Supplemental Methods

Recombination adenovirus

AdHIF-1α (a gift from Dr. G. Semenza) is a replication-defective adenovirus with tandem CMV promoters driving the expression of GFP and a stabilized HIF-1α resulting from a deletion of amino acids 392-520 and a replacement of proline residues at 567 and 685. AdFlk1-Fc (AdFlk1) is a replication defective adenovirus in which a single CMV promoter drives the expression of the murine Flk1 cDNA sequence encoding the signal peptide and the ectodomain fused to a murine IgG2α Fc fragment. This gene product binds to and traps endogenous VEGF. A recombinant adenovirus expressing eGFP (AdGFP) driven by CMV promoter (Pittsburgh Experimental Gene Therapy, Pittsburgh, PA) was injected as a control.

Assays for apoptosis

The chicken hearts harvested at stage 25 and 30 were incubated with 2.5 μM LTR (Invitrogen Corporation, Carlsbad, CA) solution diluted in PBS at room temperature for 15 min followed by several washings in PBS. The embryos were fixed in freshly made 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and photographed in whole mount. Image J and Cell Counter programs downloaded on NIH website were used to quantify the number of LTR particles. A frame was drawn to encompass the OFT at 32x magnification and used to count the number of LTR particles by Cell Counter in Image J. The number of LTR positive particles in the OFT was also scored blindly by two independent observers in whole mount of intact embryos in each group. A reference scale was established in which 1= LTR in normal stage 25 OFT, 2.5 = LTR in normal stage 30 OFT, 5= highest level observed in embryos infected with AdFasL. The

number of LTR particles in each embryo's OFT was rated on this scale and average scores for each group calculated.

For the assay of active Caspase 3, the hearts dissected from stage 25 or 30 embryos were immediately fixed in freshly prepared 4% paraformaldehyde for 1 or 2 hours, respectively, followed by extensive washing and dehydration in serial concentrations of sucrose. After embedded in OCT, sagittal sections (7μm) were collected and immunohistochemistry was performed to determine the protein level of active Caspase 3. The slides were first blocked in PBS with 5% normal goat serum, and then incubated with specific anti-active Caspase 3 antibody (R&D Systems Inc, Minneapolis, MN) for 1 hour at room temperature followed by PBS washing. The slides were incubated with secondary antibody conjugated with rhodamine or fluorescein for 30 min. The number of Caspase-3 positive cells was counted throughout the entire OFT using the Image J program. Sections were observed at 200x magnifcation. Alternate sections were counted to avoid duplicate counting of cells (6-8 sections/embryo). The data is presented as the mean number of active Caspase-3 positive cells in the OFT myocardium within a single section.

Caspase 3 activity was measured in homogenates of pooled OFT from each group as per the manufacturer's instructions (Caspase-Glo 3/7 Assay kit, Promega, Madison, WI). The OFT were dissected and immediately homogenized in cell culture lysis reagent (CCLR, Promega, Madison, WI), centrifuged to remove insoluble proteins (10,000 x g, 5 min, 4 C), and stored at -80 °C. Twenty μg of protein was assayed for Caspase 3 activity by incubating with the same volume of Caspase-3 luminogenic substrate at room temperature. The luminescence was measured within 30-60 min. The activity was measured in 3 separate experiments and each sample measured in duplicate.

FACS and real time PCR

To determine the effects of forced expression of HIF-1α on the expression of downstream genes, the transcripts encoding several cell survival and death related genes was examined. Briefly, embryonic hearts injected with AdHIF-1α or AdGFP at stage 25 were dissected, GFP labeled green tissues were collected into cold DEPC treated PBS. The tissues were digested with 0.25% trypsin-EDTA at 37 °C for 15 minutes followed by gently trituration to disassociate cells. After being washed and re-suspended in PBS, the green cells were selected through BD FACSAria Cell-Sorting System (BD Bioscience, San Jose, CA). Both fluorescein labeled and unlabeled cells were collected and total RNA was isolated by PicoPure RNA Isolation Kit (Arcturus Bioscience, Inc., Mountain View, CA) per manufacturer's protocol. Ten ng of total RNA was used to perform real time RT-PCR by standard methods. Data were first normalized to GAPDH, and then to the GFP+ group.

AdHIF-1 α and AdFlk co-infection assays

Chciken embryos at stage 17-18 were first injected with AdFlk1. AdHIF-1 α was injected 6 hours later at a ratio of 3:1 (AdFlk1 versus AdHIF) based on the ability of AdHIF-1 α to induce VEGF expression by 3-fold as determined by real time PCR (Figure. 4). Chicken hearts were harvested at stage 25 and 30, respectively, and LTR whole mount staining was performed. For morphological observation, chicken embryos were harvested at stage 34 and the hearts were photographed in whole mount.

Antibodies

Rabbit polyclonal anti-HIF-1 α antibody was generously provided by Dr. Faton Agani. Rat anti-mouse Flk1 and biotin polyclonal goat anti-rat IgG (BD Biosciences, San Jose, CA) were used to detect the expression of VEGFR2 delivered by AdFlk-Fc adenovirus. Rhodamine phalloidin (Invitrogen Corporation, Carlsbad, CA) or MF20 (anti-cardiac myosin) antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) was used to detect myocardium.

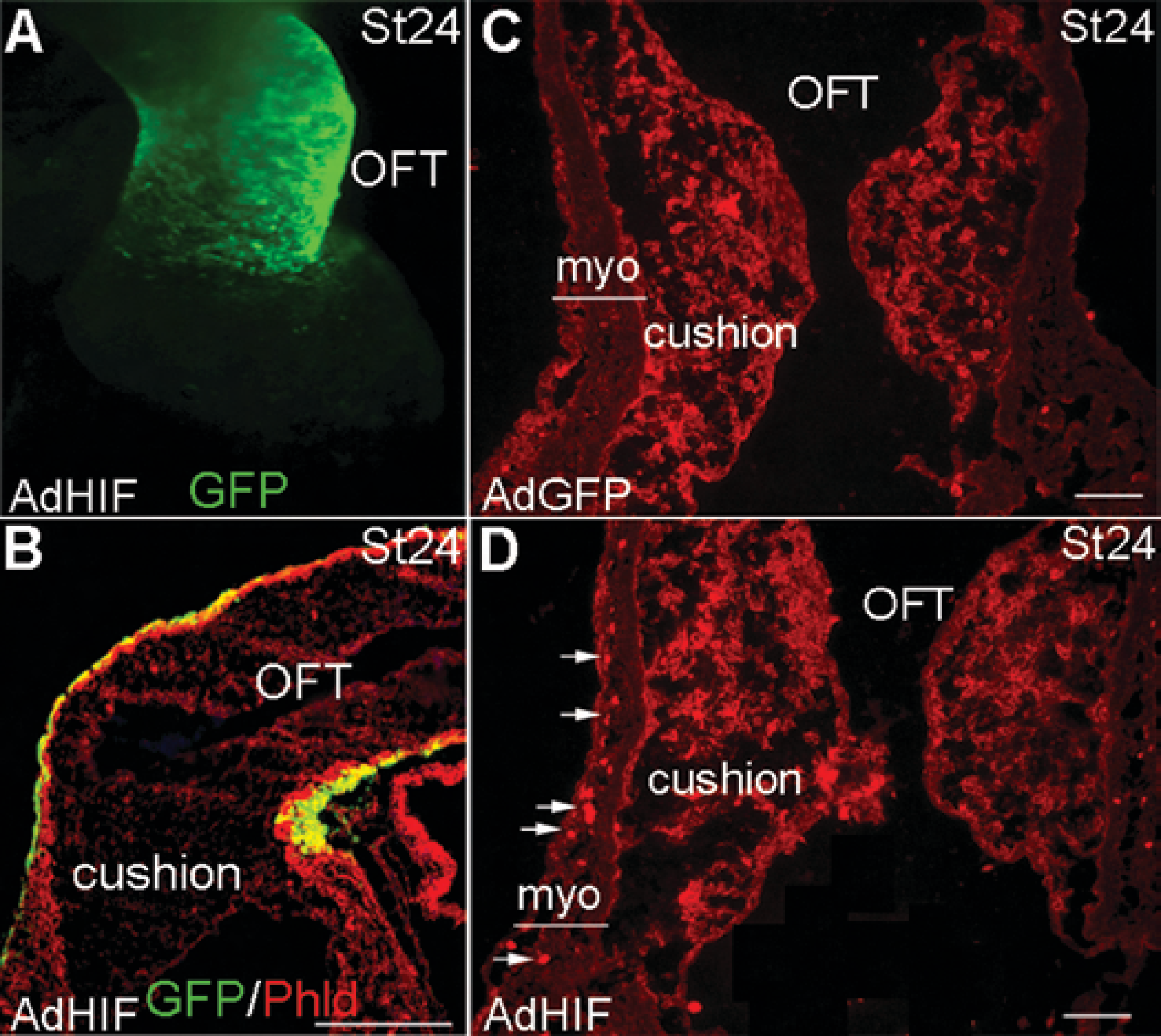
Supplemental Figure Legends

Supplemental Figure 1. Adenoviral mediated expression of HIF-1α in OFT myocardium. Chicken embryos injected with AdHIF-1α (A, B and D) or AdGFP (C) at stage 17 were harvested at stage 24. (A) The distribution of HIF-1α in the cardiac OFT as reflected by GFP expression is shown in a representative embryo in whole mount. (B) Sagittal sections were stained with to define the myocardium (GFP: green, phalloidin: red). (C,D) Sagittal sections show expression of HIF-1α in nuclei of OFT myocardium of AdHIF transduced heart but not in stage matched control transduced with AdGFP. Phld, phalloidin. Scale bar: 250μm in B; 10μm in C and D.

Supplemental Figure 2. Adenoviral mediated expression of truncated VEGFR2 (Flk1-Fc) in OFT myocardium. Chicken hearts injected with AdFlk1 at stage 17 were harvested at stage 25 and stained with anti-VEGFR2 antibody to define the expression of Flk1 (B), or MF20 to define the myocardium (C). Un-injected hearts served as control (A). Scale bar: 250μm.

Supplemental Figure 3. Co-transduction of hearts with AdHIF-1α and AdFlk1-Fc results in intermediate levels of apoptosis. The level of LTR staining (arrows) is higher in the OFT in hearts injected with AdFlk1+AdHIF-1α (B and E) as compared to AdHIF-1α alone (A and D) at stage 25 and 30, and less than that of hearts injected with AdFlk1 alone (C and F). (G) Quantification of LTR positive particles. * $P<0.05$, AdHIF+AdFlk1 versus AdHIF; ** $P<0.01$, AdHIF+AdFlk1 versus AdFlk1. OFT, outflow tract; Vent, ventricles.

Supplemental Figure 4. Co-transduction of hearts with AdHIF-1α and AdFlk1-Fc results in heart defects similar to those of AdFlk1 alone. Of 8 embryos co-infected, 6 exhibited dys-morphologies similar to that of the AdFlk1 group and the remaining two appeared to be normal. Hearts representative of the two distinct AdFlk1 phenotypes are shown here; these phenotypes are described in detail in the body of the manuscript. Scale bar: 500μm.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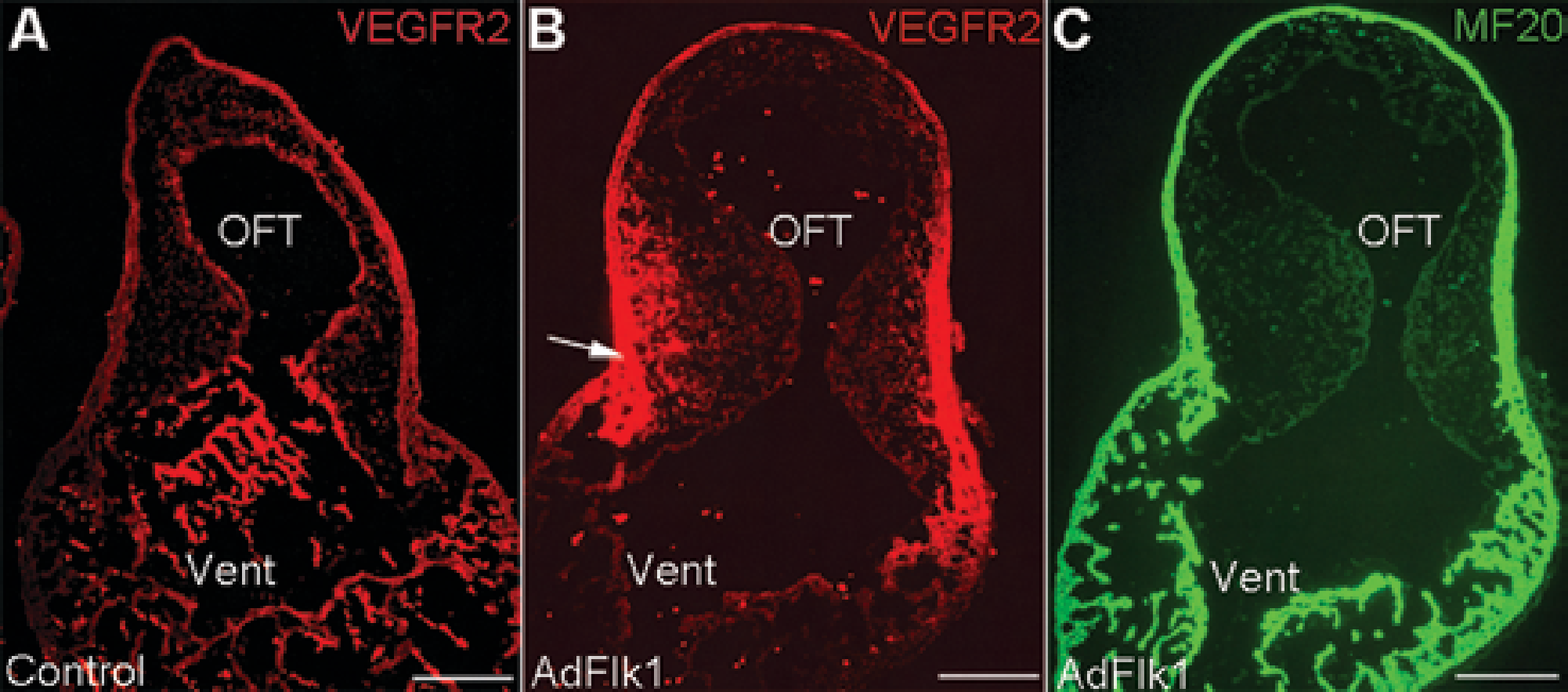
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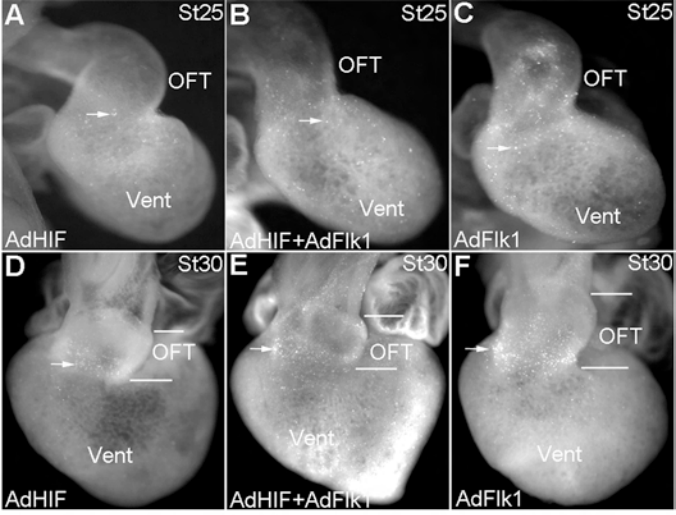
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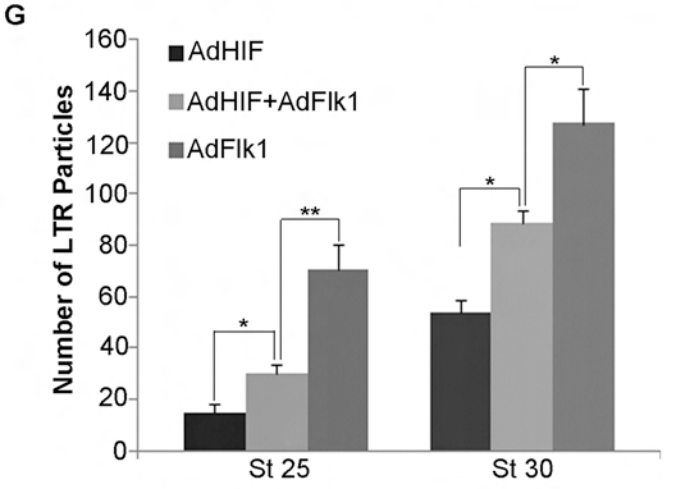
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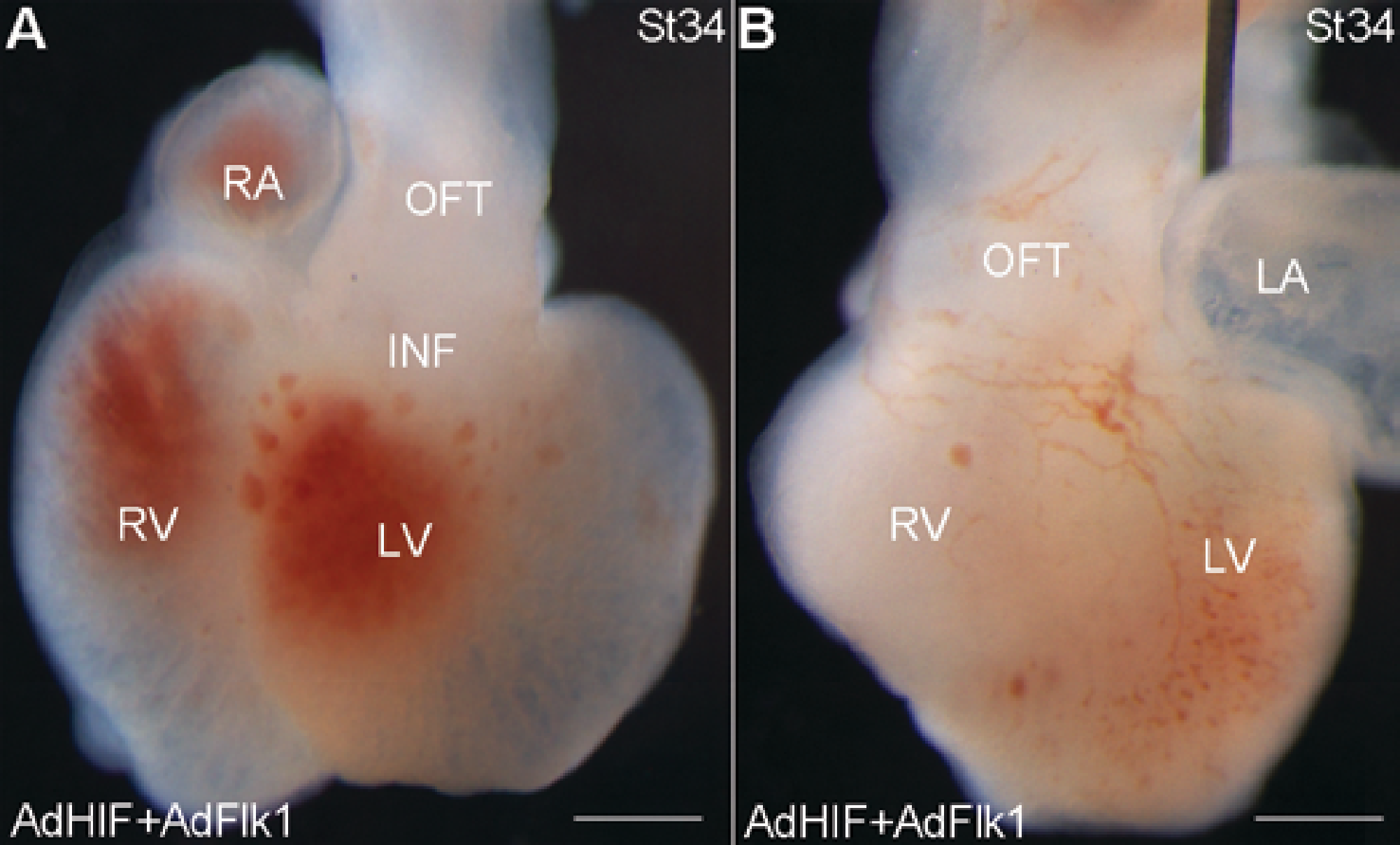
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