the autofluorescence mask from the previously obtained masks. (**F, G**) The *Fgf8* mask is used to outline the mesenchyme. A mask for the *Axin2* epidermal expression domain is obtained subtracting the *Fgf8* mask from the *Axin2* mask. To obtain intensity measurements the 3D masks are applied to the raw data after background subtraction.