Methods

Antibodies

Rabbit polyclonal antibodies against Cdk9 (C-20), cyclin T1 (H-245), Cdk7 (N-19), cyclin H (FL-323), MAT-1 (FL-309), RNAPII (N-20), TBP (SL-1), Gq (C-19), actin (C-11), HSP70 (B-6), PARP (H-250), PGC-1 (H-300), Bcl-2 (N-19) and caspase-3 (H277) were obtained from Santa Cruz Biotechnology. Mouse monoclonal antibodies against phosphoserine-5 (H14) or phosphoserine-2 (H5) of the RNAPII CTD were purchased from Covance.

Histology

Hearts were fixed in 10% formalin overnight at 4˚C, dehydrated with 70% ethanol, mounted in paraffin, and sectioned (5 μm). Sections were stained with hematoxylin and eosin or Gomori-trichrome. Myocyte diameter was measured using transnuclear width at the mid-ventricular level (N = 200 for each condition). For transmission electron microscopy, samples were prepared by standard procedures, sectioned using a RMC MT6000 ultramicrotome and visualized using a Hitachi H7500 electron microscope and 2Kx2K Gatan CCD camera.

Mitochondrial enzyme assays

Hearts from 3 month-old transgenic mice and non-transgenic littermate controls were homogenized on ice in 9 vol of 150 mM sucrose, 2 mM EDTA, 100 mM Tris-HCl, pH 7.45, using a motorized Glas-Col homogenizer, centrifuged at 700 x g for 20 min, and the supernatant was collected. Citrate synthase activity was assayed in 1 mM 5,5'-dithiobis(2-nitrobenzoic acid), 10 mM acetyl CoA, 10 mM oxaloacetate, as the increase in absorbance over two min at 412 nm (Srere, 1969). Complex IV (cytochrome oxidase) activity was determined in 0.01 M potassium phosphate, pH 7.0, 1% reduced cytochrome c, as the decrease in absorbance over three min at 550 nm (Yonetani, 1967). Complex II

(succinate dehydrogenase) activity was assayed in 0.1 M potassium phosphate, pH 7.0, 400 mM succinate, 10 mM KCN, 0.015% 2,6-dichlorophenol-indophenol as the decrease in absorbance over one min at 600 nm (King, 1967). Complex II + III (succinate cytochrome c reductase) activity was assayed in 0.1 M potassium phosphate, pH 7.5, 30 mM succinate, 10 mM KCN, 1 mM cytochrome c as the increase in absorbance over two min at 550nm (King, 1967). Complex I (NADH dehydrogenase) activity was assayed in 0.1 potassium phosphate buffer, pH 7.5, 0.01 M $K_3Fe(CN)_6$, 2mM NADH, as the decrease in absorbance over 2.5 min at 340 nm (King & Howard, 1967). Complex I + III activity (NADH cytochrome c reductase) was measured via the increase in absorbance at 550 nm. The reaction mixture contained 0.1 M potassium phosphate, pH 7.5, 2 mM NADH, 10mM KCN, and 1 mM cytochrome c. The reaction was initiated by the addition of 2 mM NADH and monitored for one min; rotenone was then added to a final concentration of 10 μM and the activity measured for an additional two min. Complex I+III activity was determined as the rotenone-sensitive NADH:cytochrome c reductase activity (King & Howard, 1967).

Mitochondrial DNA

DNA from 3 month-old transgenic mice and non-transgenic littermate controls was analyzed by PCR amplification of a mtDNA fragment corresponding to cytochrome oxidase subunit I (mtDNA), vs a genomic DNA fragment corresponding to the 5S ribosomal subunit, used to control for DNA loading (Huo & Scarpulla, 2001). PCR products were resolved by electrophoresis through 1.2% agarose and visualized with ethidium bromide.

Apoptosis

Hypodiploid cardiomyocytes were identified by two-color flow cytometry, using propidium iodide plus FITC-conjugated MF20 antibody to sarcomeric MHC (Oh *et al*, 2003). To detect the dissipation of mitochondrial membrane potential ($\Delta\Psi m$), cells were incubated for 20 min in 5 μg/ml 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolyl carbocyanine iodide (DePsipher; R & D Systems), then imaged by epifluorescence microscopy (Axioplan 2, Zeiss) using a FITC/Texas Red dual band filter (51006, Chroma). For caspase 3 activity, lysates were incubated with 10 nM DEVD-*p*-nitroaniline (pNA) (Clontech, Palo Alto, CA) in the presence of 1mM DTT for 2 hr at 37 °C. Substrate cleavage was

detected as pNA release using a Beckmann spectrophotometer at 405 nm, calibrated by comparison to known amounts of pNA, and normalized for protein concentration. Full-length and cleaved caspase-3 and PARP were detected by Western blotting. Apoptosis was detected in tissue sections as TUNEL staining, using FITC-conjugated MF20 antibody and the ApopTag in situ apoptosis detection kit (Serologicals Corp.; N = 10,000 myocyte nuclei for each condition).

Chromatin immunoprecipitation

After denaturation at 95°C for 2 min, PCR was performed for 35 cycles (95°C, 1 min; 58 °C, 2 min; 72°C, 1 min) using the following forward and reverse primers, designated by the gene and position: PGC-1 (-83/+81), 5'-GTGAGTGACAGCCCAGCC-3' and 5'-CCAGCTCCTGAATGACGCC-3'; PGC-1 (+655/+778), 5'-CCCACAGAACACAAAACGAC-3' and 5'-ACAGAAGCAAACAGAAAGCC-3'; ANP (-198/-25), 5'-CTTTTCTGCTCTTCTCACACC-3' and 5'-CCTCCCCATTCTGTCACTTG-3'; SERCA2 (-75/+60), 5'-TCTCTCGGCCAATGAGCGG-3' and 5'-GACAGCGGCGGAGGAAAC-3'; albumin (-166/+172), 5'-TGGCAAACATACGCAAGG-3' and 5'-GAAGAGGAGGAGGAGAAAGG-3'.

Supplementary References

Huo L, Scarpulla RC (2001) Mitochondrial DNA instability and peri-implantation lethality associated with targeted disruption of nuclear respiratory factor 1 in mice. *Mol Cell Biol* **21:** 644-654

King TE (1967) Preparation of succinate dehydrogenase and reconstitution of succinate oxidase. *Methods Enzymol* **10:** 322-331

King TE, Howard RL (1967) Preparation and properties of NADH dehydrogenase from cardiac muscle. *Methods Enzymol* **10:** 275-294

Oh H, Wang SC, Prahash A, Sano M, Moravec CS, Taffet GE, Michael LH, Youker KA, Entman ML, Schneider MD (2003) Telomere attrition and Chk2 activation in human heart failure. *Proc Natl Acad Sci U S A* **100:** 5378-5383

Srere PA (1969) Citrate synthase. *Methods Enzymol* **13:** 3-11

Yonetani T (1967) Cytochrome oxidase: Beef heart. *Methods Enzymol* **10:** 332-335

Table I. Echocardiographic findings

Parameter	Genotype		
	ntg	αMHC-cyc T1	αMHC-Gq
Heart rate (min^{-1})	409 ± 28	421 ± 34	292 ± 26*
LV end-diastolic diameter (mm)	3.44 ± 0.12	3.98 ± 0.05*	3.90 ± 0.08*
LV end-systolic diameter (mm)	2.15 ± 0.12	2.3 ± 0.13	2.49 ± 0.10
Fractional shortening (%)	38 ± 2	42 ± 3	36 ± 2
Peak aortic velocity ($cm \cdot sec^{-1}$)	108 ± 3	109 ± 4	111 ± 7
E/A ratio	1.5 ± 0.1	2.6 ± 0.1*†	4.2 ± 0.4*

Age, 3 months; N ≥ 5; * $p < 0.05$ vs control (ntg); † $p < 0.05$ vs αMHC-Gq

Table II. Sequences of the QRT-PCR probes and primers used for this study

Symbol	Forward primer	Probe	Reverse primer
Mouse			
Gapd	ACTGGCATGGCCTTCCG	TTCCTACCCCCAATGTGTCCGTCGT	CAGGCGGCACGTCAGATC
Nppa	GTGTACAGTGCGGTGTCCAA	TGATGGATTTCAAGAACCTGCTAGACCACC	ACCTCATCTTCTACCGGCATC
Nppb	TCACCGCTGGGAGGTCAC	CCCATCCTCTGGGAAGTCCTAGCCA	CCAGCAGCTTCTGCATCTTG
Myh6	GCTGACAGATCGGGAGAATCAG	TCCTGATCACCGGAGAATCCGGAG	TGCAATGCTGGCAAAGTACTG
Myh7	GCATTCTCCTGCTGTTTCCTT	CTCAGGTGGCTCCGAGAAAGGAAGC	TGGATTCTCAAACGTGTCTAGTGA
Acta1	GCTATCCAGGCGGTGCTG	CCCTCTATGCTTCCGGCCGTACCA	AGAATCCAACACGATGCCG
Atp2a2	GTCAAGAAGCTCAAGGAGAGATGG	TCCAACGAATTGCCGGCTGA	CAAGTTCCAGCAAGGTCTTTCC
Ryr2	ATGCTGACGTGTTACATGTTCCA	ATGTATGTGGGCGTCCGTGCTGG	AATTTCATCACCAATGCCCC
Pln	TGCAACATGCCAACTCAGCT	AAAGCCGAGCACTCCGTCATGGG	CAGCGGTGCGTTGCTTC
Gja1	AGGCCGGAAGCACCATC	CAACTCCCACGCCCAGCCGT	GGCTGTCGTCAGGGAAATCA
Hspa1b	GGTGGTGCAGTCCGACATG	CACTGGCCCTTCCAGGTGGTGAA	TTGGGCTTGTCGCCGT
Actc1	CGCCCCTAGCACGCCTA	AGAACCCACCAAAGCTGTGCCAGG	GGTGGTCTCCTCGTCGTCACA
Nkx2-5	CCAGCAGACCCGGCATC	AGCTCAGGCCTCGGGAATGCGA	TGTGGCTGTAATCAAATGGAGG
Mef2c	TCCACTCCCCCATTGGACT	ACCAGACCTTCGCCGGACGAAAG	TGCGCTTGACTGAAGGACTTT
Tbx5	CAGGCTGCCTTCACCCAG	AGGGCATGGAAGGAATCAAGGTGTTTCT	CAGCCACAGTTCACGTTCATG
Srf	GCGGCGCTACACGACCT	CAGCAAGAGGAAGACGGGCATCA	GCGTGGACAGCTCATAGGC
Gata4	CGAGATGGGACGGGACAC	ACCTGTGCAATGCCTGTGGCCTC	TTGATGCCGTTCATCTTGTGA
Ppargc1a	AACCACACCCACAGGATCAGA	CAAACCCTGCCATTGTTAAGACCGAGAA	TCTTCGCTTTATTGCTCCATGA
Nrf1	GATGCTAATGGCCTGGTCCA	TCCCTGTGAGCATGTACCAGACTGTGGT	CCTGGGCGAGGCTGGT
Nrf2	CAGGAGCGCCTTGGCA	CCCCTATGATCCTATACGCTGGTCCACG	CCACCCAATGCAGGACTTG
Tfam	CCACAGAACAGCTACCCAAATTT	AAACACCCAGATGCAAAACTTTCAGAATTGGT	TCCACAGGGCTGCAATTTTC
Ppara	CCTCAGGGTACCACTACGGAGT	CACGCATGTGAAGGCTGTAAGGGCTT	GCCGAATAGTTCGCCGAAA
Cpt1b	CATGGGACTGGTCGATTGC	TCCAGAGATGCCTCCCGGAAAGGT	GGGTCCCAAAGTGGCCA
Cycs	GCGGAAGACAGGCCAGG	TGCTGGATTCTCTTACACAGATGCCAACA	CCCAGGTGATGCCTTTGTTC
Cox5a	GGGATGAATACACTTGTTGGCTATG	TCTGGTTCCTGAGCCCAAAATCATTGA	ACATGCTCGCAATGCAGC
Cox6a2	ACCACGAGCGCCCAGAG	CATCCCGTATCACCACCTCCGCAT	AGGCGAAGGGCTTGGTTC
Atp5c1	TGCCTCGGCCCCTCA	CCCTGAAAATGTACGCCTGCTCCAAG	AGGGAGCGGGTAGAGACGA
Atp5g1	TCGGGCTGTCGGCCT	CGCCGTGCAGCCGCAATG	TTGCCATGTTTCGAACTTGG
Slc25a4	ATCGAGAGGGTCAAACTGCTG	TGCAGGTCCAGCATGCCAGCA	TGTACTGCTTCTCTGCACTGATCTG
Sod2	GCGGTCGTGTAAACCTCAATAAT	TTGTGTCGGGCGGCGTGC	AGCCTCCTGCCCGTGC
Rmrp	GCTCGCTCTGAAGGCCTGT	CCTAGGCTACATACGAGGGACATGTTCCTTATCC	GGGACTTTCCCCTAGGCG
Tfb1m	TGCGTTTCAGTTTCGAAGGA	TATTGCCACCGAGGGCTTGGAATGT	GGCGTTGTGCTTCAGGGA
Tfb2m	TAGAGCCGTTGCCTGATTCTG	TTTGGAGGAGTCGTCCCCGTGG	GCTCCGATCGATTCCTGGA

mt-Co1	TGATTCCCATTATTTTCAGGCTTC	CCCTAGATGACACATGAGCAAAAGCCCA	ACTCCTACGAATATGATGGCGAA
Cyb5	TCGCGGTCCTAGCAATCG	TCACCTCCTCTTCCTCCACGAAACAGG	GAGTTTAATCCTGTTGGGTTGTTTG
Atp5f1	TCTCATCACAAACATTCCCACTG	CACCTTCACCAAAATCACTAACAACCATAAAAGTAA	TTTCGTTCATTTTAATTCTCAAGGG
Bcl2	ACTTCGCAGAGATGTCCAGTCAGC	TGCACCTGACGCCCTTCACCG	TGGCAAAGCGTCCCCTC
Bax	GGAGCAGCTTGGGAGCG	CGGGCCCACCAGCTCTGAACA	AAAAGGCCCCTGTCTTCATGA

Rat

Ppargc1	TGCAGCCAAGACTCTGTATGGA	TGACATAGAGTGTGCTGCCCTGGTTGG	GGCAAAGAGGCTGGTCCTC
NRF-1	TGGAAGACCTGGAGTCTGCG	TGGCAGAGCACGCCCCTGC	GCAGTTCTGAATTAACCTCCTGTG
Tfam	TGTGCGCGGGCTGC	CGCATACCCTCGCCTGTCAGCC	TGGAAAAACACTTCGGAATACAGA
Cycs	AAGGAGGCAAGCATAAGACTGG	CCAAACCTCCATGGTCTGTTTGGGC	GCAGCCTGGCCTGTCTTC
Cox5b	TGTCATCTGGTTCTGGCTGC	CCAAGGCGAGAGCCAGCGATG	TTGTAATGTGTTCCACAGTTGGG
Atp2a2	CGAGCTGTTAATCAAGACAAAAAGAA	TGCTCTTTTCTGGCACAAACATCGCTG	CCACTCCCATAGCTTTGCCA
Nppa	CCGAGACAGCAAACATCAGATC	TGCCCCGACCCACGCCA	TGATGGAGAAGGAGCCCATG
Sod2	TCAGGACCCACTGCAAGGA	CCACAGGCCTTATTCCACTGCTGGG	GCGTGCTCCCACACATCA