Supplementary Material

Figure S1. WT1, NFATC1, and Tbx18 are expressed in overlapping and distinct populations in chick E7 epicardial cells and EPDCs. (A-C) Analysis of TF expression in the epicardium (arrows) and EPDCs (arrowheads) was performed by double IF using the following antibodies: (A) anti-NFATC1 (green) + anti-WT1 (red); (B) anti-NFATC1 (green) + anti-Tbx18 (red); (C) anti-WT1 (green) + anti-Tbx18 (red). Immunoreactivity for each TF was detected differentially in EPDCs (insets). (D) Quantification of individual TF expression is represented as a percentage of total EPDCs. Error bars indicate s.e.m.

Figure S2. Individual fluorescent channels of IF images in Figure 1 are shown. (A'-D") Panels (A'-B") depict Pod1, Tbx18, or NFATC1 expression in sections of chick E7 hearts, while panels (C'-D") depict Pod1, Tbx18, or NFATC1 expression in sections of mouse E14.5 hearts. A'-A'' correspond to Figure 1A, B'-B" correspond to Figure 1B, C'-C" correspond to Figure 1D, and D'-D" correspond to Figure 1E. Pod1 (A',B',C',D'), Tbx18 (A",C"), and NFATC1 (B",D") are expressed in epicardial cells (arrows) and EPDCs (arrowheads), as indicated. Pod1 is expressed in epicardium (arrows) and EPDCs (arrowheads).

Figure S3. Pod1, Tbx18, and WT1 are expressed in isolated chicken embryo EPDCs in culture. (A) AVC explants were dissected from the indicated region (boxed area) of chick E7 hearts for in vitro analysis. *Tbx18* RNA ISH of a chick E7 heart section labels the EPDC-rich explanted region. (B-D) In EPDCs isolated from AVC explants, Pod1 (B), Tbx18 (C), and WT1 (D) are expressed (arrows), as detected by colorimetric immunocytochemistry (brown). Note that not all SEMCs express Pod1 (B) or Tbx18 (C) (arrowheads).

Figure S4. Manipulation of BMP, FGF, or Wnt signaling differentially affects TF gene expression in cultured EPDCs. (A-C) TF expression was assessed in isolated EPDCs treated with (A) BSA as vehicle control (Ctrl), BMP2, Noggin, or BMP2+Noggin; (B) BSA+DMSO as vehicle controls (Ctrl), FGF2, U0126, or FGF2+U0126; or (C) BSA as vehicle control (Ctrl), Wnt3A, sFRP3, or Wnt3A+sFRP3. Fold change in TF gene expression was quantified by qPCR relative to the control set to 1.0. Statistical significance of observed differences was determined by Student's t-test (n=3-6). *$P \leq 0.01$.

Figure S5. Inhibition of RA synthesis leads to decreased Pod1 and increased Calponin expression in intact chick E7 hearts. (A-L) Chick E7 whole hearts were treated with vehicle controls (Ctrl) MeOH+DMSO (A,E,I), RA (B,F,J), DEAB (C,G,K), or RA+DEAB (D,H,L) in vitro. Expression of Pod1 and Calponin was detected by immunohistochemistry (IHC) using anti-Pod1 antibody or anti-Calponin antibody, respectively. (A-D) Pod1 (brown) is expressed in epicardium and EPDCs (black arrowheads). Pod1-negative cells are indicated with white arrowheads. (E-H) Expression of the smooth muscle marker Calponin (brown) is indicated in the subepicardium (black arrowheads). Calponin-negative cells are indicated with white arrowheads. (I-L) Negative (no primary) controls are shown. (M,N) Quantification of the average the number of $Pod1^+$ cells (M) and the number of $Calponin^+$ cells (N) per microscopic field is shown. Panels depicted in (A-L) are cropped and magnified portions of the larger microscopic fields used for quantification. Statistical significance of observed differences relative to control was determined by Student's t-test (n=6). *$P \leq 0.01$.

Figure S6. RA treatment does not affect EPDC invasion in cultured E7 chick hearts. (A-D) The epicardium of chick E7 hearts was fluorescently labeled by incubation with carboxyfluorescein succinidyl diester (CFSE) prior to treatment with MeOH+DMSO (Ctrl), RA, DEAB, or RA+DEAB. (E,F) RA treatment did not affect EPDC invasion as indicated by (E) average distance of invasion (in μm) or (F) the average number of invading EPDCs. Error bars indicate s.e.m.

Figure S7. E-Cadherin is expressed uniformly in *Pod1*$^{-/-}$ epicardium at E14.5. X-Gal staining (blue) and IHC using anti-E-Cadherin antibody (brown) were performed on E14.5 mouse heart sections. (A,B) βGal expression from the Pod1 locus is detected in the epicardium (black arrowheads) and EPDCs (arrows) of E14.5 *Pod1*$^{+/-}$ (A') and *Pod1*$^{-/-}$ (B') mouse hearts. The *Pod1*$^{-/-}$ epicardium is detached (asterisk in B') but expresses E-Cadherin (red arrowhead in B') in the epicardial epithelial layer similar to *Pod1*$^{+/-}$ littermate control (red arrowhead in A').

Figure S8. Endomucin is expressed normally in the *Pod1*$^{-/-}$ E18.5 heart. IHC was performed on E18.5 mouse heart sections using anti-Endomucin (Emcn) antibody (brown) to visualize endothelial cells. (A,B) Emcn expression is detected in endothelial cells of the capillaries and venules (arrows) in *Pod1*$^{+/-}$ (A') and *Pod1*$^{-/-}$ (B') mouse hearts at E18.5. Emcn-negative coronary arteries (arrowheads) are also present in both genotypes.

Figure S9. Expression of the smooth muscle markers αSmooth Muscle Actin and Calponin is increased in the subepicardium of *Pod1*$^{-/-}$ hearts at E17.5. IHC was performed on E17.5 heart sections using anti-Alpha Smooth Muscle Actin (αSMA) and anti-Calponin antibodies (brown). (A,B) While occasionally expressed in the *Pod1*$^{+/-}$ subepicardium (arrowheads in A'), αSMA is robustly expressed in the subepicardium (arrowheads in B') of the *Pod1*$^{-/-}$ mouse heart at E17.5. (C,D) The smooth muscle marker Calponin is expressed in the majority of EPDCs of the *Pod1*$^{-/-}$ heart (arrowheads in D'), but is detected in few cells of the *Pod1*$^{+/-}$ heart (arrowhead in C') at E17.5.

Figure S10. SM22α is expressed aberrantly on or near the surface of *Pod1*$^{-/-}$ hearts at E17.5. Whole mount IHC was performed on E17.5 mouse hearts using anti-SM22α antibody (brown). (A,C) Large SM22α^{+} coronary vessels (arrowheads) are present on the ventral (A) and dorsal (C) sides of the *Pod1*$^{+/-}$ heart. (B,D) Although a large coronary vessel (arrowhead) is present on the dorsal side of the *Pod1*$^{-/-}$ heart (D), aberrant SM22α expression (arrow) is present throughout much of the subepicardium of the *Pod1*$^{-/-}$ heart.

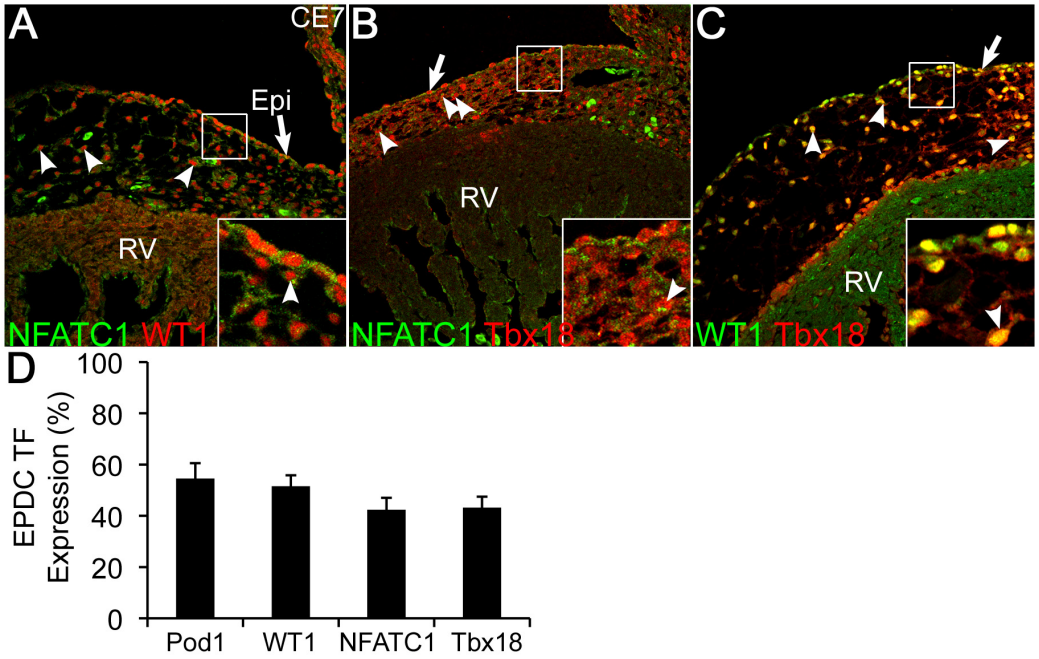
A
CE7
Epi
RV
NFATC1 WT1
B
RV
NFATC1 Tbx18
C
RV
WT1 Tbx18
D
EPDC TF Expression (%)
100
80
60
40
20
0
Pod1
WT1
NFATC1
Tbx18

Figure S1

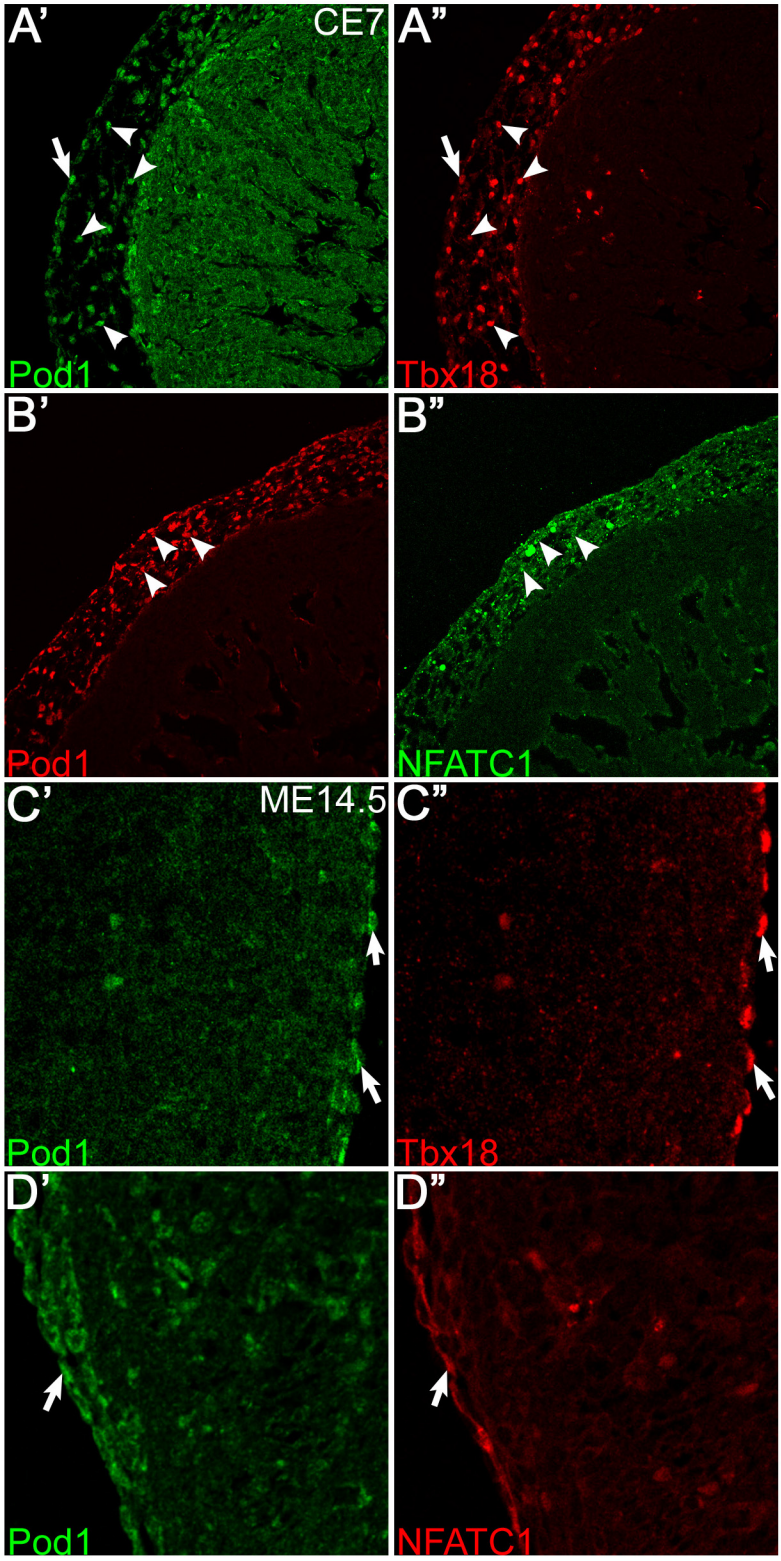
A'
CE7
Pod1
A"
Tbx18
B'
Pod1
B"
NFATC1
C'
ME14.5
Pod1
C"
Tbx18
D'
Pod1
D"
NFATC1

Figure S2

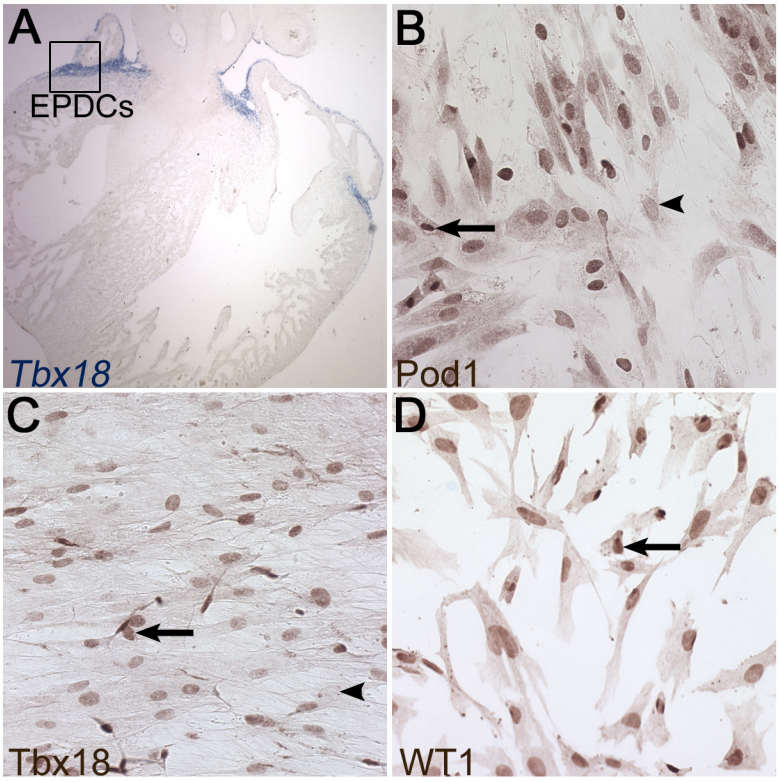

Figure S3

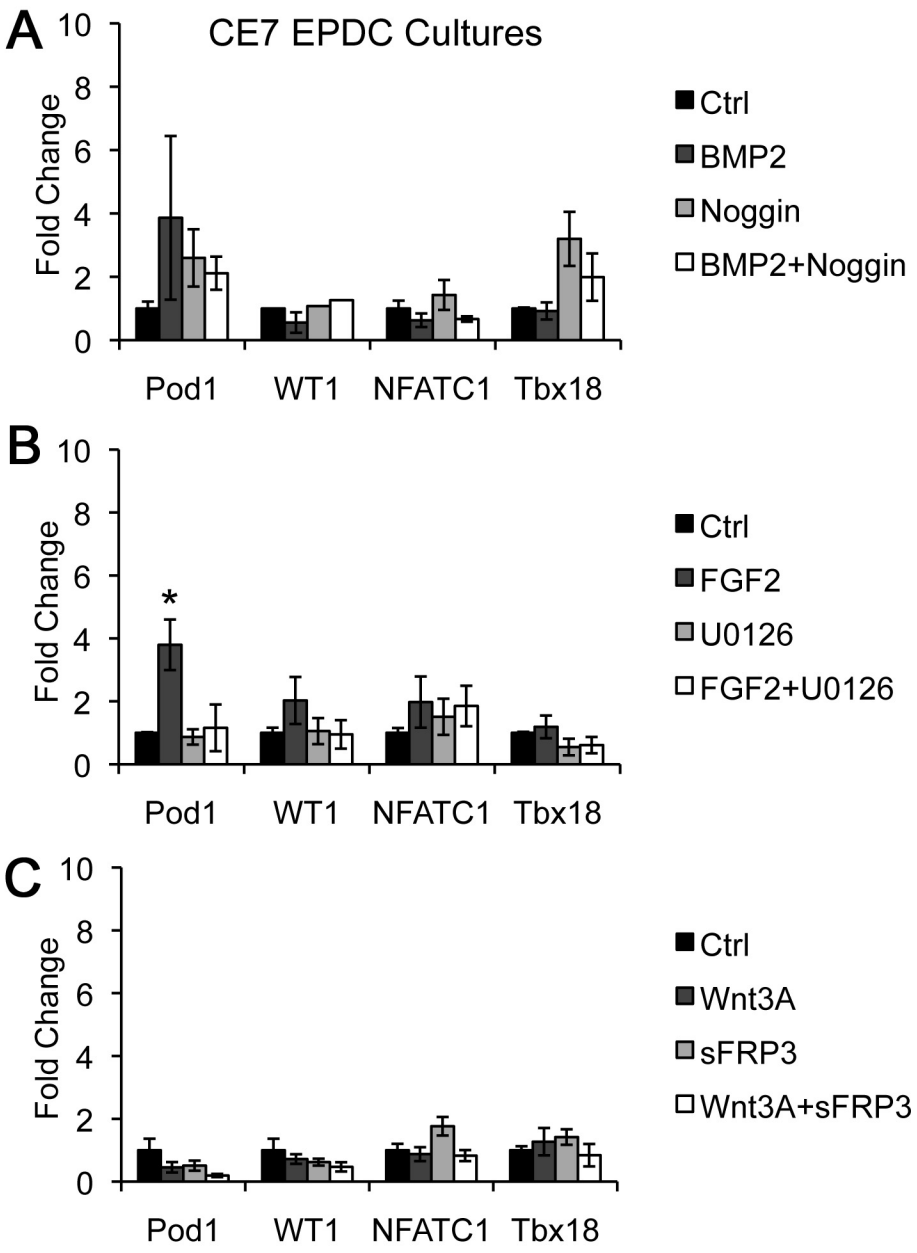
A
CE7 EPDC Cultures
Fold Change
10
8
6
4
2
0
Pod1
WT1
NFATC1
Tbx18
Ctrl
BMP2
Noggin
BMP2+Noggin
B
Fold Change
*
Pod1
WT1
NFATC1
Tbx18
Ctrl
FGF2
U0126
FGF2+U0126
C
Fold Change
Pod1
WT1
NFATC1
Tbx18
Ctrl
Wnt3A
sFRP3
Wnt3A+sFRP3

Figure S4

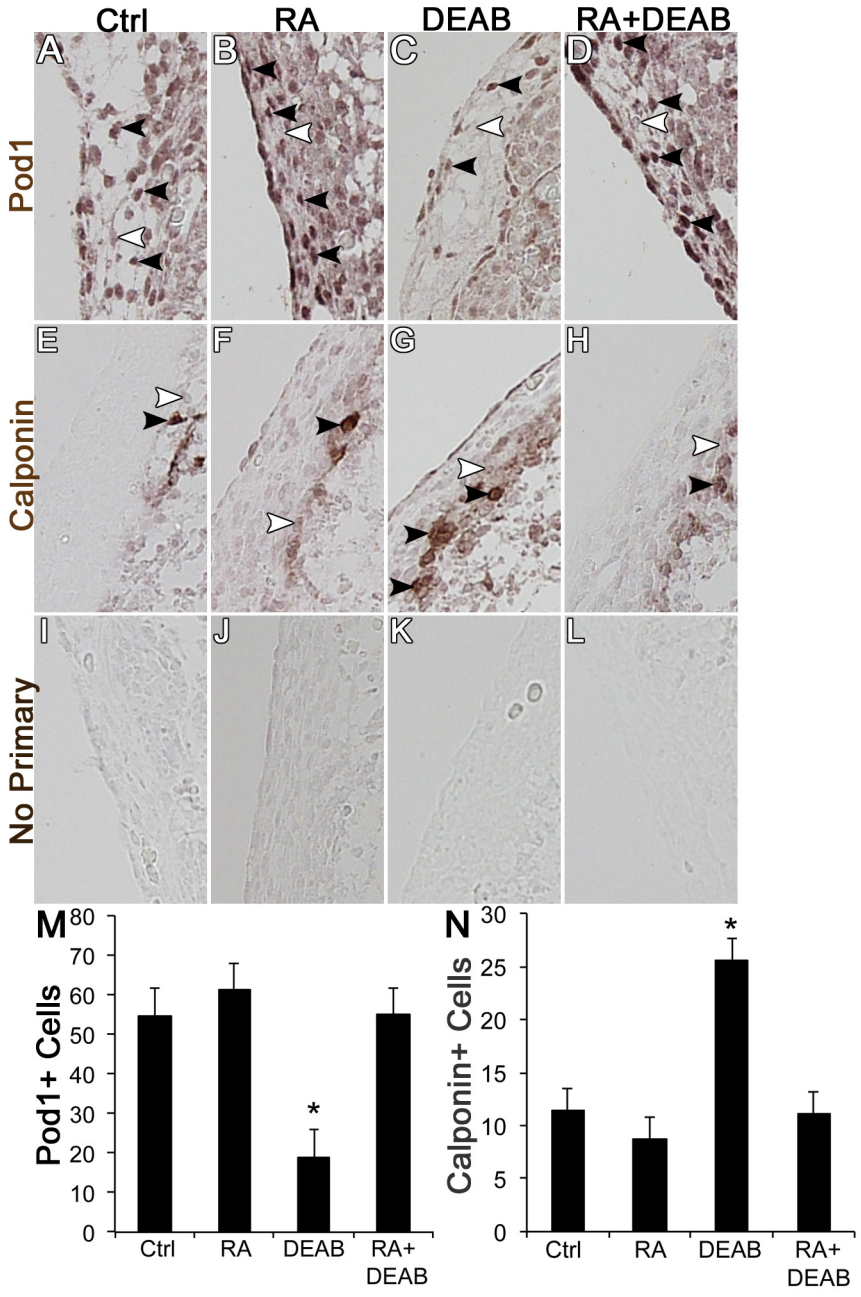
Ctrl
RA
DEAB
RA+DEAB
A
B
C
D
Pod1
E
F
G
H
Calponin
I
J
K
L
No Primary
M
Pod1+ Cells
80
70
60
50
40
30
20
10
0
*
Ctrl
RA
DEAB
RA+ DEAB
N
Calponin+ Cells
30
25
20
15
10
5
0
*
Ctrl
RA
DEAB
RA+ DEAB

Figure S5

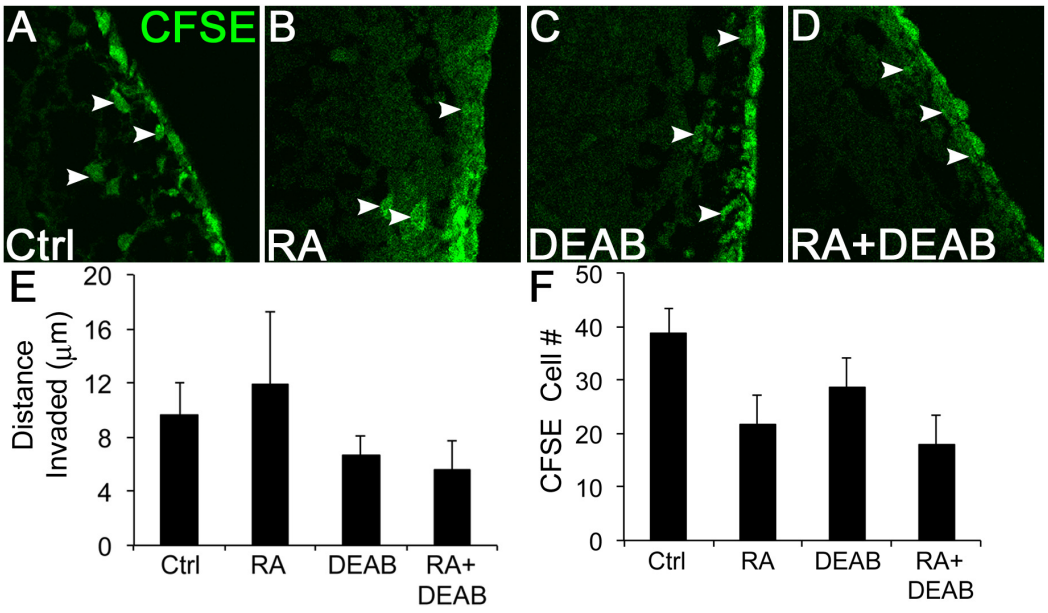
A
CFSE
Ctrl
B
RA
C
DEAB
D
RA+DEAB
E
Distance Invaded (μm)
20
16
12
8
4
0
Ctrl
RA
DEAB
RA+ DEAB
F
CFSE Cell #
50
40
30
20
10
0
Ctrl
RA
DEAB
RA+ DEAB

Figure S6

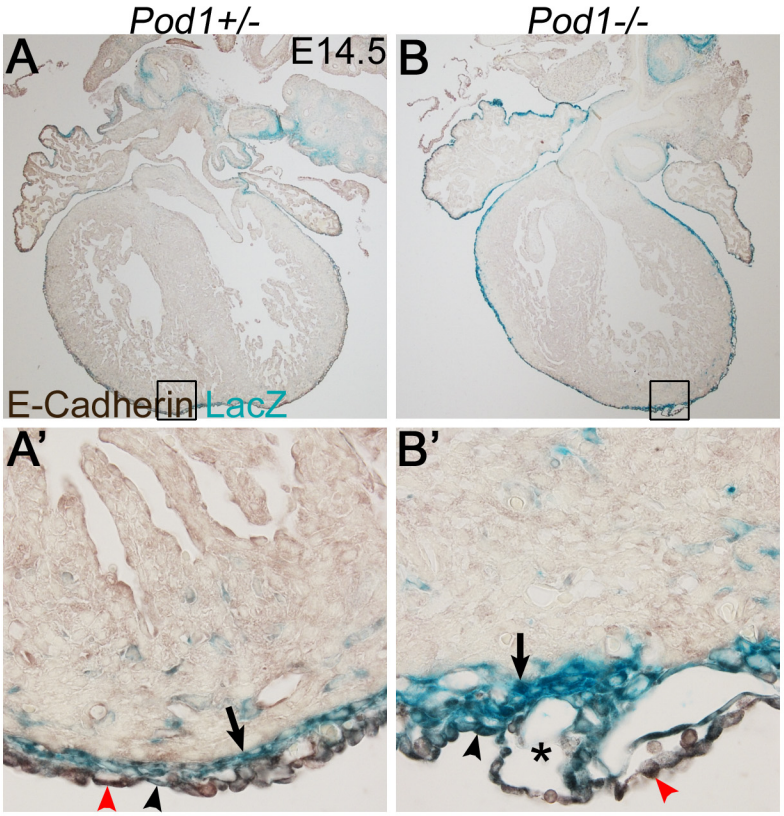

Figure S7

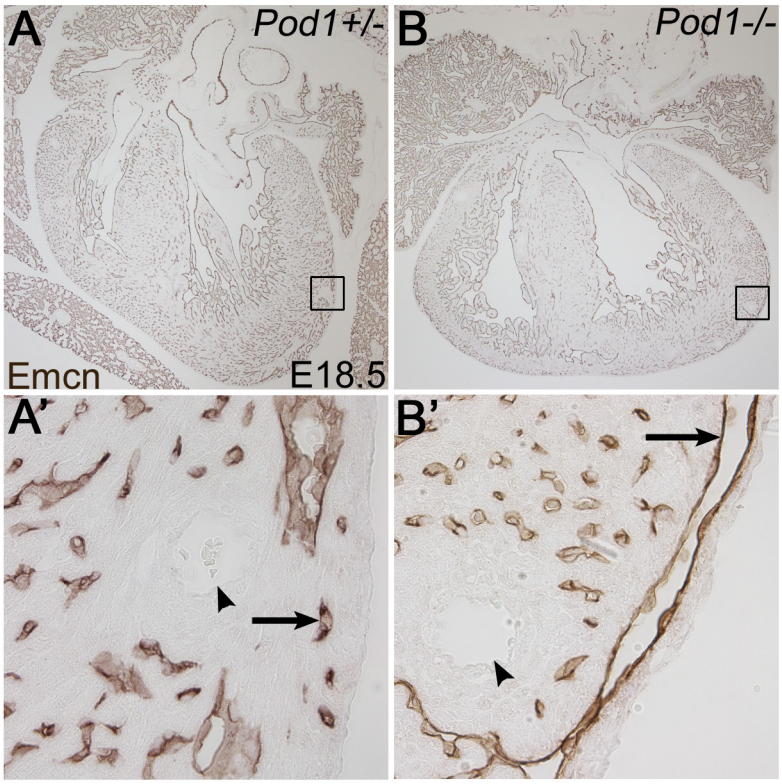

Figure S8

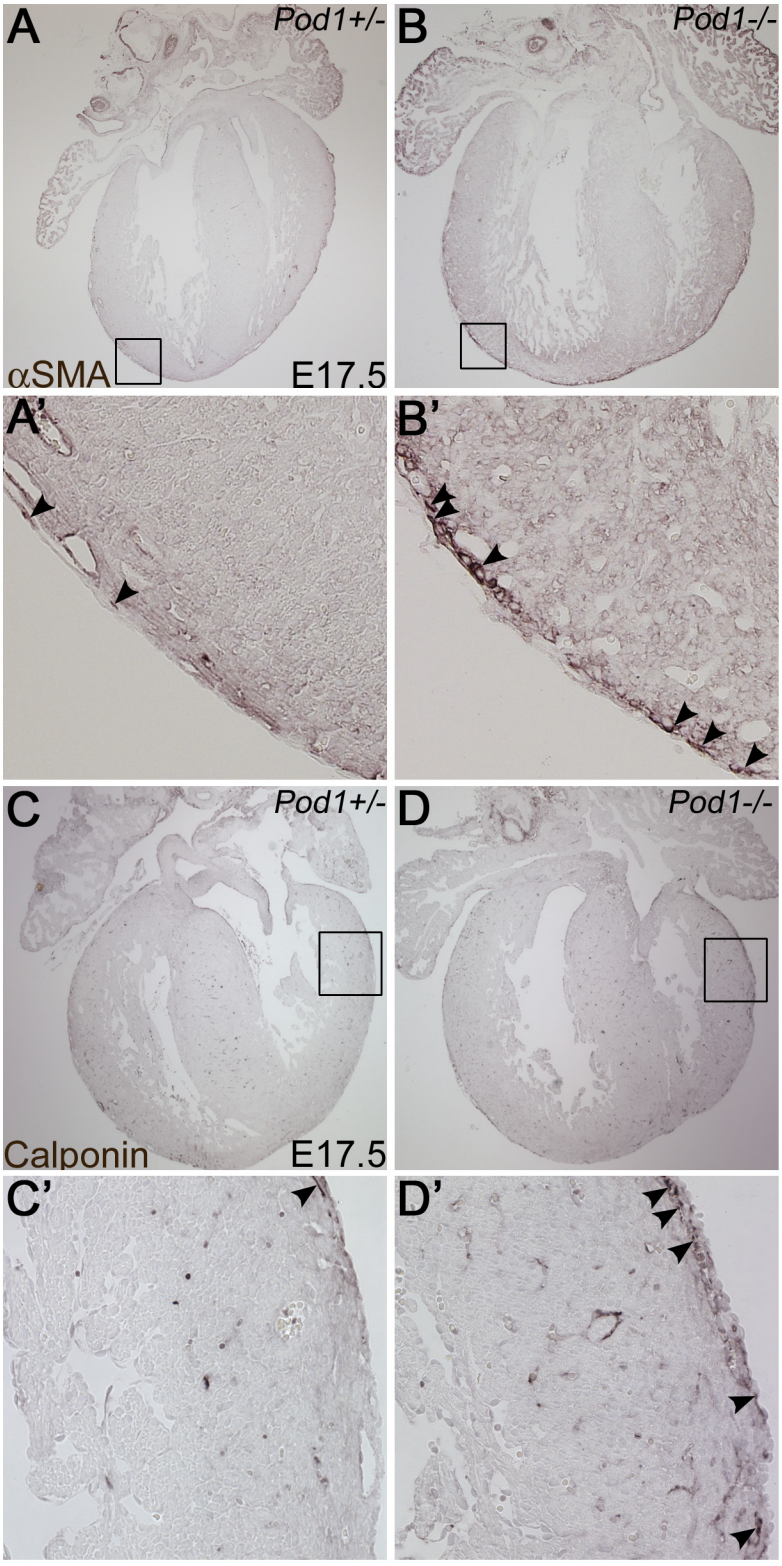

Figure S9

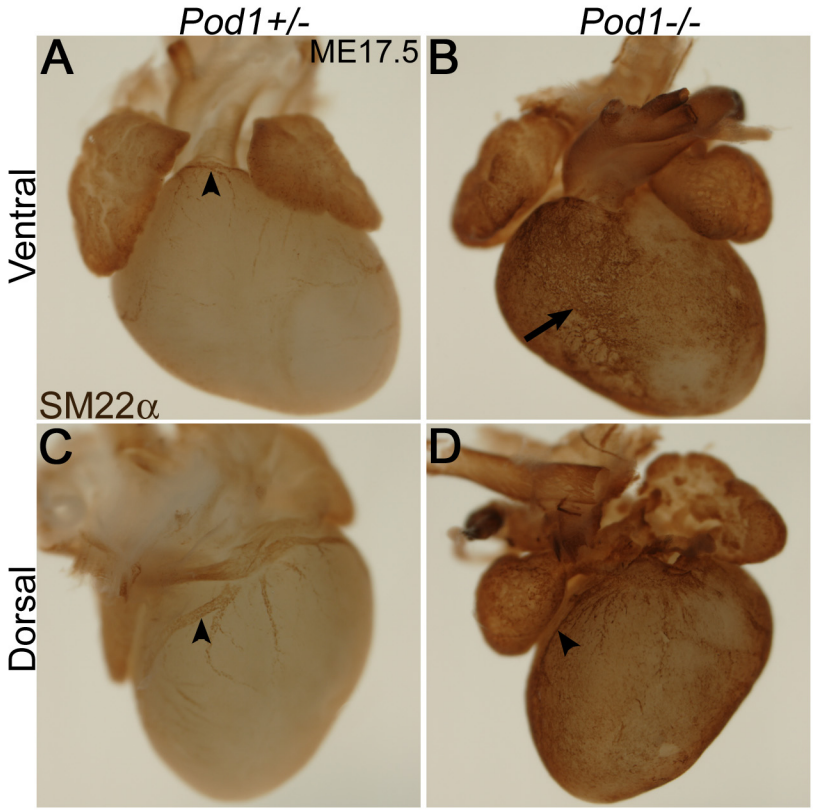

Figure S10