Supplemental information

Contains 7 Supplemental Figures and Figure legends, 4 Supplemental Tables and Table legends, 3 supplemental Movie legends and supplemental experimental procedures and supplemental references.

Supplemental Data

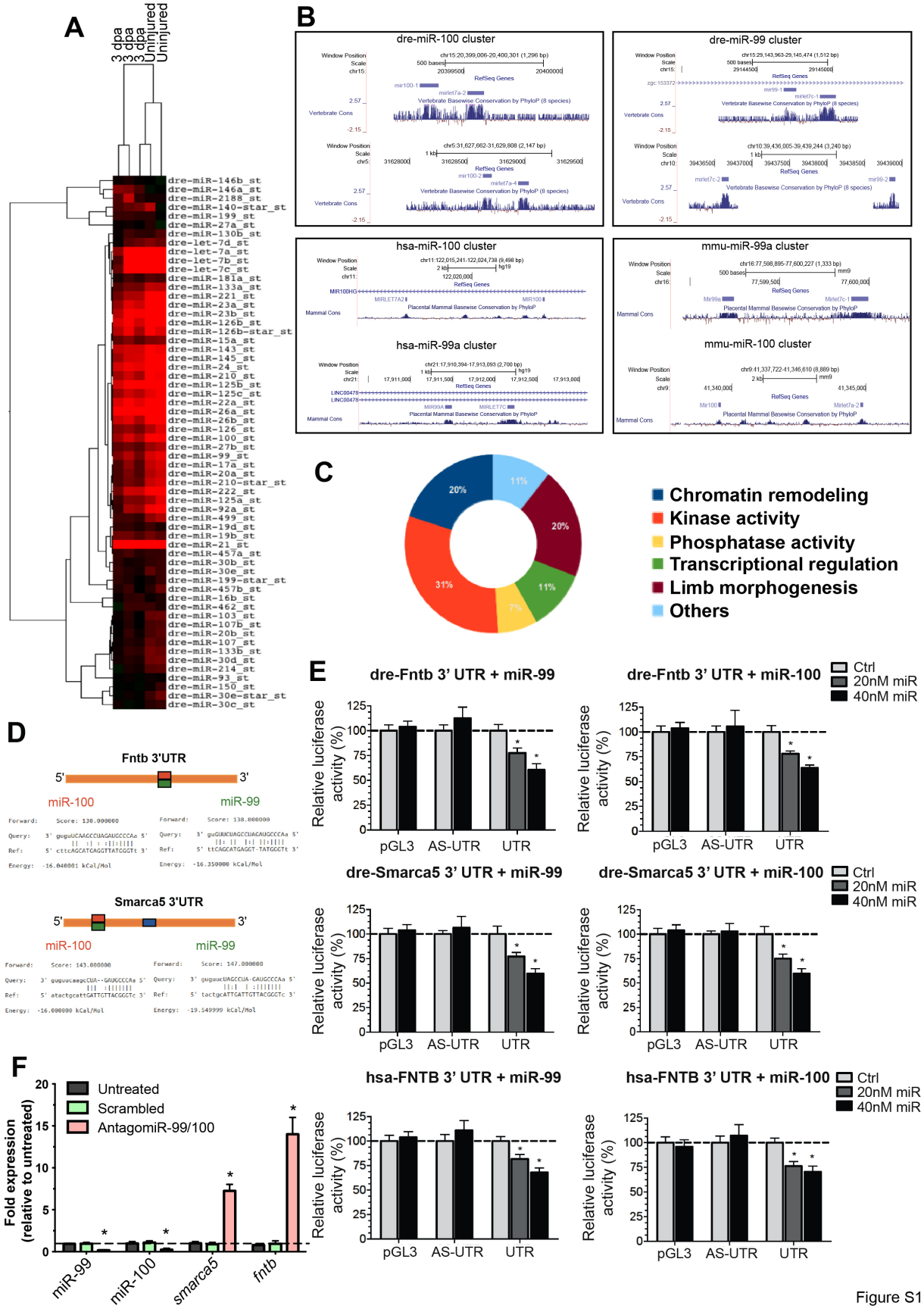


Figure S1

Figure S1, related to Figure 1. miR-99/100 and Let-7a/c are highly conserved in sequence and genomic location across vertebrates. (A) Microarray analysis identified a subset of differentially regulated microRNAs during early stages of regeneration (3 days post amputation, dpa) in the zebrafish heart. Interestingly, most of them were consistently downregulated. (B) Bioinformatic analysis of the most relevant signaling pathways targeted by miR-99/100 and Let-7a/c. (C) Genomic organization and conservation of miR-99/100 and Let-7a/c in zebrafish, mouse and human. **(**D) Miranda-based binding predictions of miR-99/100 to zebrafish Fntb and Smarca5 3' UTRs. (E) Luciferase assay to determine biochemical binding of miR-99/100 to the predicted targets Fnt and Smarca5 for zebrafish (dre) and human (hsa) 3'UTRs. (F) qRT-PCR illustrating the effects of miR-99/100 antagomiR-mediated knockdown in fntb and smarca5 expression in uninjured adult zebrafish hearts (n=5).

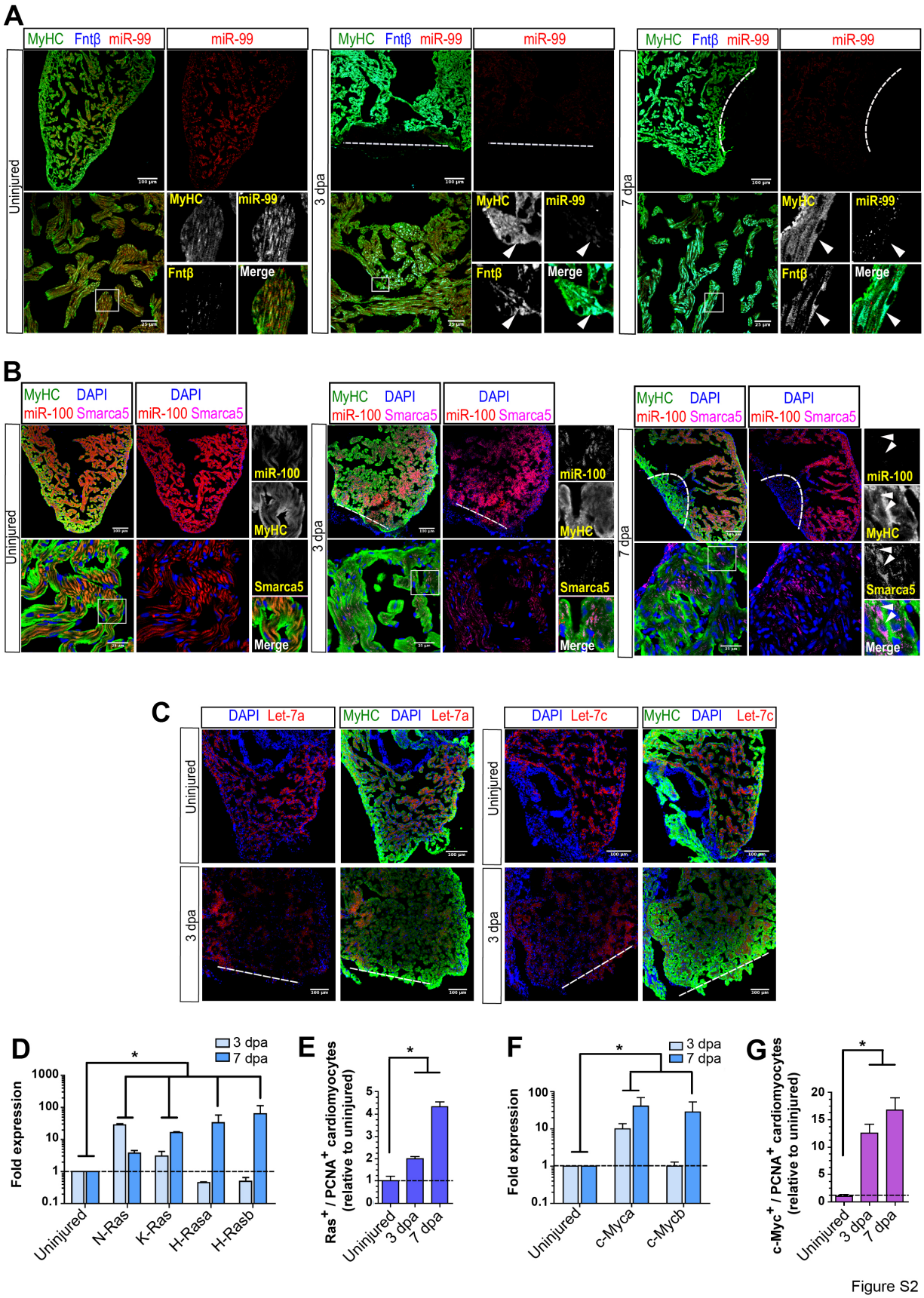
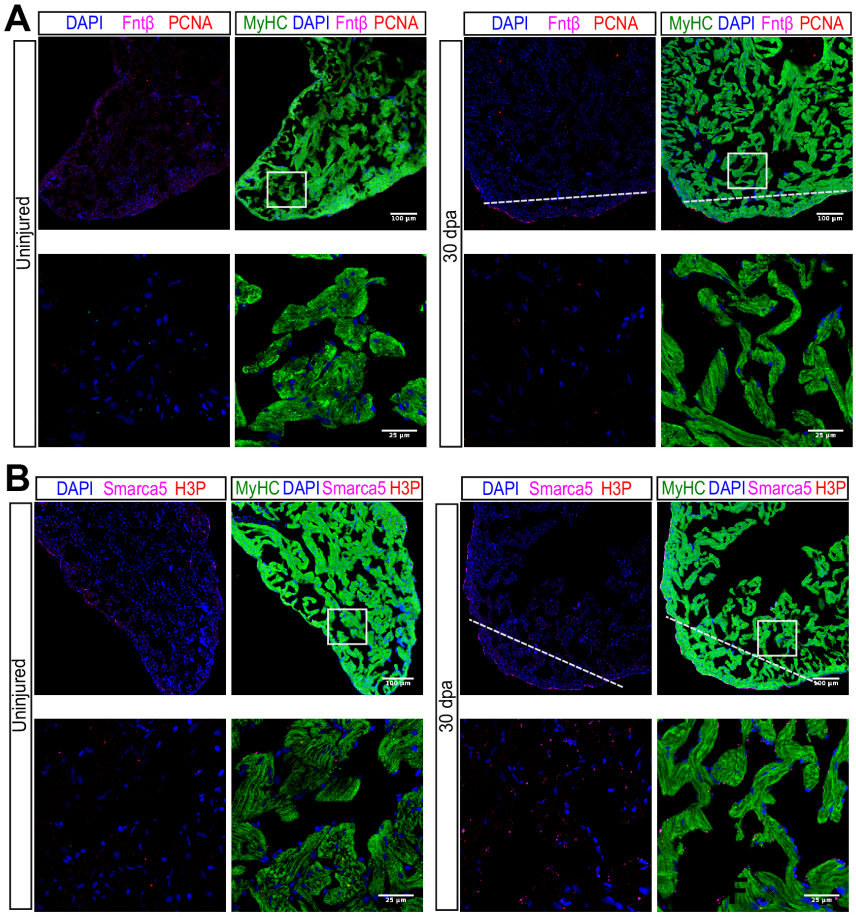

A
MyHC Fntβ miR-99
miR-99
Uninjured
3 dpa
7 dpa
MyHC
miR-99
Fntβ
Merge
B
MyHC DAPI
miR-100 Smarca5
DAPI
miR-100 Smarca5
miR-100
MyHC
Smarca5
Merge
C
DAPI Let-7a
MyHC DAPI Let-7a
DAPI Let-7c
MyHC DAPI Let-7c
Uninjured
3 dpa
D
3 dpa
7 dpa
Fold expression
1000
100
10
1
0.1
Uninjured
N-Ras
K-Ras
H-Rasa
H-Rasb
E
Ras+ / PCNA+ cardiomyocytes (relative to uninjured)
5
4
3
2
1
0
Uninjured
3 dpa
7 dpa
F
3 dpa
7 dpa
Fold expression
100
10
1
0.1
Uninjured
c-Myca
c-Mycb
G
c-Myc+ / PCNA+ cardiomyocytes (relative to uninjured)
20
15
10
5
0
Uninjured
3 dpa
7 dpa

Figure S2

Figure S2, related to Figure 2. Expression of miR-99/100 and Let-7a/c is restricted to cardiomyocytes and inversely correlates with Fntβ and Smarca5. FISH/immunofluorescence were used to determine cardiomyocyte specific expression (MyHC) of miR-99, miR-100, Let-7a/c, Fntβ and Smarca5 in uninjured and regenerating zebrafish hearts at 3 and 7dpa. (A-C) Representative pictures for miR-99 and its protein target Fntβ (A), miR-100 and its protein target Smarca5 (B) and Let-7a and Let-7c (C, see also Figure 1). (D-G) Ras expression was significantly up-regulated after amputation (D) in dedifferentiated, proliferative cardiomyocytes (E), as well as c-Myc (F, G). Dashed line: amputation plane. Boxed area: magnified field. Arrowheads indicate cells of interest. n = 5 animals, three different sections/animal.


A
DAPI Fntβ PCNA
MyHC DAPI Fntβ PCNA
Uninjured
30 dpa
B
DAPI Smarca5 H3P
MyHC DAPI Smarca5 H3P
Uninjured
30 dpa

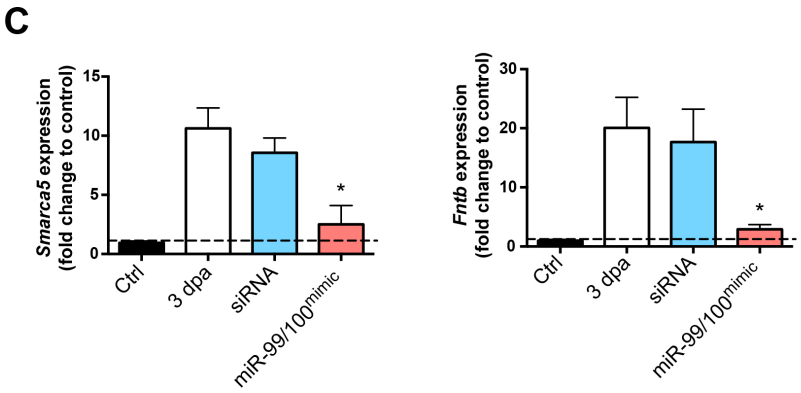

C
Smarca5 expression (fold change to control)
15
10
5
0
Ctrl
3 dpa
siRNA
miR-99/100mimic
*
Fntb expression (fold change to control)
30
20
10
0
Ctrl
3 dpa
siRNA
miR-99/100mimic
*

Figure S3

Figure S3, related to Figure 2. Chromatin remodeling is a necessary step in cardiomyocyte dedifferentiation. (A,B) Representative pictures of immunofluorescent stainings at 30 dpa showing that Fntβ (A) and Smarca5 (B) expression in the myocardium returned to pre-amputation levels at 30 dpa, when zebrafish heart regeneration is mostly completed. Boxed area: magnified section. Arrowheads: cells of interest. n = 4 animals, 3 sections/animal. (C) Expression of fntb and smarca5 in regenerating zebrafish hearts at 3 dpa with or without exogenous administration of miR-99/100 mimics. Expression levels in uninjured animals were used as controls (baseline values were set at one and are indicated by dashed lines). Notice how exogenous administration of miR-99/100 mimics strongly and specifically inhibits the expression of fntb and smarca5 at 3dpa as compared to the siRNA control (n=5).

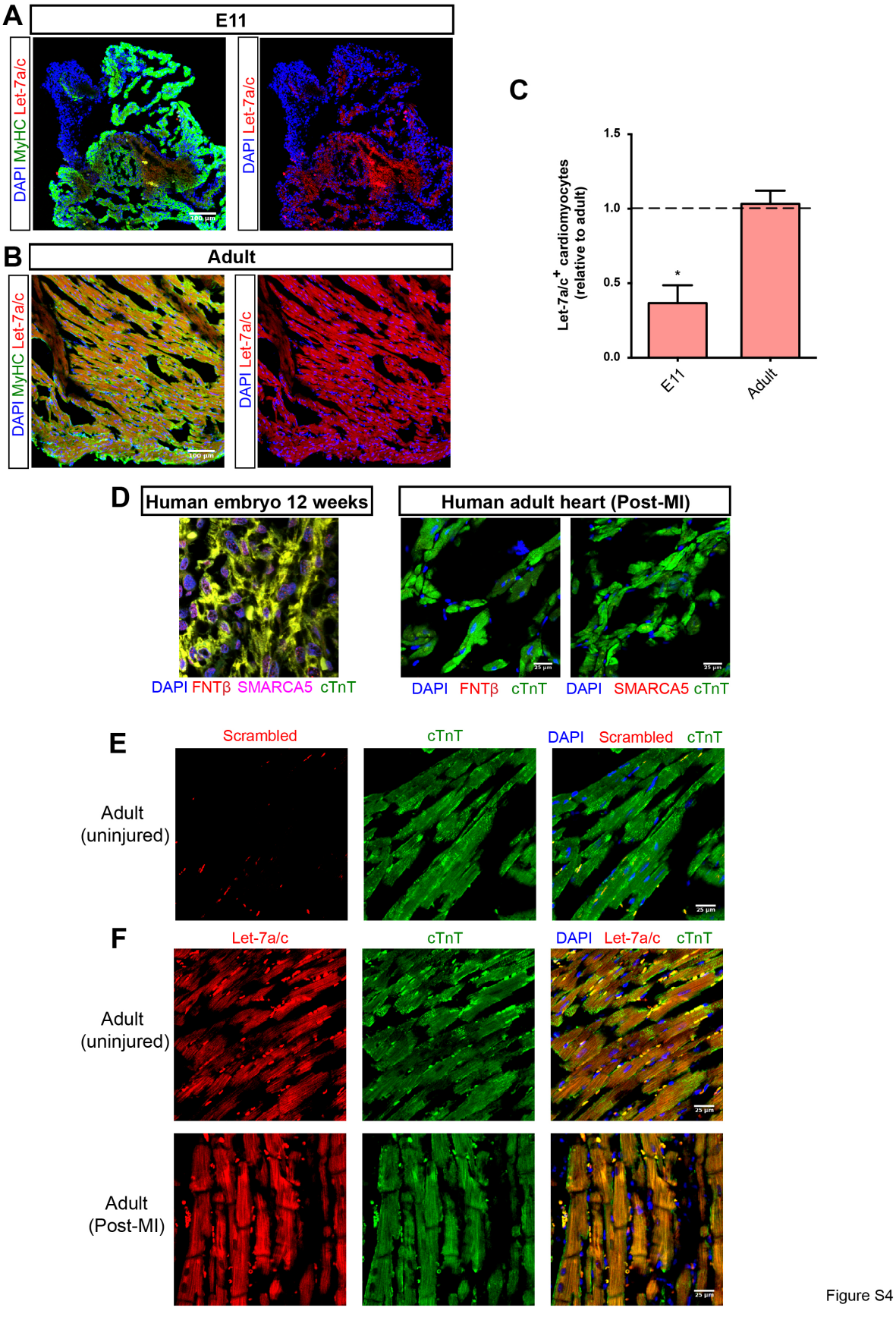

A
E11
DAPI MyHC Let-7a/c
DAPI Let-7a/c
B
Adult
DAPI MyHC Let-7a/c
DAPI Let-7a/c
C
Let-7a/c+ cardiomyocytes (relative to adult)
1.5
1.0
0.5
0.0
*
E11
Adult
D
Human embryo 12 weeks
Human adult heart (Post-MI)
DAPI FNTβ SMARCA5 cTnT
DAPI FNTβ cTnT
DAPI SMARCA5 cTnT
E
Scrambled
cTnT
DAPI Scrambled cTnT
Adult (uninjured)
F
Let-7a/c
cTnT
DAPI Let-7a/c cTnT
Adult (uninjured)
Adult (Post-MI)

Figure S4

Figure S4, related to Figure 4. The miR-99/100 mechanism is present during mammalian heart development but fails to activate during heart injury. (A-C) Representative pictures of FISH/immunofluorescence (A, B) and quantitative analysis (C) demonstrating low levels of Let-7a/c in E11 murine embryonic hearts as opposed to adult hearts, which showed high levels of Let-7a/c. (D) FNTβ and SMARCA5 are present in embryonic human heart, but are completely silenced in adult cardiac tissue after myocardial infarction (MI). (E) FISH/Immunofluorescence analysis using a scrambled probe to control the specificity of the probes used across the study to detect miR-99/100 and Let-7a/c. (F) FISH/immunofluorescence in adult mouse heart before (upper panels) and after myocardial infarction (lower panels) highlighted a failure to downregulate Let- 7a/c upon injury in the murine heart. n = 5 animals, 3 sections/animal.

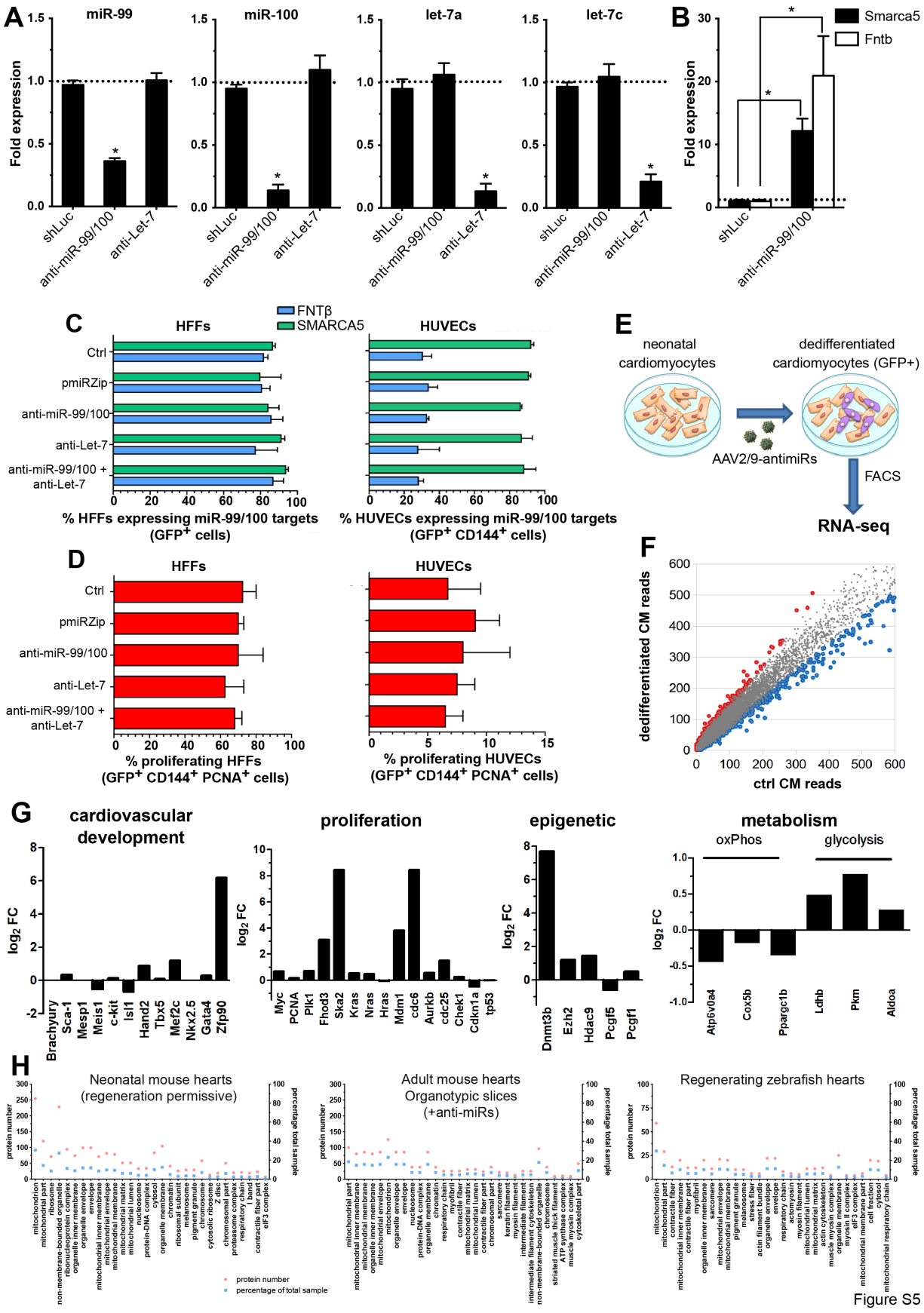

A
miR-99
miR-100
let-7a
let-7c
Fold expression
shLuc
anti-miR-99/100
anti-Let-7
B
Smarca5
Fntb
C
FNTβ
SMARCA5
HFFs
HUVECs
Ctrl
pmiRZip
anti-miR-99/100
anti-Let-7
anti-miR-99/100 + anti-Let-7
% HFFs expressing miR-99/100 targets (GFP+ cells)
% HUVECs expressing miR-99/100 targets (GFP+ CD144+ cells)
D
% proliferating HFFs (GFP+ CD144+ PCNA+ cells)
% proliferating HUVECs (GFP+ CD144+ PCNA+ cells)
E
neonatal cardiomyocytes
dedifferentiated cardiomyocytes (GFP+)
AAV2/9-antimiRs
FACS
RNA-seq
F
dedifferentiated CM reads
ctrl CM reads
G
cardiovascular development
proliferation
epigenetic
metabolism
oxPhos
glycolysis
log2 FC
Brachyury
Sca-1
Mesp1
Meis1
c-kit
Isl1
Hand2
Tbx5
Mef2c
Nkx2.5
Gata4
Zfp90
Myc
PCNA
Plk1
Fhod3
Ska2
Kras
Nras
Hras
Mdm1
cdc6
Aurkb
cdc25
Chek1
Cdkn1a
tp53
Dnmt3b
Ezh2
Hdac9
Pcgf5
Pcgf1
Atp6v0a4
Cox5b
Ppargc1b
Ldhb
Pkm
Aldoa
H
Neonatal mouse hearts (regeneration permissive)
Adult mouse hearts Organotypic slices (+anti-miRs)
Regenerating zebrafish hearts
protein number
percentage of total sample

Figure S5

Figure S5, related to Figure 5. RNA-seq of dedifferentiating cardiomyocytes uncovers novel protein targets. (A) Knockdown of miR-99/100 and Let-7a/c in neonatal murine cardiomyocytes by lentiviral expression of anti-miR shRNAs. (B) Increment in Fntβ/Smarca5 expression due to knockdown of miR-99/100. (C) Human Fibroblasts or (D) human endothelial cells expressed basal levels of FNTβ, SMARCA5 and negligible levels of miR-99/100 and Let-7a/c (data not shown), and were thus insensitive to miR silencing, indicating specificity of the treatment in a heart setting. (E) Scheme depicting the strategy used to identify critical regulators of cardiomyocyte dedifferentiation. (F) Upregulated and downregulated transcripts (Cut-off: 1.25-fold): Gene expression changes were relatively small in general, and mostly centered along small groups of proteins, suggesting that dedifferentiation involves subtle rather than dramatic cell identity changes. (G) Handpicked genes of interest potentially involved in cardiomyocyte dedifferentiation. Gene expression changes and cluster analysis suggested mitochondrial metabolic routes as novel potential regulators of cardiomyocyte dedifferentiation. n = 3 independent experiment/condition. (H) Comparative proteomic analysis of the heart during regenerative stages and bioinformatic analysis of the most abundant GO processes identified in each group. For more detailed protein and processes information please refer to Table S3.

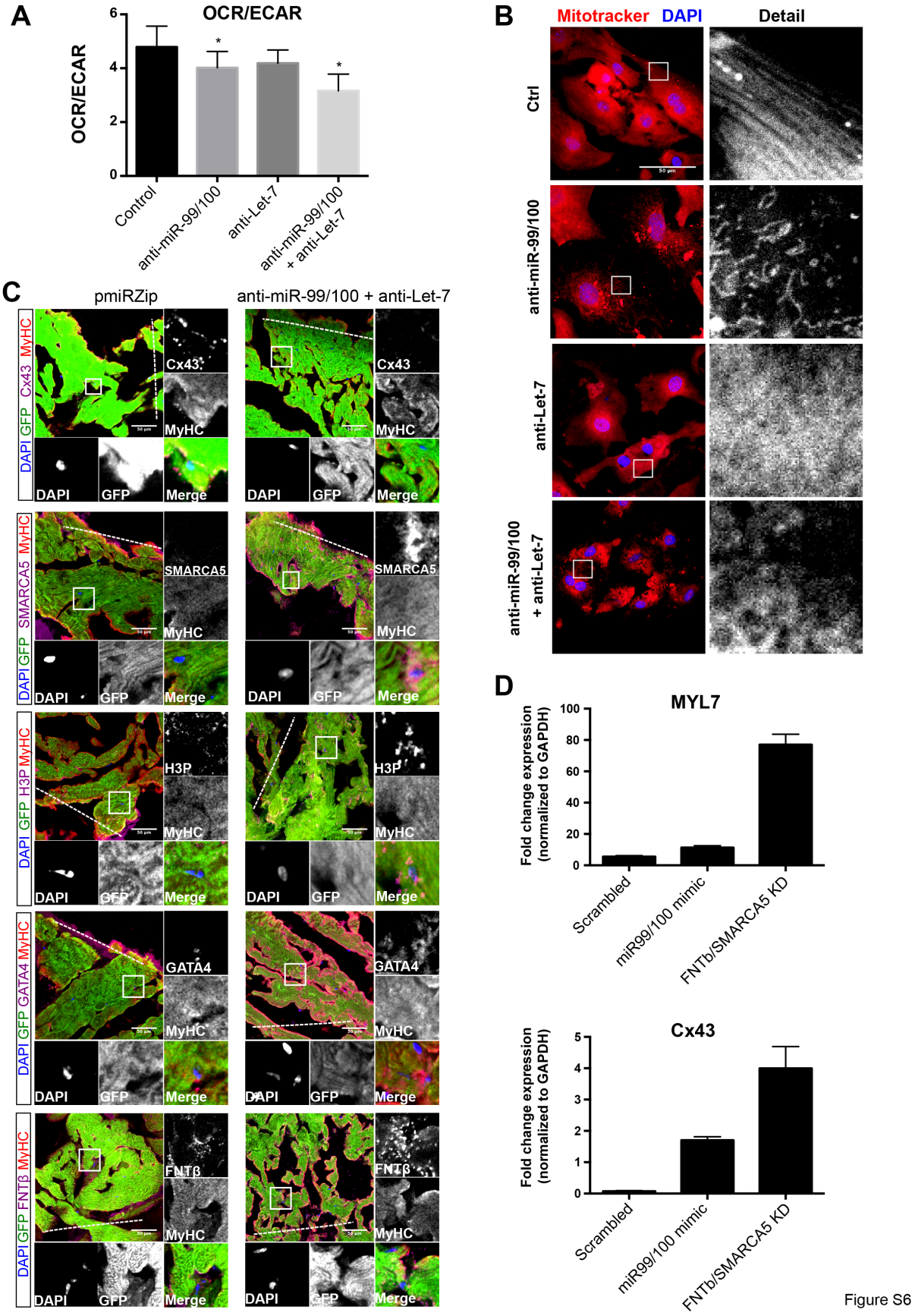

A
OCR/ECAR
OCR/ECAR
6
4
2
0
*
*
Control
anti-miR-99/100
anti-Let-7
anti-miR-99/100 + anti-Let-7
B
Mitotracker DAPI
Detail
Ctrl
anti-miR-99/100
anti-Let-7
anti-miR-99/100 + anti-Let-7
C
pmiRZip
anti-miR-99/100 + anti-Let-7
DAPI GFP Cx43 MyHC
Cx43
MyHC
DAPI
GFP
Merge
DAPI GFP SMARCA5 MyHC
SMARCA5
MyHC
DAPI GFP H3P MyHC
H3P
MyHC
DAPI GFP GATA4 MyHC
GATA4
MyHC
DAPI GFP FNTβ MyHC
FNTβ
MyHC
D
MYL7
Fold change expression (normalized to GAPDH)
100
80
60
40
20
0
Scrambled
miR99/100 mimic
FNTb/SMARCA5 KD
Cx43
Fold change expression (normalized to GAPDH)
5
4
3
2
1
0
Scrambled
miR99/100 mimic
FNTb/SMARCA5 KD

Figure S6

Figure S6, related to Figure 6. Dedifferentiating cardiomyocytes undergo a glycolytic transition and mitochondrial network fragmentation. (A) Seahorse analysis revealed that dedifferentiated cardiomyocytes, consecutively to anti-miR treatments, diminished oxidative phosphorylation routes in favor of a glycolytic state. (B) Mitotracker staining indicated mitochondrial fragmentation upon dedifferentiation with anti-miRs and suggested a glycolytic shift. (C) Confocal analysis of myocardial tissue after 7 days of miR silencing led to significant increases in FNTβ and SMARCA5, enhanced numbers of dedifferentiated cardiomyocytes - determined by Cx43 and GATA4 expression- and significantly increased numbers of proliferating cells (H3P). (D) qRT-PCR analysis for two mature cardiomyocyte markers (MYL7, Cx43), in human ESC-derived cardiomyocytes, showed that treatment with either miR-99/100 mimics or siRNA-mediated knockdown of FNTb/SMARCA5 sharply promoted cardiomyocyte differentiation. Data are represented as mean +/- SD. *$p<0.05$. n = 3 independent experiments/condition.

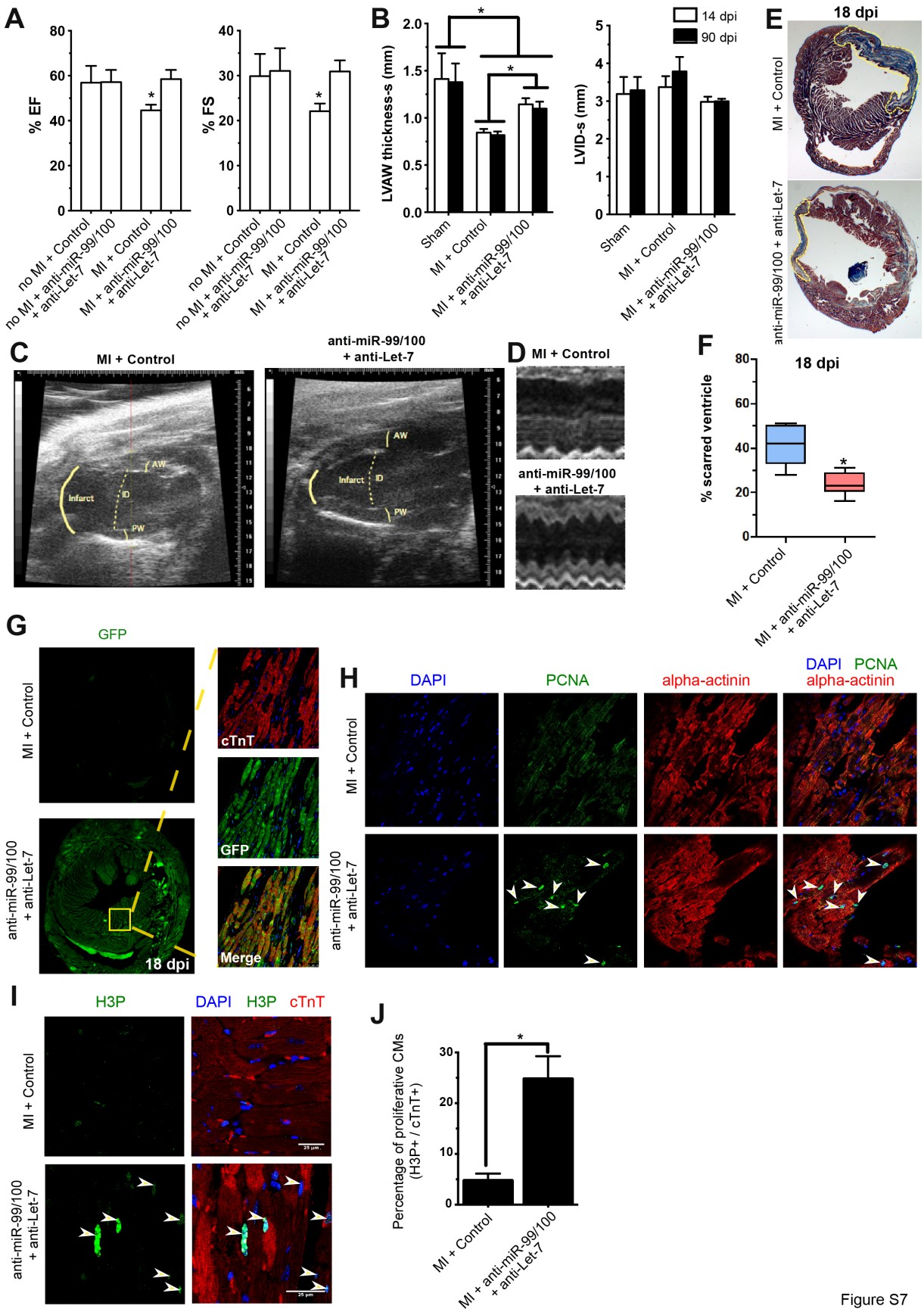

A
% EF
% FS
no MI + Control
no MI + anti-miR-99/100 + anti-Let-7
MI + Control
MI + anti-miR-99/100 + anti-Let-7
B
LVAW thickness-s (mm)
LVID-s (mm)
14 dpi
90 dpi
Sham
MI + Control
MI + anti-miR-99/100 + anti-Let-7
E
18 dpi
MI + Control
anti-miR-99/100 + anti-Let-7
C
MI + Control
anti-miR-99/100 + anti-Let-7
AW
ID
PW
Infarct
D
MI + Control
anti-miR-99/100 + anti-Let-7
F
18 dpi
% scarred ventricle
MI + Control
MI + anti-miR-99/100 + anti-Let-7
G
GFP
MI + Control
anti-miR-99/100 + anti-Let-7
18 dpi
cTnT
GFP
Merge
H
DAPI
PCNA
alpha-actinin
DAPI PCNA alpha-actinin
MI + Control
anti-miR-99/100 + anti-Let-7
I
H3P
DAPI H3P cTnT
MI + Control
anti-miR-99/100 + anti-Let-7
J
Percentage of proliferative CMs (H3P+ / cTnT+)
MI + Control
MI + anti-miR-99/100 + anti-Let-7

Figure S7

Figure S7, related to Figure 7. *In vivo* anti-miR administration improved heart function, cardiomyocyte proliferation and reduced infarct size. (A) anti-miR-99/100 and anti-Let-7 lentivirus-mediated delivery in a mouse model of myocardial infarction (MI) resulted in the significant improvement of ejection fraction (EF, left panel) and fractional shortening (FS, right panel), at 15 days post-infarction (dpi) as compared to infarcted animals injected with lentiviruses coding for an empty vector. Note that non-infarcted/lentiviruses injected animals were used as control groups. (B) Left ventricular end-systolic anterior wall thickness (LVAW thickness-s, left panel) and left ventricular end-systolic internal diameter (LVID-s, right panel) of mice transduced with AAV2/9 control, or AAV9-anti-miR-99/100 and anti-Let-7, analyzed by echocardiography at 14 and 90 days after myocardial infarction (MI). (C) Representative snapshot of an echocardiography at 14 dpi in control and anti-miR treated animals. (D) M-mode echocardiography demonstrated improved heart function in anti-miR treated mice. (E-F) Reduced infarct size in anti-miR treated animals was confirmed by Masson's trichromic staining at 18 dpi. (G) AAV2/9 anti-miR viruses efficiently transduced most cardiomyocytes in the adult heart. (H-J) Representative picture and quantitative analysis showing the higher number of proliferative cardiomyocytes as indicated by PCNA staining (G) and increased Histone 3 phosphorylation (H3P) (I, J) upon anti-miR delivery. Data are represented as mean +/- s.e.m. *$p<0.05$. Arrowheads: cells of interest. n = 8 animals/group (14dpi); n = 7 animals/group (90dpi). In all cases, three different sections per animal were utilized for quantitative analyses.

Supplemental Tables and legends

Table S1, related to Figure 1. miR-99/100 and Let-7a/c predicted targets (see attached excel file table S1).

Table S2, related to Figure 5. Differential gene expression from RNA-seq data (see attached excel file table S2).

Table S3, related to Figure 5. Raw data, differential expression and GO analysis of all different groups from the proteomic analysis. (see attached excel file table S3)

Table S4, related to supplemental experimental procedures. List of antibodies used in this study.

Primary antibody	**Specificity**	**Manufacturer**	**Dilution**
MF20	Human, mouse, zebrafish	Developmental Studies HybrydomaBanK	1:100-1:200
PCNA	Human, mouse, zebrafish	Santa Cruz	1:100-1:200
H3P	Human, mouse, zebrafish	Millipore	1:200
GFP	N/A	Abcam	1:200
Ras	Human, mouse, zebrafish	Santa Cruz	1:200-1:200
Myc	Human, mouse, zebrafish	Santa Cruz	1:100-1:200
FNTB	Human, mouse	Santa Cruz	1:200

Fntb	Human, mouse, zebrafish	Santa Cruz	1:100-1:200
Fnta	Human, mouse, zebrafish	Santa Cruz	1:100-1:200
Farnesylated protein	Human, mouse, zebrafish	Millipore	1:100
GATA4	Human, mouse	Santa Cruz	1:200
Smarca5	Human, mouse, zebrafish	Santa Cruz	1:100-1:200
SMARCA5	Human, mouse	Santa Cruz	1:200
Cx43	Human, mouse	Santa Cruzz	1:200
Anillin	Human, mouse	Abcam	1:200
ARK-2	Human, mouse	Abcam	1:200
Donkey Alexa 488	Mouse, rabbit, goat	Molecular Probes	1:200
Donkey Alexa 567	Mouse, rabbit, goat	Molecular Probes	1:200
DonekyAlexa 594	Mouse, rabbit, goat	Molecular Probes	1:200
Donkey Alexa 647	Mouse, rabbit, goat	Molecular Probes	1:200

Supplemental Movies legends

Movie S1, related to Figure 5. Untreated human cardiomyocytes.

Movie S2, related to Figure 5. Human cardiomyocytes treated with anti-miR-99/100 lentiviral vector for 1 week.

Movie S3, related to Figure 5. Human cardiomyocytes treated with both anti-miR-99/100 and

anti-Let-7 lentiviral vectors for 1 week.

Supplemental Experimental procedures

Human material. Human adult heart cDNA was purchased from Biochain (total heart, left ventricle, right ventricle), and was employed in expression analysis. Human heart samples were obtained following signed donor consent with the approval of the Institutional Review Boards of the CMRB (Barcelona, Spain) and ICGEB (Trieste, Italy).

Culture and isolation of adult and neonatal mouse ventricular myocytes. The method has been described before (Eulalio et al., 2012). Briefly, ventricles from neonatal mice (C57BL) (post-natal day 0) were separated from the atria, cut into pieces and then dissociated in calcium and bicarbonate-free Hanks with HEPES (CBFHH) buffer containing 1.75 mg ml−1 trypsin (BD Difco) and 10 μg ml−1 DNase II (Sigma), under constant stirring, at room temperature in eight to ten 10-min steps, collecting the supernatant to fetal bovine serum (FBS, Life Technologies) after each step. The collected supernatant was centrifuged to separate the cells, which were then resuspended in Dulbecco's modified Eagle medium 4.5 g l−1 glucose (DMEM, Life Technologies) supplemented with 5% FBS, 20 μg ml−1 vitamin B12 (Sigma) and with 100 U ml−1 of penicillin and 100 μg ml−1 of streptomycin (Sigma). The collected cells were passed through a cell strainer (40 μm, BD Falcon) and then seeded onto uncoated 100-mm plastic dishes for 2 h at 37 °C in 5% CO2and humidified atmosphere. The supernatant, composed mostly of CMs, was then collected and pelleted. Cells were resuspended in antibiotic-free media, counted and plated at the appropriate density; cultures of neonatal rat or mouse ventricular CMs prepared using this procedure yielded consistently a purity of >90%. Adult cardiomyocytes were obtained as follows: hearts were extracted and perfused retrogradely with calcium-free Krebs–Henseleit bicarbonate (KHB) buffer, and were then perfused with KHB buffer containing 1 mg ml−1 Liberase (Roche) for 10 min. Following removal of the atria and great vessels, the

hearts were minced in KHB buffer and the cell mixture was filtered through a cell strainer (100 µm, BD Falcon). The cells were then pelleted by centrifugation at 530g for 3 min at room temperature. The cell pellet was resuspended in a mixture DMEM 1.0 g l−1 glucose (Life Technologies) and perfusion buffer (1:1) and the separation of CMs from other cell types was achieved by sedimentation on a 6% bovine serum albumin (BSA, Sigma) cushion for 15 min. The CM pellet was resuspended and plated in DMEM 1.0 g l−1 glucose supplemented with 2 g l−1 BSA, 2 mM l-carnitine (Sigma), 5 mMcreatine (Sigma), 5 mM taurine (Sigma), 1 mM 2,3-butanedione monoxime (BDM; Sigma) and with 100 U ml−1 of penicillin and 100 µg ml−1 of streptomycin. Cells were plated on 24-well plates coated with laminin (Sigma), and kept at 37 °C in 5% CO2 and humidified atmosphere. The medium was exchanged 24 h later to DMEM 4.5 g l−1 glucose supplemented with 5% FBS, 20 µg ml−1 vitamin B12. Adult cardiomyocytes were kept in culture no more than 1 week.

RNA-seq and transcriptomic analysis. Neonatal cardiomyocytes were isolated and mock-transduced or transduced with anti-miR-99/100 and anti-Let-7a/c containing AAV2/9. Total RNA was extracted 7 days after transduction and subjected to total RNA deep sequencing. Samples were processed using TruSeq RNA-seq sample prep kit from Illumina. A pool of 3 samples was loaded on each lane of an Illumina flowcell and clusters created by Illumina cBot. The clusters were sequenced in an Illumina HiSeq2000. Read densities were visualized by preparing normalized BigWig files using HOMER and uploading them to the UCSC Genome Browser (http://genome.ucsc.edu/http://genome.ucsc.edu/). Gene expression values were determined by identifying reads on the appropriate strand in the genic regions defined by GENCODE and RefSeq using HOMER. Annotation of transcript type was based on GENCODE.

Gene Ontology functional enrichment and clustering calculations were provided by DAVID. A fold-enrichment of 1.25 was used.

Multidimensional protein identification technology. Digested proteins from adult regenerating zebrafish hearts (uninjured, 3 dpa, 7dpa), neonatal regeneration-permissive mouse hearts (P0, P7, adult) and anti-miR treated adult myocardial mouse tissue (control, anti-miR-99/100, anti-Let-7a/c, anti-miR-99/100+anti-Let-7a/c) were collected and analyzed by MudPIT as previously described (Ambatipudi et al, 2012; Washburn et al., 2001).

Proteomics Data Analysis. Counts were normalized using a global mean normalization method. Proteins with a fold change of two or more were considered significantly different. Uniprot accession ids were converted to their corresponding gene symbol. Overlap between the murine neonatal, ogranotypic slices and zebrafish datasets was performed following orthologous conversion to murine gene symbols (Mudunuri et al., 2009). Genes with significant differences were analyzed for GO enrichments using DAVID (Huang et al., 2008). GO enrichments with a FDR less than 0.01 were considered significant.

Echocardiography analysis. To evaluate left ventricular function and dimensions, transthoracic two-dimensional echocardiography was performed on mice sedated with 5% isoflurane at 12, 30 and 60 days after myocardial infarction, using a Visual Sonics Vevo 770 Ultrasound (Visual Sonics) equipped with a 30-MHz linear array transducer. M-mode tracings in parasternal short axis view were used to measure left ventricular anterior and posterior wall thickness and left ventricular internal diameter at end-systole and end-diastole, which were used to calculate left

ventricular fractional shortening and ejection fraction.

Heart collection and histological analysis. At the end of the studies, animals were anaesthetized with 5% isoflurane and then killed by injection of 10% KCl, to stop the heart at diastole. The heart was excised, briefly washed in PBS, weighted, fixed in 4% PFA or 10% formalin at room temperature, embedded in paraffin and further processed for histology or immunofluorescence. Haematoxylin–eosin and Masson's trichrome staining were performed according to standard procedures, and analysed for regular morphology and extent of fibrosis. Infarct size was measured as the percentage of the total left ventricular area showing fibrosis. Histomorphometric analysis were performed with ImageJ. For cryosectioned samples, hearts were removed, washed in PBS-EDTA 0.4% and fixed for 20 min in 4% paraformaldehyde at 4 °C. Afterwards, they were washed several times in PBS, equilibrated in 30% sucrose, and then frozen for cryosectioning. 10 μm slices were obtained with a cryostat (Leica).

BrdU labeling. Fish were anaesthetized in 0.4 % Tricaine, and 10 μl of a 10 mg/ml solution of BrdU (in PBS) was injected into the abdominal cavity once every 2 days for 14 days. At that point, hearts were removed and fixed overnight in 4 % paraformaldehyde at 4 °C, washed in PBS, equilibrated in 30 % sucrose in PBS and frozen for cryosectioning.

Real time RT-PCR. For RNA, tissue was obtained from adult heart ventricles from different time points and conditions, extensively washed in PBS-EDTA 0.4% to remove blood, and then mechanically homogenized and processed using RNeasy kit (Qiagen) as per manufacturer's instructions. RT and PCR were performed using Quantitect Reverse Transcription Kit (Qiagen).

Total cellular RNA from cultured cardiomyocyte (adult and neonatal) was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's recommendations. 1µgs of DNAse1 (Invitrogen) treated total RNA was used for cDNA synthesis using the iScript^{TM} cDNA synthesis kit for RT-PCR (BioRad). Real-time PCR was performed using the SYBR Green Supermix (BioRad). The levels of expression of respective genes were normalized to corresponding GAPDH values and are shown as fold change relative to the value of the control sample. All the samples were done in triplicate.

For miRNAs, small RNA (<200 pb) was obtained employing the miRNeasy mini kit (Qiagen) using the same procedure as before. RT and PCR reactions were carried out employing miRCURY LNA RT and PCR kits (Exiqon) and stem-loop LNA primers (Exiqon).

MicroRNA microarrays. RNA was obtained as for PCR applications. GenechipmiRNA 2.0 microarrays were purchased from Affymetrix and small RNA labeling was performed using FlashTag HSR labeling kit (Genesphere). 200 ng of small RNA was polyA-tailed and biotin conjugated. After labelling, RNA was hybridized using GeneChip reagents (Affymetrix) and protocols as indicated by the manufacturer. The chip contains hybridization probes for the miRbase v15 annotations, including 248 zebrafishmiRNAs. MicroRNA data was analyzed by using the R package.

Bioinformatic analysis of miRNA targets and sequence conservation. Signaling pathways and downstream target prediction related to the identified miRNAs were determined by using DIANA, Miranda and TargetScan databases. Gene ontology analysis was performed with DAVID software. Sequence alignments for zebrafish, mouse and human 3' UTRs were

performed with ClustalX2, 3' UTRs were obtained from ENSEMBL.

Fluorescence in situ hybridization. 10 μm heart slices were further fixed in 4 % PFA for 10 min at room temperature, washed in PBS and acetylated for 10 min in acetylation solution. After washing in PBS, samples were treated with proteinase K, prehybridized for 4 h and hybrydized overnight at the appropriate temperature with LNA DIG-labeled probes for the corresponding miRNAs (Exiqon). The next day slides were washed and immunolabeled with anti-DIG-alkaline phosphatase antibodies (1:2,000) and antibodies against cardiomyocytic proteins of interest (1:100) overnight at 4 °C. Secondary antibody incubation was performed as for immunofluorescence experiments. Alkaline phosphatase activity was detected by incubating samples in a Fast Red solution (Dako) for 2 hours. Samples were then washed, mounted in Vecta-shield and imaged in a confocal microscope. Fast Red fluorescence was detected with Cy3 settings.

Immunofluorescence. Tissue slices were fixed for 15 min in 4% paraformaldehyde, washed in PBS-gly 0.3 M, and blocked in PBS-10% donkey serum, 0.5 % TX-100, 0.5 % BSA for 1 hour. Primary antibodies were diluted at the appropriate concentrations in PBS-1% donkey serum, 0.5 % TX-100, 0.5 % BSA and incubated overnight. After washing, slices were incubated overnight with secondary antibodies, washed and mounted in Vecta-shield. Antibodies employed are listed in table S3.

Cell transfections. On-Target siRNA pools against mouse Fntb and Smarca5 were obtained from Dharmacon. siRNAs were diluted in OPTI-MEM with transfection reagent Lipofectamine (Life

Technologies) to a final concentration of 25 nM and administered to neonatal mouse cardiomyocytes or human ESC-derived cardiomyocytes. Screening was performed in quadruplicate. Twenty-four hours after transfection, culture medium was replaced by fresh medium. Cells were fixed at 5 days after plating and processed for immunofluorescence analysis and cell counting, or RNA was extracted for RT-PCR analysis as described before.

Cell culture. COS7 cells were maintained in DMEM (high glucose) supplemented with 10 % FBS, L-Glutamine and non-essential amino acids (Invitrogen). Human ES cells, H1 and H9 (WA1 and WA9, WiCell), were cultured in chemically defined hES/hiPS growth media, mTeSR1 on growth factor reduced matrigel (BD biosciences) coated plates. Briefly, 70-80% confluent hES/iPS cells were treated with dispase (Invitrogen) for 7 minutes at 37°C and the colonies were dispersed to small clusters and lifted carefully using a 5 ml glass pipette, at a ratio of ~1:4. Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Promocell and cultivated in EBM medium supplemented with EGM singlequots (Lonza), 2% FBS, hEGF 10 µg/ml, Heparin 100 µg/ml (Sigma). Neonatal human fibroblasts (HFF-1; ATCC) and 293T cells were cultivated in DMEM containing 10% FBS, 2 mM GlutaMAX (Invitrogen), 50 U ml−1 penicillin and 50 mg.ml−1 streptomycin (Invitrogen). HUVEC and HFF were grown in collagen I coated plates (BD biosciences). All cell lines were maintained in an incubator (37°C, 5% CO2) with media changes every second day.

Differentiation of Human ES cells to immature cardiomyocytes. Human ES cells grown on matrigel dots (BD biosciences) were carefully dissociated using dispase and were plated on low attachment plates in EB media (IMDM, 20% FBS, 2.25nM L-Glutamine and non-essential

aminoacids). After 6 days of suspension in culture, the EBs were seeded on gelatin-coated plates in EB media. Spontaneously beating EBs were manually picked and used for further analysis. For directed differentiation, human ES cells grown in mTeSR on matrigel coated plates were treated with 12μM GSK3β inhibitor CHIR 99021 (Stemgent) in cardiomyocyte differentiation base media (RPMI 1640 supplemented with 125μg/ml human holo-transferrin (Sigma-Aldrich)) for 24 hours, followed by 24 hour of rest in the base media. On day 3, the cells were treated with 5μM WNT inhibitor, IWP4 (Stemgent) for 48 hours, followed by treatment with Cardiac differentiation base media supplemented with 20μg/ml human Insulin (SAFC) until colonies started beating.

Luciferase constructs and microRNA binding validation. 3' UTR of human and zebrafish FNTB and SMARCA5 were amplified with the indicated primers using genomic DNA as a template and were cloned into PGL3 vector (Promega) at the Xho1 site downstream of luciferase gene. COS7 cells (seeded at $3x10^4$ cells per well of a 12 well plate and grown for 24 hours) were transfected with 50 ng each of indicated luciferase reporter vectors, pRL TK (Renilla luciferase control vector, Promega) either in the presence or absence of 20nM or 40nM of double stranded DNA oligonucleotide mimics of miR-99 or miR-100 (Dharmacon) using Lipofectamine (Invitrogen) following manufacturer's protocol. 12 -16 hours post-transfection, cells were lysed using passive lysis buffer (Promega). Luminescent signals arising from the cell lysates obtained 12 hours post transfection of COS7 cells with appropriate luciferase constructs were measured using the Dual Luciferase assay system (Promega) in a Synergy H1 hybrid reader (BioTek). The relative luminescence intensity of each sample was calculated after normalization with corresponding *Renilla* luciferase activity, and were represented as % values compared to the

corresponding sample without the miR mimic.

Confocal microscopy. Samples were imaged using a Zeiss L710 confocal microscope. For every sample, at least two different fields were examined at two different magnifications (using a 20x objective and a 63x oil-immersion objective). Z-stacks were obtained for further analysis and 3D reconstruction. For intensity comparison purposes, images were taken with the same settings (pinhole size, laser intensity, etc). Cell counting was performed with ImageJ.

Morpholino and microRNA mimic injections in zebrafish embryos. Morpholinos (Gene Tools) were dissolved in water at a 2 mM stock concentration and diluted to a 2 ng/nl working concentration in PBS/phenol red solution. Embryo injections were performed by injecting ~1 nl morpholino solution at the 1-cell stage using a FemtoJet (Eppendorf). For microRNA mimic injection, a miR-99/100 equimolar mixture at 2 ng/nl in PBS was employed. For rescue experiments, human FNTB and SMARCA5 ORFs were cloned into pCS2 plasmid, *in vitro* transcribed using the Megascript T6 kit (Life Tech) and co-injected with their corresponding morpholinos at a 100 pg/nl. Morphants were evaluated at 24, 48 and 72 h in a StereoLumar stereoscope (Zeiss). miR mimics, morpholinos and antagomiR sequences are listed below : Dre-miR-100 mimic: 5'-AACCCGUAGAUCCGAACUUGUG-3' and 5'-CAAGCUUGUAUCUAUAGGUAUC-3' ; Dre-miR-99 mimic: 5'-AACCCGUAGAUCCGAUCUUGUG-3' and 5'-CAAGCUCGAUUCUAUGGGUCUC-3' ; Dre-miR-99 inhibitor: 5’-ACAAGTTCGGATCTACGGGT-3’ ; Dre-miR-100 inhibitor: 5’-ACAAGATCGGATCTACGGGT-3’ ; Fntb morpholino (translation-blocking): 5’-GCGCCTCTTCCATGATGAGCTCTCA-3’ ; Smarca5 morpholino (translation-blocking): 5’-

CTTCTTCCCGCTGCTGCTCCATGCT-3'. In addition, control non-targeting morpholinos were injected as controls in all cases.

In vivo microRNA delivery. MicroRNA siRNA mimics without chemical modifications were purchased from Life Technologies, dissolved in nuclease-free water and complexed to jetPEI (10 N/P ratio) for *in vivo*, intra-cardiac administration. 0.2 μg siRNA was injected per animal every 2 days. To determine the efficiency of the delivery, a control Cy5-labeled siRNA directed against GFP was used in cmlc2:GFP animals. MicroRNA inhibitors against the miR-99/100 family were purchased from Exiqon and used at 0.2 μg siRNA per animal every 2 days.

Tipifarnib injections. Tipifarnib was dissolved in DMSO at 10 mg/ml and 2 μl were administered by intraperitoneal injection (final concentration 0.02 mg/animal) every 2 days for 14 days. Control animals were administered DMSO.

Metabolic flux analysis. Analysis of oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) was performed using a Seahorse extracellular flux XF96 analyzer (Seahorse Bioscience) in accordance with manufacturer's instructions. Briefly, cardiomyocytes were seeded in extracellular flux 96-well culture microplates in 150 μl of corresponding cell culture media, and infected with anti-miR lentivirus. 5 days after infection, prior analysis, culture media was replaced with 150 μl of unbuffered assay media and cardiomyocytes were incubated for 1 h at 37 °C for pH and temperature stabilization. Analysis of OCR and ECAR was performed simultaneously both at basal conditions and after injections of the inhibitors in the XF Cell Mito Stress Test Kit (Sigma, St. Louise, MO).

Mitochondrial staining. For mitochondrial staining, cardiomyocytes were seeded on cover slips and infected with anti-miR lentivirus. 7 days after infection, cardiomyocytes were incubated for 30 min at 37 °C in regular media in the presence of 200 nM MitoTracker Red (Invitrogen) for staining of mitochondria and 1 μg/ml DAPI to stain the cell nuclei. Thereafter, cells were fixed in 4% paraformaldehyde and visualized by confocal microscopy (Zeiss).

Supplemental References

Ambatipudi, K.S., Swatkoski, S., Moresco, J.J., Tu, P.G., Coca, A., Anolik, J.H., Gucek, M., Sanz, I., Yates, J.R., 3rd, and Melvin, J.E. (2012). Quantitative proteomics of parotid saliva in primary Sjogren's syndrome. Proteomics *12*, 3113-3120.

Huang, D. W., Sherman, B. T. & Lempicki, R. A. (2008) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protocols *4*, 44-57,

Mudunuri, U., Che, A., Yi, M. & Stephens, R. M. (2009) bioDBnet: the biological database network. Bioinformatics *25*, 555-556.

Washburn, M.P., Wolters, D., and Yates, J.R., 3rd (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat Biotechnol *19*, 242-247.

Eulalio, A., Mano, M., Ferro, M.D., Zentilin, L., Sinagra, G., Zacchigna, S., and Giacca, M. (2012). Functional screening identifies miRNAs inducing cardiac regeneration. Nature. *492,* 376-381.