SUPPLEMENTAL DATA

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cardiomyocyte isolation and culture. All experimental procedures complied with the Guidelines of the Italian National Institutes of Health and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee. Neonatal cardiomyocytes were isolated from 1-2-days-old SWISS CD1 mice as previously described (1). Briefly, hearts were dissected, minced and trypsinized. After 2 pre-plating steps (30 min each) to remove fibroblasts from the preparation, the resultant cell suspension was plated in 35-mm culture dishes coated with fibronectin (20 µg/mL, F895 Sigma) in MEM containing 10% FBS. To minimize the number of residual proliferating cardiomyocytes and of contaminating fibroblasts, cell cultures were cultured in the presence 20 µM cytosine β-D-arabinofuranoside (AraC, C1768 Sigma) for 48h. After a 3 day recovery time in AraC-free medium neonatal cardiomyocytes were transfected.

Left ventricular cardiomyocytes from 8-12 week-old Wistar rats (250 to 350 g) were isolated as described before (2,3). Briefly, adult hearts were rapidly excised and retrogradely perfused through the aorta as previously described (4,5). Briefly, After mechanical dissection of the left ventricle, adult cardiomyocytes were cultured on laminin (10 µg/mL, L2020 Sigma)-coated dishes (3). Cells were cultured for 1 day in standard free-serum medium (DMEM, 25mM HEPES, 5mM taurine, 5mM creatine, 2mM L-carnitine, 20 U/mL insulin (Sigma), 0.2% BSA, penicillin (100 U/mL), and streptomycin (100 µg/mL)). Then, cardiomyocytes were transfected and fresh medium (without BSA containing 2mM L-glutamine) was added every 3 days.

RNA interference and cardiomyocyte transfection. Both neonatal and adult cardiomyocytes were transfected with HiPerfect transfection reagent (QIAGEN) complexed with small interfering RNAs (siRNAs, Dharmacon). Specifically, HiPerfect and 5nM siRNA were used, as previously described (6). The following siGenome duplexes and SMART pool reagents were used for mouse cells: CDKN1A (p21 D-058636-03-0005), CDKN1B (p27 D-040178-01-0005) and CDKN1C (p57 mouse M-062494-01-005). The following reagents were used for rat: CDKN1A (p21 rat M-091552-00-0010), CDKN1B (p27 rat M-090938-00-0010)

and CDKN1C (p57 rat M-098880-00-0010). The siCONTROL Non-targeting siRNA (D-001210-02-05) was used as a control for both mouse and rat cells.

Western Blotting.

Western blotting was performed as previously described (7). Briefly, cells were lysed in 2x Laemmli buffer and boiled for 5 minutes. Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose by standard procedures. Proteins of interest were detected with the following specific antibodies: rabbit polyclonal anti-p21 (c-19; Santa Cruz), rabbit polyclonal anti-p27 (c-19; Santa Cruz), rabbit polyclonal anti-p57 (H-91-sc8298; Santa Cruz), monoclonal anti-Human Retinoblastoma Protein (Rb) (554136; Pharmigen), rabbit polyclonal anti-Retinoblastoma Protein (Rb) {pT826}phosphospecific antibody (44- 576G; Biosurce), monoclonal anti-Troponin T-C (CT3) (sc 20025; Santa Cruz), α-actinin (sarcomeric) (EA-53; Sigma), and anti-α-tubulin (Ab-1; Oncogene Research Prodouct). Membranes were stained with Red Ponceau (Sigma) to assess equal protein loading and even transfer.

*Indirect immunofluorescence staining and BrdU assay***.** Immunofluorescence stainings were performed as previously described (8). Briefly, cardiomyocytes were fixed with 4% paraformaldehyde and immunofluorescences were performed using following antibodies: permeabilized with 0.2% Triton X-100/PBS for 10 min. Samples were blocked with 5% BSA in 0.1% Triton X-100/PBS for 15 min and incubated with the following primary antibodies: anti-AuroraB (AIM-1; BD transduction laboratories), anti-phospho-Histone-H3 (Ser10) (RR002; Upstate), anti-BrdU (ab1893; Abcam), anti-γH2AX antibody (clone JBW301; Upstate Biotechnology, Lake Placid, NY), monoclonal anti-troponin T-C (CT3) (sc 20025; Santa Cruz), and anti-α-actinin (sarcomeric) (EA-53; Sigma). Cells were then washed and incubated with secondary antibodies/PBS for 45 min. Secondary antibodies are: Polyclonal Rabbit anti-mouse /FITC (F0232; 1:100; DAKO), Texas Red anti-mouse IgG (H+L) (TI-2000; 1:100; VECTOR), and Rabbit polyclonal to Sheep IgG (H+L)/ FITC (ab 6743; 1:100; Abcam). Cells were counter-stained with Hoechst 33342 (Sigma-Aldrich). The same number of optical fields was counted for each sample, totalling 70-200 neonatal cardiomyocytes/sample and 50-100 adult cardiomyocytes/sample. In order to compare the results of different experiments, data were expressed as % of control. Images were acquired by a fluorescence microscope (Axioplan2; Carl Zeiss). Images were analyzed by IAS-software (Delta System), processed and

overlayed using Adobe Photoshop CS2 (Adobe). Cells were counted by two blinded readers obtaining similar results.

*Apoptosis assay***.** Apoptosis was assessed by measuring the amount of cytoplasmic nucleosomes generated during the apoptotic fragmentation of cellular DNA by Cell Death Detection Elisa (Roche), according to the manufacturer instructions.

In brief, cardiomyocytes were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized for 2 min with 0.1% Triton X-100 in sodium citrate. Samples were then rinsed with PBS and incubated in the TUNEL reaction mix according to the manufacturer's instructions (Roche). Cells were counterstained with Hoechst 33342 before analysis and quantification.

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SUPPLEMENTAL FIGURE LEGENDS

Fig.S1: Neonatal cardiomyocyte characterization. A) After AraC treatment, cell cultures were allowed to recover in AraC-free medium for 3 days and assayed by immunofluorescence for the expression of the cardiac specific markers α-sarcomeric actinin and troponin T. Nuclei were stained using Hoechst 33342 (blue). Representative overlays showing that cardiomyocytes displayed the characteristic pattern of expression for both troponin T (green) and α-sarcomeric actinin (red). Calibration bar= 30μm. B) Representative western blotting showing α-sarcomeric actinin and troponin T expression in

whole cell extracts derived from neonatal cardiomyocyte cultures. NIH-3T3 fibroblasts were used as negative control. Tubulin shows equal loading in each lane. C) Neonatal cardiomyocytes cultures were assayed for α-sarcomeric actinin and nuclei were detected by Hoechst 33342 staining. Then, α-sarcomeric actinin/Hoechs-double positive cardiomyocytes and α-sarcomeric actinin-negative/Hoechst positive cells were counted and the relative proportions were calculated. The bar graph shows that the contamination of non-cardiomyocyte cells (fibroblasts), was below 13% (n= 20; *p<0.0005). D) Differentiation marker expression in cardiomyocytes and in NIH-3T3 fibroblast cultures was analyzed by qPCR. The mRNA levels of cardiac-muscle α-actin 1 (Actc1) and cardiac-muscle α-myosin (Myh6), two cardiac markers, and of vimentin, that is highly expressed in fibroblasts, were assayed. The bar graph shows that Actc1 and Myh6 were highly expressed in cardiomyocytes cultures and undetectable in fibroblasts (n=10; *$p<10^{-8}$). Reciprocally, vimentin was detected only in fibroblasts (n=10; p*$<10^{-11}$).

Fig.S2: Adult rat cardiomyocyte characterization. Cell cultures were assayed by immunofluorescence for the expression of the cardiac specific marker α-sarcomeric actinin and nuclei were stained using Hoechst 33342 (blue). A) Representative overlay showing that adult cardiomyocytes displayed the characteristic rod shape and the expected pattern of expression of α-sarcomeric actinin (red). Calibration bar= 30µm. B) α-sarcomeric actinin/Hoechs-double positive cardiomyocytes and α-sarcomeric actinin-negative/Hoechst positive cells were counted and the relative proportions were calculated. The bar graph shows that the contamination of non-cardiomyocyte cells (fibroblasts), was below 2% (n= 20; *p<0.0005). C) Differentiation marker expression in cardiomyocytes and in RAT1 fibroblast cultures was analyzed by qPCR. The mRNA levels of cardiac-muscle α-actin 1 (Actc1) and cardiac-muscle α-myosin (Myh6), two cardiac markers, and of vimentin, that is highly expressed in fibroblasts, were assayed. The bar graph shows that Actc1 and Myh6 were highly expressed in cardiomyocytes cultures and undetectable in fibroblasts (n=10; *$p<10^{-6}$). Reciprocally, vimentin was detected only in fibroblasts (n=10; p*$<10^{-10}$).

Fig.S3: p21 and p27 knock-down did not increase cardiomyocyte apoptosis and micronucleation**.** A) Neonatal cardiomyocytes were transfected with p21 and p27 or control siRNA. Then, cells were fixed at 2, 4 and 6 days after transfection and apoptosis cell death was assayed by TUNEL assay. The bar graph shows percentage of TUNEL positive cells. Differences are not statistically significant (n= 4). B) and C) Neonatal cardiomyocytes were transfected with p21 and p27 or control siRNA. BrdU was added at the time of transfection to label all cells that underwent DNA replication in the following 2 days. Then, BrdU

incorporating cells were detected by immunofluorescence using a FITC-conjugated specific antibody, while cardiomyocytes were stained using a texas red-conjugated antibody to α-sarcomeric actinin. Micronuclei identity was confirmed by Hoechst 33342 (blue) staining. B) Bar graph showing the percentage of BrdU positive cardiomyocytes displaying overt micronucleation. Differences are not statistically significant. C) Representative α-sarcomeric actinin/Hoechst (top panel) and α-sarcomeric actinin/BrdU overlays are shown. Calibration bar= 30μm.

Fig.S4: CKI knock-down did not increase DNA damage in neonatal cardiomyocytes. Neonatal cardiomyocytes were transfected with p21and p27 siRNAs in the presence or absence of p57 siRNA or with control siRNA. BrdU was added at the time of transfection and was constantly present throughout the time course to monitor all cells that underwent DNA replication. Then, BrdU incorporating cells were detected by immunofluorescence using a FITC-conjugated specific antibody, while γH2AX was detected using a texas red-conjugated antibody. Nuclei were stained using Hoechst 33342 (blue). A) The bar graph shows the percentage of γH2AX positive cells. Differences are not statistically significant (n= 4). B) Bar graph showing the percentage of γH2AX negative cells among BrdU positive cardiomyocytes (n=4; *$p<0.0001$; #$p<0.002$).

Fig.S5: Upon p21 and p27 knock-down, only p57 was induced. Neonatal cardiomyocytes were transfected with p21 and p27 siRNAs. RNA was extracted 2, 4 or 6 days after transfection and the levels of the indicated CKIs were measured. The bar graph shows that p15, p16, p18 and p19 were not affected significantly, while p57 was induced over time and peaked at 6 days after transfection (n= 6; *$p< 0.001$).

Fig.S6: hTERT expression increases cell proliferation and BrdU incorporation induced by CKI RNAi in neonatal but not in adult cardiomyocytes. Neonatal and adult cardiomyocytes were infected with Ad-hTERT or Ad-LacZ and, 8 hours later, cells were transfected with p21, p27, and p57 or control siRNAs. Cardiomyocytes were stained by α-sarcomeric actinin and Hoechst 33342, and counted. A) Bar graph shows that hTERT overexpression further up-regulates cardiomyocyte increase induced by CKI knock-down at the indicated time points (n=5; *$p<0.001$), while C) it had no significant effect on adult cardiomyocytes counted at day 10 (n=3; ns= not significant). B)Two, 4, or 6 days after transfection, neonatal cardiomyocytes were pulse labeled with BrdU for 8 hrs, stained by α-sarcomeric actinin and Hoechst 33342, and counted. The bar graph shows that hTERT over-expression further up-regulates cardiomyocyte increase induced by CKI knock-down at indicated time points (n=5; #$p<0.05$).D) Nine days after transfection, adult cardiomyocytes

were pulse labeled with BrdU for 24 hrs, stained by α-sarcomeric actinin and Hoechst 33342, and counted. The bar graph shows that hTERT over-expression it had no significant effect on adult cardiomyocytes counted (n=3; ns= not significant).

TABLE S1. Percentage of adult cardiomyocytes displaying 1, 2, or >2 nuclei for cells following siRNA transfection (n=3).

	MONONUCLEATED	**p**	**BINUCLEATED**	**p**	**>2 NUCLEI**	**P**
Control siRNA	1.7 ± 0.2		97.3 ± 0.3		0.9 ± 0.3	
p21/p27 siRNA	0.8 ± 0.3	0.039	94.4 ± 1.4	ns	4.7± 1.1	0.016
p21/p27/p57 siRNA	0.8 ± 0.2	0.019	94.6 ± 0.8	ns	4.6 ± 0.6	0.002

FIGURE S1

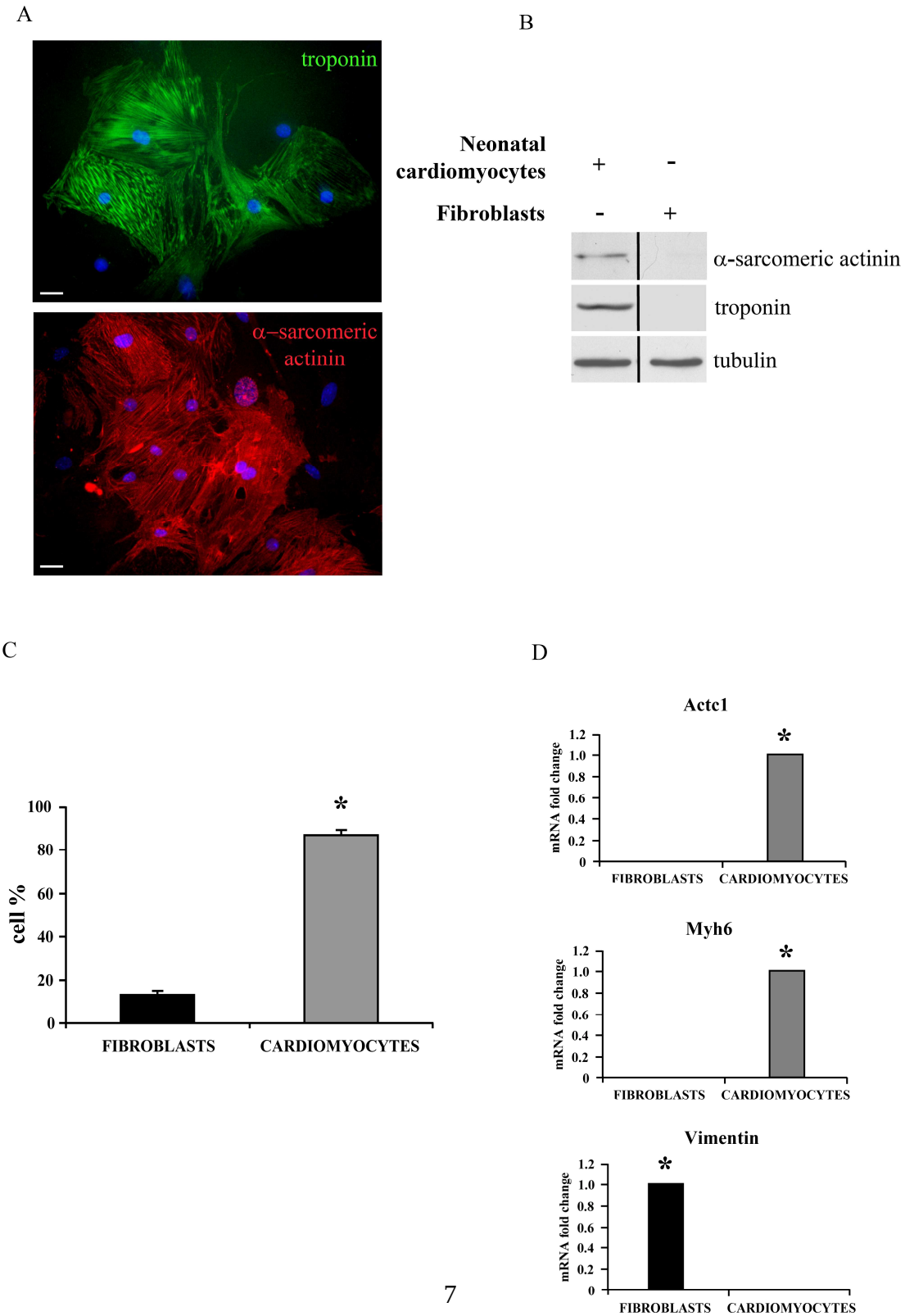

A
troponin
α–sarcomeric actinin
B
Neonatal cardiomyocytes
+
-
Fibroblasts
-
+
α-sarcomeric actinin
troponin
tubulin
C
cell %
100
80
60
40
20
0
*
FIBROBLASTS
CARDIOMYOCYTES
D
Actc1
mRNA fold change
1.2
1.0
0.8
0.6
0.4
0.2
0
*
FIBROBLASTS
CARDIOMYOCYTES
Myh6
mRNA fold change
1.2
1.0
0.8
0.6
0.4
0.2
0
*
FIBROBLASTS
CARDIOMYOCYTES
Vimentin
mRNA fold change
1.2
1.0
0.8
0.6
0.4
0.2
0
*
FIBROBLASTS
CARDIOMYOCYTES

FIGURE S2

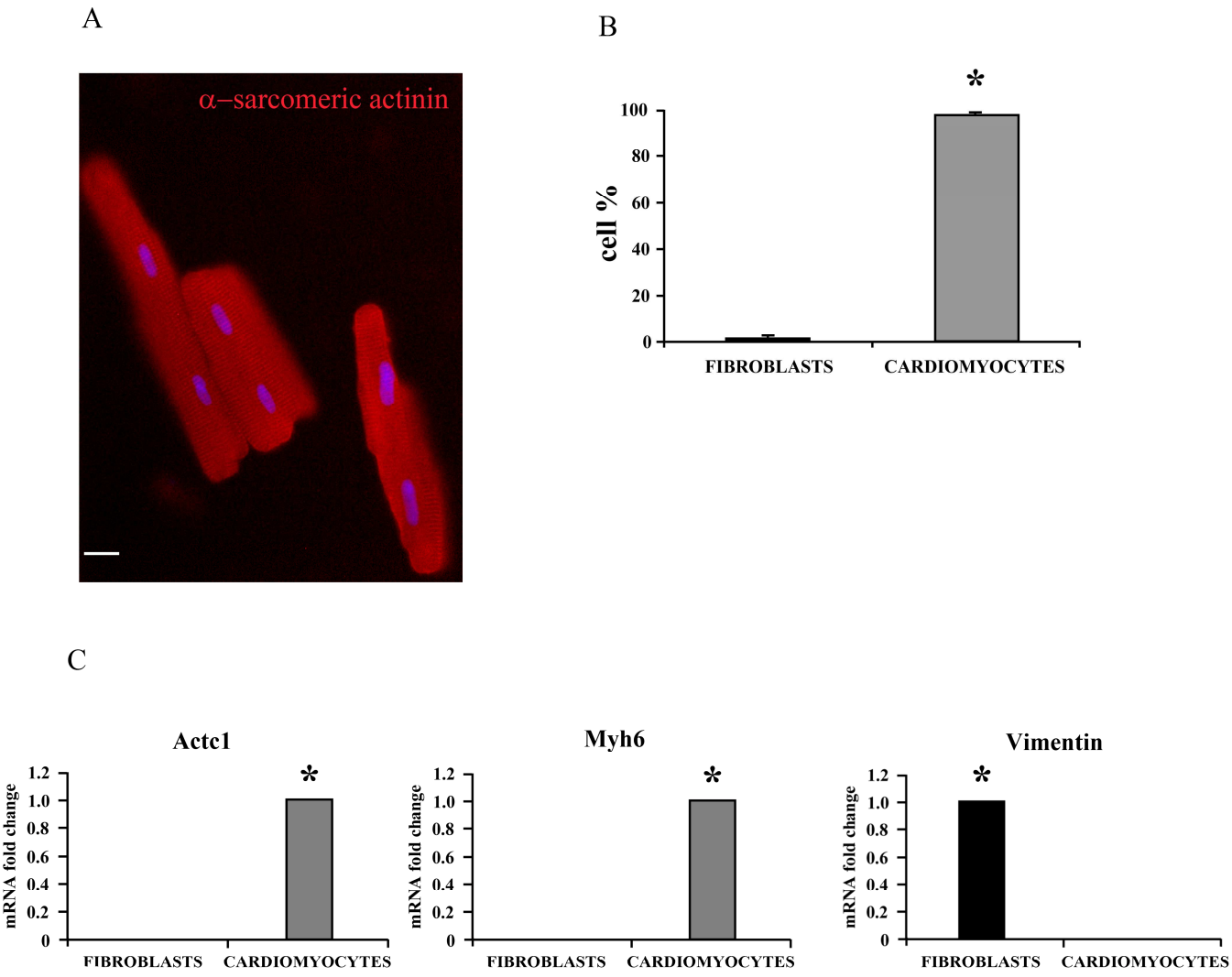

A
α–sarcomeric actinin
B
cell %
100
80
60
40
20
0
*
FIBROBLASTS
CARDIOMYOCYTES
C
Actc1
Myh6
Vimentin
mRNA fold change
1.2
1.0
0.8
0.6
0.4
0.2
0
*
FIBROBLASTS
CARDIOMYOCYTES

FIGURE S3

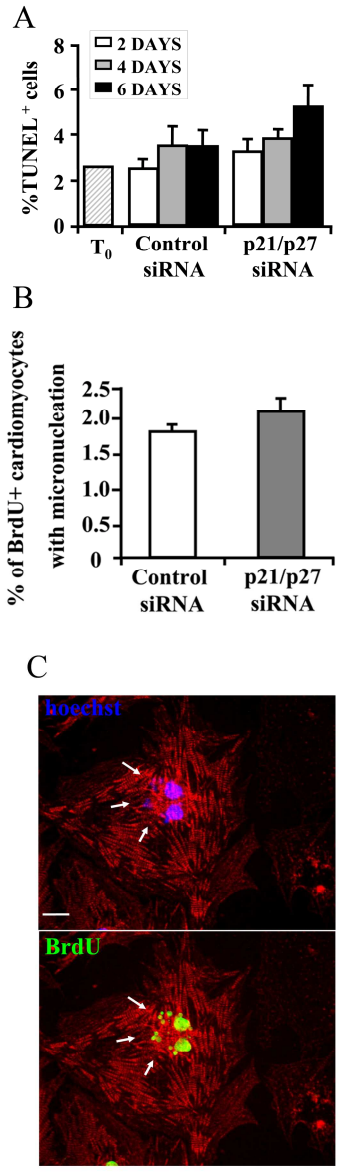

A
2 DAYS
4 DAYS
6 DAYS
%TUNEL+ cells
8
6
4
2
0
T0
Control siRNA
p21/p27 siRNA
B
% of BrdU+ cardiomyocytes with micronucleation
2.5
2.0
1.5
1.0
0.5
0
Control siRNA
p21/p27 siRNA
C
hoechst
BrdU

FIGURE S4

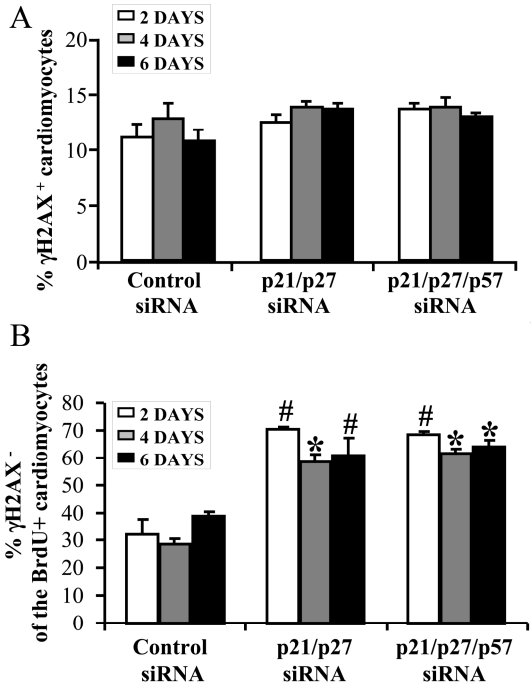

A
2 DAYS
4 DAYS
6 DAYS
% γH2AX+ cardiomyocytes
20
15
10
5
0
Control siRNA
p21/p27 siRNA
p21/p27/p57 siRNA
B
% γH2AX- of the BrdU+ cardiomyocytes
80
70
60
50
40
30
20
10
0
#
*

FIGURE S5

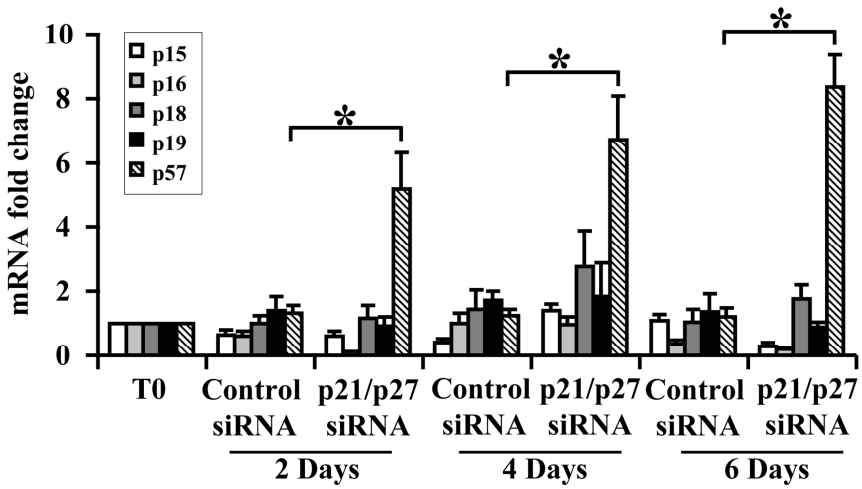
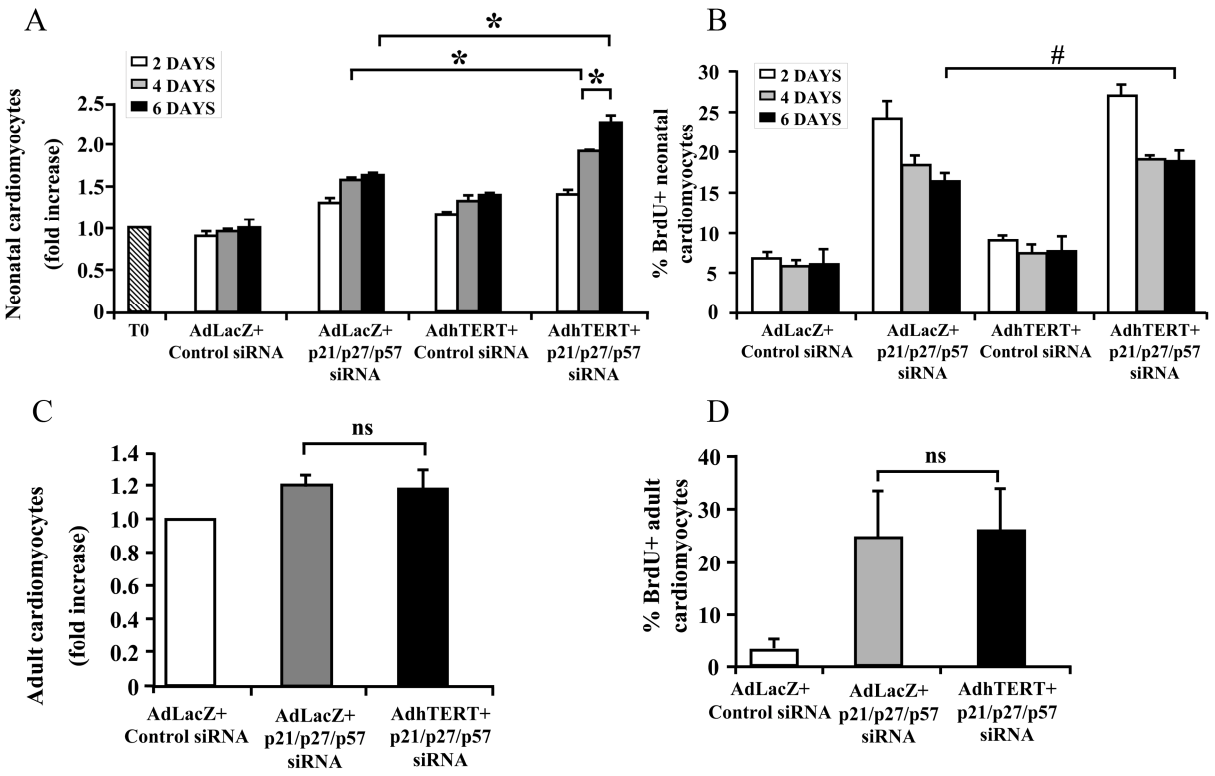

mRNA fold change
10
8
6
4
2
0
p15
p16
p18
p19
p57
*
*
*
T0
Control siRNA
p21/p27 siRNA
Control siRNA
p21/p27 siRNA
Control siRNA
p21/p27 siRNA
2 Days
4 Days
6 Days

FIGURE S6


A
2 DAYS
4 DAYS
6 DAYS
Neonatal cardiomyocytes (fold increase)
T0
AdLacZ+ Control siRNA
AdLacZ+ p21/p27/p57 siRNA
AdhTERT+ Control siRNA
AdhTERT+ p21/p27/p57 siRNA
B
% BrdU+ neonatal cardiomyocytes
#
C
Adult cardiomyocytes (fold increase)
ns
D
% BrdU+ adult cardiomyocytes