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Figures and figure supplements

Precardiac deletion of Numb and Numblike reveals renewal of cardiac progenitors

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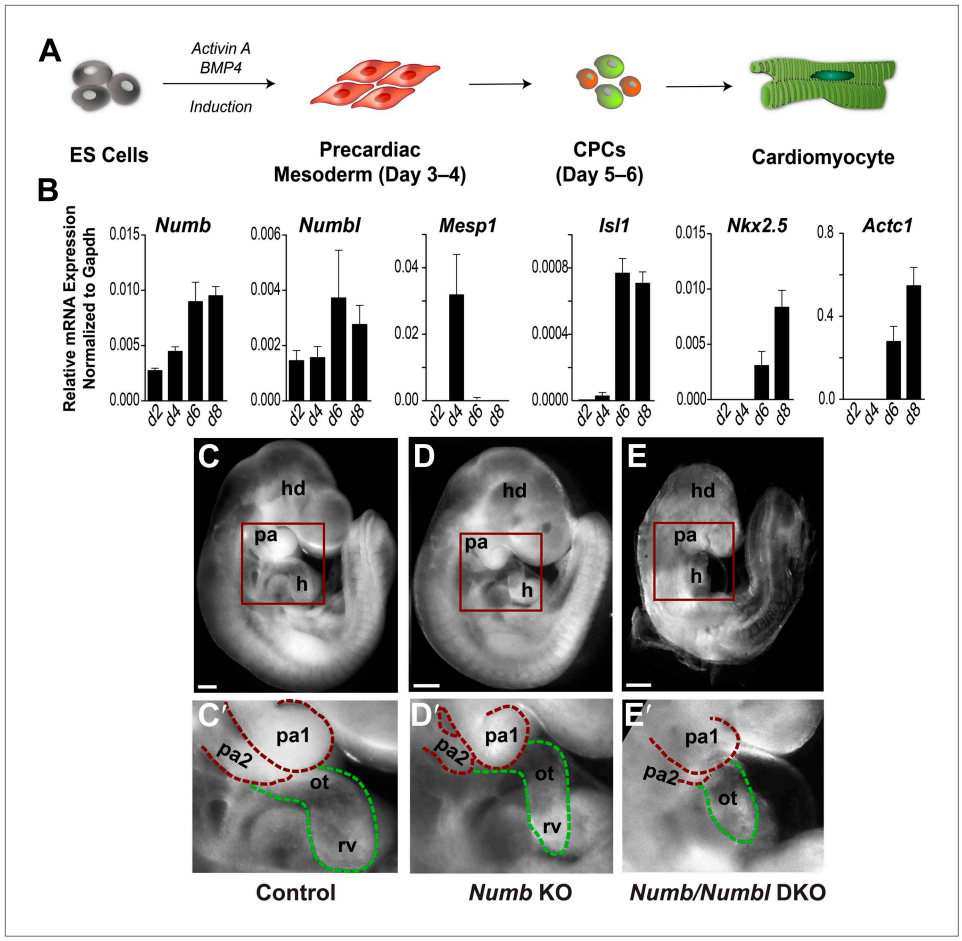

Figure 1. Numb and Numbl are required for PA2 and heart development. (**A**) Schema of cardiac differentiation in ES cell system. (**B**) Expression profiles of genes indicated during cardiac differentiation of ES cells. Gene expression was analyzed by qPCR. Data are mean ± SD; n = 4; d, day. (**C**–**E**) Lateral views of control (**C**), *Mesp1*^{Cre}; *Numb*$^{flox/flox}$ (*Numb* KO, **D**), *Mesp1*^{Cre}; *Numb*$^{flox/flox}$; *Numbl*$^{-/-}$ (*Numb/Numbl* DKO, **E**) embryos. (**C′**–**E′**) Enlargement of boxed areas in (**C**–**E**), showing normal, hypoplastic or atrophic PA2 and heart in control (**C′**), *Numb* KO (**D′**) or *Numb/Numbl* DKO (**E′**) embryos, respectively. Pharyngeal arches (red) and outflow tract/right ventricle (green) are outlined in dashes. Scale bars, 150 μm. hd, head; pa, pharyngeal arch; h, heart; ot, outflow tract; ra, right atrium; rv, right ventricle.
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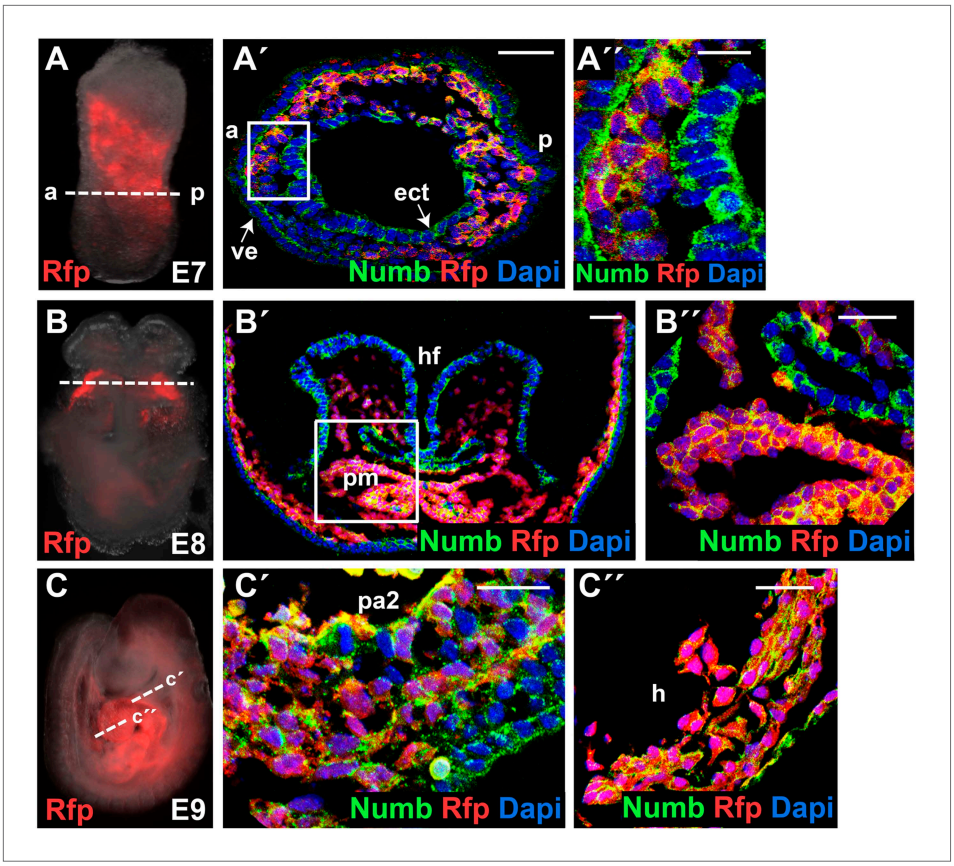

Figure 1—figure supplement 1. Numb is ubiquitously expressed in developing embryos.

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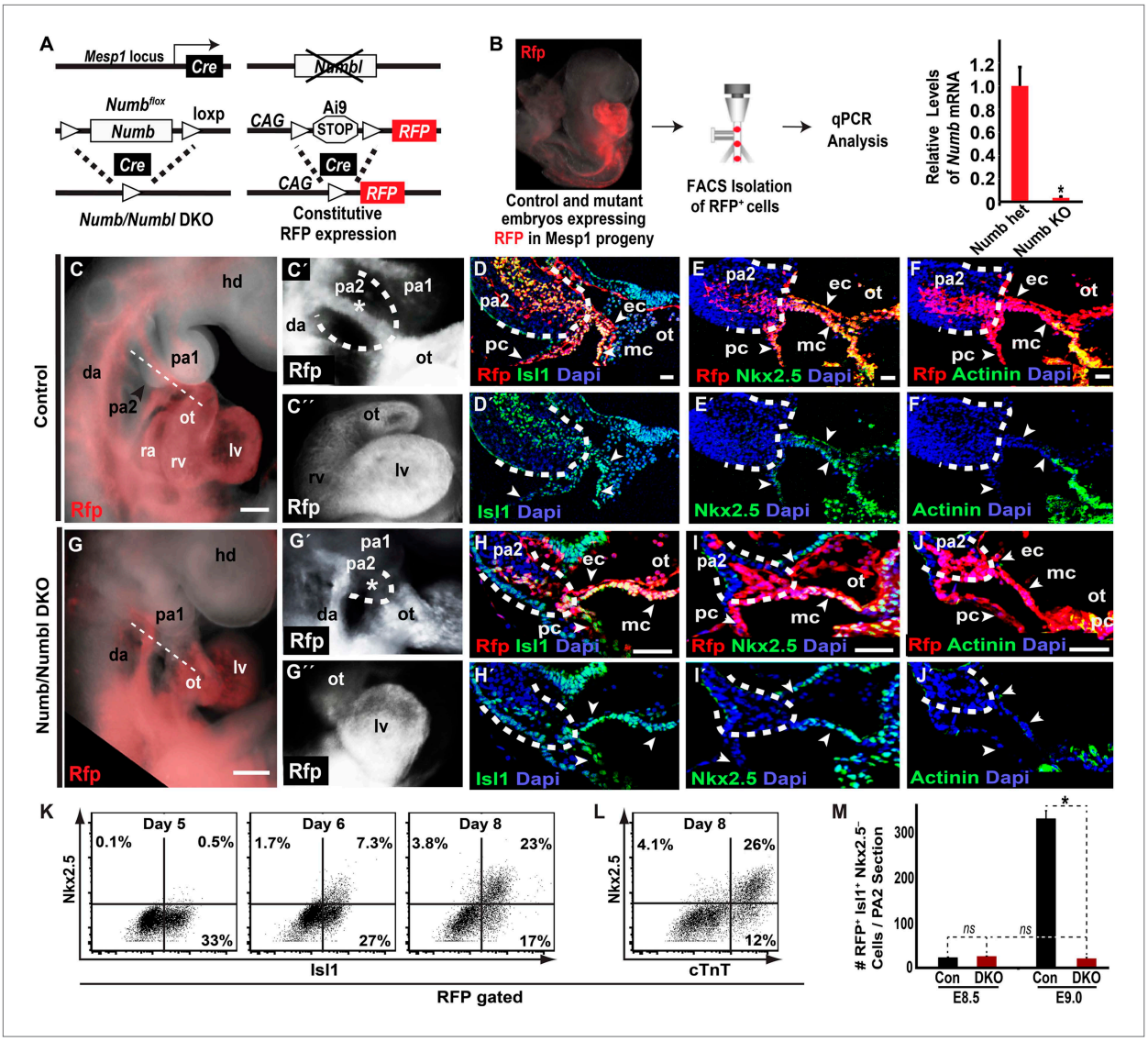

Figure 2. Deletion of Numb and Numbl depletes Mesp1+ progenitor-derived Isl1+ Nkx2.5– cells in PA2. (**A**) Generation of *Numb/Numbl* DKO embryos expressing RFP in Mesp1 progeny (*Mesp1*^{Cre}; *Numb*$^{flox/flox}$; *Numbl*$^{-/-}$; *Ai9*). (**B**) Relative mRNA levels of *Numb* in RFP^{+} cells isolated from control and *Numb/Numbl* DKO embryos. Data are mean ± SD; n = 9. (**C**, **C′**, **G**, **G′**) Lateral views of Mesp1^{+} cell-traced control (**C** and **C′**) or *Numb/Numbl* DKO (**G** and **G′**) embryos analyzed at E9.0. RFP marks Mesp1 progeny. Control embryos show continuous RFP expression from second pharyngeal arch (PA2, outlined) to heart (asterisk, **C′**), but the arch (outlined) is severely underdeveloped in *Numb/Numbl* DKO embryos without noticeable RFP expression (asterisk, **G′**). (**C″** and **G″**) Frontal views of control (**C″**) or *Numb/Numbl* DKO (**G″**) heart. (**D**–**F′**), (**H**–**J′**), Representative confocal images of transverse sections through PA2 and outflow tract (OT) of control (**D**–**F′**) and *Numb/Numbl* DKO (**H**–**J′**) embryos. Cutting planes are shown in (**C**) and (**G**). Internal boundaries of PA2 are outlined in dashes. Dapi (blue) was used to counterstain the nuclei. (**K** and **L**) Representative plots of intracellular staining of RFP-gated cells. Isl1 and Nkx2.5 staining during day 5–8 (**K**) and cardiac troponin T (cTnT) and Nkx2.5 staining at day 8 (**L**). (**M**) Average number of RFP^{+} Isl1^{+} Mef2c^{-}Nkx2.5^{-} cells in PA2 section (12-micron) of indicated embryos and stages. Data are mean ± SD; n = 5; *p<0.05; *ns*, not significant. p values were determined using the paired Student *t* test. Scale bars, 25 μm (**D**–**F** and **H**–**J**), 150 μm (**C** and **G**). hd, head; pa, pharyngeal arch; ot, outflow tract; da, dorsal aorta; fe, foregut endoderm; ra, right atrium; rv, right ventricle; lv, left ventricle; ec, endocardial layer; mc, myocardial layer; pc, pericardial layer.
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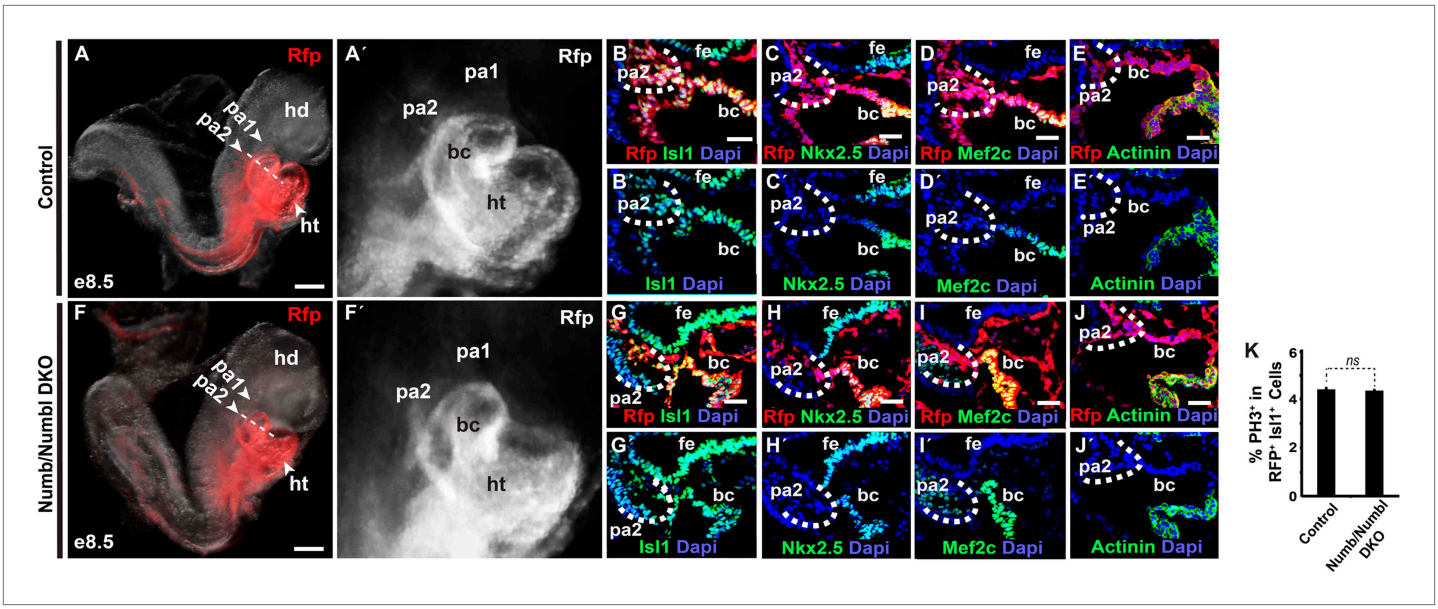

Figure 2—figure supplement 1. Numb/Numbl DKO embryos are grossly normal at E8.5.

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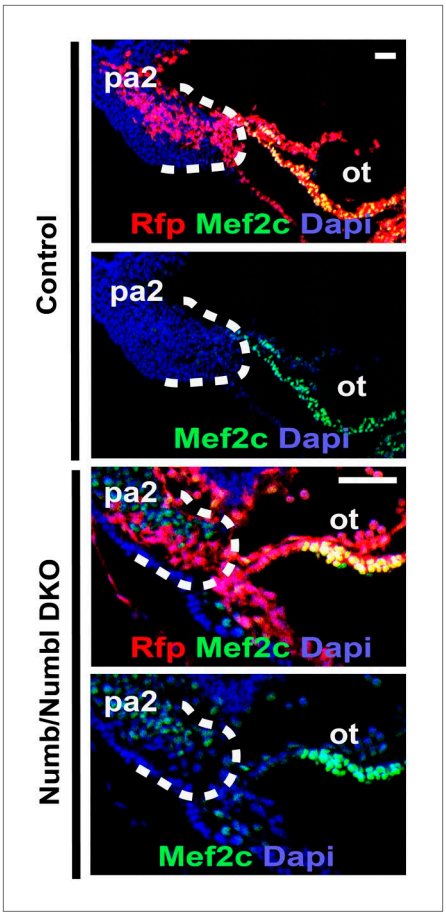

Figure 2—figure supplement 2. Mef2c expression in PA2 and OT of control and Numb/Numbl DKO embryos.
DOI: 10.7554/eLife.02164.007

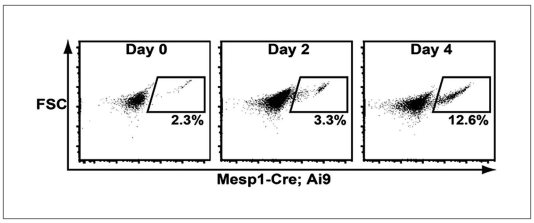

Figure 2—figure supplement 3. Histogram of RFP + cell induction from Mesp-Cre; Ai9 ES Cells.
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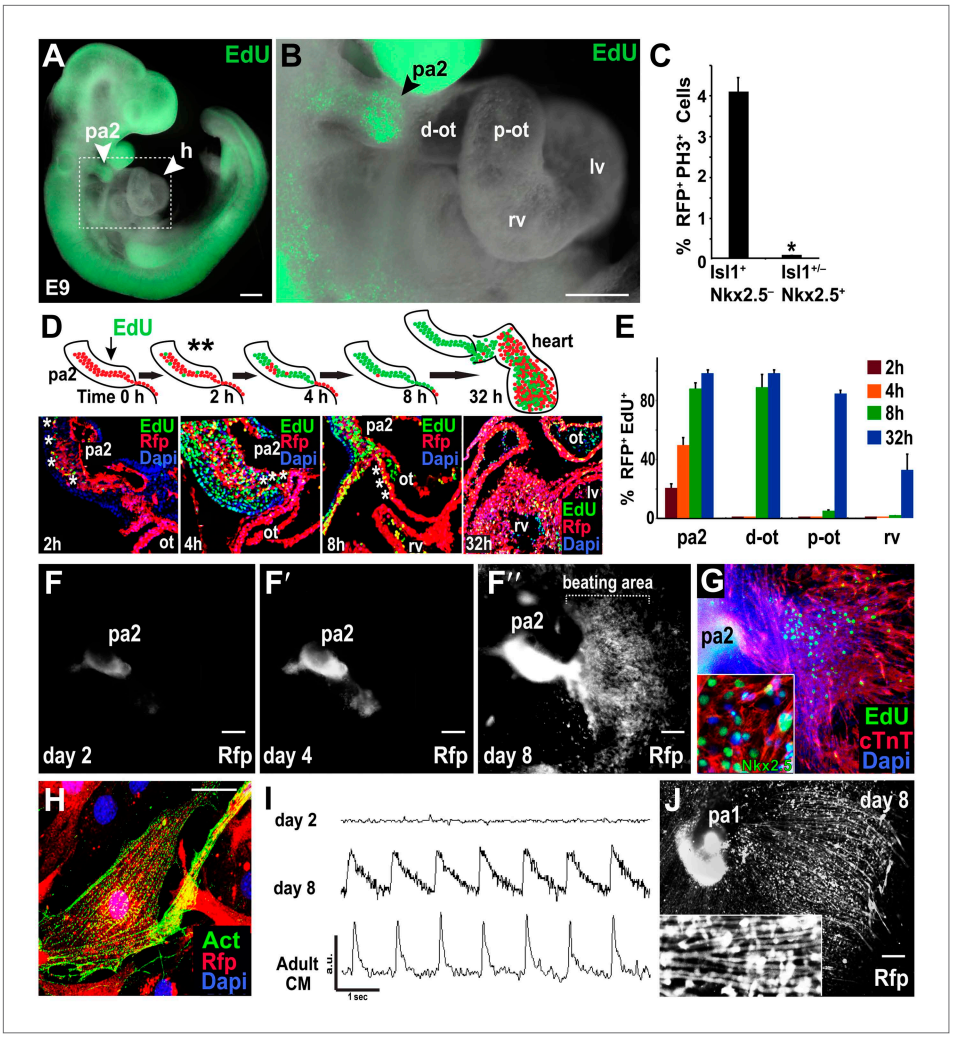

Figure 3. Mesp1+ progenitor-derived Isl1+ Nkx2.5– cells expand in PA2 and differentiate into Nkx2.5+ heart cells after leaving PA2. (**A**) Whole-mount view of EdU (green)-treated embryo at E9.0. (**B**) Enlargement of the boxed area in (**A**), showing enrichment or lack of EdU$^+$ cells in the PA2 or heart region, respectively. (**C**) Percentage of RFP$^+$ PH3$^+$ cells in Isl1$^+$ Mef2c$^-$Nkx2.5$^-$ cells and Isl1$^{+/-}$ Mef2c$^+$ Nkx2.5$^+$ cells. Data are mean ± SD; n = 4. (**D**) EdU pulse experiment. Top, EdU experiment schema. PA2s were also dissected out for ex vivo culture after 2 hr (**). Bottom, progeny of EdU$^+$ cells at 2, 4, 8, and 32 hr after single pulse of EdU injection at E9.0 demonstrating that Mesp1 progeny in PA2 proliferate and migrate to form the OT/RV. (**E**) Quantification of RFP$^+$ EdU$^+$ cell progeny shown in (**D**). Data are mean ± SD; n = 3. (**F**–**F**″), Cultured PA2 explants in 2D culture form a sheet of beating cardiomyocytes (see ***Video 1***). (**G**) EdU-pulsed PA2-derived sheet of beating cells stained with cTnT, EdU, or Nkx2.5 (inset). (**H**) Confocal image of RFP$^+$ cells migrated from PA2 stained with cardiac α-actinin (Act). (**I**) Intracellular Ca2$^+$ transients from day 2 and day 8 PA2 explants and adult cardiomyocyte (CM). a.u., arbitrary unit. (**J**) Cultured PA1 explants form myotube-like cells. Inset shows a magnified view. *p<0.05. D, day; Heart, E10.5 embryonic heart; *ACTC1*, actin, alpha, cardiac muscle 1. Dapi (blue) was used to counterstain the nuclei. p values were determined using the paired Student *t*-test. Scale bars, 10 μm (**H**), 150 μm (**A** and **B**), 250 μm (**F**–**F**″ and **J**). pa, pharyngeal arch; h, heart; d-ot, distal outflow tract; p-ot, proximal outflow tract; rv, right ventricle; lv, left ventricle.

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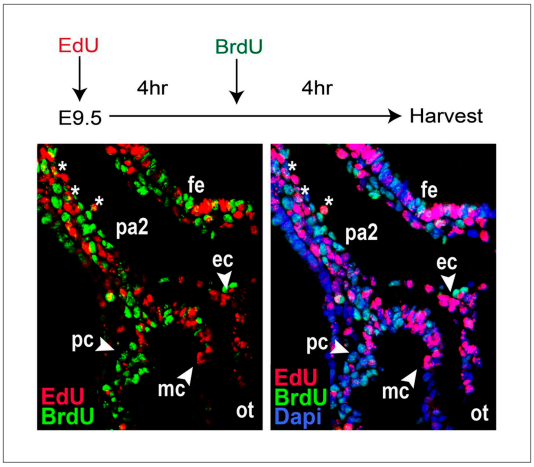

Figure 3—figure supplement 1. Dual Injection of EdU and BrdU.

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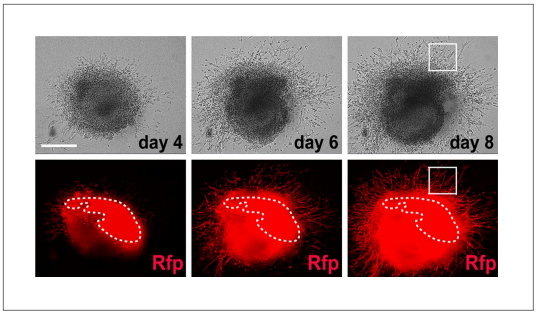

Figure 3—figure supplement 2. Time lapse images of 3-D matrigel PA2 culture.

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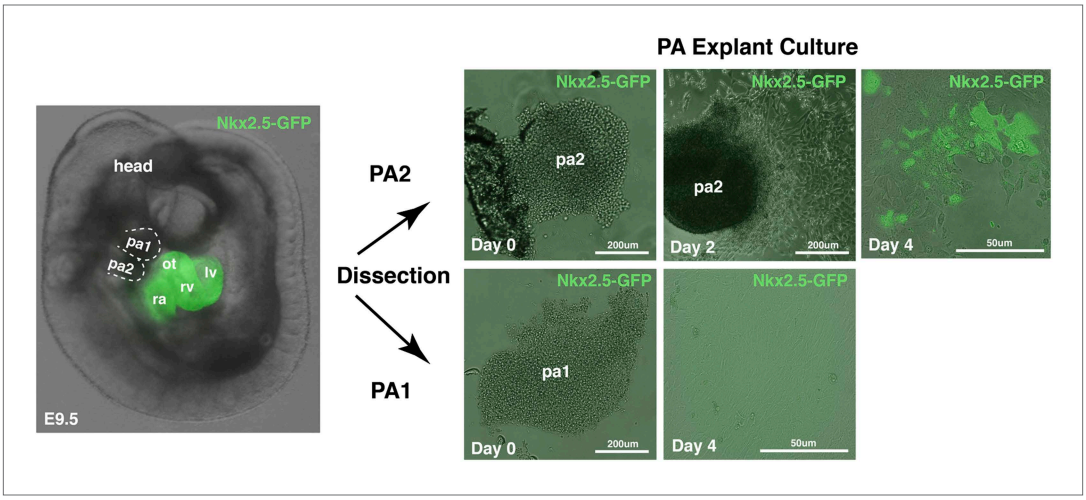

Figure 3—figure supplement 3. Explant culture of PA1 and PA2 dissected from Nkx2.5GFP embryo.

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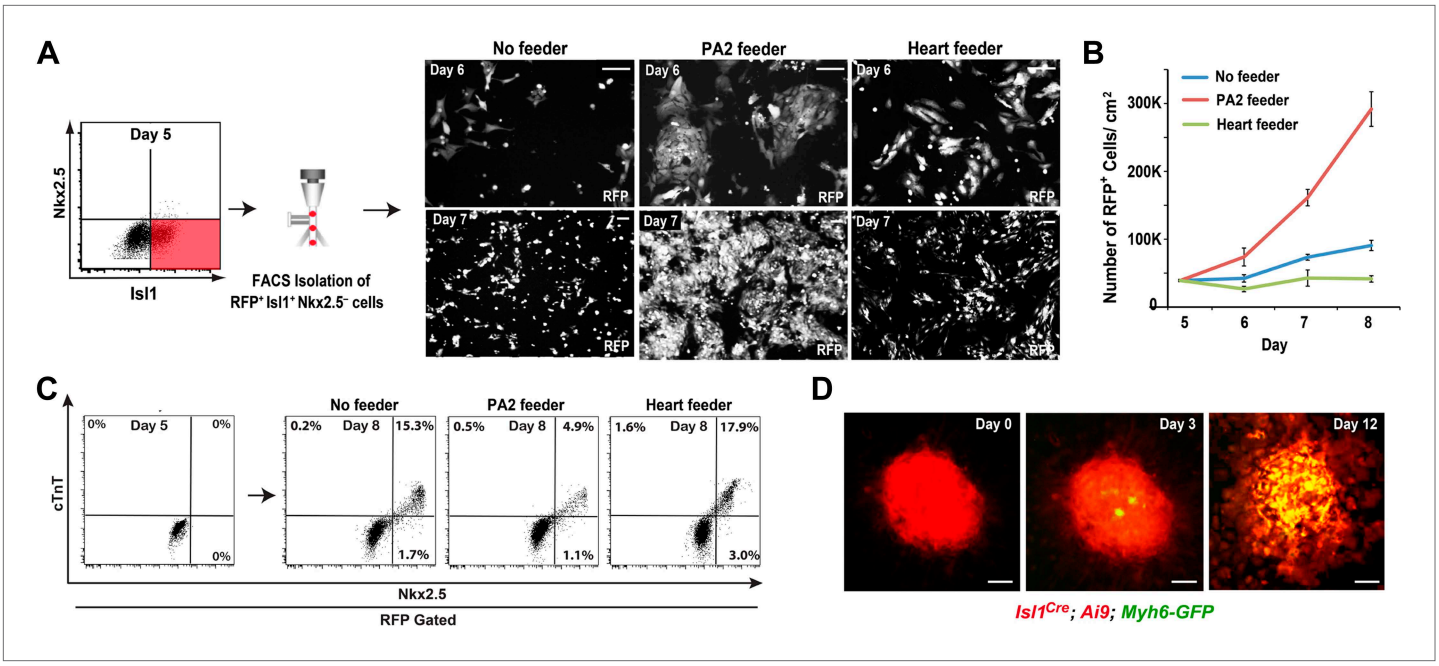

Figure 4. PA2 cells promote CPC expansion and suppress cardiac differentiation. (**A**) FACS-purification of RFP⁺ Isl1⁺ Nkx2.5⁻ CPCs induced from ES cell-derived precardiac mesoderm and their culture with no, embryonic PA2, or embryonic heart feeders. Images show RFP⁺ cells at day 6 and day 7. *Isl1*^{Cre}*; Ai9* ES cells were used to purify the CPCs at day 5 of cardiac differentiation, when Nkx2.5 is not expressed in Isl1⁺ CPCs. (**B**) Quantification of numbers of RFP⁺ cells cultured with no, embryonic PA2, or embryonic heart feeders. Data are mean ± SD; n = 3. (**C**) FACS plot of RFP⁺ Isl1⁺ Nkx2.5⁻ CPCs differentiating into Nkx2.5⁺/cTnT⁺ cells with no, embryonic PA2, or embryonic heart feeders, determined at day 8. (**D**) Time-lapse images of Isl1⁺ Nkx2.5⁻ CPC colony showing cardiac differentiation after removal of PA2 cells, indicated by GFP expression driven by *Myh6* promoter. Scale bars, 50 μm.

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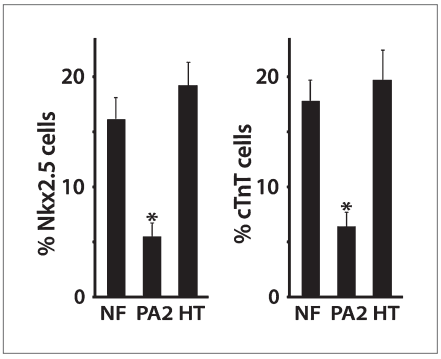

Figure 4—figure supplement 1. Percentages of Nkx2.5+ or cTnT + cells cultured with No, PA2, or embryonic heart feeders at day 8.

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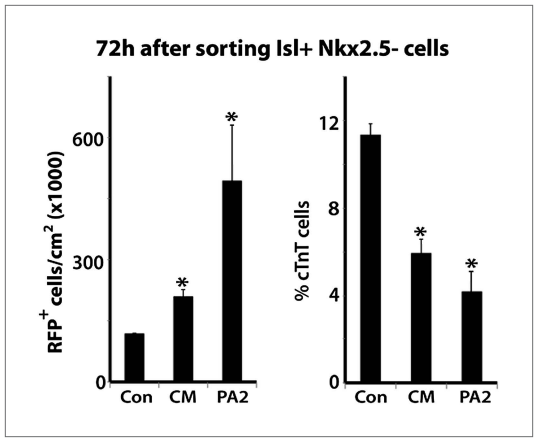

Figure 4—figure supplement 2. PA2 conditioned medium mimics PA2 co-culture.
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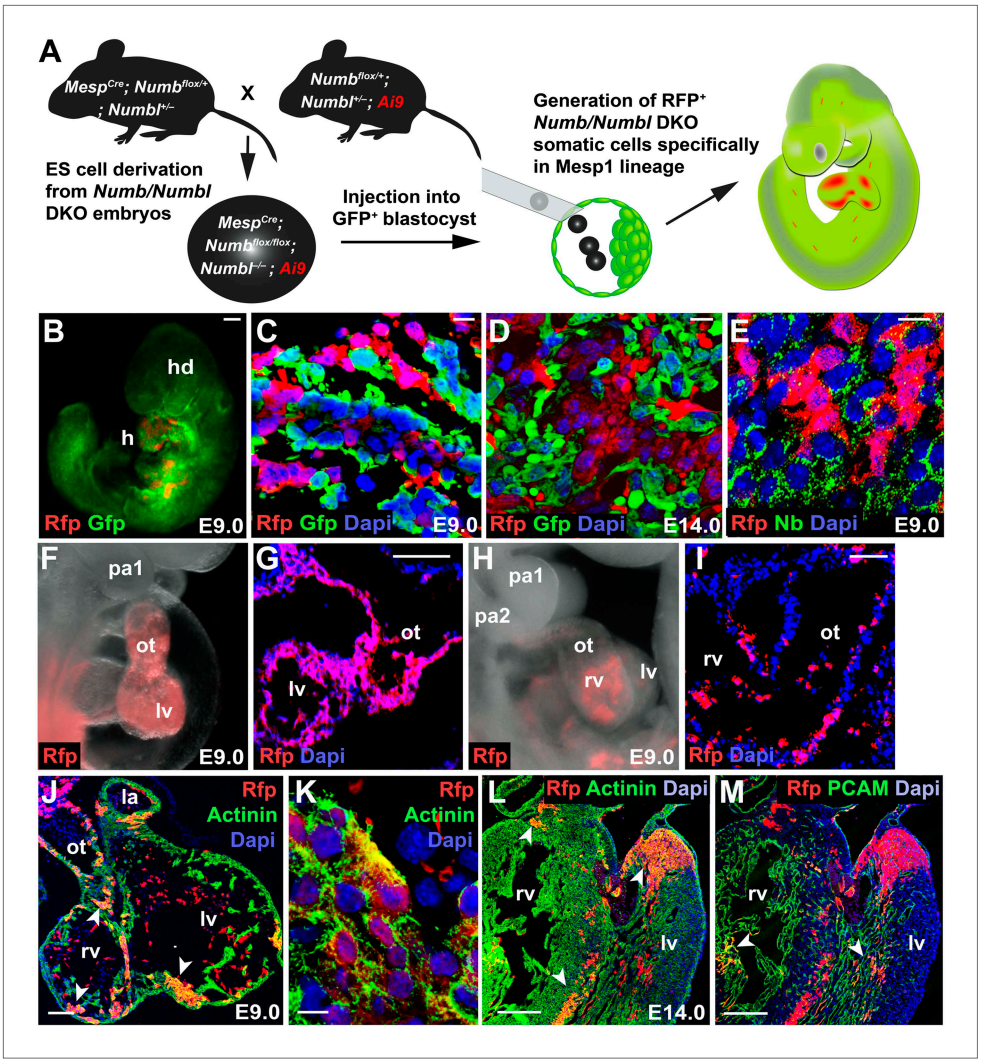

Figure 5. Generation of Mesp1 lineage-specific somatic cells lacking Numb and Numbl in vivo. (**A**) Scheme for generation of RFP⁺ *Numb/Numbl* DKO cells in Mesp1 lineage. In this study, three independent sets of blastocyst injection were carried out and 16 chimeras were obtained from 132 embryos. (**B**–**E**) Chimeric embryos at E9.0 (**B**) generated with GFP⁺ host cells. *Numb/Numbl* DKO cells are shown in red. Sections were made transversely through cardiac region (**C**–**E**) and immunostained with RFP and GFP (**C** and **D**) or RFP and Numb (**E**) antibodies at corresponding stages. (**F**–**I**) Lateral views of chimera and sections, focused on PA and heart, showing contribution of RFP⁺ cells. Major contribution of RFP⁺ cells causes a phenotype similar to *Numb/Numbl* DKO embryos (**F** and **G**). (**J**–**M**) Confocal images of heart transverse sections at indicated stages. α-Actinin and PCAM are properly expressed in RFP⁺ cells (arrowheads). RFP⁺ area in (**J**) is enlarged in (**K**). Dapi (blue) was used to counterstain the nuclei. Scale bars, 10 μm (**C**, **D**, **E**, **K**), 100 μm (**B**, **G**, **I**, **J**), and 200 μm (**L** and **M**). a, anterior; p, posterior; hd, head; h, heart; pa, pharyngeal arch; ot, outflow tract; rv, right ventricle; lv, left ventricle; la, left atrium.

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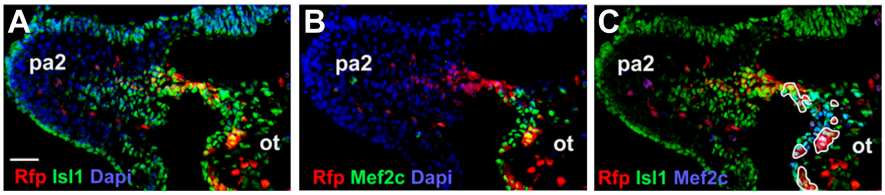

Figure 5—figure supplement 1. Numb/Numbl DKO cells are normally specified into OT cells.

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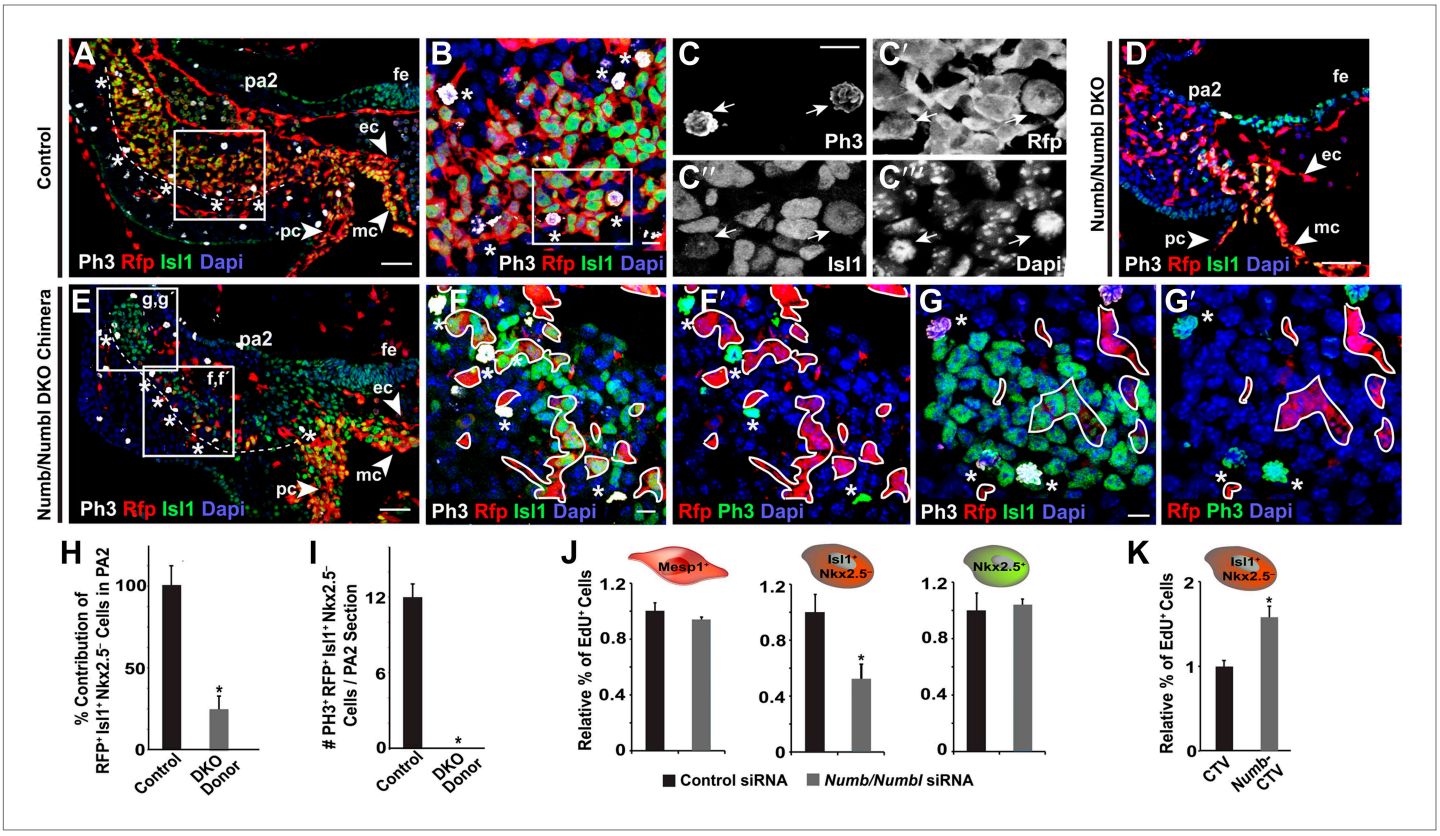

Figure 6. Numb and Numbl are required for proliferation of Mesp1+ progenitor-derived Isl1+ Nkx2.5– cells in PA2. (**A**–**G**′) Confocal images of PA2 sections of control (**A**–**C**‴), *Numb/Numbl* DKO (**D**), and chimeric (**E**–**G**′) embryos, immunostained with PH3, RFP, Isl1 antibodies. Asterisks indicate $PH3^+$ RFP^+ $Isl1^+$ (triple positive) cells located in outer layers of RFP^+ $Isl1^+$ cell population (outlined, **A** and **E**). Boxed areas in (**A**), (**B**), and (**E**) are shown in higher magnification in (**B**), (**C**–**C**‴), and (**F**–**G**′), respectively. No $PH3^+$ cells are found in RFP^+ cells (asterisks, **F**–**G**′). RFP^+ cells were outlined in white (**F**–**G**′). (**H** and **I**) Percentage of donor-derived $Isl1^+$ $Nkx2.5^-$ cells in PA2 is shown in (**H**) and number of $PH3^+$ RFP^+ $Isl1^+$ cells per 12-micron PA2 section is shown (**I**). Data are mean ± SD; n = 10; *p<0.05. (**J** and **K**) Relative percentage of EdU^+ cells in ES cell-derived $Mesp1^+$ progenitor, $Isl1^+$ $Nkx2.5^-$ CPCs or $Nkx2.5^+$ CPCs transfected with control or *Numb/Numbl* DKO siRNA (**J**) or $Isl1^+$ $Nkx2.5^-$ CPCs transfected with control (CTV) or Numb overexpression construct (CTV-Numb) (**K**). Data are mean ± SD; n = 3; *p<0.05. The $Mesp1^+$, $Isl1^+$ $Nkx2.5^-$, or $Nkx2.5^+$ cells were FACS-purified from day 4 *Mesp1*^{Cre}; *Ai9*, day 5 *Isl1*^{Cre}; *Ai9*, or day 6 *Nkx2.5*^{GFP} ES cells, respectively. Dapi (blue) was used to counterstain the nuclei. p values were determined using the paired Student *t* test. Scale bars, 10 μm (**B**, **C**, **F**, **G**). 50 μm (**A**, **D**, **E**). fe, foregut endoderm; pa, pharyngeal arch ec, endocardial layer; mc, myocardial layer; pc, pericardial layer.

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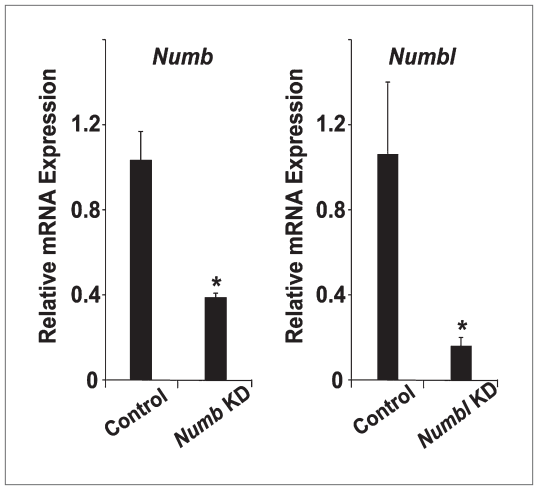

Figure 6—figure supplement 1. Knockdown efficiency of Numb siRNA and Numbl siRNA.

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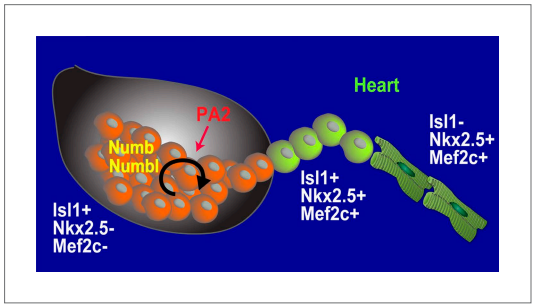

Figure 7. Model for Renewal and Niche of Mesp1+ progenitor-derived CPCs.

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