Materials and Methods:

Histological analysis and Western blotting

The following primary antibodies or cell death detection system were utilized: ANF pAb (Millipore NMM1717334), cardiac MLCK,[1] cleaved caspase-3 (Cell Signaling 9664), GAPDH (Research Diagnostics TRK5G4-6C5), Nav1.5(α)(Alomone Labs ASC-005,) Nkx2-5 pAb,[2] Palloidin-TRIC (Fluka 77418), phospho-histone H3 (serine 10)(Millipore 06-570), troponin T (Sigma T6277), and TUNEL (Roche In Situ Cell Death Detection kit).

Acetylcholine esterase staining was performed as follows: unfixed frozen heart sections (10 μm) were incubated with the reaction solution (see below) at 37°C overnight, followed by developing with 1% $(NH_4)_2S$ for 5 min at room temperature. Stained tissue sections were briefly washed with PBS, fixed with 4% paraformaldehyde, dehydrated with ethanol (70% and 95%), stained with 10-fold diluted eosin in 95% ethanol, dehydrated with 100% ethanol, and mounted with Permount. Reaction solutions were made as follows. Solution A; addition of 10 ml 0.1M Na-acetate (pH 5.3), 7 ml ddH_2O and 0.8 ml Cu-glycine solution (glycine 3.75 g + $CuSO_45H_2O$ 2.5 g in 100 ml ddH_2O). Solution B; mixture of acetylcholine iodide 29 mg in 1.5 ml ddH_2O and 0.5 ml of 0.1 M $CuSO_4$, followed by centrifuge at 2,000 rpm for 10 min. Solution C; Promethazine 25 mg in 10 ml dH_2O. Solution A was combined with the supernatant of Solution B and 0.5 ml of Solution C to create the reaction solution.

Ultrasound imaging

Fetal positions were marked on the surface of the shaved-abdominal skin. As mouse fetal positions in-utero affected the imaging planes, some embryos not suitable for imaging were

eliminated. Immediately after echocardiography, the pregnant mice were euthanized, and the abdominal cavity was carefully opened to maintain embryo positions during dissection.

Real-time RT-PCR

Real-time RT-PCR was performed using inventoried Taqman Gene Expression Assays (Applied Biosystems): Nkx2-5 Mm00657783, Scn5a Mm00451971, KcneI Mm01215533, T-type Ca channel α1G Mm00486549, T-type Ca channel α1H Mm00445369, connexin40 00433619, ANF Mm01255748, BNP Mm00435304, HOP/HOD Mm00558629, cardiac MLCK Mm00615292, RyR2 Mm0046587, SERCA2a Mm00437634, phospholamban Mm00433182, βMHC Mm00600555, α2-actinin Mm00473657, cardiac actin Mm01333821, skeletal actin Mm0080218, BMP10 Mm03024178, sarcolipin Mm00481536, and HCN1 Mm01308021. Data were normalized to β-actin expression (No. 4352933E).

Simultaneous recording of cardiac contraction and Ca^{2+}

E15.5 embryonic hearts were isolated and kept in buffer (in mmol/L: NaCl 137, KCl 5.36, $MgSO_4$ 0.81, HEPES 10, dextrose 5.55, KH_2PO_4 0.44, Na_2PO_4 0.34, pH 7.4) on ice during genotyping (2-3 hrs). Multiple flox/flox or flox/flox/Cre hearts were combined for serial trypsin-digestion in buffer, and isolated cardiomyocytes were cultured for 2 days (equivalent to E17.5). Only isolated cardiomyocytes with clear surface membranes free from blebs were studied for simultaneous measurements of cardiomyocyte contraction and Ca^{2+} measurement as described previously.[1]

References:

1. Chan JY, Takeda M, Briggs LE, Graham ML, Lu JT, Horikoshi N *et al*. Identification of cardiac-specific myosin light chain kinase. *Circ Res* 2008;**102**:571-580.

2. Kasahara H, Bartunkova S, Schinke M, Tanaka M, Izumo S. Cardiac and extracardiac expression of Csx/Nkx2.5 homeodomain protein. *Circ Res* 1998;**82**:936-946.

Figure S1. Tamoxifen-injection at E10.5 results in lethality by E14.5 with increased ventricular trabeculation and thinner compact layer. (A) E14.5 flox/flox (left) and flox/flox/Cre (right) littermates. **(B)** Crown rump length is slightly decreased in flox/flox/Cre compared to littermates. **(C)** Histological sections from flox/flox and flox/flox/Cre hearts at E14.5 (n=3 each). **(D)** Histological sections from flox/flox and flox/flox/Cre hearts at E13.5 (n=2 each).

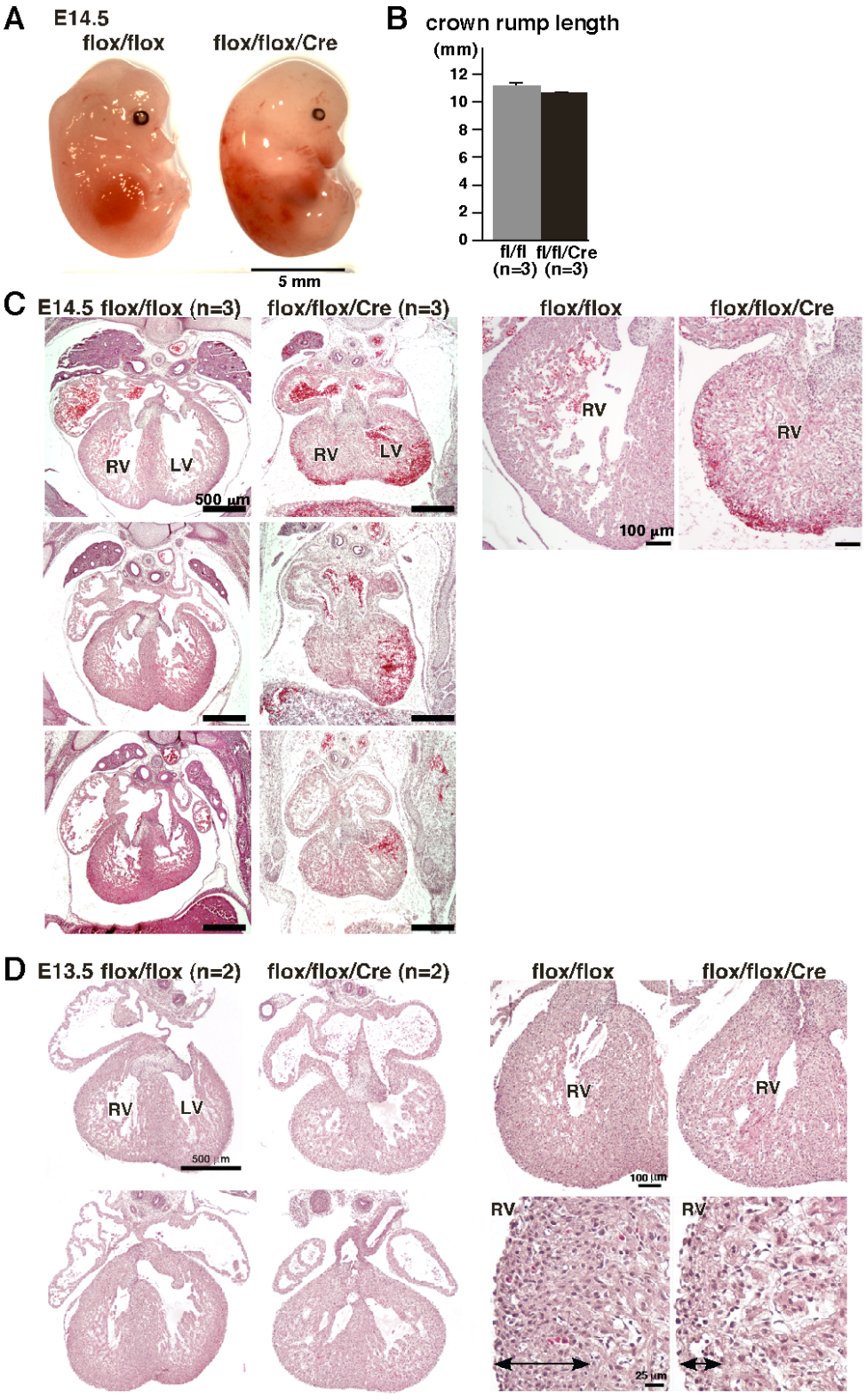

Figure S2. Distribution of AchE activity in E16.5 embryos predominantly in the ventricular trabeculae and inner layer of atria both in flox/flox (E1-3, n=3) and flox/flox/Cre heart (E4-6, n=3).