Supplemental Material

Detailed Methods

Mice

All animal protocols were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. Mice were maintained on a mixed genetic background. *Isl1*$^{Cre/+}$ [1,2], *Pax3*^{Cre} [3], *Mef2c-AHF-Cre* [4] and *R26*$^{LacZ/+}$ [5] mice were genotyped as previously described. *Wnt1::Flpe* mice [6,7] were genotyped using the following primers as described: [7]

Forward: 5' GGTCCTGGTTCGTCAGTTTGTG 3'

Reverse: 5' TCCCTTATCTGCTTCTTCCGATG 3'

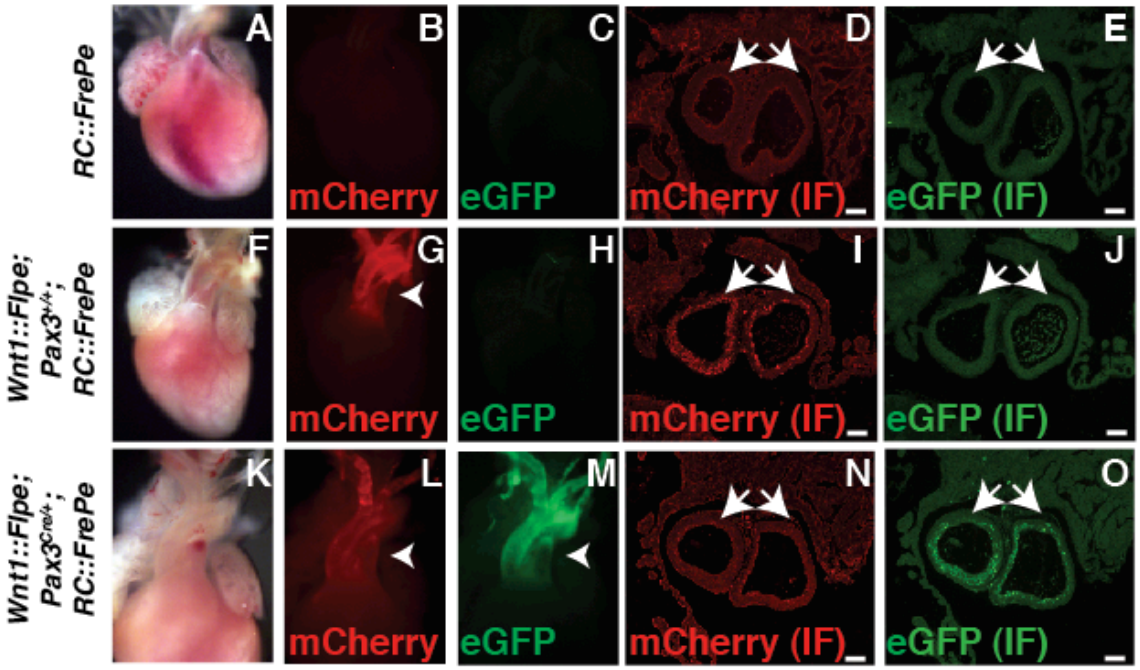
The dual reporter allele *RC::FrePe* was genotyped with the following primers for the *Rosa26* locus:

Forward: 5' CACTTGCTCTCCCAAAGTCG 3'

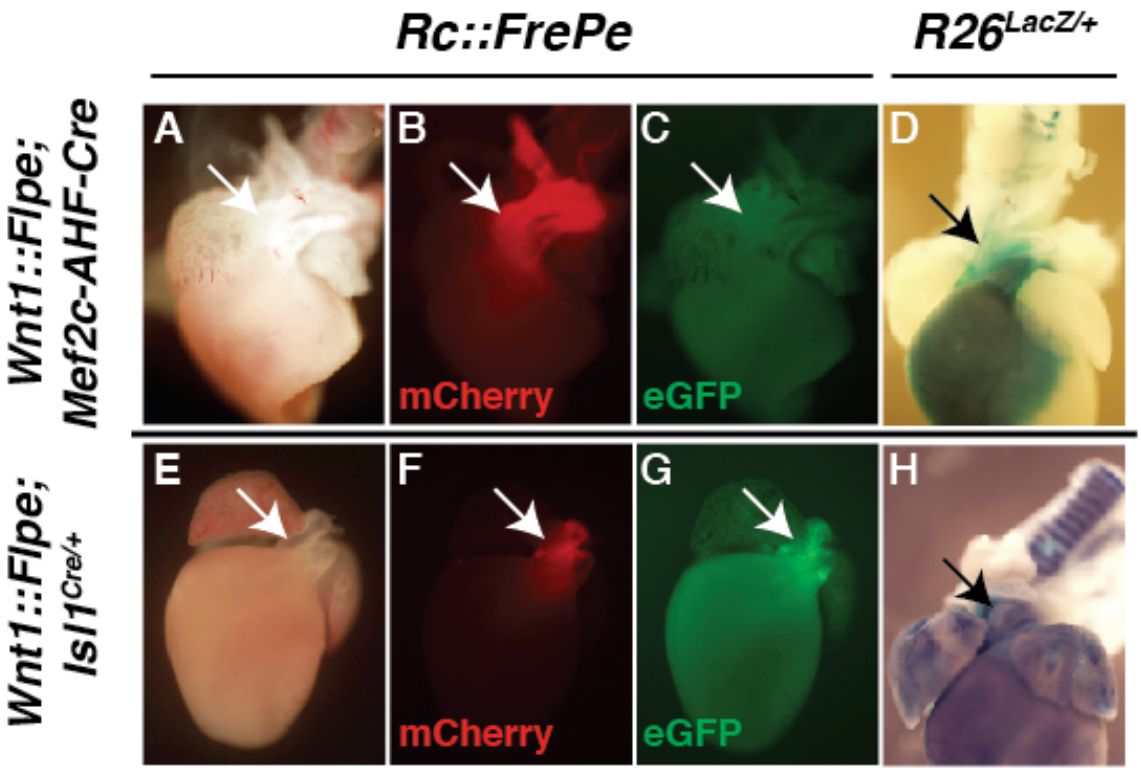
Wild-type Reverse: 5' TAGTCTAACTCGCGACACTG 3'

Mutant Reverse: 5' GTTATGTAACGCGGAACTCC 3'

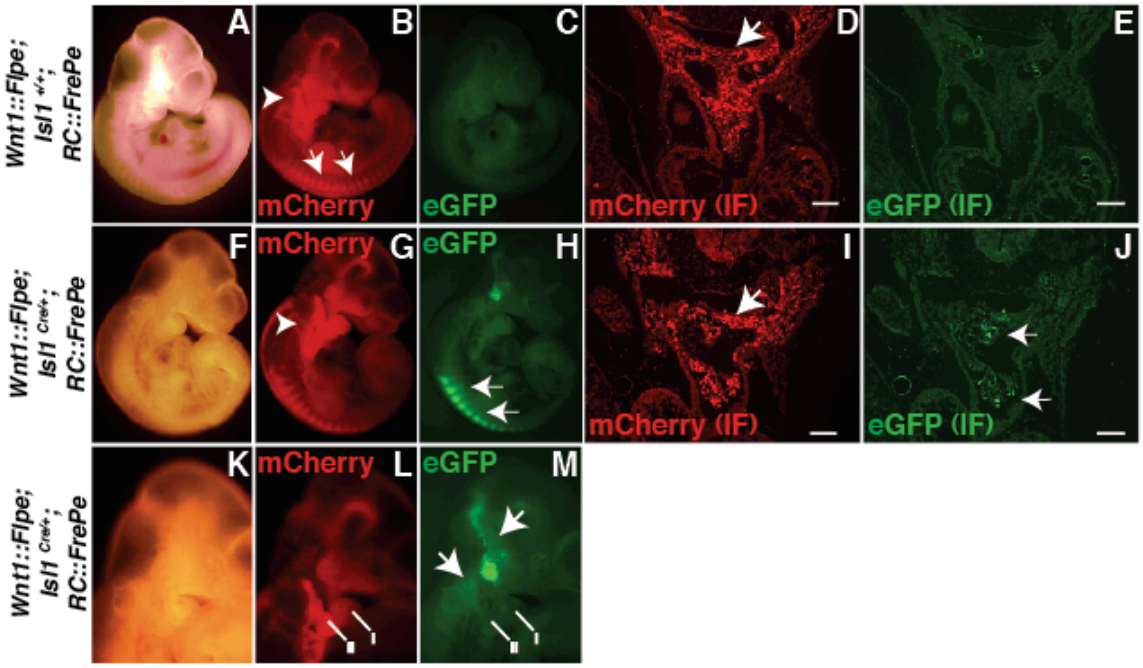
Supplemental Figures and Figure Legends



Online Figure I. Validation of the dual reporter *RC::FrePe* mouse in neural crest. *RC::FrePe* is knocked into the *Rosa26* locus as per previously designed intersectional reporter alleles. [6, 8, 9] **A-E,** Postnatal (P) day 0 control *RC::FrePe* whole hearts **(A-C)** and cross sections through the outflow tract **(D, E)**. mCherry and eGFP are not detected. **F-J,** P0 hearts from *Wnt1::Flpe; Pax3*$^{+/+}$; *RC::FrePe* embryos. mCherry expression is detected in the outflow tract **(G, arrowhead)** and is confirmed by immunofluorescence of cross sections **(I)**. eGFP is not detected **(J)**. **K-O,** P0 hearts from *Wnt1::Flpe; Pax3*$^{Cre/+}$; *RC::FrePe* pups. Strong eGFP expression is now evident in the outflow tract **(M, arrowhead, and O)** while only trace amounts of mCherry expression are detected **(L, N)**. Scale bars: 100μm. BF-Bright field. IF-Immunofluorescence



Online Figure II. ***Mef2c-AHF-Cre*** **and** ***Isl1***$^{Cre/+}$ **derivatives in newborn hearts include the area of** ***RC::FrePe*** **dual-reporter expression.** **(A-C)** Post-natal day 0 (P0) *Wnt1::Flpe; Mef2c-AHF-Cre*; *RC::FrePe* heart showing mCherry fluorescence in the outflow tract **(B, arrow)**. eGFP is not detected **(C, arrow)**. **D**, P0 *Wnt1::Flpe; Mef2c-AHF-Cre*; *R26*$^{LacZ/+}$ whole-mount X-Gal-stained hearts demonstrating *LacZ* expression in the outflow tract in comparable regions of the outflow tract highlighted in **A-C** (**D, arrow**). **(E-G)** P0 *Wnt1::Flpe; Isl1*$^{Cre/+}$; *RC::FrePe* hearts showing mCherry **(F, arrow)** and eGFP **(G, arrow)** fluorescence in the cardiac outflow tract. **H**, P0 *Wnt1::Flpe; Isl1*$^{Cre/+}$; *R26*$^{LacZ/+}$ whole-mount X-gal stained heart demonstrating *LacZ* expression in the outflow tract in comparable regions of the outflow tract highlighted in **E-G** (**H, arrow**).



Online Figure III. **Dual fate mapping identifies *Isl1*$^{Cre/+}$*/Wnt1::Flpe*-derived cells in the heart at E10.5. A-E,** E10.5 *Wnt1::Flpe*; *Isl1*$^{+/+}$; *RC::FrePe* embryos **(A-C)** and immunofluorescence (IF) for mCherry **(D)** and eGFP **(E)** of cross sections through the region of the developing cardiac outflow tract **(D,E)**. *Wnt1::Flpe*-derived craniofacial neural crest **(B, arrowhead)** and dorsal root ganglia **(B, arrows)** express mCherry, which is also detected by IF in the developing outflow tract **(D)**. **F-J,** *Wnt1::Flpe; Isl1*$^{Cre/+}$; *RC::FrePe* embryos. mCherry is detected in craniofacial mesenchyme **(G, arrowhead)** and eGFP is now expressed by dorsal root ganglia **(H, arrows)**. mCherry **(I)** and eGFP **(J)** are both detected by IF in the developing outflow tract. **K-M,** Higher magnification of the *Wnt1::Flpe; Isl1*$^{Cre/+}$; *RC::FrePe* embryo shown in **F-H**. mCherry is detected in the first (I) and second (II) pharyngeal arches **(L)** while eGFP is expressed by cells near the pharyngeal arches **(M, arrows)**. Scale bars: 100μm.

Supplemental Tables and supporting information

Reports Utilizing Isl1 for Identification of Second Heart Field Precursors

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