**SUPPLEMENTAL MATERIAL:**

## Supplemental Materials and Methods:

### Phospho-histone H3, BrdU, and TUNEL Staining Quantitation

Embryos were dox-treated, harvested, sectioned, and stained as outlined in the Materials and Methods. To score cells, the anterior mitral valve leaflet was first outlined to delineate the appropriate area to be counted. Then, both the number of positive cells and the number of nuclei (stained with Hoechst) were counted in each area using Advanced Elements software (Nikon). The fraction of positive cells was determined for three *Actin-rtTA;TRE-VEGFR2T* and three paired wildtype littermates. Three different litters were used for the combined analysis. The mean of each set of three samples was determined and P-values calculated using two-tailed t-tests.

### NFATc1 Staining Quantitation

Paraffin sections of dox-treated *Actin-rtTA;TRE-VEGFR2T* and control littermate embryos were stained with anti-NFATc1 antibodies as described in the main text. The average NFATc1 signal intensity within the nucleus (overlaying with Hoechst nuclear stain) and cytoplasm (average of one or two neighboring areas) was determined for between eight and ten mitral valve endocardial cells of each embryo using Advanced Elements software (Nikon). For each endocardial cell, a ratio of nuclear to cytoplasmic NFATc1 staining intensity was determined, which was then averaged across all cells scored for a given embryo. The mean ratio of three *Actin-rtTA;TRE-VEGFR2T* and

matched littermate control embryos, representing three different litters, was then calculated and the two groups compared using a two-tailed t-test.

**Supplemental Figure Legends:**

Supplemental Figure 1. VEGFR2 and VEGFR1 have dynamic expression patterns in the developing heart. (**A, B**) Antibody staining for VEGFR2 (green) in *wildtype* sections of E9.5 (A) and E12.5 (B) hearts that include the outflow tract (OFT). Arrowheads point to OFT cushion endocardial cells. Arrows indicate ventricular endocardial cells. Nuclei are counterstained blue with Hoechst. (**C-F**) In situ hybridizations for *VEGFR2* (C, D) and *VEGFR1* (E, F) transcripts (brown) in *wildtype* embryonic heart sections. Heart sections including the atrioventricular canal (AVC) from E9.5 wildtype embryos are shown in (C) and (E) and from E10.5 in (D) and (F). Arrowheads point to AVC cushion endocardium. Arrows denote ventricular endocardial cells. The sections are counterstained with hematoxylin.

Supplemental Figure 2. Blocking VEGF signaling with VEGFR2T does not affect outflow tract (OFT) or atrioventricular canal (AVC) EMT while blocking with sFlt does not disrupt AVC EMT. **(A, B)** H&E stained sections of OFTs of *wildtype* (A) and *Actin-rtTA; TRE-VEGFR2T* (B) E10.5 embryos treated with doxycycline (dox) from E8.5-10.5. **(C-F)** H&E stained sections of AVC cushions of *wildtype* (C, E), *Actin-rtTA; TRE-sFlt* (D), and *Actin-rtTA; TRE-VEGFR2T* (F) E10.5 dox-treated embryos from either E9.5-10.5 (C, D) or E8.5-10.5 (E, F).

Supplemental Figure 3. Blocking VEGF signaling with sFlt during OFT EMT does not affect proliferation or cell death. **(A,B)** Immunofluorescent staining for phospho-histone H3 (green) in OFT cushions of *wildtype* (A) and *Actin-rtTA; TRE-sFlt* (B) E10.5 dox-treated embryos from E9.5-10.5. Nuclei are stained with Hoechst (purple). **(C, D)** Immunostaining for incorporation of BrdU (5 hour exposure *in utero*; brown) in OFT cushions of *wildtype* (C) and *Actin-rtTA; TRE-sFlt* (D) E10.5 embryos treated with dox from E9.5-10.5. The nuclear counterstain is hematoxylin (blue). **(E)** Measurements o f the percentage of BrdU positive mesenchymal cells in the OFT cushions of *wildtype* and *Actin-rtTA; TRE-sFlt* E10.5 embryos treated with dox from E9.5-10.5. n=5 embryos **(F, G)** TUNEL staining (green) of OFT cushions of *wildtype* (F) and *Actin-rtTA; TRE-sFlt* (G) E10.5 embryos treated with dox from E9.5-10.5. Nuclei are stained with Hoechst (purple).

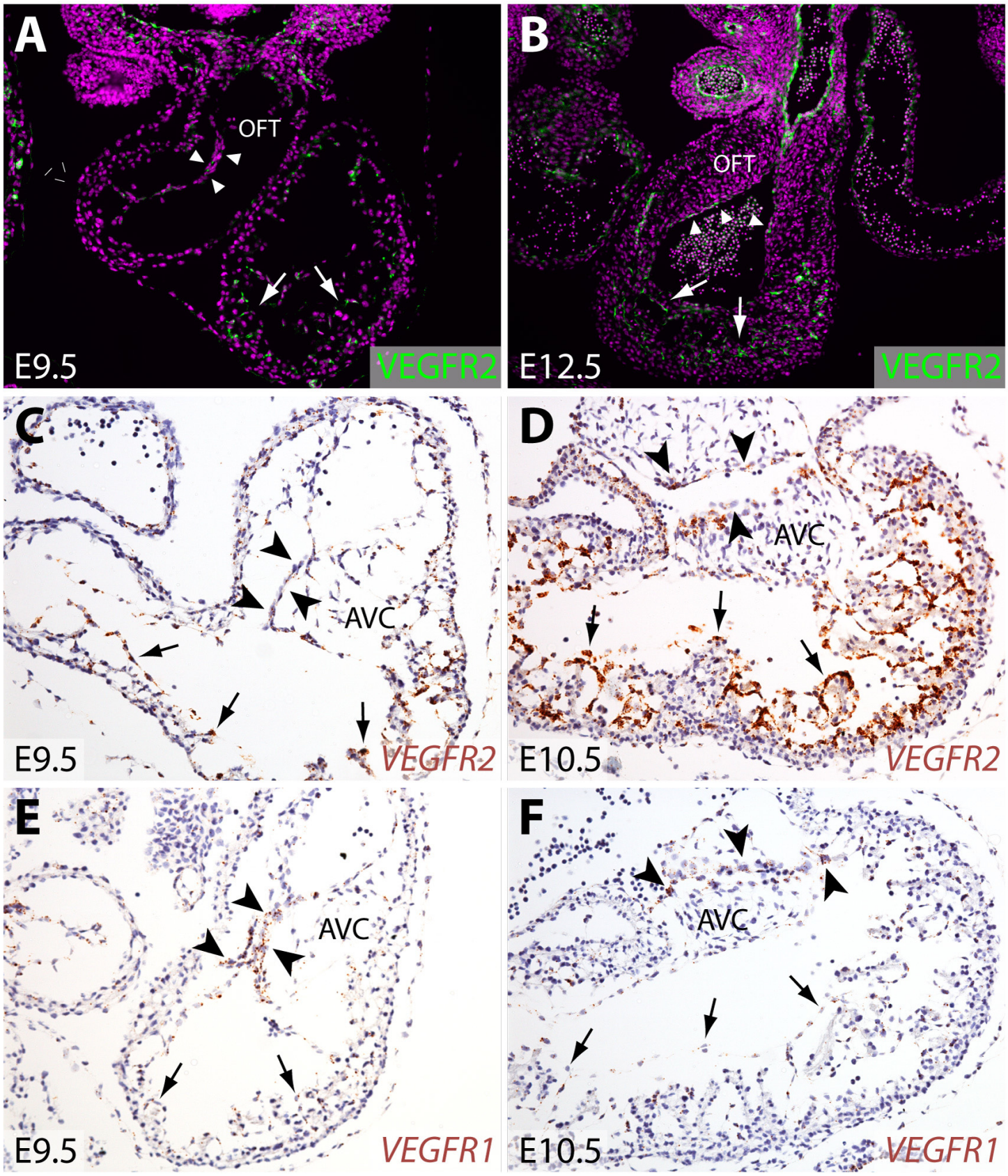
Supplemental Figure 4. Sections used for the morphometric analysis of mitral valve(MV) elongation defects caused by VEGFR2T inhibition of VEGF signaling. **(A-R)** H&E stained sections of MVs of *wildtype* (A-I) and *Actin-rtTA; TRE-VEGFR2T* (J-R) E13.5 embryos treated with doxycycline from E10.5-13.5. The green lines show MV leaflet length and width (mean taken) measurements used to derive length:width ratios.

Supplemental Figure 5. Morphometric analysis of pulmonic valve (PV) leaflet elongation upon VEGFR2T-mediated inhibition of VEGF signaling. **(A-L)** H&E stained sections of PVs of *wildtype* (A-F) and *Actin-rtTA; TRE-VEGFR2T* (G-L) E13.5 embryos

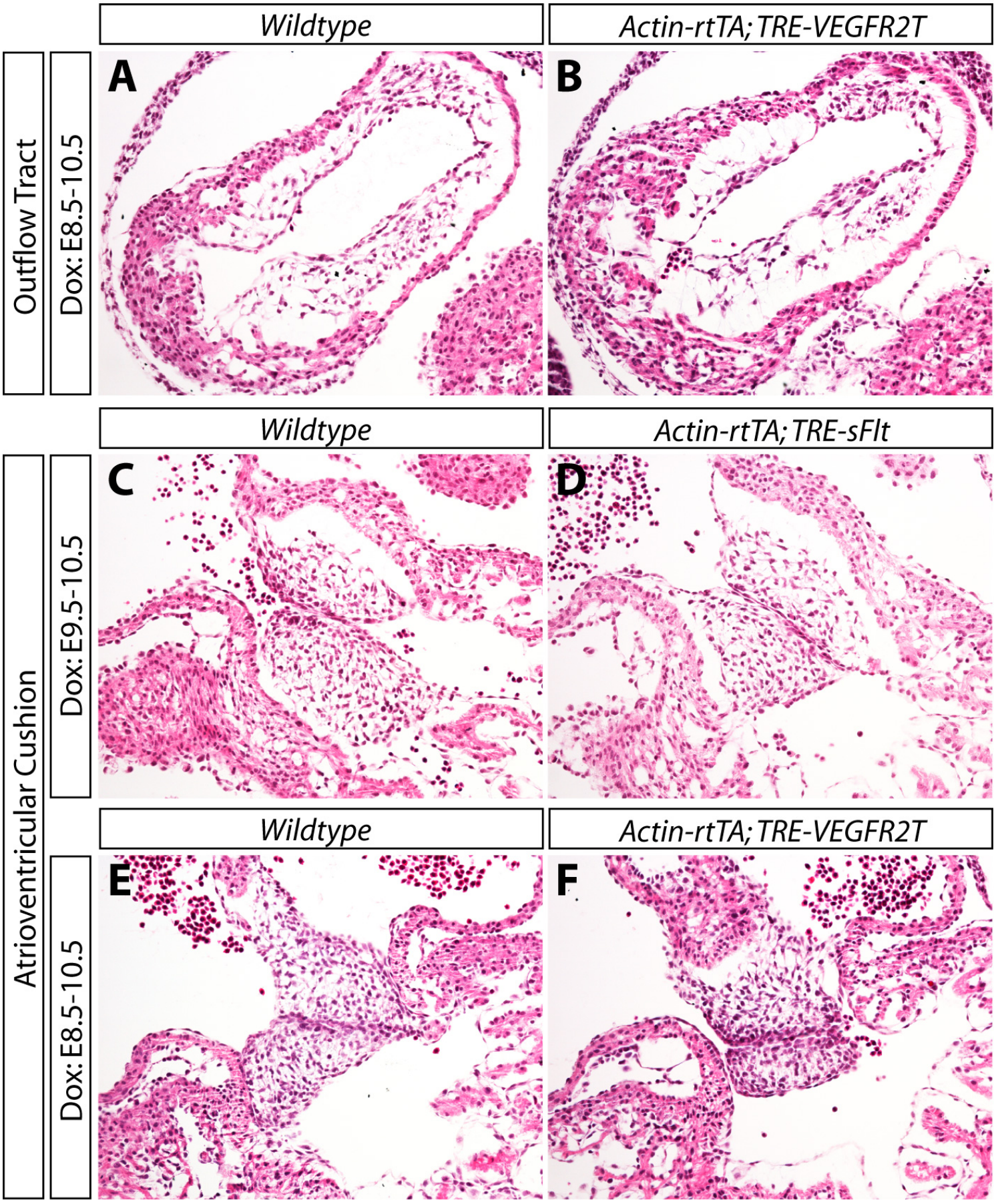
treated with doxycycline from E10.5-13.5. Green lines represent the length and width measurements used to determine length:width ratios.

Supplemental Figure 6. VEGFR2 signaling is not required for cell proliferation or cell survival nor nuclear translocation of NFATc1 in E12.5 mitral valve leaflets. **(A-C)** Bar graphs showing the fraction of **(A)** phospho-histone H3 positive cells, **(B)** BrdU incorporated cells, and **(C)** TUNEL positive cells within the anterior mitral valve leaflets of wildtype and *Actin-rtTA; TRE-VEGFR2T* embryos. **(D)** A bar graph of the ratio of the average nuclear:cytoplasmic anti-NFATc1 antibody staining signal intensity of mitral valve endocardial cells. For all graphs, three wildtype and three littermate paired *Actin-rtTA; TRE-VEGFR2T* E12.5 embryos treated with doxycycline from E10.5 to E12.5 were scored. Errors bars represent one standard deviation.

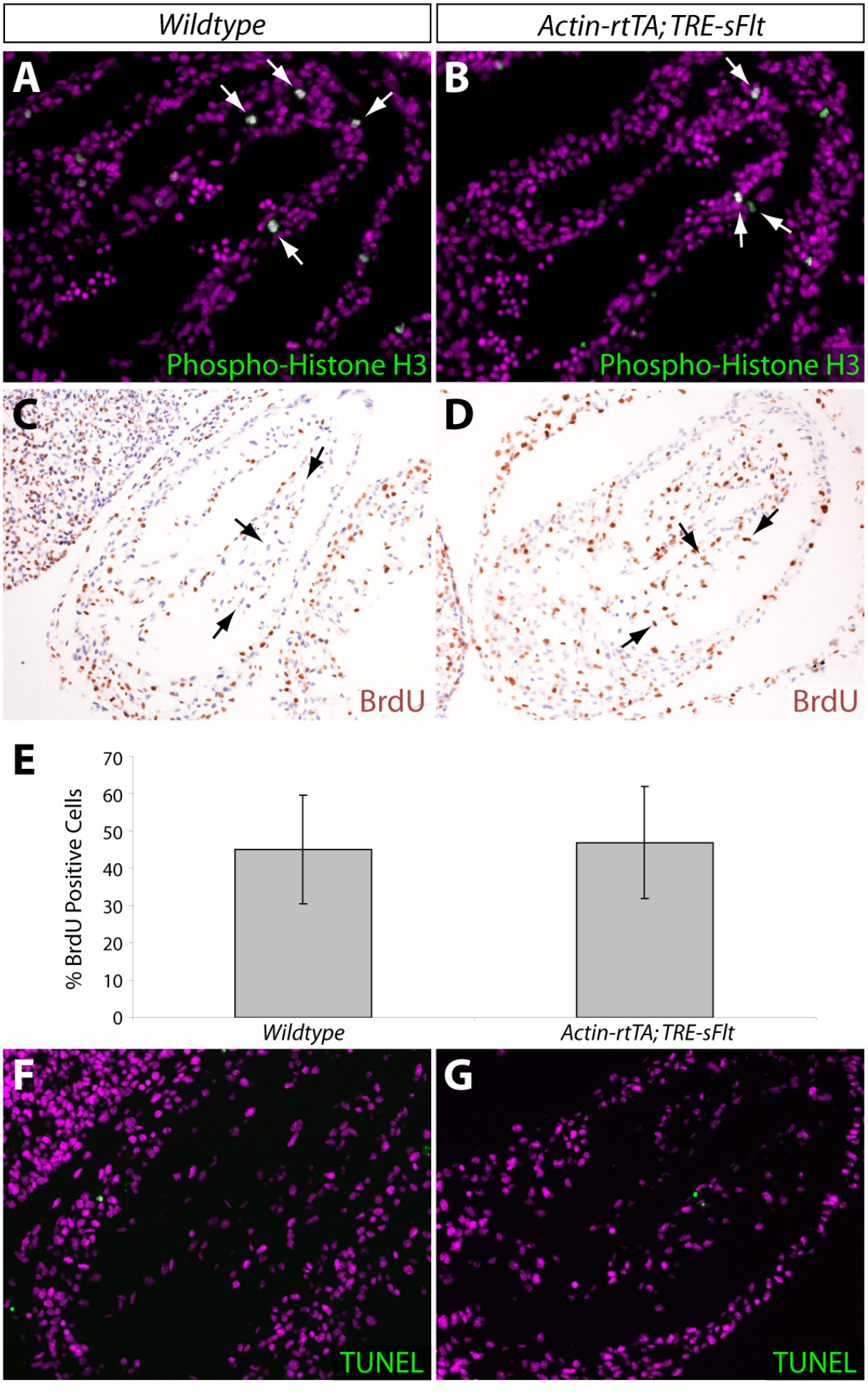
Supplemental Figure 7. miR-126 is not required for nuclear translocation of NFATc1 in endocardial cells of elongating mitral valves. **(A-D)** Immunofluorescent staining of NFATc1 (green) in MVs of *wildtype* (A, C) and *miR-126*$^{\Delta/\Delta}$ (B, D) E11.5 embryos. Nuclei are stained with Hoechst (purple). Arrows point to endocardial cells.



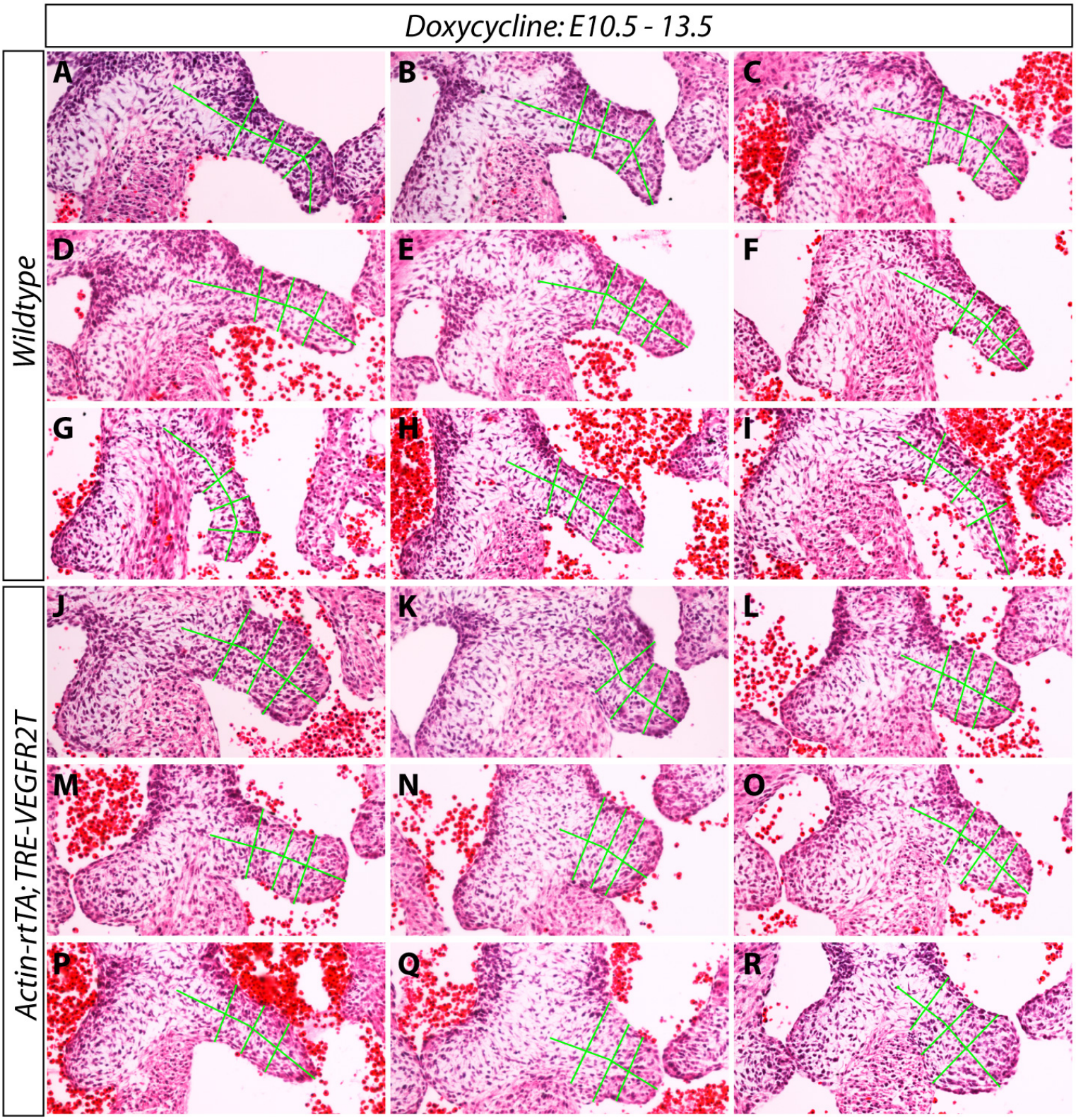

Supplemental Figure 1



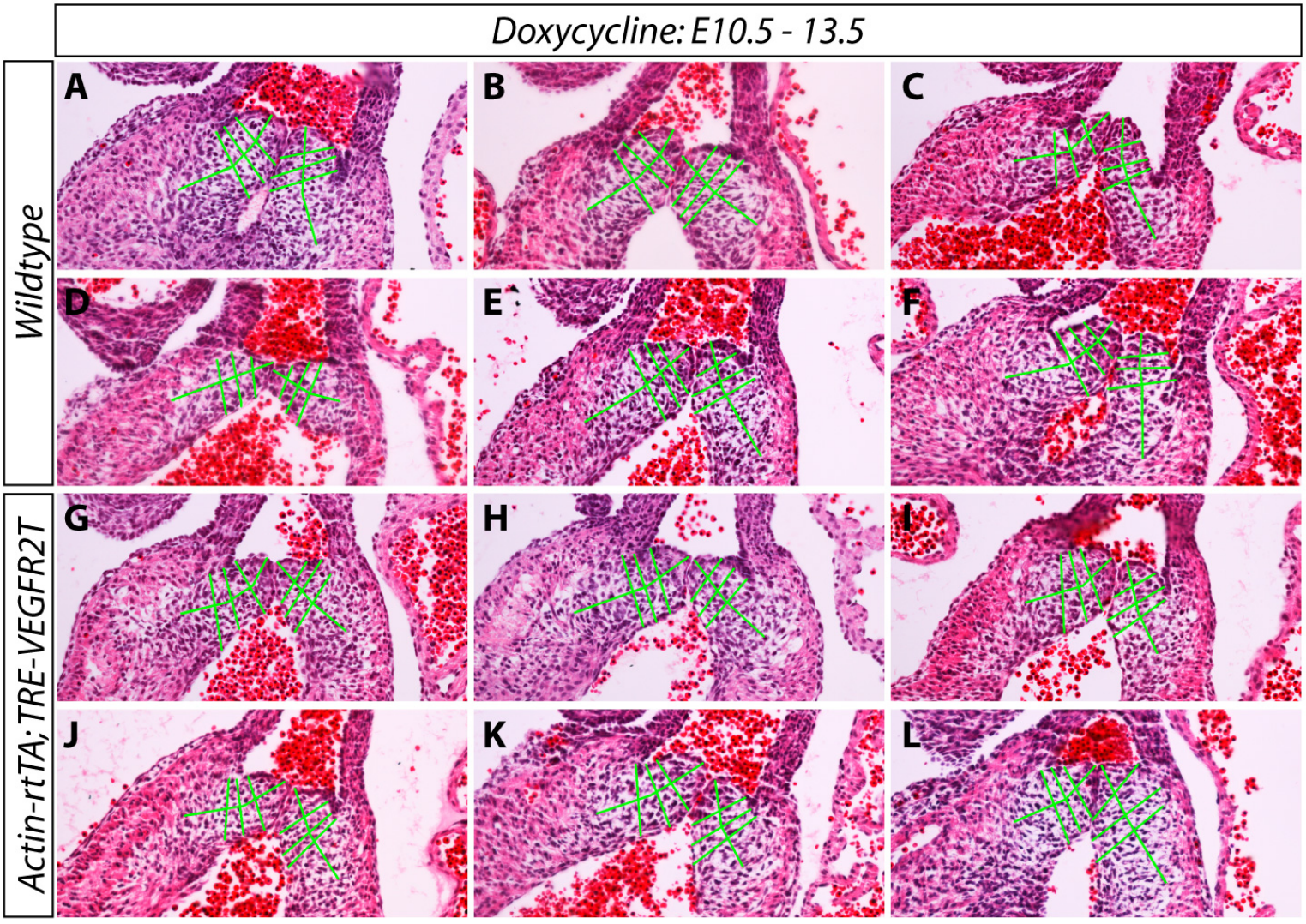

Supplemental Figure 2



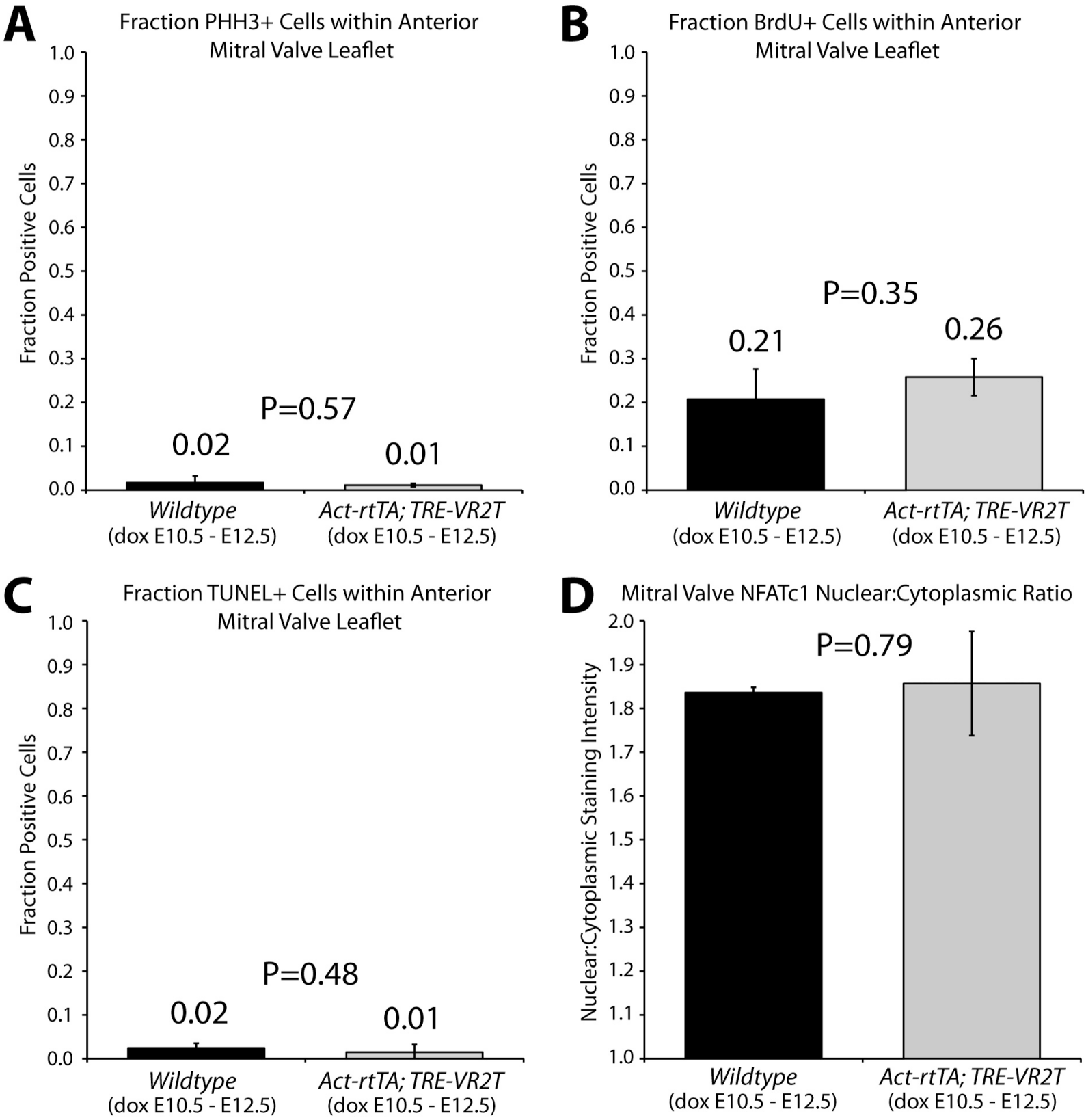

Supplemental Figure 3



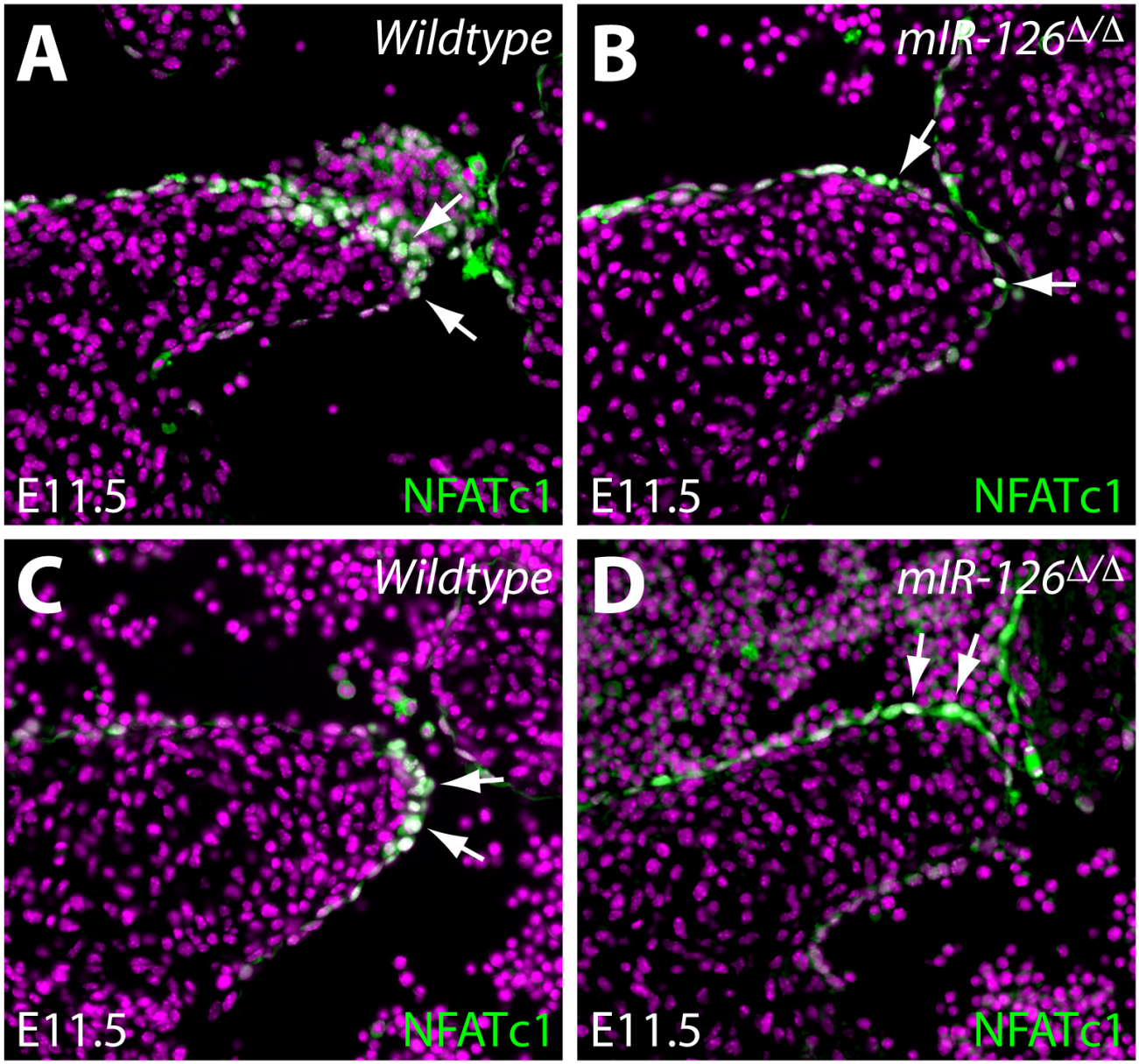

Supplemental Figure 4




Supplemental Figure 5




Supplemental Figure 6




Supplemental Figure 7