Supplemental Materials and Methods

Mouse strains

The following strains of mice were used in our studies: *Tfap2a*^{IRESCre} knock-in mice (a gift from Dr Anne Moon, Geisinger Institute, Danville, PA) (Macatee et al., 2003), *P3Pro-Cre* transgenic mice (generated by Dr Jonathan Epstein, University of Pennsylvania, Philadelphia, PA and obtained from Jeff Miner) (Li et al., 2000). The *Wnt1-Cre1* (Jiang et al., 2000) and *Wnt1-Cre2* (Lewis et al., 2013) strains were obtained from Jackson Labs (3829 and 22137, respectively). *Fn1*$^{+/-}$, *Itga5*$^{+/-}$ and *Itga5*$^{flox/flox}$ mice were a gift from Dr Richard Hynes, MIT, Cambridge, MA (George et al., 1997; van der Flier et al., 2010; Yang et al., 1993). *Fn1*$^{flox/flox}$ mice were a gift from Dr Reinhard Fässler, Max Planck Institute of Biochemistry, Martinsried, Germany (Sakai et al., 2001). *R26R* and *ROSA*^{mTmG} reporter mice were obtained from the Jackson Labs (Muzumdar et al., 2007; Soriano, 1999). *Cre*$^{+}$ mice were crossed with *Fn1*$^{+/-}$ mice to generate *Fn1*$^{+/-}$*;Cre*$^{+}$ mice. *Fn1*$^{flox/flox}$*; R26R/R26R* or *Fn1*$^{flox/flox}$*;ROSA*^{mTmG} mice were generated by mating *Fn1*$^{flox/flox}$ mice with *R26R* or *ROSA*^{mTmG} reporter mice. Similarly, *Itga5*$^{flox/flox}$*; ROSA*^{mTmG} mice were generated by mating reporter strains with *Itga5*$^{flox/flox}$ mice. *Fn1*$^{flox/flox}$*; R26R/R26R*, *Fn1*$^{flox/flox}$*; ROSA*^{mTmG} or *Itga5*$^{flox/flox}$*; ROSA*^{mTmG} females carrying two copies of the reporter allele were mated with *Fn1*$^{+/-}$*;Cre*$^{+}$ or *Itga5*$^{+/-}$*; Cre*$^{+}$ males, respectively, and the day of vaginal plug was designated as E0.5. All mice were on a mixed *129-129S4;C57BL/6J* background.

Histology, immunohistochemistry and β-gal staining

Samples were fixed overnight with 4% paraformaldehyde (PFA), embedded into paraffin and stained using hematoxylin and eosin (H&E). For immunohistochemistry, sections were deparaffinized, rehydrated and treated with 10 mM Sodium Citrate (pH 6.0) for 10 minutes in microwave. After incubating in blocking buffer (PBS containing 5% normal donkey serum, Sigma, catalog # D9663-10ml), sections were treated with primary antibodies overnight at 4^{o}C. The following primary antibodies were used on paraffin (or frozen, if noted) sections: anti-GFP (1:500, catalog # GFP-1020, Aves labs), anti-Sox10 (1:50, catalog # sc-17342, Santa Cruz Biotechnology), rabbit polyclonal anti-fibronectin (a gift from Dr. Richard Hynes, MIT), anti-TFAp2α (1:50, catalog # 3B5, The Developmental Studies Hybridoma Bank), anti-αSMA (1:300, catalog # A5228 or # SAB2500963, both from Sigma), anti SM22α (1:100, catalog # ab1406, Abcam), ant-Myh11 (1:100 on frozen sections, catalog # ab53219, Abcam), anti-Jagged1 (1:10, catalog # sc8303, Santa Cruz Biotechnology), anti-NICD (1:30, catalog# 4147, Cell Signaling Technology), anti-Calponin (1:150, catalog # ab46794, Abcam), anti-integrin α5 (1:15 on isolated cells, catalog #sc-10729, Santa Cruz Biotechnology), the anti-FLAG tag (1:300, catalog # A00187, Genscript, NJ, USA), anti-V5 tag (1:300, catalog # A01724, Genscript, NJ, USA), anti-laminin gamma 1 (1:100 on frozen sections, catalog # L9393, Sigma), and anti-Fibulin1 (1:50 on frozen sections, rabbit polyclonal antibody #2954, a gift from Dr Marion Cooley). The following secondary antibodies were applied the next day: anti-mouse, anti-goat, anti-chicken, or anti-rabbit conjugated to Alexa-488, Alexa-555, or Alexa 647 (1:300, Invitrogen). Nuclei were stained with either DAPI (1:1000 dilution of 5 mg/ml stock solution in H_2O, Sigma, cat # 32670-5MG-F) or

DRAQ5 (1:1000 dilution of the stock solution supplied by the manufacturer, catalog # 4048, Cell Signaling Technology). Slides were mounted using ProLong® Gold antifade (Invitrogen). The native GFP and tdTomato fluorescence in paraffin sections was extinguished by boiling the sections in 10 mM Sodium Citrate, pH6.0 for 10 min; for frozen sections, slides were incubated in graded series of methanol, and cover slipped in 50/50 solution of methanol/glycerol. For TUNEL, tissue sections were boiled in 10 mM Sodium Citrate, pH6.0 for 10 minutes, permeabilized in PBS with 0.5% tween-20 for 10 minutes, and incubated TUNEL reaction mixture (In Situ Cell Death Detection Kit, Roche) 1 hour at 37^{o}C. Double or triple immunohistochemistry staining was performed afterwards. For the analysis of proliferation using BrdU, pregnant mice received intraperitoneal injection of BrdU (10mg/ml in saline, cat # BP2508250, Fisher Scientific) at 100mg/kg mouse body weight. Mice were sacrificed 20 minutes later and E11.5 embryos were harvested and fixed in 4% PFA overnight. Embryos were then dehydrated and embedded into paraffin. An antigen retrieval step was performed by boiling the slides in 10 mM Sodium Citrate buffer for 10 minutes before applying the blocking buffer (PBS containing 5% normal donkey serum, Sigma, cat # D9663-10ml). Sections were then incubated with anti-BrdU antibody (1:500, cat # G3G4, Iowa Developmental Studies Hybridoma Bank) overnight at 4^{o}C, followed by Alexa-conjugated secondary antibody. For β-gal staining, embryos were fixed in 0.2% glutaraldehyde and stained using bluo-gal as the substrate (catalog # B2904, Sigma), as described previously (Mittal et al., 2010). Sections were imaged using Zeiss 510 LSM or Olympus FV500 confocal microscopes, whole mount stained embryos were

imaged using Olympus FV500. Bright field images were acquired using Zeiss Stemi 2000-C or Zeiss Axio Observer microscopes with DFC420 digital camera (Leica).

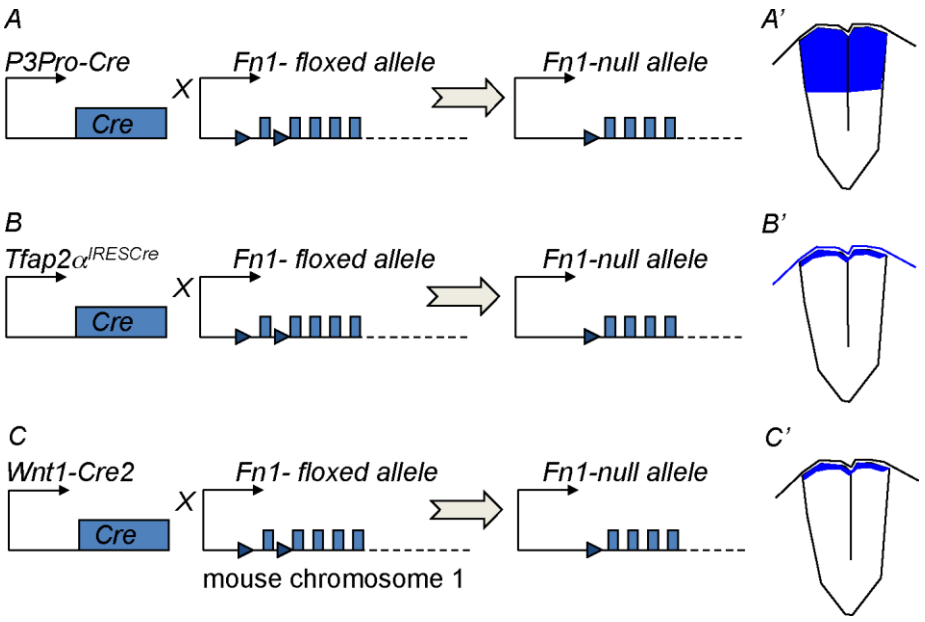

Fig. S1. Deletion of *Fn1* in the NC. A – C. Schematics of mating schemes used for the experiments described in this manuscript. **A' – C'.** Schematics of the Cre-expression domains in the three strains used. Blue color in **A'** – **C'** marks domains of Cre expression.

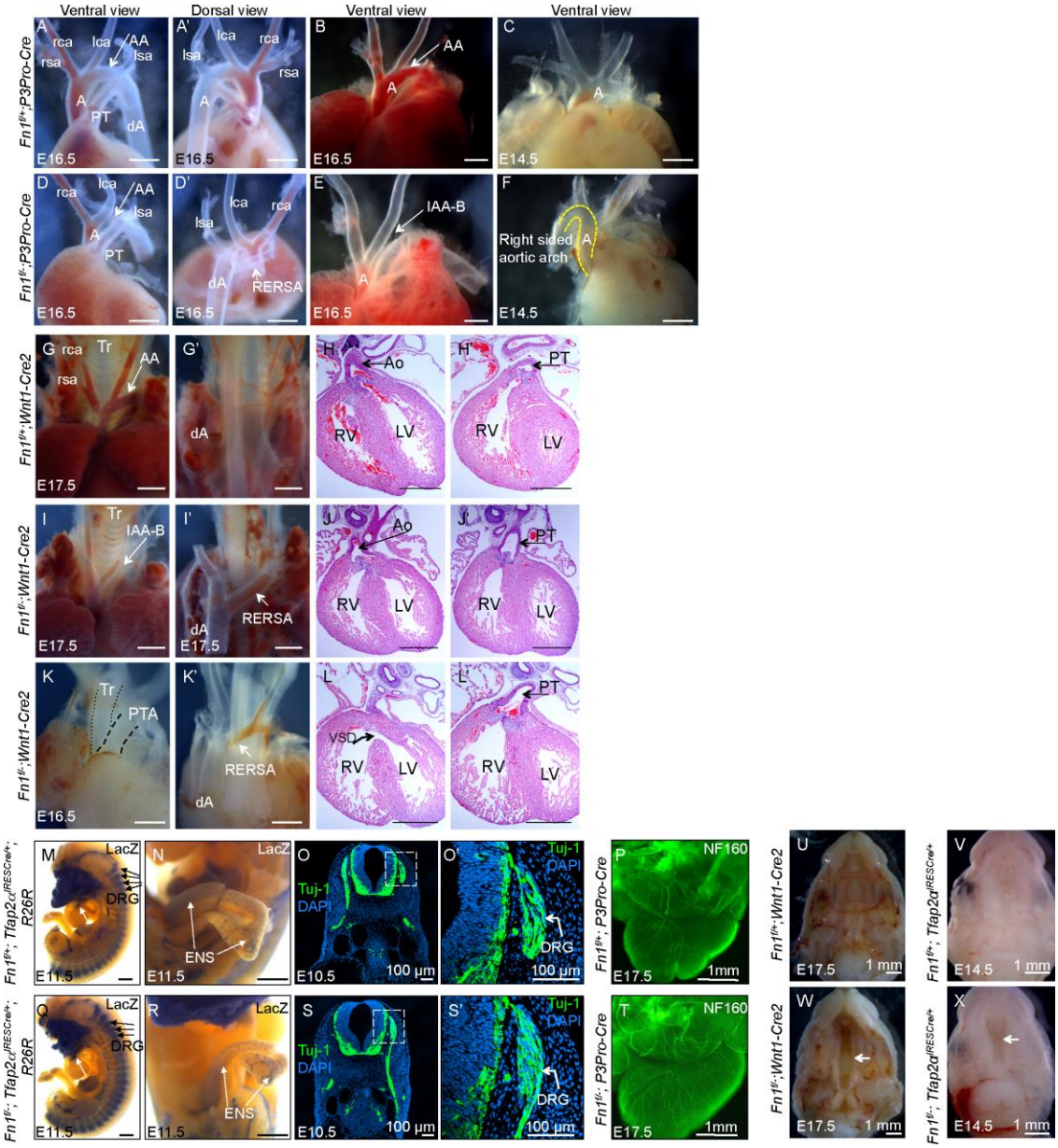

Fig. S2. Cardiovascular defects in *Fn1*$^{flox/-}$; *P3Pro-Cre* and *Fn1*$^{flox/-}$; *Wnt1-Cre2* mutants; Normal development of NC-derived neuronal lineages in *Fn1*$^{flox/-}$; *Cre*$^{+}$ mutants; and Defects in palate development in *Fn1*$^{flox/-}$; *Wnt1-Cre2* and *Fn1*$^{flox/-}$; *Tfap2α*^{IRESCre} mutants. Fn1$^{flox/+}$; P3Pro-Cre Controls (**A – C**); Fn1$^{flox/-}$; P3Pro-Cre Mutants (**D – F**). **G, G', H, H'**: *Fn1*$^{flox/+}$; *Wnt1-Cre2* controls; **I – L'**: *Fn1*$^{flox/-}$; *Wnt1-Cre2* mutants. Abbreviations are as in Figure 2. dA – descending aorta. Note the presence of hypoplastic aortic arch (AA) in **D**, arrow; RERSA in **D'**, arrow; interrupted aortic arch type B, IAA-B, in **E**, arrow; right-sided aortic arch, dotted line (**F**) in *Fn1*$^{flox/-}$; *P3-Pro-Cre*; IAA-B (**I**), RERSA (**I', K'**) and persistent truncus arteriosus (PTA) in (**K**) in *Fn1*$^{flox/-}$; *Wnt1-Cre2* mutants. **H, H', J, J', L, L'** are coronal sections of the hearts shown on the left and stained with H&E. Note that the aorta in the control (**H**) is connected to the left

ventricle (LV) and the pulmonary trunk (PT) in the control (**H'**) is connected to the right ventricle (RV). In the mutant with the double outlet right ventricle (DORV) shown in **J – J'**, both the aorta (**J**) and the PT (**J**') are connected to the RV. In the mutant with PTA, the aorta is absent (**L**) and is accompanied by the VSD (curved arrow in **L**); a single outflow blood vessel, the PT, is connected to the RV (**L'**).
M – P: Control embryos; **Q – T**: Mutants. DRG – dorsal root ganglia (**M, O - O' and Q, S – S'**), ENS – enteric nervous system (**N, R**), and cardiac ganglia (**P, T**) develop in mutants and controls alike, and express Tuj1 and NF160. White arrows in **M** and **Q** point to NC-derived cells within the cardiac outflow tract; black arrows point to DRG. White arrows in **N** and **R** point to the ENS (blue, LacZ staining within the gut). The blue color in **M, N, Q,** and **R** is due to the LacZ activity of the ROSA reporter. **U – V:** Controls; **W, X**: *Fn1*$^{flox/-}$*; Wnt1-Cre2* and *Fn1*$^{flox/-}$*; Tfap2*$\alpha^{IRESCre}$ mutants develop cleft palates (arrows in **W** and **X**). Scale bars are 500 microns unless noted otherwise.

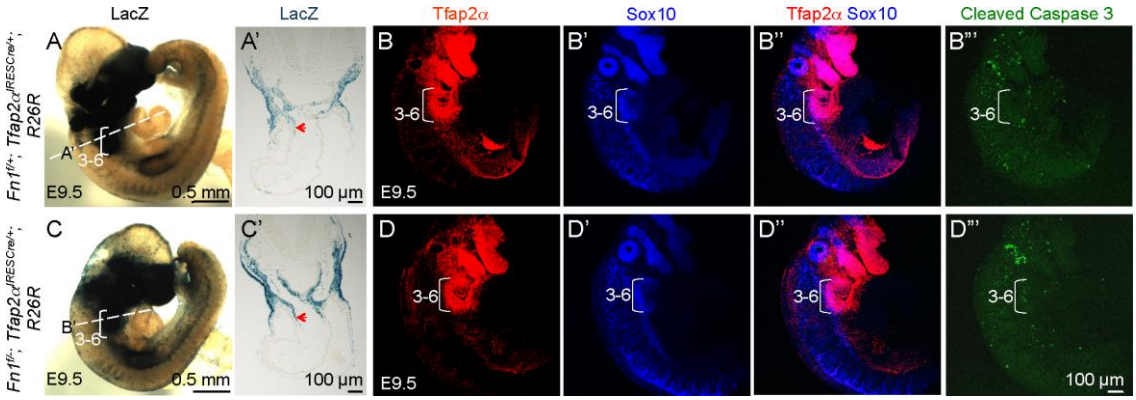

Fig. S3. NC-derived Fn1 is not required for NC migration and survival. A – B’’’: Control and **C – D’’’**: mutant E9.5 embryos. **A, A’, C, C’**: Fate mapping shows that NC-derived cells (blue) migrate into the pharyngeal arches (**A, C**) and into the cardiac outflow tract, red arrows in **A’, C’**, in control and mutant embryos comparably. Dashed lines in **A** and **C** show the planes of section in **A’** and **C’**. The blue color is due to the LacZ activity of the ROSA reporter. Pharyngeal arches 3 – 6 are labeled with brackets. **B – B’’’** 3D reconstructions of confocal whole mount immunofluorescence staining of whole E9.5 control and **D – D’’’** mutant embryos co-stained to detect NC markers Tfap2α (**B, D**), Sox10 (**B’, D’**), merged in **B’’** and **D’’**; and cleaved caspase 3 (**B’’’, C’’’**). Magnifications are the same in **B – B’’’** and **D – D’’’**.

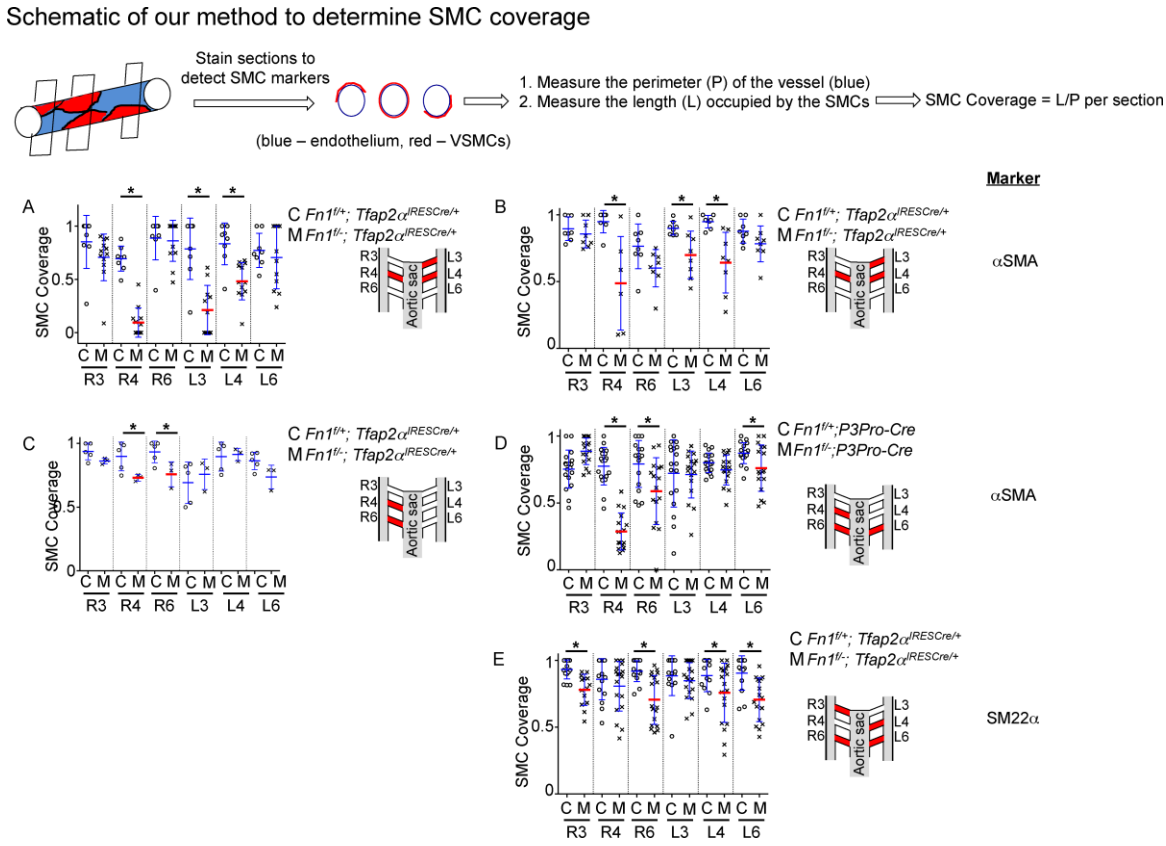

Fig. S4. VSMC coverage around the PAAs in controls and *Fn1$^{flox/-}$; Cre^{+}* mutants. Method Schematic: At the time of our assay (E11.5), PAA endothelium (blue) is not fully surrounded by αSMA^{+} or SM22α^{+} cells (red). To gain insight into the extent of NC-to-VSMC differentiation along the length of the PAA, we sectioned each E11.5 embryo along the entire length of the PAAs and measured VSMC coverage in each section, as depicted. Measurements from sections derived from 5 independent control-mutant pairs are presented in **A – E**; genotypes are indicated next to each graph. PAAs with deficient VSMC coverage are marked in red in the cartoon next to each graph. Mean +/- SD are plotted. Each data point is VSMC coverage measured in one section. Data were analyzed using two-tailed, unpaired Student's t test, * $p < 0.05$. PAAs on the left, right or both sides were affected.

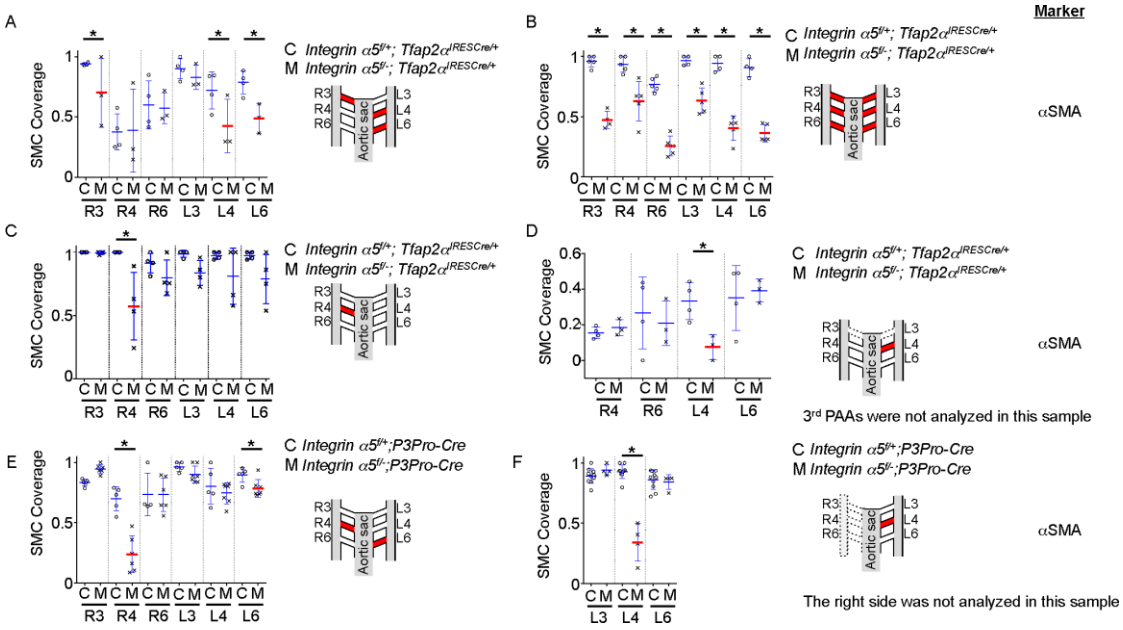

Fig. S5. VSMC coverage around the PAAs in controls and *integrin α5$^{flox/-}$; Cre^{+}* mutants. VSMC coverage was determined, as shown in supplemental figure 4. Measurements from sections derived from 6 independent control-mutant pairs are presented; genotypes are indicated next to each graph. PAAs with deficient VSMC coverage are marked in red in the cartoon next to each graph. Each data point is VSMC coverage measured in one section. Mean +/- SD are plotted. Data were analyzed using two-tailed, unpaired Student's t test, * $p < 0.05$. PAAs on the left, right or both sides were affected.

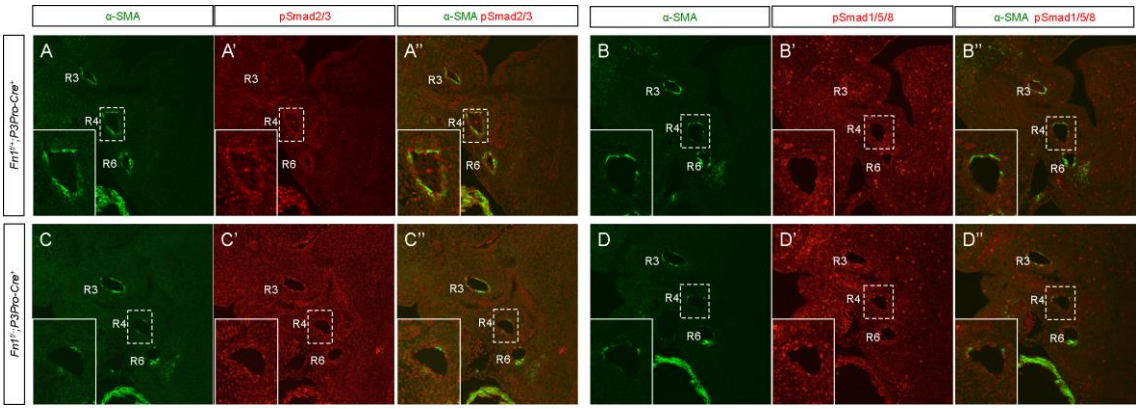

Fig. S6. NC-synthesized Fn1 is not required for the activation of SMADs 2, 3 and SMADs 1, 5, 8 around the PAAs. Controls (**A – A”**, **B – B”**) and mutants (**C – C”**, **D – D”**) were stained to detect αSMA and either the phosphorylated forms of SMADs 2 and 3 (**A – A’**, **C – C’**) or phosphorylated forms of SMADs 1, 5, 8 (**B – B”**, **D – D”**). PAAs are numbered. The regions of the 4th PAA in dashed boxes is expanded in the inset of each panel. Magnification is the same in all panels, scale bars are 100 microns.

Table S1. Genotypes of progeny at weaning

	Fn1$^{flox/+}$; *Cre*$^{-}$	*Fn1*$^{flox/+}$; *Cre*$^{+}$	*Fn1*$^{flox/-}$; *Cre*$^{-}$	*Fn1*$^{flox/-}$; *Cre*$^{+}$	Total # genotyped
Tfap2α^{JRESCre}	108 (40.7%)	81 (30.6%)	76 (28.7%)	0*	265
P3Pro-Cre	81 (28.2%)	110 (38.3%)	83 (29%)	13 (4.5%)	287
	α5$^{flox/+}$; Cre^{-}	α5$^{flox/+}$; Cre^{+}	α5$^{flox/-}$; Cre^{-}	α5$^{flox/-}$; Cre^{+}	Total # genotyped
Tfap2α^{JRESCre}	13 (27%)	24 (50%)	11 (23%)	0*	48

*In addition to vascular defects, 100% of *Fn1*$^{flox/-}$; *Tfap2α*^{JRESCre} and *α5*$^{flox/-}$; *Tfap2α*^{JRESCre} mutants developed cleft palate, a neonatal lethal defect. None of the mutants generated with the *P3Pro-Cre* strain showed cleft palate. This is likely due to a more caudally restricted contribution of Cre-labeled cells in the *P3Pro-Cre* strain compared with the *Tfap2α*^{JRESCre} strain [32, 33, 45].

Table S2. Cardiovascular phenotypes in $Fn1^{flox/-}$; Cre^{+} mutants

Phenotypes	*Fn1*$^{flox/-}$; *Tfap2α*^{IRESCre}		*Fn1*$^{flox/-}$; *P3Pro-Cre*	*Fn1*$^{flox/-}$; *Wnt1-Cre2*
	E14.5 (n=17)	E16.5 (n=11)	E16.5 (n=7)	E14.5 – E17.5 (n=8)*
Retroesophageal right subclavian artery (RERSA)	70.59% (12/17)	72.73% (8/11)	57.14%(4/7)	50% (4/8)
Interrupted aortic arch, type B (IAA-B)	0	9.09%(1/11)	57.14%(4/7)	12.5% (1/8)
Hypoplasitc aortic arch	0	9.09%(1/11)	28.57%(2/7)	0/8
Right sided aortic arch	5.88% (1/17)	0%(0/13)	14.29%(1/7)	0/8
VSD	28.57%(4/14)	36.36%(4/11)	42.86%(3/7)	31.25% (2/5*)
PTA	0	0	14.29%(1/7)	12.5% (1/8)
DORV	0	9.09%(1/11)	14.29%(1/7)	12.5% (1/8)
Intra-cardiac cartilage	NA	88.89%(9/11)	(2 of 2 examined)	31.25% (2/5*)
Incidence‡ of AAA defects (RERSA, IAA-B, hypoplastic aortic arch, or right sided aortic arch)	82% (23/28)#		57% (4/7)#	62.5% (5/8)#

VSD – ventricular septal defect, PTA – persistent trucnus arteriosus, DORV – double outlet right ventricle, AAA – aortic arch artery; NA – not applicable (intracardiac nodules become apparent after E14.5). NE – not examined; ‡ Some embryos had both RERSA and IAA-B.

The difference in penetrance of AAA defects among *Fn1*$^{flox/-}$; *Tfap2α*^{IRESCre}, *Fn1*$^{flox/-}$; *P3Pro-Cre*, and *Fn1*$^{flox/-}$; *Wnt1-Cre2* mutants is not statistically significant (p=0.33, 2-tailed Fisher exact test). *3 of 8 embryos were dissected at E14.5 and 5 of 8 were dissected at E16.5-E17.5; These five embryos were sectioned and analyzed at a histological level.

Table S3. Neural crest-derived cells in the 4th pair of pharyngeal arches

	Control *Fn1*$^{flox/+}$; *Tfap2α*^{IRESCre}	Mutant *Fn1*$^{flox/-}$; *Tfap2α*^{IRESCre}	p
Proliferation (%BrdU^{+} NC cells)	40+/-8	34+/-7	0.16
NC cell density (number of NC cells per 10^4 mm^2)	110+/-11	102+/-9	0.19