Supplementary Table 1.

M-mode ecocardiographic data of FVB/BL-6 WT, myo-Tg and p53 $^{-/-}$/myo$^{+/+}$ mice

	FVB/BL6-WT	myo-Tg	p53$^{-/-}$/myo$^{+/+}$
IVSd: *Interventricular septal thickness end diastole (mm)*	1.0±0.1	0.9±0.08	1.0±0.05
IVSs: *Interventricular septal thickness end systole (mm)*	1.7±0.1	1.3±0.1	1.6±0.06
LVEDD: *Left ventricular end diastolic dimension (mm)*	3.2±0.2	4.8±0.1	3.5±0.2
LVESD: *Left ventricular end systolic dimension (mm)*	1.4±0.1	3.6±0.1	1.5±0.05
PWd: *Posterior wall end diastole (mm)*	1.2±0.1	1.0±0.1	1.1±0.06
PWs: *Posterior wall end systole (mm)*	1.9±0.1	1.4±0.08	1.7±0.05
FS: *Fractional shortening (%)*	54.7±2.9	24.8±2.3	53.2±3.2
EF: *Ejection fraction*	0.93±0.017	0.57±0.08	0.93±0.008
LVmass: *Left ventricular mass (mg)*	120±6.4	200±4.3	120±15.7

Supplementary Methods

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Influence of p53 in the transition of myotrophin-induced cardiac hypertrophy to heart failure

Methods

Animals used

Animals required (*Mus musculus* and *Rattus norvegicus*) for the proposed study were housed and cared for in the AAALAC-approved animal facilities of the Cleveland Clinic, and the studies conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Heath (NIH Publication No. 85-23, revised 1996). All animal studies have been approved by the Institutional Animal Care and Use Committee. For isolation of neonatal rat (*Rattus norvegicus*) cardiomyocytes, 3- to 4-day-old rat pups were obtained from timed pregnant American Wistar rats purchased from Harlan, Inc. (Indianapolis, IN). The rats were fed Teklad regular rat chow (Harlan); mice were fed with Teklad regular mouse chow (Harlan) and given water ad libitum.

To evaluate p53 expression levels and other experiments, we used the following different hypertrophy/HF mouse models and their cardiac tissues. (1) The myo-Tg HF mouse model, in which mice overexpress myotrophin in the heart under the transcriptional regulation of the α-myosin heavy chain promoter. This myo-Tg ($myo^{+/+}$) mouse model was developed in our laboratory, as described previously.[1] These myo-Tg mice show hypertrophy as early as 4 wks of age that progressively transits to HF with severely compromised cardiac function, mimicking the symptoms of human HF;[1,2] (2) the

transverse aortic constriction (TAC) model, an *in vivo* pressure overload mouse model, was obtained from our colleague Dr. Sathyamangala V. Naga Prasad (Department of Molecular Cardiology, Cleveland Clinic, Cleveland, Ohio). To generate the pressure-overload model, TAC was performed as described previously;[3] (3) For conditional overexpression of myotrophin in a cardiac-specific manner, we used a unique Tg mouse model (binary myo-Tg mice) having controlled myo overexpression with a cardiac-specific tetracycline-responsive system (Tet-on/Tet-off; Sudhiranjan Gupta and Subha Sen, unpublished data [2009]). In brief, rat myo cDNA was placed downstream of a promoter responsive to a doxycycline (Dox, a semi-synthetic tetracycline)-regulated transcriptional activator (tTA), and a Tg mouse line ($myo^{+/+}$) was generated. These $myo^{+/+}$ mice were bred with another line harboring cardiac-specific expression of tTA (α-MHC-tTA), and thus binary Tg mice (α-MHC-tTA/myo) were generated. Gene expression was regulated by either administering or removing Dox in the drinking water. Gene expression initiation was achieved by removing Dox (Dox-off; myo gene-on) at three different time points during the progression of CH; these results were compared with results from Dox-on (myo gene-off) binary Tg mice (Sudhiranjan Gupta and Subha Sen, unpublished data, [2009]); (4) p53-null ($p53^{-/-}$) mice[4] were received as a generous gift from Dr. Andrei V. Gudkov (presently at Roswell Park Cancer Institute, Buffalo, NY), and these p53-null mice were used to generate our double-Tg mice ($p53^{-/-}/myo^{+/+}$) as described below.

Generation of double-Tg mouse lines and determination of their genotyping

All required myo-Tg ($myo^{+/+}$) and p53-null ($p53^{-/-}$) mice and wild-type (WT) controls were housed at the Cleveland Clinic's vivarium. We developed our $p53^{-/-}/myo^{+/+}$ double-Tg line by breeding $myo^{+/+}$ and $p53^{-/-}$ mice. We also performed breeding of FVB-WT and BL-6 WT mice to generate crossbred FVB/BL-6 WT mice for our study because the parental

background of myo-Tg and $p53^{-/-}$ mice are FVB and BL-6, respectively. Following the Institutional Animal Care and Use Committee-approved protocol and guidelines, genomic DNA obtained from cells obtained from the tails of of 3-wk-old mice were used as a template for PCR genotype screening with the help of the following primers: p53 primers were designed [see http://www.jaxmice.jax.org] that included (1) Neo1 forward primer: 5' CTT ggg Tgg AgA ggC TAT TC 3'; (2) Neo2 reverse primer: 5' Agg TgA gAT gAC Agg AgA TC 3'; (3) p53 exon 7 reverse primer: 5' ATA ggT Cgg Cgg TTC AT 3'; and (4) p53 exon 6 forward primer: 5' CCC gAg TAT CTg gAA gAC Ag 3'. Myotrophin primers (based on the GenBank accession number U21661) designed for 350 bp amplicon, included (1) forward primer: 5' ATg TgC gAC AAg gAg TTC ATg Tgg gC 3' and (2) reverse primer: 5' TCA CTg gAg AAg AgC TTT gAT TgC 3'. When the p53 exon primers were used, a PCR-amplified product at 600 bp only indicated the genotype for p53 as +/+. Amplicons at both 600 bp and 280 bp were indicative of the +/- genotype, whereas a single product of 280 bp was for the -/- genotype.

Collection of human tissues

All heart tissues used in this study were obtained from the Cleveland Clinic's Cardiac Transplant Program. Protocols (Protocol No. 2378) for tissue procurement were approved by the Cleveland Clinic Institutional Review Board (Principal Investigator Christine S. Moravec, PhD, Director of Basic Research, Kaufman Center for Heart Failure, Cleveland Clinic). The investigation conforms with the principles outlined in the Declaration of Helsinki. Nonfailing human hearts were obtained from unmatched donors who had no history of cardiac disease. Primarily, they were from victims of motor vehicle accidents, gunshot wounds, or cerebral vascular accidents without any known hemodynamic abnormalities. Failing human hearts were obtained from transplant patients who had been diagnosed with dilated cardiac myopathy. Ventricular tissues

were then frozen in liquid nitrogen for storage at –80ºC. The clinical parameters are as described previously.[5]

Preparation of neonatal rat ventricular myocytes

Neonatal rat (*Rattus norvegicus*) cardiomyocytes were isolated from ventricular tissues of 3- to 4-day-old American Wistar rat pups. Timed pregnant American Wistar rats were obtained from Harlan, Inc. The rats were fed TEKLAD regular rat chow, given water ad libitum, and housed in AAALAC-certified facilities. The experimental procedures for animals were performed following institutional guidelines and the investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Heath (NIH Publication No. 85-23, revised 1996).

Media and other reagents for tissue culture were purchased either from Gibco*/* Invitrogen Corporation or from Sigma-Aldrich. Collagenase type-II was purchased from Worthington Biochemicals. Neonatal rat myocytes were isolated and cultured on laminin-coated wells according to the procedure described earlier.[6] In brief, hearts from 3-4 day-old normal American Wistar rat pups were aseptically taken in DMEM-F12 medium (Gibco). The ventricles were separated, minced and incubated. Isolated myocytes were plated (at a density of 10^6 cells/35-mm well) on laminin-coated (20 mg/35-mm well) glass coverslips kept inside the wells. On culture day 3 (or 4), myocytes were incubated in DMEM-F12 medium alone and were used for the experiment. Throughout the experimental procedure bromodeoxyuridine was used at a concentration of 0.1 mM/L to inhibit the growth of cardiac fibroblasts. The myocyte population (~80–90% myocytes) was confirmed by α-actinin staining.

Confocal Imaging to detect activation of p53 by myotrophin

Isolated rat neonatal ventricular myocytes were stimulated with myotrophin and 5-fluorouracil (5-FU, a potent activator of p53)[7] for 24 h; cell fixation and permeabilization were performed according to the protocol of Rouet-Benzineb et al.[8] The anti-p53 rabbit monoclonal and the anti-α-actinin (sarcomeric) mouse monoclonal primary antibody and AlexaFluor®488 goat anti–rabbit and AlexaFluor®568 goat anti-mouse IgG (HL) conjugate secondary antibody were used for staining purposes. Cells were mounted in Vectashield mounting medium containing DAPI. Fluorescence of AlexaFluor®-488 labeled p53 was observed using a Leica TCS-SP-AOBS spectral laser scanning confocal microscope (Leica Microsystems) equipped with UV1364, two argon ion NA 1.4 (488 nm) lasers and one krypton/argon ion laser (568 nm). A PlanApo 63X oil immersion objective lens was used. To compare the staining patterns, images of AlexaFluor®-488 (green), AlexaFluor®-568 (red) and DAPI (blue) channels were merged to determine overlap in the staining patterns of p53, α-actinin, and nucleus, respectively. Three independent experiments (with three replicas for each experiment) were performed.

Quantitation of cell surface area

For measurement of cell surface area of neonatal rat cardiomyocyte [untreated or treated with myotrohin, 5-FU and Ang II(Sar^1)] images were acquired from different areas of the slide using a Leica DMR upright microscope (Heidelberg, Germany), a 20X objective, Retiga EXi CCD digital camera (Q-Imaging, Burnaby, B.C., Canada). For measurement of cell surface area of untreated and treated cardiomyocytes, Image-Pro Plus 6.1 software was used.

Histology

All mice hearts were fixed, paraffin-embedded and cut into 5-μm sections for histological analysis. Sections (taken from same area of the heart of FVB/BL6-WT, myo-Tg and $p53^{-/-}/myo^{+/+}$ double-Tg mice) were stained for hematoxylin/eosin and Masson's trichrome by standard techniques. Images of histological cross-sections (stained for hematoxylin/eosin and Masson's trichrome) were acquired using a Leica DMR upright microscope (Heidelberg, Germany), a 20X objective, RGB color filter, and a Retiga 2000R CCD digital camera (Q-Imaging, Burnaby, B.C., Canada). In all the cases (FVB/BL6-WT, myo-Tg and $p53^{-/-}/myo^{+/+}$ double-Tg mice), the area of cardiac cells was measured in randomly selected fields within the left ventricular area using Image-Pro Plus 6.1 software.

RNA isolation and reverse transcription PCR for gene expression profiling

Total RNA was isolated from heart tissues of mice (three independent samples from myo-Tg and $p53^{-/-}/myo^{+/+}$ mice) by using the TRIzol® (Invitrogen, Carlsbad, CA) extraction method following the company-supplied protocol. Following the isolation, total RNA was purified and subjected to DNAse treatment using the Qiagen RNeasy Kit (Valencia, CA). After analyzing the samples both spectrophotometrically and in formaldehyde-agarose gel to check the quality and integrity of the RNA, the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) was used to synthesize the first strand with oligo-dT primer. For each sample, a total of 5 μg RNA was used. Synthesis of the second strand was performed in a Bio-Rad iCycler (BioRad, Hercules, CA) by using gene-specific primers for myotrophin and atrial natriuretic factor with a Roche PCR Core Kit.[9] A profile of gene expression was then determined by analysis on a 1% agarose gel after equi-volume sample loading using expression of GAPDH as an internal control.

Expression levels were quantified with NIH ImageJ software, and obtained values were expressed in arbitrary units.

p53 Real-Time Reverse Transcription PCR pathway array

A real-time reverse transcription polymerase chain reaction (RT^2-PCR) array was performed at SuperArray Biosciences Corp. (Frederick, MD) for p53 signaling pathway analyses in myo-Tg and $p53^{-/-}/myo^{+/+}$ mice using the RNA obtained from the heart tissues. The RT^2 Profiler PCR Array takes advantage of real-time PCR performance and combines it with the ability of microarrays to detect the expression of many genes simultaneously. RT^2 Profiler PCR Arrays are designed to analyze a panel of genes related to a disease state or biological pathway. To complete the PCR Array procedure, experimental RNA samples were first converted into first-strand cDNA, the template for the polymerase chain reaction, using the RT^2 First Strand Kit. Then templates were mixed with ready-to-use RT^2 qPCR Master Mixes. The mixture was aliquoted into each well of the same plate containing pre-dispensed gene-specific primer sets. After performing PCR, relative expression was determined using the ΔΔCt method. Each array contained a panel of 96 primer sets for a thoroughly researched set of 84 mouse p53 signaling pathway molecules, plus five housekeeping genes and three RNA and PCR quality controls.

Ingenuity Pathway Analysis™ for generating the functional network

Values (fold increase/decrease) obtained from RT^2-PCR array analysis were critically examined by comparing the dataset of WT and $p53^{-/-}/myo^{+/+}$ double Tg mice. A specific cut-off value of 2 was set to identify the gene products with significantly and differentially regulated expression. Those candidate genes, called focus genes, were uploaded to the Ingenuity Pathway Analysis™ (IPA) server to build a functional network based on the

information contained in the Ingenuity Pathways Knowledge Base. Networks of those focus genes, along with other related genes/molecules generated by the Knowledge Base, and were then algorithmically generated based on their connectivity. The network pathway is a graphical representation of the molecular relationships between genes/gene products/molecules, which are represented as nodes. The biological relationship between two nodes is represented as an edge (line). All edges were supported by at least one reference from the literature, textbooks, or from canonical information stored in the Knowledge Base. Nodes are displayed by various shapes that represent their functional class. Edges are displayed with various styles that describe the nature of relationship (such as direct or indirect) between two nodes.[10] From this functional IPA network, nodal molecules were selected for their validation and to postulate the functional role.

Protein isolation and immunoblot analysis for protein expression profiling

Left ventricular tissue from WT and Tg (myo-Tg and $p53^{-/-}$/$myo^{+/+}$ double Tg) mouse hearts (from three independent individuals in each case) was used to isolate the total proteins. After sacrifice, left ventricular tissue of WT and Tg mouse hearts was aseptically excised and washed with cold phosphate-buffered saline (PBS) to remove the blood. The tissue was then minced with a sterile blade and lysed on ice in a buffer containing 10 mM Tris-Cl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, 10% glycerol, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton-X-100, 1 µg/ml leupeptin, 10 µg/ml aprotinin and 1 µg/ml pepstatin. The lysate was centrifuged at 4,000 X *g* for 20 min at 4 °C. The total protein concentration, present in the supernatant fraction, was measured by the Bradford method. Then 50 µg protein samples were separated on 10% sodium dodecylsulfate polyacrylamide gels, and western blotting was performed as described

previously.[5] Membranes were probed with myo primary antibody (BD Transduction Laboratories, Lexington, KY, at 1:250 dilution) and p53 primary antibody (Cell Signaling Technology, Beverly, MA, at 1:1000 dilution) overnight at 4 °C and also with other desired primary antibodies such as Cdkn1a, Bcl2, c-*Myc*, active caspase3 and IκB-α, depending on the experimental requirement. The immunoreactive protein band was visualized using chemiluminescence according to the manufacturer's protocol (NEN). GAPDH primary antibody was used as an internal protein loading control. Expression levels were quantified with NIH ImageJ software, and obtained values were expressed in arbitrary units.

Nuclear protein extraction and electrophoretic mobility shift assay

WT and Tg mouse hearts (three individuals in each experiment for each group) were aseptically removed and washed with cold PBS to remove the blood. Hearts were minced with a sterile blade, and nuclear protein extracts were made according to the method described previously by Dignam et al.[11] All buffers were kept on ice unless stated otherwise. PMSF, DTT, and a protease inhibitor cocktail were added just before use. The nuclear extract was normalized for protein amounts determined by Bradford assay using bovine serum albumin as a standard (Bio-Rad Protein Assay Kit). By using the obtained nuclear protein, an electrophoretic mobility shift assay (EMSA) was performed using a double-stranded p53 and NF-κB oligonucleotide as a probe, as described previously.[12,13] The sequence of p53 probe used was 5' TAC AGA ACA TGT CTA AGC ATG CTG GGG ACT 3' and NF-κB probe was 5' AGT TGA GGG GAC TTT CCC AGG C 3'. A binding reaction was initiated using 25 μg of nuclear extract for p53 EMSA and 10 μg of nuclear extract for NF-κB EMSA made from heart tissue of the WT and Tg mice. Competition assays were performed using a 100-fold molar excess of cold p53 and NF-κB oligonucleotide for p53 and NF-κB EMSA respectively. Supershift

analysis was performed using anti-p53 antibody or anti-p65 antibody as per the experimental requirement. The EMSA profile obtained from three independent experiments was quantified with NIH ImageJ software, and obtained values were documented as arbitrary units.

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