Materials and methods

Illumina/Solexa sequencing

The Illumina/Solexa libraries were made as described[1] except the 3' ligation was performed separately with both commercially derived T4RNA ligase (GE Health Care) and with a mutant ligase (Rnl2(1-249)K->Q) [2] and pooled prior to 5' ligation. We sequenced small RNAs derived from total RNA obtained from the hearts of 6-8 week old mice (one female and one male) and we present the data for known miRNAs (Sanger release 10). The percentage of reads corresponding to known microRNA hairpins was ~95% with much smaller fractions comprising tRNAs, snRNAs, rRNAs and srpRNAs (Online **Fig. S1A**). The high percentage of microRNA reads is consistent with size fractionation we employed and the requirement of an unmodified 5' phosphate for adapter ligation. Since we sampled the same set of tissues from two different animals (one male and one female), we also compared the hairpin reads (defined as the total number of reads from each known microRNA locus); between the two samples and obtained a high degree of reproducibility (Online **Fig. S1B;** R^2= 0.97). The read lengths obtained from our library sequencing (and mapping to known microRNA loci) showed a peak at a length of 22 nucleotides for both libraries (Online **Fig. S2**). This was true even if the most dominant hairpin read (miR-1; see below) was omitted from the analysis. Smaller, but substantial read percentages were obtained from 19, 20, 21, 23 and 24-mers and they followed a Gaussian pattern with a peak at 22 nucleotides. This distribution pattern is in agreement with the expectations of microRNA lengths**.** We summed all sequenced species between 16-27 nucleotides long with the same 5' dominant end (after normalizing for the number of times that particular sequence occurs in the genome) to

identify a 5' dominant read (see Online **Fig. S3** for an illustrative example). This places special emphasis on the 5' end, as it is believed to be the most important in determining functionality. We included all species between 16-27 nucleotides since it is as yet unclear as to what the specific length limitations are with regard to mRNA targeting. For comparing to miRBase, we still used the most sequenced species (within the 5' dominant read subset) as the mature miRNA.

Animals

Mice bearing a floxed allele of *dgcr8* were generated using a previously described construct [3] targeted into B6/129SvJ F1 ES cells. Following targeting, the selection cassette was removed by transient transfection of a ubiquitously expressing cre recombinase construct. Resulting 2lox/+ ES cells were injected into B6/DBA F1 blastocysts and transplanted into surrogate mothers. Resulting offspring were backcrossed to B6 mice and then crossed with cre positive mice to produce mutant and control litters. MCK-Cre mice have been previously described[4]. No differences in lethality were observed between wt/wt; Cre positive and 2lox/wt; Cre positive mice. The following primers were used to genotype mice: 5`primer: cagatgatcaaatgccatcag and 3` primer: catctccaccttctcaaaccc and the amplicon size was 1011 bp and 1241 bp for the wild type and 2lox/2lox modified loci respectively. If moribund mice with significant weight loss were detected, they were sacrificed and treated as 'dead' for the survival curve analysis. Heart tissue was isolated from anaesthetized mice after perfusion with heparin/PBS (10 units/ml). Isolated hearts were immersed in Krebs-Hanseleit solution lacking Ca^{2+}(118 mM NaCl, 25 mM $NaHCO_3$, 1.2mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7mM

KCl, 11 mM glucose, pH 7.4) before formalin fixation. Tissue was then paraffin embedded and stained with H & E or Masson's Trichrome (Center for Cancer Research, MIT Histology facility). For ECG analysis, subcutaneous electrodes with three leads were surgically implanted in mice and directly connected via a comutator system to a signal processor that records ECG signals and allows the mouse to move freely for a long period without anesthesia (Krieger et al, unpublished). This system also streams the data to the network for remote visualization. (Oefinger, M.B., Krieger, M., Mark, R.G. *Computers in Cardiology*, 695 - 698(2005)). Heart rates were obtained from the ECG readings. Trans-Thoracic Echocardiography was performed on mice in light isoflurane anaesthesia using a Vevo770 machine (Visualsonics, Toronto, ON) with a 30Mhz probe (RMV 707B, Visualsonics, Toronto, ON). Mice were anaesthetized using 2% isoflurane/air mixture in a plexiglas chamber, fixed on a heated stable platform and anaesthesia maintained at light levels using 0.5% isoflurane/air mixture. Hair was removed using a chemical depilant (Surgi-Prep™, Integra LifeSciences, Plainsboro, NJ). The heart was imaged in a parasternal long-axis view and after obtaining a satisfying image quality switched to a short axis view. An M-Mode echocardiogram of the mid-ventricle was recorded at the level of the papillary muscles. LV fractional shortening was calculated as the end-diastolic diameter minus the end-systolic diameter normalized for the end-diastolic diameter and was used as a parameter for cardiac function. The overall wall thickness is obtained by averaging the anterior and posterior diastolic wall thickness. MIT's and BWH's Committee on Animal Care approved all animal protocols.

RNA isolation and analysis:

For generating the Solexa library, RNA from heart was isolated from 6-8 week old C57/BL6 mice using the Trizol method. Tissue was harvested, chopped into small pieces and either stored in RNAlater (Ambion, The Woodlands, TX) or Tri-Reagent (Sigma Chemical Company, St. Louis, MO). If stored in RNAlater, they were processed for RNA extraction using Tri-Reagent following the manufacturer's instructions. Northern Blot analysis for small RNAs was performed using standard protocols[5]. Probes used for ^{32}P end labeling are: miR1 (5`..catacttctttacattccatttca..3`), miR-133 (5`..ggttgaaggggacca..3'), miR208 (5`..acaagctttttgctcgtcttat..3'), miR-206 (5`..ccacacacttccttacattcca..3'), miR-122 (5`..caaacaccattgtcacactcca..3') and U6 (5`.. ttgcgtgtcatccttgcgcagg..3`). 5-10 micrograms were used for Northern Blot analyses and the bands were quantified by exposing the membranes to a phosphoimager plate and scanning. Q-RT-PCR for mRNAs assayed in Fig 4 was performed using Superscript II (Invitrogen, Carlsbad, CA) using random primers for reverse transcription and Taqman probes (Applied Biosystems, Foster City, CA) or Quantitect primers (Qiagen, Valencia, CA). For quantification of mature miRNAs using RT-PCR , Taqman based mature microRNA assays from Applied Biosytems were used. Input RNA levels were normalized using U6 or snoRNA202 levels. Fold changes were calculated using the $\Delta\Delta C_t$ method using Ct values obtained from age and sex-matched mutant and control mice RNA.

For microRNA microarrays, in-house printed arrays using LNA probes from Exiqon were used. Control RNA was labeled with Hy5 and mutant RNA was labeled with Hy3 in two sets of samples and dyes were swapped (Hy3 labeled control RNA competitively labeled with Hy5 labeled mutant RNA) for a third set. The labeled samples were hybridized to the miRNA arrays printed using miRCURY™ LNA oligoset version 8.1 (Exiqon, Denmark). Each miRNA was printed in duplicate, on codelink slides (GE), using GeneMachines Omnigrid 100. The hybridization was performed at 60C overnight using the Agilent Hybridization system - SurHyb, after which the slides were washed using the miRCURY™ LNA washing buffer kit (Exiqon, Denmark) following the procedure described by the manufacturer. The slides were then scanned using Axon 4000B scanner and the image analysis was performed using Genepix Pro 6.0. The raw data was normalized and quantified using limma library, part of the Bioconductor project, using the R statistical environment[6-8]. Differences in the levels of miRNA expression, and their significance, were assessed using linear model and empirical Bayes method as implemented in the limma library.

REFERENCES

1. Grimson A, Srivastava M, Fahey B, Woodcroft BJ, Chiang HR, King N, Degnan BM, Rokhsar DS, Bartel DP. Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. *Nature*. 2008.
2. Ho CK, Wang LK, Lima CD, Shuman S. Structure and mechanism of RNA ligase. *Structure*. 2004;12:327-39.

3. Wang Y, Medvid R, Melton C, Jaenisch R, Blelloch R. DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat Genet*. 2007;39:380-5.

4. Bruning JC, Michael MD, Winnay JN, Hayashi T, Horsch D, Accili D, Goodyear LJ, Kahn CR. A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance. *Mol Cell*. 1998;2:559-69.

5. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science*. 2001;294:858-62.

6. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol*. 2004;5:R80.

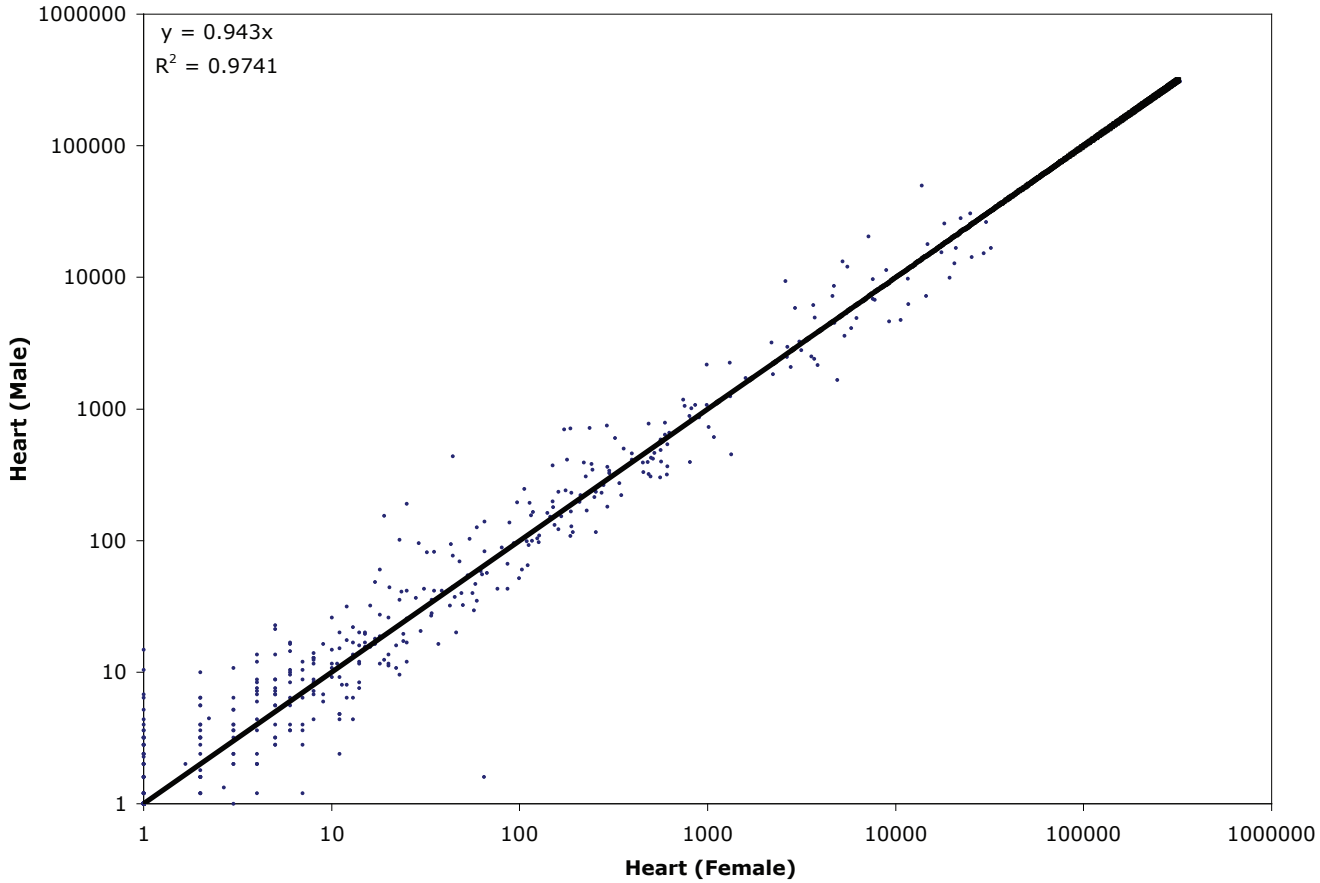
7. Smyth GK, Speed T. Normalization of cDNA microarray data. *Methods*. 2003;31:265-73.

8. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3:Article3.

FIG. S1A

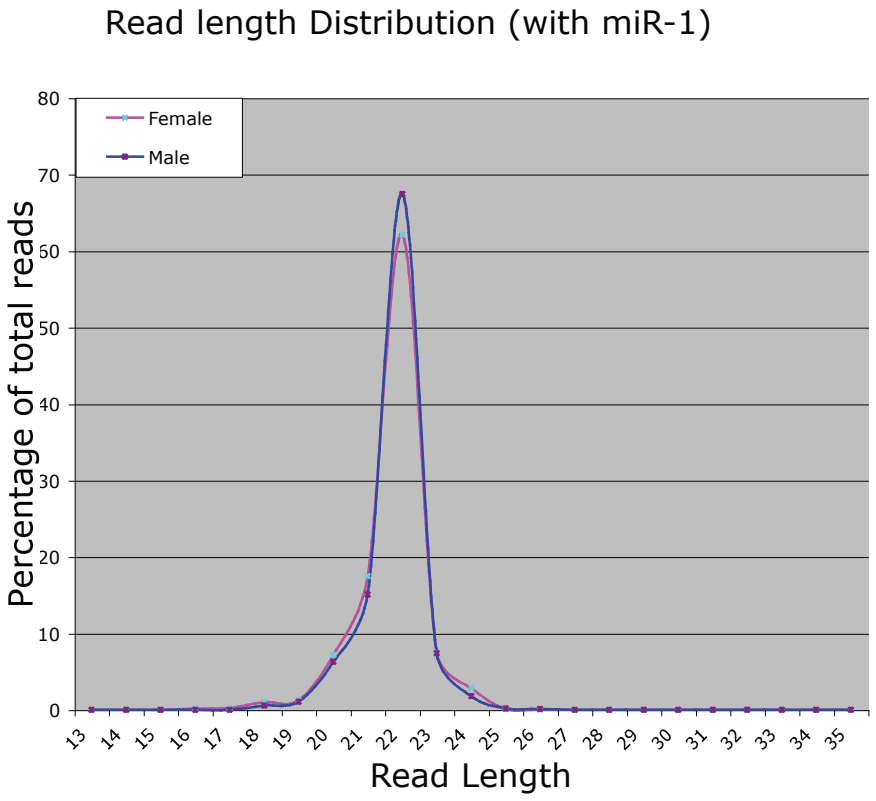
Reads	heart_f	%age	heart_m	%age
Total	919,574.79	99.9999995	2,282,711.48	100
rRNA	15,068.49	1.63863721	24,707.92	1.08239347
tRNA	19,119.12	2.07912623	47,779.85	2.0931183
snRNA	2,556.47	0.27800594	4,723.60	0.20692927
srpRNA	321.17	0.03492576	537.89	0.02356348
miRNA	882,509.53	95.9693044	2,204,962.22	96.5939954

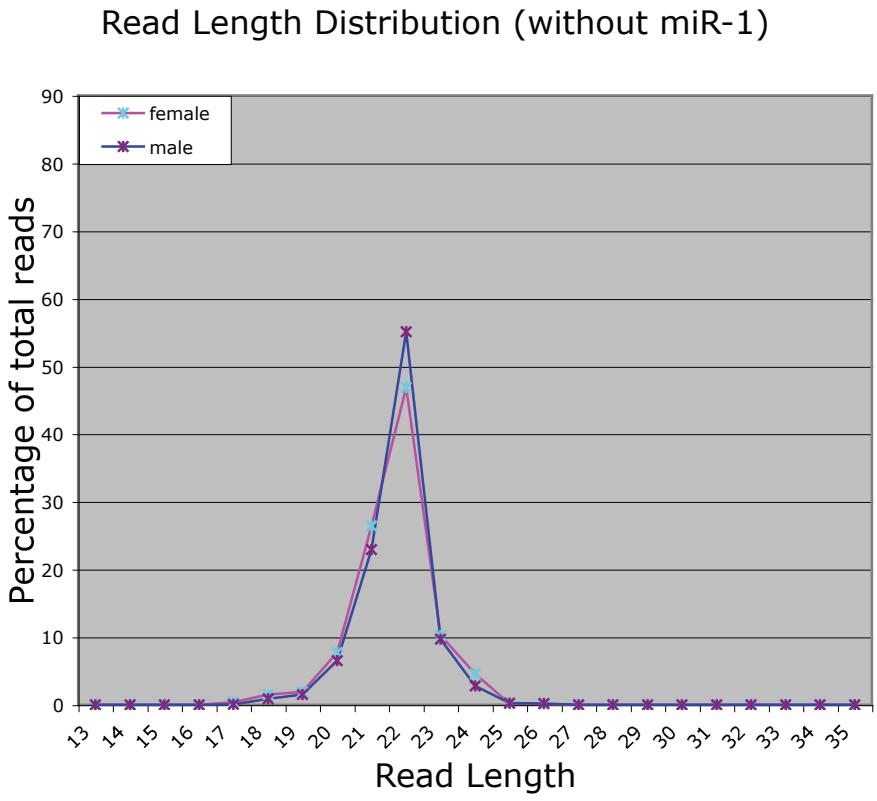
FIG. S1B



Online Fig. S1A. Enrichment of miRNAs. Alignable counts corresponding to the various classes of RNA species for the heart-derived small RNA library showing an enrichment for microRNAs.

Online Fig. S1B. Reproducibility of Sequencing Data. Scatter plot between the normalized total counts from all the know miRNA hairpins from normal female heart (x axis) and normal male heart (y axis). The regression line using the data points is shown in black.

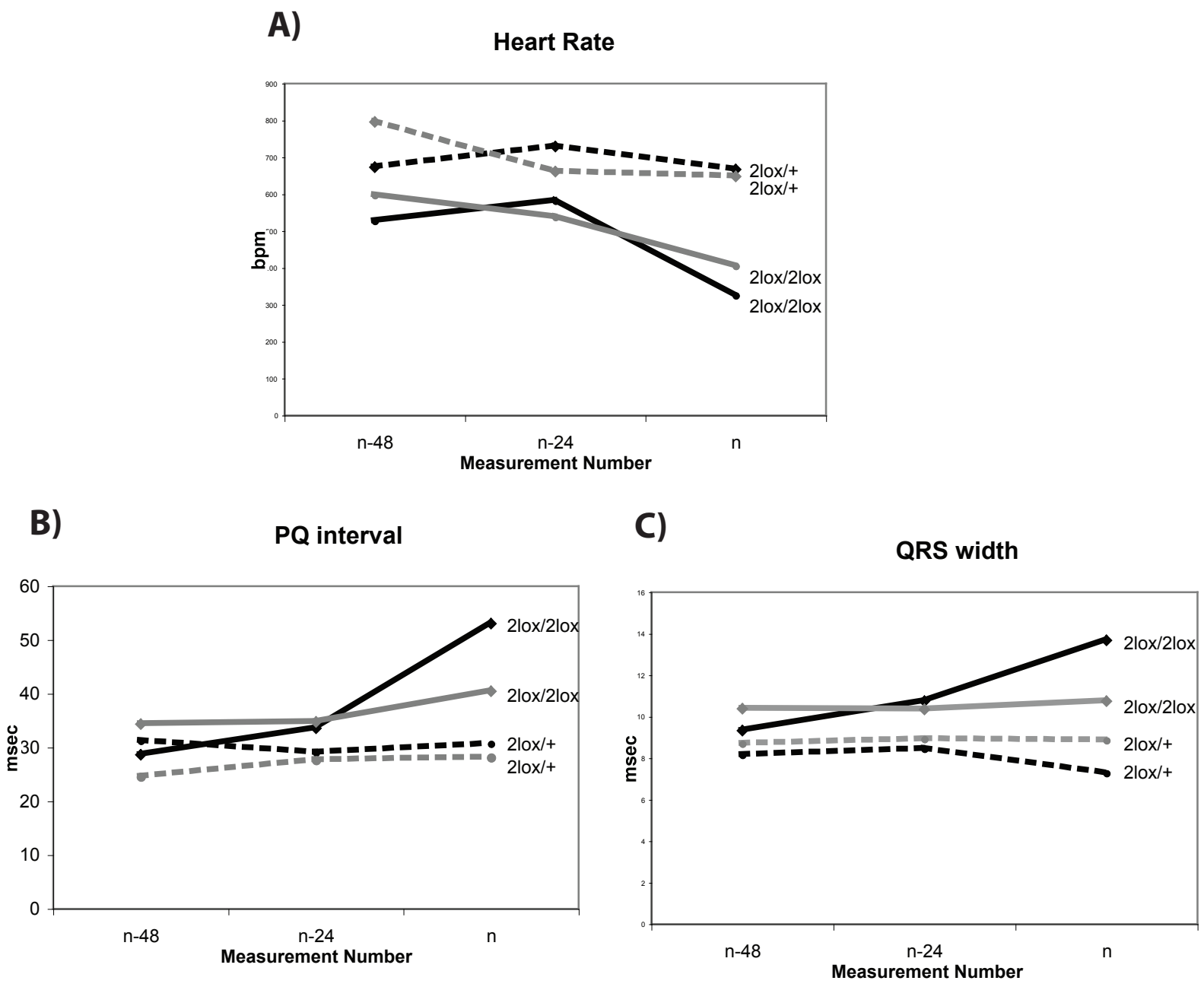




Online Fig S2: Read Length Distribution. Line plot between the read length (x axis) and percentage of reads (y-axis) with that length, mapping to known miRNA hairpins, in the normal heart male and female Solexa data set. The same distribution is seen if miR-1 is included (top) or excluded (bottom).

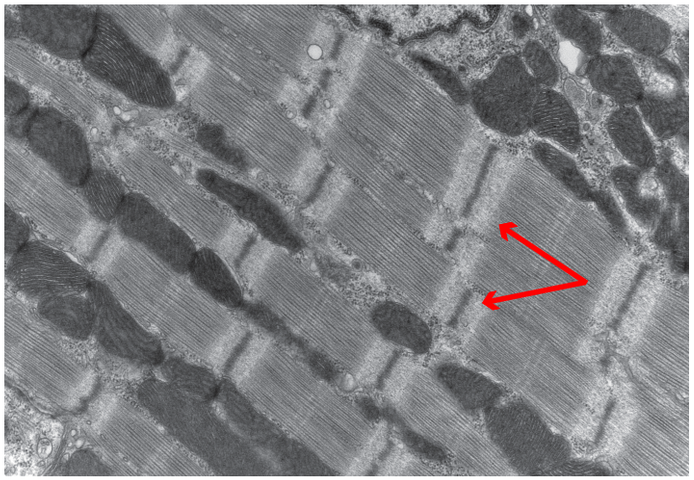
```
mir: mmu-let-7c-1      chr16(+):77482248-77482342
chr16(+):77482248-77482342
TGTGTGCATCCGGGTTGAGGTAGTAGGTTGTATGGTTTAGAGTTACACCCTGGGAGTTAACTGTACAACCTTCTAGCTTTCCTTGGAGCACACTT
. (( ((( (. ((( (( (. . (((  .(( (.( ( (((((((( (( ((. . (( .(.. (. . . . . ) . .) . )))) ))))) )) )) )). ))). . ))). . .) ))) )) )) ) ))).. 
.....TTGAGGTAGTAGGTTGTATGGTT..... 7    1
.....TTGAGGTAGTAGGTTGTATGGT..... 8    1
.....TTGAGGTAGTAGGTTGTATGG.....3    1
.....TGAGGTAGTAGGTTGTATGGTTTAGAG.....1    1
.....TGAGGTAGTAGGTTGTATGGTTTA.....35    1
.....TGAGGTAGTAGGTTGTATGGTT..... 20027 2
.....TGAGGTAGTAGGTTGTAT..... 447  4
.....TGAGGTAGTAGGTTGTATG..... 118  2
.....TGAGGTAGTAGGTTGTATGG..... 1336 2
.....TGAGGTAGTAGGTTGTA..... 68   4
.....TGAGGTAGTAGGTTGTATGGTTT..... 2636 2
.....TGAGGTAGTAGGTTGT.....18   8
.....TGAGGTAGTAGGTTGTATGGT..... 5600 2
.....GAGGTAGTAGGTTGTATGG..... 3  2
.....GAGGTAGTAGGTTGTATGGTTT.....6  2
.....GAGGTAGTAGGTTGTATGGTT.....116 2
.....GAGGTAGTAGGTTGTATGGT.....16 2
.....AGGTAGTAGGTTGTATGGTT.....11 2
.....AGGTAGTAGGTTGTATGGT.....1  2
.....GGTAGTAGGTTGTATGGTT.....8  2
.....GGTAGTAGGTTGTATGGT.....7  2
.....GGTAGTAGGTTGTATGGTTT..... 2  2
.....GGTAGTAGGTTGTATGG.....1  2
.....GTAGTAGGTTGTATGGTT..... 3  2
.....GTAGTAGGTTGTATGGT..... 1  2
.....TAGTAGGTTGTATGGTT..... 2  3
.....AGTAGGTTGTATGGTTT..... 1  2
.....AGTAGGTTGTATGGTT..... 1  3
.....TAGAGTTACACCCTGGGAGTTAA.....1  1
.....TAGAGTTACACCCTGGGAGTTA.....1  1
.....TAGAGTTACACCCTGGGAGTT..... 1  1
.....CTGTACAACCTTCTAGCTTTCC.....4  1
```

Online Fig S3. Determination of mature microRNA counts. Step 1: Normalize counts between libraries (using total known hairpin reads to compute normalization factor between libraries). Step 2: Identify 5'-dominant species with the same 5' end AND with maximum number of normalized reads (normalized to genome occurrence): shown in blue above. Step 3: Identify all species between 16 nucleotides and 25 nucleotides long with the same 5' end as the one with the maximum number of normalized reads (boxed above) . i.e. remove those less than 16 nucleotides and greater than 26 nucleotides. Step 4: Get a cumulative count of all reads for the various species identified in steps 1) & 2).

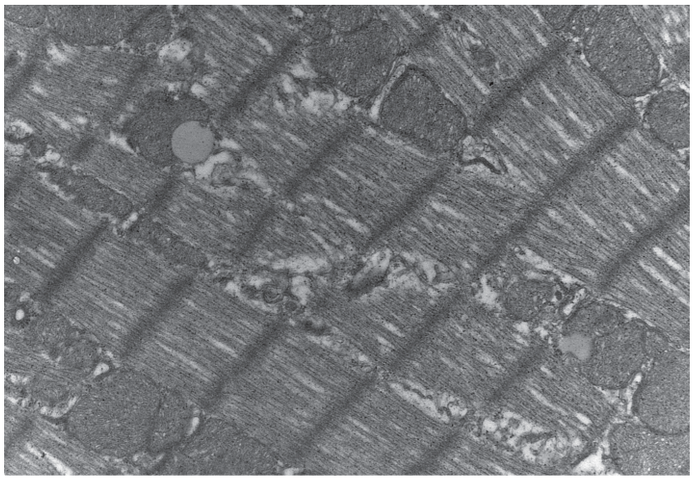


Online Fig S4. Electrophysiological measurements: A). Heart beat rates at the indicated time points (exactly 24 hours apart where n is the time of death or sacrifice) were calculated from electrocardiograms from two sets of Cre positive mutant (2lox/2lox) and control mice (2lox/+). A steep decline in heart rate was seen in mutant mice during the 24 hours prior to sacrifice (red arrows point to values immediately prior to sacrifice). B). PQ interval was notably higher in mutant mice from both pairs at the same time that the heart rate decreased; C). QRS width was increased in one mutant. Further ECG readings (derived from mice with stable heart beat rates) are necessary to determine whether significant conduction defects occur in the absence of overt heart failure.

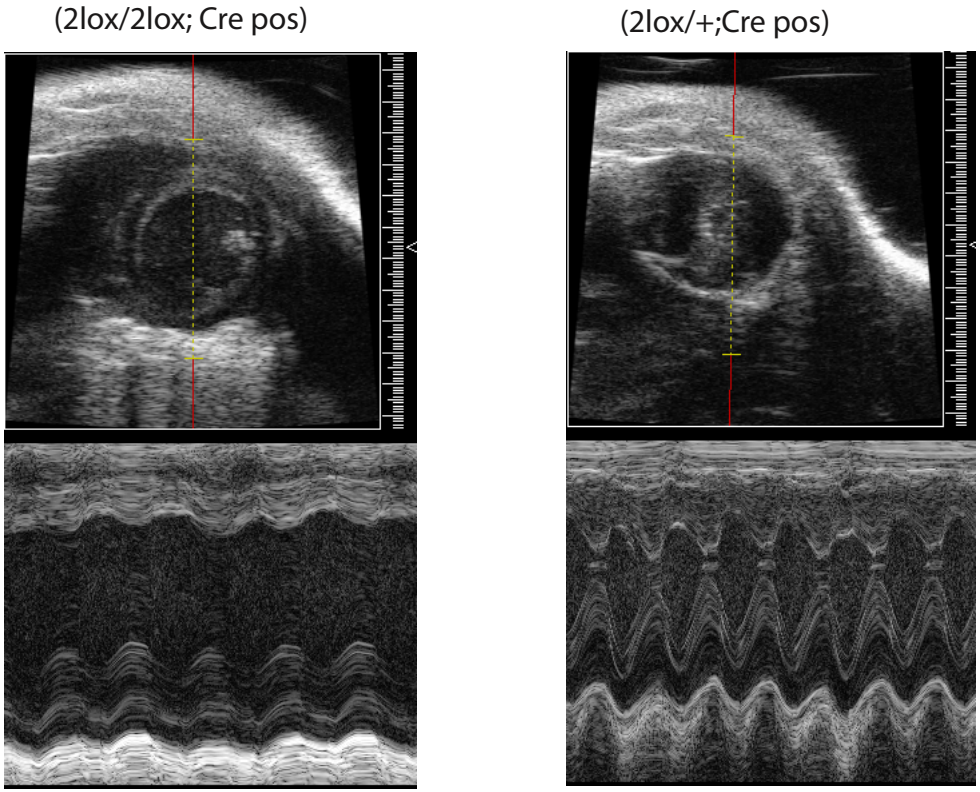
mutant



control



Online Fig S5. Ultrastructure of cardiac muscle from mutant and control mice reveals the mild myofibrillar disarray as seen in the misalignment of adjacent sarcomeres (see arrows) in the section obtained from mutant mice (left). Both images were taken at same magnification (18500X) allowing for a direct comparison.



Online Fig S6. Trans-Thoracic Echocardiography. (A) Representative short axis B and M mode images for both mutant (left) and control (right) mice at four weeks of age showing extensive ventricular dilation in the mutant mice.

microRNA	Fold change (log2)	adj P value
mmu-miR-422b	-3.62982341	8.29E-06
mmu-miR-1	-3.00296272	9.75E-07
mmu-miR-133a*	-2.99057569	4.97E-05
mmu-miR-133a-133b	-2.60462301	0.00035654
mmu-miR-499	-2.16649157	0.00064897
mmu-miR-208	-2.05498929	0.00384307
mmu-miR-378	-1.95554702	0.00016538
mmu-miR-22	-1.4290778	4.30E-05
mmu-miR-30b	-1.39193484	0.00062223
mmu-miR-486	-1.3526117	0.00010939
mmu-miR-30c	-1.34692339	0.00030721
mmu-miR-30e*	-1.30565939	0.00214884
mmu-miR-149	-1.23631614	0.00231666
mmu-miR-709	-1.16246592	0.02112764
mmu-miR-345	-1.07778498	0.00106424
mmu-miR-29b	-0.99169338	0.00523785
mmu-miR-691	-0.93794605	0.03479406
mmu-miR-99a	-0.91812002	0.00011651
mmu-miR-29c	-0.88502083	0.00592418
mmu-miR-100	-0.81267368	0.00054988
mmu-miR-708	-0.78046751	0.03281587
mmu-let-7g	-0.778488	0.00191197
mmu-let-7e	-0.70020297	0.00395975
mmu-miR-181c	-0.66661239	0.00242741
mmu-miR-148a	-0.6627879	0.04379085
mmu-miR-30a-3p	-0.63757405	0.01531949
mmu-miR-684	-0.62275918	0.00558271
mmu-miR-29a	-0.61189379	0.02683102
mmu-miR-350	-0.59898948	0.00562685
mmu-miR-411	-0.58727732	0.02577598
mmu-miR-101a	-0.5671441	0.04168894
mmu-miR-128a	-0.56270581	0.00733114
mmu-miR-717	-0.56203284	0.00520757
mmu-miR-707	-0.53457485	0.01332789
mmu-miR-128b	-0.4969575	0.00095941
mmu-miR-181a*	-0.49174634	0.00074969
mmu-miR-488	-0.47964508	0.00824777
mmu-miR-181b	-0.45438911	0.00216597
mmu-miR-31	-0.44847016	0.00153385
mmu-miR-10a	-0.43516922	0.00153014
mmu-miR-145	-0.40565085	0.01786797
mmu-miR-107	-0.38690929	0.00573767
mmu-miR-331	-0.38079738	0.03876503
mmu-miR-328	-0.36673681	0.0077787
mmu-let-7f	-0.36288581	0.02331131
mmu-miR-467a	-0.3577968	0.01933442
mmu-miR-490	-0.34767875	0.03647629
mmu-miR-412	-0.33300455	0.00315222
mmu-miR-142-5p	-0.32918782	0.01340981
mmu-miR-134	-0.32569707	0.01016595
mmu-miR-434-3p	-0.32364942	0.03647629
mmu-miR-329	-0.32266973	0.03163038
mmu-miR-191	-0.31158511	0.04108819
mmu-miR-377	-0.30682598	0.03655519
mmu-miR-103	-0.28815337	0.02335659
mmu-miR-98	-0.28362496	0.0332177
mmu-miR-146b	-0.27415315	0.01444873
mmu-miR-339	-0.2697622	0.01069352
mmu-miR-193	-0.24354615	0.04231637
mmu-miR-125a	-0.20954425	0.01615212

Online Fig. S7. microRNA microarray to detect global changes in relative microRNA levels in RNA derived from dgcr8 deficient hearts (as compared to RNA from control hearts). Aside from the anticipated decrease in cardiomyocyte-specific microRNAs (miR-1, miR-133 and miR-208) levels of a number of other microRNAs are also decreased due to dgcr8 loss in the cardiomyocytes. The second column depicts the log ratio in the signal intensity obtained from mutant RNA over control RNA and hence negative numbers indicate depletion in the mutant. The microRNAs listed here are those that are down regulated significantly (corrected p value less than or equal to 0.05) from results obtained in three separate experiments including one with a dye-swap.