SUPPLEMENTAL MATERIAL

Supplemental Methods

Fluorescent activated cell sorting (FACS):

(1) Adult mouse cardiac progenitor cells (CPCs):

Single cell suspension was obtained from 8 to 12 week-old male C57BL/6 mice as previously described [1]. The procedure was performed according to the approved UC Davis Animal Care and Use protocol. Briefly, mice were injected with 0.1 ml heparin (1000 units ml^{-1}) 10 min prior to heart excision, then anesthetized with pentobarbital intraperitoneally (80 mg kg^{-1}). Hearts were removed and placed in sterile Tyrode's solution (mmol l^{-1}: NaCl 140, KCl 5.4, $MgCl_2$ 1.2, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES) 5 and glucose 5, pH 7.4). All chemicals were obtained from Sigma Chemicals (St. Louis, MO) unless stated otherwise. The aorta was cannulated under a dissecting microscope and mounted on the Langendorff apparatus. The coronary arteries were retrogradely perfused with Tyrode's solution gassed with O_2 at 37°C for 3 min at a flow rate of ~3 ml min^{-1}. The solution was switched to Tyrode's solution containing collagenase type 2 (1 mg ml^{-1}, 330 units mg^{-1}, Worthington Biochemical Corporation). After ~12 min of enzyme perfusion, hearts were removed from the perfusion apparatus and gently teased in high-K^+ solution (mmol l^{-1}: potassium glutamate 120, KCl 20, $MgCl_2$ 1, EGTA 0.3, glucose 10 and HEPES 10, pH 7.4 with KOH). The cells were filtered through 100 μm cell strainer, re-suspended in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS), treated with phytoerythrin-conjugated anti-c-kit antibody (2 μg ml^{-1}, BD Bioscience, San Diego, CA) and lineage antibodies (CD3e, CD11b, Cd45R, Ly-6C, Ly-6G and TER-119, 1:100 dilution, BD Bioscience) at room temperature for 30 min under sterile conditions. Cells were washed with PBS and stained with streptavidin Alexa Fluor 750-

allophycocyanin conjugate (1:1500 dilution, Invitrogen) for 15 min at room temperature. Lin^{neg} c-kit^{pos} CPCs were sorted using a Cytomation MoFlo Cell sorter (UC Davis Optical Biology CORE). Data was acquired using Summit software (Cytomation) and analyzed using FlowJo software (ver9.0.1 Treestar Inc., San Carlos, CA). These cells were used for miRNA microarray analysis and qPCR experiments.

(2) Neonatal mouse CPCs:

Single cardiac myocytes were isolated from 1-2 day old neonatal congenic C57BL/6 mice as previously described [2]. The procedure was performed according to the approved UC Davis Animal Care and Use protocol. Animals were decapitated and the hearts excised under sterile conditions into filter-sterilized (0.2 μm) Ca^{2+}-and bicarbonate-free Hanks' buffer with HEPES (CBFHH) solution (mmol l^{-1}: NaCl 137, KCl 5.36, $MgSO_4$ 0.81, HEPES 20.06, K_2PO_4 0.44, and Na_2HPO_4 0.34, pH 7.4) at room temperature. The hearts were washed and minced in CBFHH solution. The tissue was digested with CBFHH solution containing trypsin (Sigma, 1.5 mg ml^{-1}) with continuous stirring at 37°C. The supernatant was removed and the enzyme solution replaced at 5 min intervals until the tissue was completely dissociated. The cells were diluted in sterile FBS, centrifuged at 300 g for 3 min, filtered through 100 μm cell strainer, stained with antibodies (as mentioned above) and sorted. The sorted cells were used for microarray analysis and qPCR experiments.

Cell culture of neonatal and adult CPCs:

Sorted neonatal and adult CPCs were maintained in CPCs maintenance media [DMEM/DMEM F12 media (1:1, Invitrogen), 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 1% penicillin/streptomycin and 1% L-glutamine] at 37°C in a 5% CO_2 incubator.

To differentiate the cells into cardiomyocytes, sorted adult mouse CPCs were maintained in CPC-maintenance medium. After a week, 10 ng ml^{-1} epidermal growth factor, 20 ng ml^{-1} basic fibroblast growth factor, 40 nmol ml^{-1} cardiotrophin-1 (Biosource, Carlsbad, CA), 0.1 mmol ml^{-1} 2-β mercaptoethanol and 40 nmol ml^{-1} thrombin were added to the media [3].

MicroRNA Profiling:

Total RNA including microRNAs was isolated from neonatal and adult mouse CPCs using the *mirVana* microRNA isolation kit (Ambion, Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. As a control, microRNAs from adult mouse cardiomyocytes were also isolated. Microfluidic μParaflow expression microarrays (LC Science, Houston, TX) with probes for Sanger microRNA Database v12.0 were used to detect the differentially expressed microRNAs across biological duplicates for each CPC sample. Differentially expressed microRNAs were identified after background subtraction using regression-based background mapping method and normalization using a cyclic LOWESS method [4]. MicroRNAs with $P<0.05$ were selected for cluster analysis. The clustering was performed using hierarchical methods with average linkage and Euclidean distance metric [5]. A threshold value of 32 was used and signal intensities below the threshold were considered inaccurate. Differential expression was analyzed using one-way Analysis of Variance (ANOVA).

Real-time PCR

Total RNA including microRNAs from neonatal and adult mouse CPCs was isolated using the *mirVana* microRNA isolation kit (Ambion) according to manufacturer's protocol and measured by spectrophotometry. 100 ng of RNA was reverse transcribed into cDNA using the TaqMan microRNA reverse transcription (RT) kit (Applied Biosystems) with the microRNA-specific looped RT primer. Quantitative PCR (Q-PCR) was carried out using TaqMan Universal

PCR Master Mix (Applied Biosystems) with AmpliTaq Gold DNA polymerase in triplicates according to manufacturer's specifications. Nucleolar RNA, Sno234, was used as endogenous control as per manufacturers recommendations. The relative microRNA expression is expressed as $\Delta\Delta C_T$, where $\Delta\Delta C_T$ represents the mean difference between neonatal and adult $-\Delta C_T$ values.

For the evaluation of the Rbl2 protein, 100 ng of RNA was reverse transcribed into cDNA using the RT² First Strand Kit reverse transcription kit (SA Bioscience). Q-PCR was carried out using RT² qPCR SYBR Green Master Mixes (SA Bioscience) in triplicates according to manufacturer's specifications. β-actin was used as an endogenous control. The PCR was performed using a Bio-Rad C1000 thermocycler.

Flow cytometric analysis of human and mouse proliferating CPCs:

Human atrial appendage specimens from informed consented patients undergoing cardiac bypass surgery were obtained from UC Davis Medical Center in accordance with the approved UC Davis Institutional Review Board (IRB) protocol. The tissue was stored in cardioplegic solution at 4° C and processed within 2 hours. Human embryonic cardiac tissue was obtained from informed consented patients in accordance with the Sanford Burnham Medical Research Institute IRB protocol. To obtain a single cell suspension, the cardiac tissue was washed and minced in sterile Tyrode's solution and digested in the Tyrode's solution containing collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ, 1 $\mu g\ ml^{-1}$) with continuous stirring at 37°C and the solution was replaced every 5 min. The cells were diluted in sterile FBS, centrifuged at 300 g for 3 min, filtered through 100 μm cell strainer and stained with antibodies as described below.

Cells from human as well as mouse hearts were stained with phycoerythrin-conjugated anti-c-kit antibody. Stained cells were fixed with 0.4% paraformaldehyde (Electron Microscopy

Sciences, Hatfield, PA), treated with 0.1% Triton-X 100 and stained with monoclonal mouse anti-myosin heavy chain antibody (MF20; Developmental Studies Hybridoma Bank, Iowa), anti-troponin T antibody (Thermo Scientific) lineage antibodies (CD3e, CD11b, Cd45R, Ly-6C, Ly-6G and TER-119, 1:100 dilution, BD Bioscience), and proliferation-specific Ki67 antibody (15 μg ml^{-1}, BD Bioscience) in PBS with 5% donkey serum and 20 μg ml^{-1} DNAse-free RNAse (Sigma) overnight at 4°C [6]. Cells were washed with PBS and stained with streptavidin Alexa Fluor 750-allophycocyanin conjugate (1:1500 dilution, Invitrogen) for 15 min at room temperature. Cells were also stained with 40 μg ml^{-1} 7-amino- actinomycin D (7AAD, BD Bioscience, San Jose, CA) or propidium iodide (10 μg ml^{-1}, Invitrogen) to measure the DNA content. Data was collected using a standard FACScan cytometer (BD Biosciences, San Jose, CA) upgraded to a dual laser system with the addition of a blue laser (15mW at 488nm) and a red laser (25mW at 637nm Cytek Development, Inc, Fremont, CA). Data was acquired using CellQuest software (BD Bioscience). Cells stained with isotype-matched IgG antibodies were used as controls to determine the positive cell population. Data was analyzed using FlowJo software (ver9.0.1 Treestar Inc., San Carlos). Cell cycle data analysis for mouse CPCs was performed using the ModFit (Variety House) software.

Lentiviral packaging:

Control lentiviral (LV) expression vector with GFP (System Bioscience, Mountain View, CA) and lentiviral expression vector with miR-17 cluster (miR-17, -18, -19a, 19b, 20a, 92a, System Bioscience, Mountain View, CA) and GFP were used in the intact heart. The control and the miR-17 lentivirus were packaged separately using the HEK293 cells. The LV-vectors (2 μg each of control and miR-17), pMD.G (1 μg) and pCMVD8.9 (4 μg) plasmids were diluted in 750 ml of Opti-MEM (Sigma) separately for 5 min at room temperature. 20 μl of Lipofectamine

2000 (Invitrogen) was diluted in 750 ml Opti-MEM and incubate for 5 min at room temperature. The plasmids and the lipofectamine were mixed gently and incubate for 20 min at room temperature. HEK293 cells were washed with Opti-MEM without serum and antibiotics before adding the plasmids and incubated for 6 hr at 37 °C with 5% CO_2. Fresh DMEM medium with 10% FBS and 1% penicillin/streptomycin was added at 6 hrs and 24 hours after transfection. The media was collected on day 3, centrifuged at 1500 rpm and the lentivirus was precipitated using PEG-it (System Bioscience) according to manufacturers protocol. Transducing HEK293 cells with serial dilution of the lentivirus accessed the titer of the lentivirus. The titer **(control lentivirus $1.8x10^9$ IFU/ml; MOI - 46 and miR-17 cluster $8.3x10^8$ IFU/ml and MOI - 23)** was determined using the Ultra-rapid lentiviral global titering Kit (System Bioscience) according to the manufacturers protocol.

Assessment of excitation-contraction (EC) coupling:

Enzymatically isolated adult ventricular mouse cardiomyocytes, CPCs and CPC-derived-myocytes (CPCs-MC) were incubated with the Ca^{2+} indicator, fluo 4-AM (10 μM, Invitrogen) and stimulated at 1 Hz using MyoPacer field stimulator (IonOptix Corporation, Milton, MA). Confocal line-scan imaging was performed using a Pascal Zeiss confocal microscope and the were images acquired at sampling rates of 0.7 ms per line and 0.07 μm per pixel, with radial and axial resolutions of 0.4 and 1.0 μm, respectively as previously described [7]. Ca^{2+} transients were expressed as the normalized local fluorescence (F/F_o), where F_o refers to the fluorescence level before depolarization using OriginPro 7 software (OriginLab Corporation, Northampton, MA).

Immunofluorescence confocal laser scanning microscopy

Sorted CPCs were labeled with rat anti-c-kit antibody (0.5 μg/ml) and Alexa Fluor 488-conjugated donkey anti-rat antibody (5 μg ml^{-1}, Invitrogen). Immunofluorescence-labeled and

differential interference contrast (DIC) images were obtained using a Zeiss LSM700 confocal laser-scanning microscope. Cells stained with isotype-matched IgG antibodies were used as controls and did not show positive staining under the same experimental conditions. Identical settings were used for all the specimens.

Population doubling

Live-sorted CPCs were plated at 10-100 cells/well in 24-well cell culture dishes (Nunc, Thermo Fisher Scientific). The cells were counted in triplicates every 8 days. The cell population doubling time (T_D) was calculated using the formula: $T_D = (t_2\text{-}t_1) \times \log 2 / (\log N_2 - \log N_1)$, where N_1 is the number of cells at time point t_1 and N_2 is the number of cells at time point t_2 [8].

Western blot analysis:

Immunoblots were performed as previously described [9]. The following primary antibodies were used: (1) monoclonal anti-Rbl2 antibody (1:250 dilution, BD Bioscience) and (3) anti-GAPDH antibody (Sigma) was used as an internal loading control.

Supplemental Methods References:

[1] Li N, Timofeyev V, Tuteja D, Xu D, Lu L, Zhang Q, et al. Ablation of a Ca2+-activated K+ channel (SK2 channel) results in action potential prolongation in atrial myocytes and atrial fibrillation. The Journal of Physiology. 2009; 587(5): 1087-100.

[2] Chiamvimonvat N, Kargacin ME, Clark RB, Duff HJ. Effects of intracellular calcium on sodium current density in cultured neonatal rat cardiac myocytes. J Physiol. 1995 Mar 1; 483 (Pt 2): 307-18.

[3] Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F, et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res. 2004 Oct 29; 95(9): 911-21.

[4] Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003 Jan 22; 19(2): 185-93.

[5] Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. PNAS. 1998; 95: 14863-8.

[6] López JE MB, Swigart PM, Montgomery MD, Haynam S, Bigos M, Rodrigo M, Simpson PC. Beta-Myosin Heavy Chain Is Induced by Pressure Overload in a Minor Sub-Population of Smaller Mouse Cardiac Myocytes. Circ Res. 2011; In Press.

[7] Xu Y, Tuteja D, Zhang Z, Xu D, Zhang Y, Rodriguez J, et al. Molecular identification and functional roles of a Ca(2+)-activated K+ channel in human and mouse hearts. J Biol Chem. 2003 Dec 5; 278(49): 49085-94.

[8] Davis DR, Kizana E, Terrovitis J, Barth AS, Zhang Y, Smith RR, et al. Isolation and expansion of functionally-competent cardiac progenitor cells directly from heart biopsies. J Mol Cell Cardiol. 2010 Aug; 49(2): 312-21.

[9] Xu Y, Zhang Z, Timofeyev V, Sharma D, Xu D, Tuteja D, et al. The effects of intracellular Ca^{2+} on cardiac K^{+} channel expression and activity: novel insights from genetically altered mice. J Physiol. 2005 Feb 1; 562(Pt 3): 745-58.

Supplemental Figures

Figure S1

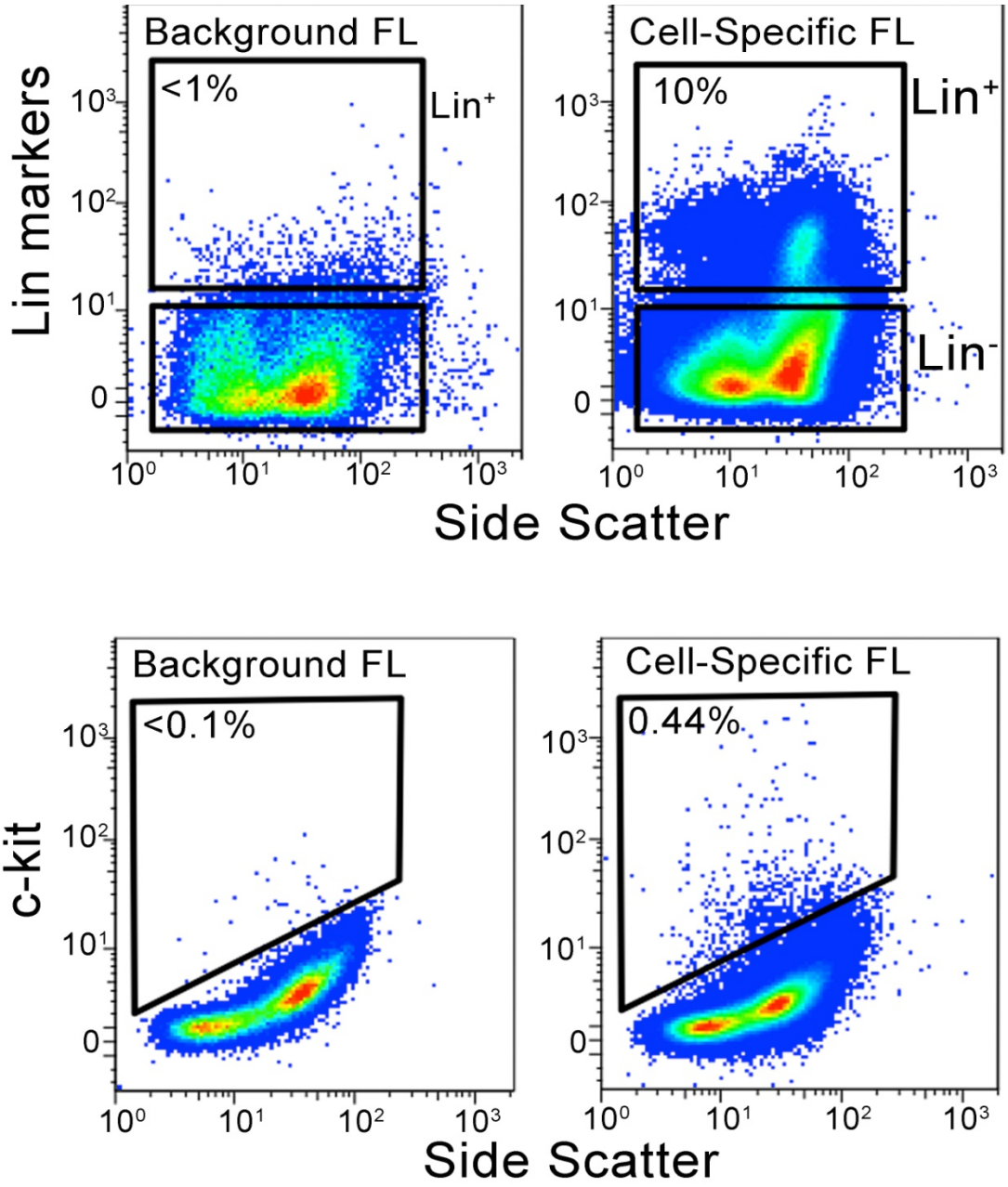

Figure S1: Identification of lin^{neg} c-kit^{pos} CPCs from mouse NMC: The lin^{neg} cells were identified from the NMC population using lineage (lin)-specific antibodies, which were 10% of all NMCs (top panels). The c-kit^{pos} cells were identified from the lin^{neg} population using c-kit antibody and were 0.44% of lin^{neg} cells (bottom panels). Isotype-matched antibodies were used to control for background fluorescence (FL) and is shown in the left panels (top and bottom). X and Y-axes represent arbitrary units. Representative results are shown. NMC: non-myocyte cells.

Figure S2

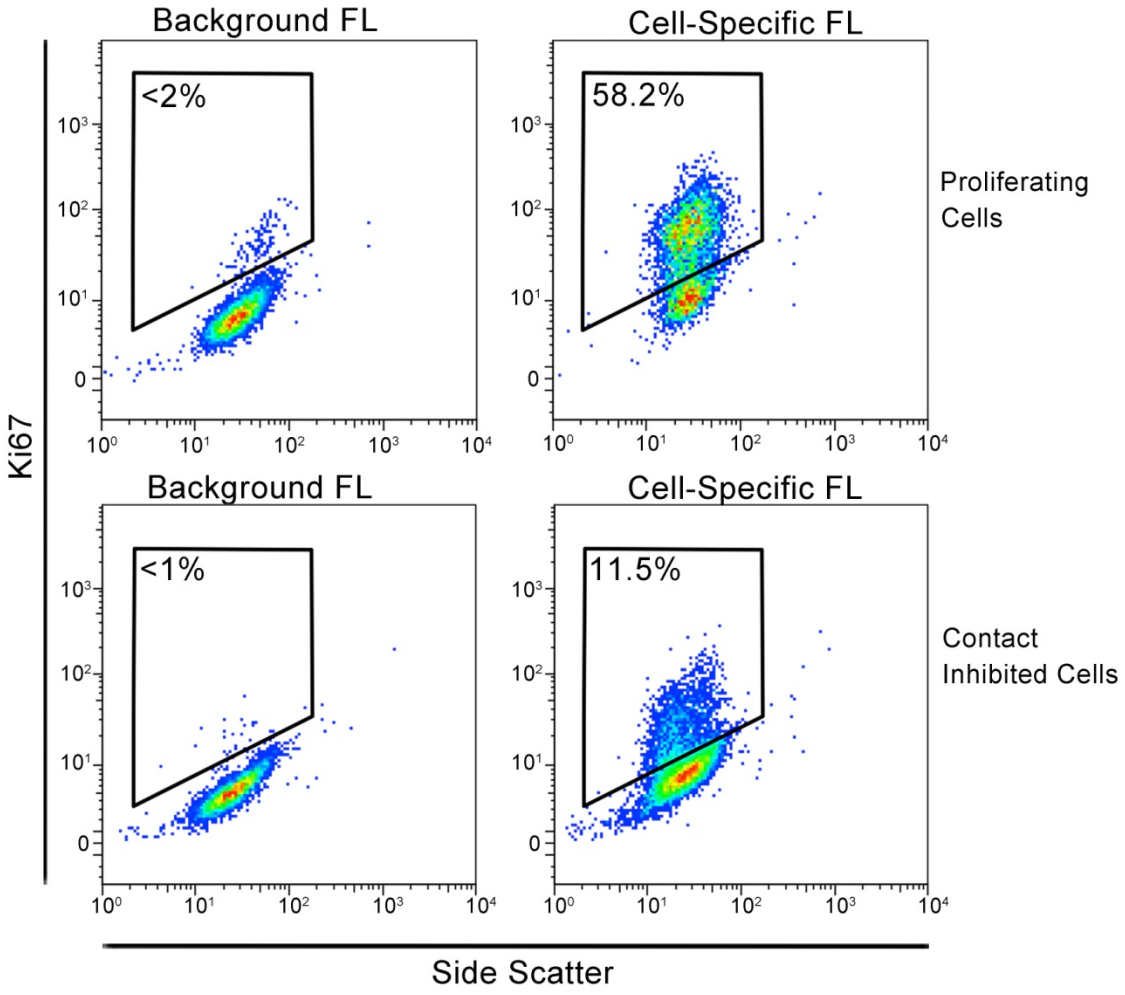

Figure S2: Flow cytometric analysis of Ki67 using low density plated proliferating fibroblasts (top 2 panels) and high density plated non-proliferating fibroblasts (bottom 2 panels). Isotype-matched antibodies were used to control for background fluorescence (FL) and is shown in the left panels (top and bottom). Note decrease fraction of Ki67 expressing cells in contact inhibited cells.

Figure S3

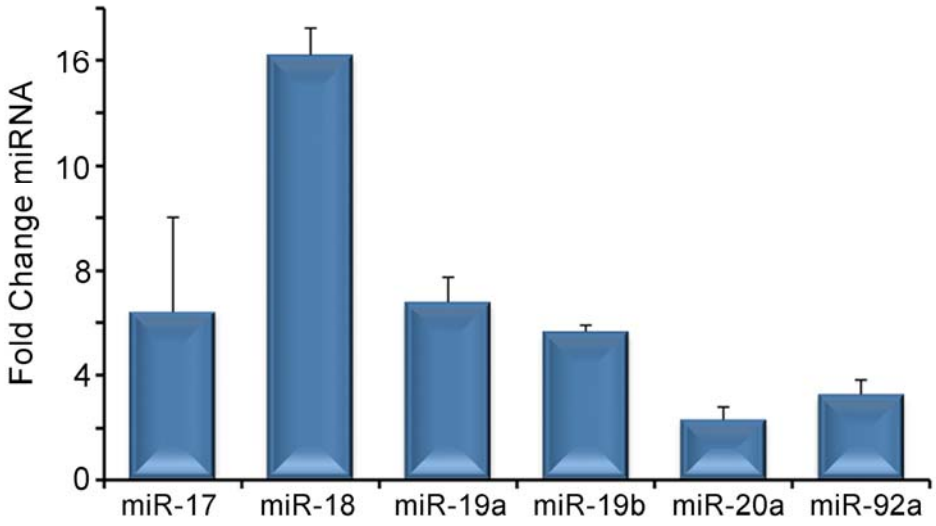

Figure S3: Q-PCR analysis of miR-17-92 cluster (miR-17, -18, -19a, -19b, -20a, -92a) in CPCs. The y-axis denotes the fold change ($2^{-\Delta\Delta CT}$) in expression of neonatal CPCs compared to adult CPCs. Error bars represent standard error.

Figure S4

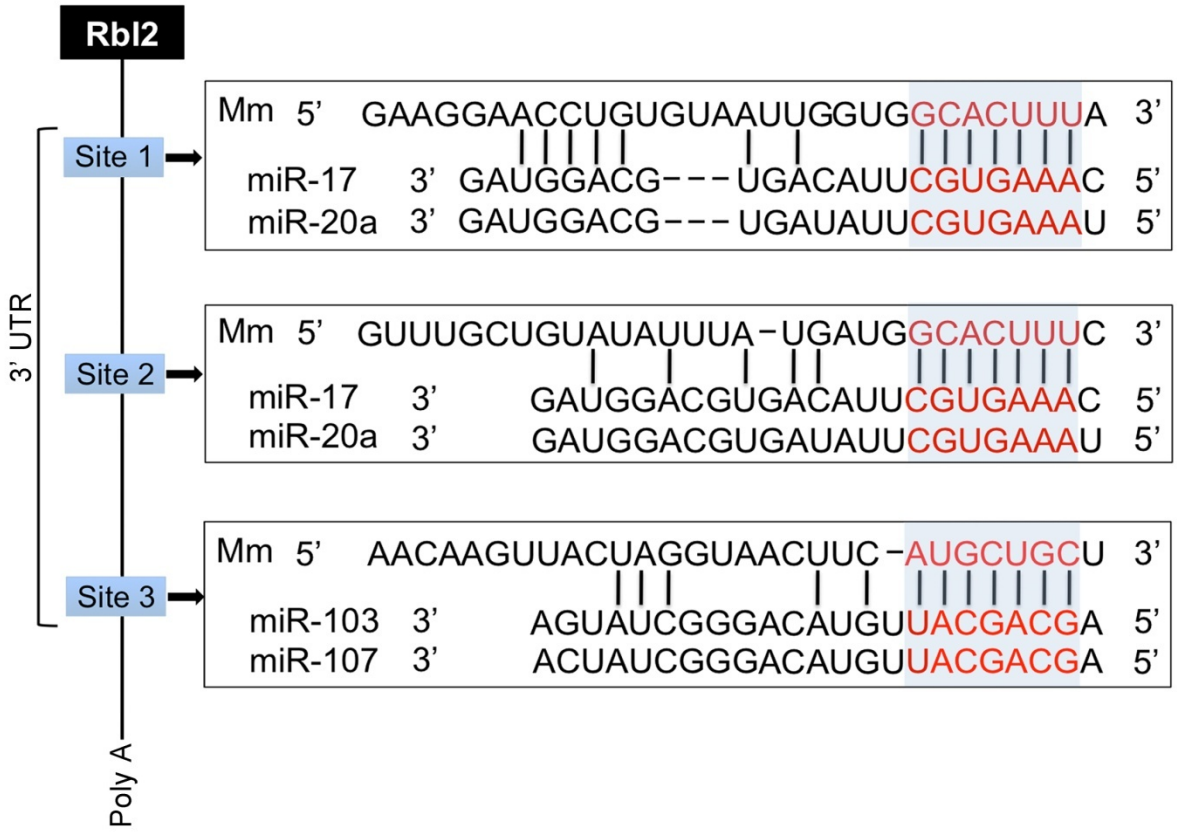

Figure S4: The 3' untranslated regions (UTR) of mouse Rbl2 (Retinoblastoma-like 2) mRNA showing sites (Site 1, 2, and 3) homologous to the differentially expressed microRNAs as shown. Mm – *Mus musculus.*