p53 Improves Aerobic Exercise Capacity and Augments Skeletal Muscle Mitochondrial DNA Content


Rationale: Exercise capacity is a physiological characteristic associated with protection from both cardiovascular and all-cause mortality. p53 regulates mitochondrial function and its deletion markedly diminishes exercise capacity, but the underlying genetic mechanism orchestrating this is unclear. Understanding the biology of how p53 improves exercise capacity may provide useful insights for improving both cardiovascular as well as general health.

Objective: The purpose of this study was to understand the genetic mechanism by which p53 regulates aerobic exercise capacity.

Methods and Results: Using a variety of physiological, metabolic, and molecular techniques, we further characterized maximum exercise capacity and the effects of training, measured various nonmitochondrial and mitochondrial determinants of exercise capacity, and examined putative regulators of mitochondrial biogenesis. As p53 did not affect baseline cardiac function or inotropic reserve, we focused on the involvement of skeletal muscle and now report a wider role for p53 in modulating skeletal muscle mitochondrial function. p53 interacts with Mitochondrial Transcription Factor A (TFAM), a nuclear-encoded gene important for mitochondrial DNA (mtDNA) transcription and maintenance, and regulates mtDNA content. The increased mtDNA in p53+/+ compared to p53−/− mice was more marked in aerobic versus glycolytic skeletal muscle groups with no significant changes in cardiac tissue. These in vivo observations were further supported by in vitro studies showing overexpression of p53 in mouse myoblasts increases both TFAM and mtDNA levels whereas depletion of TFAM by shRNA decreases mtDNA content.

Conclusions: Our current findings indicate that p53 promotes aerobic metabolism and exercise capacity by using different mitochondrial genes and mechanisms in a tissue-specific manner. (Circ Res. 2009;105:705-712.)

Key Words: aerobic ■ exercise ■ mitochondrial DNA ■ p53 ■ TFAM

Across populations aerobic exercise capacity inversely correlates with cardiovascular disease and all-cause mortality.1–3 Our report of a marked reduction in the maximal exercise capacity of p53 homozygous knockout (p53−/−) mice and subsequent confirmation by others provided physiological evidence for p53 as an important mediator of aerobic metabolism.4,5 We previously showed that p53 promotes mitochondrial respiration in human and murine cells by regulating the transcription of Synthesis of Cytochrome c Oxidase 2 (SCO2), a gene essential for oxidative phosphorylation.4,6 A concurrent report demonstrating that p53 directly suppresses glycolysis through TIGAR, a p53-dependent regulator of glycolysis and apoptosis, suggested that p53 can coordinate aerobic and glycolytic metabolism.7

Multiple factors contribute to aerobic exercise capacity, but one major determinant is the mitochondrial content of skeletal muscle as demonstrated by a genetic selection experiment.8 A recent study showed decreased mitochondrial density in the skeletal muscle of p53-deficient mice,5 but the genetic mechanism orchestrating this change has remained unclear. A number of other studies have also associated p53 with exercise response and mitochondrial function. For example, p53 levels are increased after acute exercise, and the transition from glycolysis to oxidative metabolism during development is dependent on p53.9,10 p53 can transactivate ribonucleotide reductase p53R2 (RRM2B) which is important for maintaining mtDNA in skeletal muscle.11 Additionally, 2 recent studies have shown asso-
lations between p53 and mitochondrial DNA (mtDNA) content using cell models.\textsuperscript{12,13}

Mitochondrial Transcription Factor A (TFAM) is another essential gene for mtDNA replication and transcription. p53 has been shown to physically interact with TFAM protein for mtDNA maintenance,\textsuperscript{14} however the levels of p53 in mitochondria are normally very low. Here, we report that p53 can regulate TFAM transcription by interacting with its p53 binding site in myoblasts, determines mtDNA content in skeletal muscle, and confers higher maximum exercise capacity, either at baseline or after exercise training. Taken together, these data suggest that p53 affects mitochondrial function through more than one pathway and strengthens its role as a general promoter of aerobic capacity, an important determinant of cardiovascular function and health.

### Methods

#### Animals and Cell Lines

All mice were maintained and handled in accordance with NHLBI Animal Care and Use Committee. p53\textsuperscript{−/−} mice (C57BL/6J strain) were obtained from Jackson Laboratories, and male mice were tested at 8 to 12 weeks of age unless otherwise specified. C2C12 cells were obtained from ATCC, primary myoblasts were isolated from hind limb muscles of 2-week-old mice, and mouse embryo fibroblasts (MEFs) were obtained from 13.5- to 14.5-day embryos as previously described.\textsuperscript{15,16} p53\textsuperscript{−/−} MEFs were transiently transfected with 0.4 μg wild-type or mutant (mt135) p53 plasmid in 6-well plates (Clontech) using Effectene (Qiagen), and TFAM mRNA was quantified by RT-PCR after 24 hours.

#### Mouse Exercise Characterization, Metabolic and Mitochondrial Studies

Details for mouse exercise testing, training, other phenotypic characterization, metabolic and mitochondrial studies are presented in detail in supplemental materials (available online at http://circres.ahajournals.org).

#### Antibodies and Western Blotting

Antibodies were from the following sources: rabbit control IgG serum (SC-2027), rabbit polyclonal anti-p53 (against full-length protein, FL-393, SC-6243), goat polyclonal anti-TFAM (A-17) (Santa Cruz Biotech); rabbit polyclonal anti-PGC-1α (101707, Cayman); rabbit polyclonal anti-p53R2 (Abcam); rabbit polyclonal anti-SCO2 as described\textsuperscript{a}; rabbit polyclonal anti-TFAM (a generous gift from Dr Eric A. Shoubridge, McGill University, Montreal, Canada), and mouse monoclonal antitubulin (Clone B-5-1-2, Sigma) antibodies. Proteins samples were homogenized in ice-cold RIPA lysis buffer with protease inhibitor cocktail (Roche), resolved by Tris-glycine SDS PAGE, and transferred to Immobilon-P membrane (Millipore) for standard ECL Western blotting.

#### Identification of p53 Response Elements and Luciferase Transactivation Assay

The mouse TFAM genomic sequence was obtained from UCSC Genome Browser (http://genome.ucsc.edu/). Putative p53 responsive elements (p53REs) were identified using VectorNTI Advance 10 (Invitrogen). Only those matching more than 80% of the core consensus sequence (5′-RRRCWWGYYY-3′) with 0 to 13 bases between the 2 core binding motifs were evaluated further by reporter assay (R. purine; Y, pyrimidine; W, A, or T).

40-bp-long oligonucleotides containing the putative TFAM p53RE sequence (or mutated core binding sequence RRAWWAWYY) were synthesized, annealed, and cloned into the pTa-luciferase vector (Clontech). Transactivation was measured 36 hours after Lipofectamine 2000 (Invitrogen)-mediated cotransfection with pGL4.74 containing TK promoter and Renilla luciferase as a transfection efficiency control in C2C12 myoblasts. For p53 knockdown experiments, ON-TARGET Plus mouse p53-specific and nonspecific siRNAs (Dharmacon RNAi Technologies) were mixed with the above reporter constructs and cotransfected.

#### Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) assay was carried out using ChIP-IT Express (Active Motif) according to the manufacturer’s protocol. C2C12 myoblasts were treated with 100 μg/mL 5-fluorouracil for 48 hours, fixed in 1% formaldehyde containing complete medium for 10 minutes at room temperature, and sonicated to obtain the nuclear lysates. Rabbit control IgG serum or polyclonal anti-p53 antibody was used at 10 μg/mL concentration to immunoprecipitate the fixed chromatin for PCR amplification. Primer sequences for APOE (nonspecific genomic control), TFAM, and p21 p53REs are provided in online supplemental material.

#### Real-Time PCR mRNA and mtDNA Quantification

Details for mRNA and mtDNA quantification and primer sequences are provided in the online supplemental material.

#### cDNA Transfection or Lentiviral Transduction of C2C12 Cells

Mouse myoblast C2C12 cells were transfected with empty vector or pLEX containing mutated (MUT) or wild-type (WT) p53 using FuGENE 6 (Roche Diagnostics) according to the manufacturer’s protocol and allowed to recover for 24 hours before changing the medium for myotube differentiation. Plasmids containing the sequences for nonspecific shRNA (SHC002, Sigma-Aldrich) and TFAM shRNA (TRCN0000006006, Open Biosystems) (sequences in supplemental material) were used to prepare lentivirus according to manufacturer’s protocol (Sigma-Aldrich). Cells were transduced with lentivirus (MOI ~1) for 24 hours, followed by puromycin selection. For differentiating into myotubes before mtDNA and protein analysis, cells were incubated for 3 days in DMEM containing 2% horse serum.

#### Statistical Analysis

Data are presented as mean±SEM. All probability values were calculated using 2-tailed distribution Student t test and considered to be significant if \(P<0.05\). One-way analysis of variance (ANOVA) was performed for comparisons among the different genotype groups followed by Tukey’s post hoc test using Instat v3.06 software (Graph pad).

### Results

p53 Determines Maximum Exercise Capacity via Modulation of Skeletal Muscle

To quantify the effect of p53 on aerobic exercise capacity, we examined its gene dosage effect on swimming endurance using 3 different genotypes of mice: wild-type (p53\textsuperscript{+/+}), heterozygous (p53\textsuperscript{+/−}) and homozygous (p53\textsuperscript{−/−}) knockout animals. We observed a direct relationship between p53 copy number and swimming duration; relative to p53\textsuperscript{+/+} mice, the swim times were reduced by 22% \(±\) 8% (\(P=0.14\)) and 42% \(±\) 8% (\(P<0.05\)) in p53\textsuperscript{+/−} and p53\textsuperscript{−/−} mice, respectively (1-way ANOVA, \(P<0.05\), Figure 1A). Although the p53\textsuperscript{+/−}
Thus, it appeared that the decreased aerobic capacity of either aerobic or glycolytic muscle groups (Online Figure I). We did not observe any significant differences by p53 genotype in the tibialis anterior (TA) muscle group, which is more glycolytic. Although we observed higher glycogen content in the TA muscle group, this did not correlate with the decrease in exercise capacity. Indeed, the reduction in exercise capacity was not explained by decreased mitochondrial respiration or the observed exercise phenotype. Instead, the decrease in exercise capacity was more dependent on mitochondrial function. Indeed, p53−/− mice were equally capable of high-intensity sprinting as their wild-type counterparts (Figure 1D). This suggests that the decrease in exercise capacity observed in p53−/− mice is not due to a general loss of muscle function, but rather to a specific defect in aerobic capacity.

Figure 1. Aerobic exercise capacity is p53 dependent. A, Effect of p53 gene dose on maximum swimming duration (min). p53 genotype: +/+ (black); +/- (gray); −/− (white). One-way ANOVA, P<0.05. B, Maximum treadmill running capacity in p53−/− (dark) and p53−/− (light) mice expressed as distance (km) and work (J) (n=6 to 8 each). C, Measurements of resting blood lactate levels (mmol/L) under resting conditions (left) and after submaximum exercise (fold-change of resting state; right), n=9 each, mean±SEM. D, Similar sprint capacity as measured by maximum treadmill speed tolerated by p53+/+ (dark) and p53−/− (light) mice, n=9 each.

mice were useful for demonstrating a gene-dosage effect for the swimming test, in subsequent experiments we focused our studies on p53+/+ and p53−/− animals that gave more consistent and significant changes and to avoid the more variable phenotype associated with the haploinsufficient state.

To confirm the observed change in swimming endurance, we used a treadmill running protocol as a different exercise modality and observed a similar reduction in running distance during running and work capacity of p53−/− mice compared to p53+/+ mice (Figure 1B). After submaximum exercise p53−/− mice had up to a 3-fold higher rise in blood lactate compared to p53+/+ mice, providing biochemical evidence that these isogenic mice have decreased aerobic exercise capacity (Figure 1C). Despite the decrease in endurance exercise capacity of p53−/− mice, one prediction would be that these mice have preserved sprint capability, as this form of exercise is less dependent on mitochondrial function. Indeed, p53−/− mice were equally capable of high-intensity sprinting as their wild-type counterparts (Figure 1D). Another question that arises is whether there are changes in the glycogen stores of p53−/− skeletal muscle in association with altered endurance capacity as glycolytic muscles contain higher glycogen levels. Although we observed higher glycogen content in the more glycolytic tibialis anterior (TA) muscle group, we did not observe any significant differences by p53 genotype in either aerobic or glycolytic muscle groups (Online Figure I). Thus, it appeared that the decreased aerobic capacity of p53−/− mice manifests itself only when challenged by maximum endurance testing.

Because p53−/− mice appear overtly normal, with similar body composition and mass to their p53+/+ counterparts (Online Figure II), we closely examined some of the major determinants of exercise capacity. As cardiac output is an important determinant of exercise capacity, we examined both baseline left ventricular function and inotropic reserve using dobutamine MRI (Figure 2A). No significant differences were detected between p53+/+ and p53−/− mice in cardiac function nor in the gross functioning of their neurovascular and vascular systems as assessed by motor coordination and muscle strength, fiber type composition, and capillary density of both aerobic and glycolytic skeletal muscle groups (Figure 2A through 2E). Other factors such as hematocrit and hemoglobin concentration were also similar between p53+/+ and p53−/− mice; 48±2% (14.9±0.4 g/dL) and 49±3% (15.6±0.7 g/dL), respectively. The absence of significant alterations in these important physiological parameters in p53−/− mice was striking given the profound reduction in exercise capacity, and underscored the potential contribution of p53-dependent regulation of mitochondrial respiration to the observed exercise phenotype.

Training Accentuates the Lower Exercise Capacity of p53−/− Mice
As skeletal muscle is a major determinant of aerobic exercise capacity and exercise training increases mitochondrial bio-
The relatively greater increase in blood lactate levels of p53−/− mice compared to p53+/+ mice in the trained versus untrained group further complemented the differences observed in RER (Figure 3D). Collectively, these findings reemphasized that p53 is required to complete the adaptive changes in aerobic metabolism that are necessary to increase exercise capacity in response to training. It is also notable that the relative difference in peak VO₂ between trained p53+/+ and p53−/− mice and its impact on exercise capacity were comparable to a model of exercise enhancement by the overexpression of mitochondrial biogenesis regulator PGC-1α.18

To understand the biology underlying the increase in aerobic capacity after training, we examined skeletal muscle mitochondrial oxygen consumption in untrained and trained states. When normalized to skeletal muscle mass to account for differences in tissue mitochondrial volume density, we observed increases in mitochondrial state 3 respiration in the setting of similar respiratory control ratios (RCR) that correlated well with peak work capacity and VO₂ changes (Figure 3A, 3B, and 3E). The higher oxygen consumption in p53−/− mice was consistent with the recently reported increase in mitochondrial volume density in the mixed fiber gastrocnemius muscle measured by electron microscopy,5 which we also confirmed in the more aerobic soleus muscle group (Online Figure III). In addition to increased mitochondrial density, succinate dehydrogenase staining of plantaris, another aerobic muscle group, and gastrocnemius supported relatively increased mitochondrial oxidative capacity in p53−/− compared to p53+/+ skeletal muscles (Figure 3F).

**p53 Interacts with TFAM Gene**

We had previously reported that decreased oxygen consumption in p53−/− liver mitochondria was primarily mediated by SCO2.4 However, unlike in liver, mitochondria prepared from p53−/− skeletal muscle did not show a significant decrease in SCO2 mRNA or protein levels (Online Figure IVA). These findings suggested that in skeletal muscle p53 affects mitochondrial function by regulating the expression of proteins other than SCO2. Although p53-dependent ribonucleotide reductase (RRM2B, p53R2) was an obvious candidate gene given its previous association with skeletal muscle mtDNA homeostasis,11 we did not observe significant differences in p53R2 levels or in the expression of 2 additional p53 targets ferredoxin reductase (FDXR) and Tpf3-induced glycosylation and apoptosis regulator (TIGAR) previously associated with mitochondrial and metabolic functions (Online Figure IVB to IVD).7,10 We also failed to detect a significant effect of p53 genotype on the abundance of mitochondrial biogenesis regulators PPARγ coactivator-1α (PGC-1α), PGC-1β, NRF1, and NRF220 and representative members of both nuclear- and mitochondrial-encoded respiratory chain components at baseline or after exercise (Online Figure V). These negative results led us to speculate the involvement of other factors in skeletal muscle.

Mitochondrial biogenesis is also mediated by mitochondrial transcription factors (TFAM, TFBS).21 The central importance of TFAM to the transcription and maintenance of mtDNA, which is correlated to aerobic exercise capacity in both mouse and man, made it an attractive candidate for mediating some of the effects of p53.18,22 Indeed, in silico
p53 Regulates TFAM Expression and mtDNA Content in Skeletal Muscle

Under basal (ie, no exercise) state, the absence of p53 reduced both TFAM mRNA and protein levels in soleus skeletal muscle but not in heart or liver, supporting our in vitro interaction studies (Figure 5A and 5B). TFAM protein in liver is undetectable at this level of sensitivity consistent, with its low mtDNA content shown in Figure 6A; TFAM resolves as a single or double band in mouse skeletal muscle or heart tissue, respectively.) C, Acute exercise (14 m/min for 1 hour) increases TFAM mRNA expression in soleus muscle of p53^{-/-} mice, **p<0.01 vs preexercise level; n=3 to 5. D, Relative TFAM mRNA expression in soleus 12 hours after acute exercise. *P<0.05 between corresponding p53^{+/+} (dark) and p53^{-/-} (light) mice; n=6. Mean ± SEM.

mutant p53 into p53^{-/-} MEFs. The expression of wild-type p53 significantly increased TFAM transcript levels relative to mutant p53 (Figure 4D). A corresponding increase in the transcript level of p21, the prototypical target of p53, was also observed. To establish that p53 interacts with the putative p53 binding sequences in TFAM, we performed ChIP in C2C12 murine myoblasts. In contrast to nonspecific control IgG, anti-p53 antibody pulled down the TFAM p53RE-3 genomic fragment detected by PCR amplification, with the full-length p53 (Anti-p53) were used for immunoprecipitation; INPUT, crude chromatin. *P<0.05. Mean ± SEM.

TFAM gene structure

Figure 4, p53 interacts with TFAM gene. A, Genomic structure of mouse TFAM with seven exons (black boxes). The putative p53 responsive elements (p53REs) relative to bp +1 position of the ATG start site are: 1, +2475 to +2497; 2, +6538 to +6568; 3, +7087 to +7110; and 4, +11631 to +11657. Consensus matching p53RE bases are shown in capital letters (ID, % identity), R, purine; Y, pyrimidine; W, A, or T base. B, Transcriptional activities of wild-type (WT) or mutated (MUT) p53REs were determined by luciferase reporters after transfection into C2C12 myoblasts. C, Knockdown of endogenous p53 decreases p53RE luciferase activity. The p53RE-3 luciferase construct was cotransfected with indicated p53-targeted and nonspecific (NS) siRNA (100 nmol/L total). D, TFAM mRNA response to transient transfection of exogenous wild-type or mutant p53 into transient transfection of exogenous wild-type or mutant p53 into C2C12 myoblasts. E, ChIP assay to demonstrate in vivo interaction between p53 protein and TFAM p53RE-3 in C2C12 myoblasts. Nonspecific (NS) genomic and TFAM p53RE-1 primers were used as negative controls; the nonspecific genomic region in the APOE gene and TFAM gene promoter serving as positive control (Figure 4E). A full-length p53RE-3 luciferase reporter plasmid was transfected into C2C12 myoblasts. In contrast to nonspecific control IgG, anti-p53 antibody pulled down the TFAM p53RE-3 genomic fragment detected by PCR amplification, with the p21 gene promoter serving as positive control (Figure 4E). A nonspecific genomic region in the APOE gene and TFAM p53RE-1 (unresponsive to p53 by luciferase assay) served as negative controls. In summary, these data supported the notion that p53 is capable of interacting with and regulating the expression of the TFAM gene, thereby contributing to mitochondrial biogenesis.
decreased levels of both in the content of p53 in C2C12 myoblasts which mimicked the reduced mtDNA explored by the stable shRNA-mediated depletion of TFAM. 

A, Relative mtDNA content expressed as a function of total genomic DNA (nDNA) in various skeletal muscle (SKM) groups, heart (HRT), and liver (LIV) of p53+/− (dark) and p53−/− (light) mice. Aerobic muscle groups are represented by soleus (SOL) and plantaris (PLT); glycolytic muscle groups are represented by tibialis anterior (TA) and extensor digitorum longus (EDL). n=5 each. B, Relative mtDNA content and corresponding TFAM protein levels of primary mouse myoblasts. C, Stable knock down of TFAM by shRNA concomitantly decreases TFAM protein and mtDNA levels in C2C12 cells. D, Conversely, transient overexpression of wild-type (WT) but not mutant (MUT) p53 in C2C12 cells increases TFAM protein and relative mtDNA content in parallel. *P<0.05; **P<0.01; n=3. Mean±SEM.

12 hours after exercise stimulation (Figure 5C), at which point the relative increase in TFAM mRNA was comparable in p53+/− and p53−/− mice (Figure 5D). Although these data suggest that factors other than p53 such as p38 MAPK and PGC-1α are important in initiating the exercise-induced change in TFAM expression, p53 may enhance its expression.

We measured the tissue content of mtDNA relative to nDNA and observed significantly lower levels in various skeletal muscle groups of p53−/− mice (Figure 6A). A notable pattern emerged: the relative difference in mtDNA content associated with p53 copy number was greater for aerobic muscles (soleus, plantaris) versus glycolytic muscles (tibialis anterior, extensor digitorum longus; P<0.01 between soleus and EDL), suggesting the adaptive changes for aerobic metabolism in muscle may require p53. In contrast, heart and liver mtDNA content was not significantly affected further demonstrating that p53 may modulate various aspects of mitochondrial function in a tissue-specific manner (Figure 6A). To control for the potential confounding effects associated with cellular heterogeneity in tissues, we analyzed mtDNA content and TFAM protein levels in myoblasts derived from p53+/− and p53−/− mice and observed similarly decreased levels of both in the p53−/− state (Figure 6B). The relationship between p53, TFAM, and mtDNA was further explored by the stable shRNA-mediated depletion of TFAM in C2C12 myoblasts which mimicked the reduced mtDNA content of p53−/− myoblasts, whereas the transient overexpression of wild-type, but not mutant, p53 increased TFAM protein and mtDNA levels in parallel (Figure 6C and 6D). These findings were consistent with well-established mouse genetic studies showing that deletion of the TFAM gene results in mitochondrial DNA depletion and dysfunction, whereas overexpression of TFAM leads to increased mtDNA content.24,25

Discussion

Here, we have established that p53 determines maximum aerobic exercise capacity and regulates mtDNA content in skeletal muscle. Given the tight concordance between TFAM and mtDNA,24,25 p53 interaction with the TFAM regulatory sequences may contribute to the observed differences in mtDNA content of skeletal muscle. The effect of p53 on TFAM and mtDNA appears to be tissue-specific and distinct from what we have previously described for p53 regulation of SCO2 in cytochrome c oxidase assembly in liver.4

The mechanisms underlying the tissue-specific alterations in mtDNA content are likely to be complex because of the interaction of p53-dependent and independent components expressed in tissues relevant to exercise physiology. p53 has many transcriptional target genes and indirectly influences the expression of many others,26 thus our proposed regulation of the mitochondria by p53 in mouse skeletal muscle is unlikely to be the sole mechanism. Rather than a promoter, the p53 binding site in TFAM likely serves as a regulatory element such as an enhancer given its location in an intron 8 kb downstream of the transcription start site.26,27 It should be noted that a recent publication did not detect significant differences in TFAM mRNA levels in p53 knocked down cells,12 which is not consistent with the lower levels that we observed in p53−/− adult mouse skeletal muscle. However, the knock down of p53 in cultured fibroblasts promotes cell proliferation, which likely impacts mitochondrial biogenesis including TFAM expression by known regulators such as NRF1 and NRF2.20 Thus, this discrepancy highlights the importance of considering the cellular context of gene expression in comparing in vitro to in vivo observations.

An additional layer of complexity is introduced by the translocation of p53 to the mitochondria and its potential interaction with TFAM or other components important for mtDNA maintenance.28–32 However, the greater abundance of TFAM protein compared to the relatively negligible pool of mitochondrial p53 under normal state raises questions about their stoichiometric relationship.33 Our observation that p53 can enhance TFAM transcription provides a genetic insight into skeletal muscle mitochondria biogenesis, an important determinant of exercise capacity. Although we have proposed a transcriptional mechanism for TFAM regulation by p53, there may be additional mechanisms by which p53 and TFAM interact.14

A recent study has demonstrated the proteomic diversity of tissue mitochondria which may reflect the need for mitochondria to fulfill functions that are unique to different tissues.34 Whereas liver mitochondria mainly serve biosynthetic or general metabolic functions, skeletal muscle mitochondria are suited for oxidative phosphorylation to supply energy for contractile work. Thus, mitochondria are likely to have different regulatory requirements depending on their func-
tion. We propose that the targeting of SCO2 and TFAM by p53 exemplify 2 distinct pathways by which it may influence mitochondrial biogenesis in a tissue-specific manner. p53 regulation of TFAM expression may contribute by augmenting the overall program of mitochondrial biogenesis initiated by p53-independent mediators of exercise training such as p38 MAPK and PGC-1α.

There is increasing evidence that link mitochondrial function with other well-known regulators of cell growth. These include mitochondrial regulation by ataxia-telangiectasia mutated kinase (ATM) via ribonucleotide reductase R1 subunit,35 retinoblastoma (Rb) via mitochondrial biogenesis co-activator PGC-1α,36 and PTEN via mitochondrial protein PTEN-induced kinase 1 (PINK1).37 Studies showing the role of mitochondrial uncoupling proteins in both cardiovascular diseases and cancer further indicate the importance of mitochondrial function in these processes.38,39 It is conceivable that the optimization of mitochondrial function that reduces reactive oxygen species (ROS) generation, DNA damage, or bioenergetic stress ultimately result in improvement of exercise capacity. Such mitochondrial adaptations in a wide range of chronic conditions such as atherosclerosis and congestive heart failure would appear important. Thus, understanding the molecular basis for maintaining or expanding aerobic capacity may thereby provide further insights into a host of debilitating cardiovascular diseases as well as potential therapeutic strategies against them.

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Disclosures
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References


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MATERIAL AND METHODS

Mouse Exercise Testing and Blood Lactate Measurements
Maximum duration of swimming exercise was measured in a temperature-controlled (34°C), adjustable-current tank as previously described. Briefly, mice were acclimated to swimming for 10 min/day for 3 d and swimming duration measured to the point of exhaustion. For graded maximal treadmill exercise, mice were acclimated by running for 10 min at 10 m/min for 3 d and maximum exercise capacity determined by graded increase in treadmill speed (10, 12 and 15 m/min for 3-5 min at each speed followed by 1.8 m/min increase every 3 min) on a 10% incline to exhaustion. Work (J) = Force (body weight (kg) X 9.8 m/sec²) X Vertical distance (sin (5°) x speed (m/min) x time (min)). Exercise training was initiated in 8 week-old mice at 10 m/min for 40 min on a 6-lane treadmill and gradually increased to 14 m/min for 90 min, 5 d/wk for 5 wk (final age ~14 wk old). For high intensity exercise, mice were treadmill acclimated for 3 d and sprint capacity determined by rapid ramping speed protocol (1 m/min every 30 sec on a 10% incline) to exhaustion. Blood lactate levels at rest and after sub-maximum (~70% of p53+/+ capacity, ~22 min) exercise were measured using the LactatePro analyzer (Arkray).

Body Mass Composition
Body composition (muscle, fat and free fluid) was measured in non-anesthetized mice using the Bruker Minispec NMR analyzer mq10 (Bruker Optics).

Assessment of Cardiac Function by Dobutamine MRI
Cardiac MRI was performed at rest and during dobutamine stress. Mice were anesthetized with isoflurane, placed prone on a plastic cradle, head stabilized with a holder-bite bar and nosecone for isoflurane delivery, hydrated with subcutaneous 0.9% saline (~0.5-1 ml/25 g mouse), and monitored with a pressure transducer for respiration and rectal temperature probe. Conductive leads were placed for ECG acquisition. The mouse cradle and apparatus were placed in a volume MR coil with warm air blower for body temperature control. A contrast agent (Gd-DTPA, Berlex) was delivered at 0.3 mmol/kg i.v. by tail vein cannulation. Short axis cine cardiac MRI scans were obtained: repetition time 9-10 ms, echo time 3.4 ms, 4-5 averages, 10 or more frames depending on heart rate. Cine scans were obtained on 3 slices at mid-ventricle at baseline and during dobutamine stress. Dobutamine was administered in two steps at 10 and 40 μg/kg/min by tail vein catheter. Ejection fractions were calculated for baseline and stress steps using CAAS_MRV_FARM software (Pie Medical Imaging, Netherlands).

Neuromuscular and Strength Testing
Motor coordination was assessed by latency to fall using the rotarod (Rota-Rod, UGO Basile). Rotarod performance was measured for four consecutive days with four trials per day. Rotating rod speed was gradually increased from 2-20 rpm or 4-40 rpm and endpoint was time to fall or up to 600 sec.

As a general assessment of skeletal muscle strength, mouse forelimb grip strength was assessed by using a digital grip strength meter (Columbus Instruments). Mice were acclimated for two consecutive days prior to the experiment days. Each mouse was held by the tip of the tail and lowered toward the grip triangle bar and was allowed to grasp it with its forepaws. The mouse was then pulled steadily by the tail away from the triangle bar until the mouse’s grip was broken. The force exerted on the gauge at the time the grip was broken was recorded from the strength meter. Each mouse was given three trials approximately 10 min apart. This was repeated the next day and the results were an average of the two days for a total of six trials.
Muscle Fiber Typing and Capillary Density by Immunofluorescence
Muscle tissue was frozen in Tissue-Tek OCT compound (Sakura Finetek) by placing on partially frozen isopentane in liquid nitrogen. Muscle sections (8 µm) were cut using a cryostat (Leica). For muscle fiber typing, tissue sections were fixed in 4% paraformaldehyde for 15 min, permeabilized in 0.1% Triton X-100 for 20 min and incubated with goat polyclonal antibodies against MHC I (slow) or MHC II (fast) fibers (Novocastra) at room temperature.

To assess for capillary density, tissue sections were fixed in 0.4% paraformaldehyde for 10 min, permeabilized in 0.1% Triton-X for 20 min and incubated with anti-mouse CD31 antibody (BD Pharmingen). Secondary antibodies conjugated to Alexa Fluor 594 (Molecular Probes) were used for visualization of primary antibody binding.

Peak Vo2 and Respiratory Exchange Ratio (RER)
Peak Vo2 and RER were measured using indirect calorimetry with single-lane sealed treadmill apparatus (Columbus Instruments) as previously described. The calorimeter was calibrated before each testing session using standard gases (20.5% O2 and 0.5% CO2) and air flow rate through the sealed treadmill was set at 0.6 L/min. Mice were acclimated to the sealed treadmill for 3 days. After 5 min of baseline gas collection, the maximum exercise protocol was initiated at 10 m/min at 10% incline with a graded increase in speed by 1 m/min every 1.5 min until exhaustion. Peak Vo2 was defined as O2 consumption at maximum exercise per kg body mass per h (ml/kg/h). O2 consumption and CO2 production were collected every 30 seconds during exercise to calculate the RER.

Mitochondria Purification and Oxygen Consumption
After mice were euthanized and perfused with ice-cold PBS, skeletal muscle mitochondria were isolated using standard protocols as described. Oxygen consumption was measured using a Clark-type oxygen microelectrode at 30°C as previously described. Respiration was initiated by adding 10 mM glutamate, 2 mM malate and 0.8 mM ADP. Final oxygen consumption was adjusted to the weight of skeletal muscle tissue to reflect differences in mitochondrial content (nmol O2/min/g tissue).

Antibodies and Western Blotting
Antibodies were from the following sources: rabbit control IgG serum (SC-2027), rabbit polyclonal anti-p53 (against full-length protein, FL-393, SC-6243), goat polyclonal anti-TFAM (A-17) (Santa Cruz Biotech); rabbit polyclonal anti-PGC-1α (101707, Cayman); rabbit polyclonal anti-p53R2 (Abcam); rabbit polyclonal anti-SCO2 as described; rabbit polyclonal anti-TFAM (a generous gift from Dr. Eric A. Shoubridge, McGill University), and mouse monoclonal anti-tubulin (Clone B-5-1-2, Sigma) antibodies. Proteins samples were homogenized in ice cold RIPA lysis buffer with protease inhibitor cocktail (Roche), resolved by Tris-glycine SDS PAGE, and transferred to Immobilon-P membrane (Millipore) for standard ECL western blotting.

Histochemistry
Glycogen was detected in 10% formalin fixed tissue sections with periodic acid Schiff staining using a PAS stain kit (Polysciences) with and without diastase digestion. Acidified Harris hematoxylin was used as a counterstain. Succinate dehydrogenase staining was performed using nitro blue tetrazolium in frozen tissue sections as previously described. For all histochemical techniques, the p53+/+ and p53/- muscle sections were placed on the same glass slide, thus, all pairs underwent identical incubation conditions and washes.

Electron Microscopy
Muscle tissue was fixed with glutaraldehyde and paraformaldehyde, post-fixed with osmium tetroxide, stained en block with uranyl acetate, ethanol dehydrated and Epon embedded
(Electron Microscopy Sciences). 60 nm thick sections were cut on a Sorvall MT2 ultramicrotome, stained with uranyl acetate and lead citrate and viewed with a JEM 1200 EXII electron microscope (JEOL USA) equipped with and AMT XR-60 digital camera (Advanced Microscopy Techniques). The volume density of mitochondria was estimated using the point-counting method.10,11

Chromatin Immunoprecipitation (ChIP) Assay Primer Sequences
The genomic locations (relative to bp +1 position of the ATG start site) of the putative p53 responsive elements (p53REs) in the TFAM gene are as follows: p53RE-1, +2475 to +2497; p53RE-2, +6538 to +6568; p53RE-3, +7087 to +7110; and p53RE-4, +11631 to 11657. Real-time PCR primer pair sequences:
TFAM p53RE-1 (intron 3) nonspecific control,
Forward (F) 5' - GCTTCCTGACAGTTGTAGATAGGG-3'
Reverse (R) 5' - GCTCTGAGATGCTCAGGGTC-3';
TFAM p53RE-3 (intron 5),
F (+7017 to +7037) 5' - CTGCAAACACCCCCACCCAGAC-3',
R (+7197 to +7172) 5' - CTATGTGAGGGATATACAACCTGCTGAC-3';
APOE nonspecific control,
F 5' - GCCTAGCGAGGGAGAGCCG-3',
R 5' - TGACTCTGGGAGCTGCAGC-3';
p21 p53RE,
F (-7600 to -7580) 5' - GCAAGGCTGCATCAGTCCTCC-3',
R (-7351 to -7373) 5' - GGCTCTGCTCTCCATTCTGCT-3'.

Real-time PCR mRNA Quantification
Total RNA was isolated from tissues using the RNeasy Kit (QIAGEN), cDNA synthesized on poly-dT magnetic beads by reverse transcription (Superscript II, Invitrogen) and quantified by real-time RT-PCR using SYBR green fluorescence on the 7900HT Sequence Detection System (Applied Biosystems) as previously described.12 Cycle threshold (Ct) values were normalized to the housekeeping gene eukaryotic translation initiation factor EIF35S (TIF). Primer sequences are provided below:

TIF,
F 5' - CTGAGGATGTGCTGTCTGGGAA-3',
R 5' - CTTTTTGCTCCACTCTGGT-3';
TFAM,
F 5' - CTGATACCAGCCATCTTTCTGGTC-3';
R 5' - CCAACTTTGCTCCACTCTGCTTC-3';
p53R2,
F 5' - CGGTTACCTGATTTGTAAGG-3',
R 5' - CCAGTGCTCAGTCTCC-3';
SCO2,
F 5' - CAGCCTGTCTTCATCAGTCTTGGA-3',
R 5' - GACACTGGAAGCCAGCTATAGTGCC-3';
FDXR,
F 5' - CATGAGACAGACAGCCTTCTCAC-3',
R 5' - CAGCAGCCAGCAGCATCTCTTC-3';
TIGAR,
F 5' - ACTGAGAAGGAGCACTGAGTG-3',
R 5' - CGGATGCTGCAAGCCTCAATGTC-3';
PGC-1α,
F 5’-ACGGTTTACATGAACACAGCTGC-3’,
R 5’-CTTGTTCGTTCTGGTCCACAGAGCTGC-3’;
PGC-1β,
F 5’-ATACCTCAGACAGACCCCTTCC-3’,
R 5’-ACAGAAGGAATTCAGGTTCCAGGC-3’;
NRF1,
F 5’-GAACCACCCGGATTTCACTGTC-3’,
R 5’-CCCTACCACCCCACTGAACTTG-3’;
NRF2,
5’-GGCACAGTCGCTTATGCCGTG-3’,
5’-CCAGCTCGACATGTTCTGGTCGAC-3’;
CYCS,
F 5’-TTGACCAGGCCGCAAGCAGA-3’
R 5’-GCTATTAGGTCTGCCTCTCTCCC-3’
COX4i1,
F 5’-TCGAGAGCTTCGCCGAGATGAAC-3’,
R 5’-GCAGCTCTCCAGATGCCGAGG-3’;
ND1,
F 5’-AATCGCCATAGGCTTCTCTACATGTAAT-3’,
R 5’-GGCGTCTGCAAATGGTTGTAA-3’;
COX2 (MTCO2),
F 5’-CCATAGGGCCATAGGATGATGATTGATCAT-3’
R 5’-AGTCCGGGCTTGGATGGATTCAT-3’

mtDNA Content Quantification
Mitochondrial DNA was co-purified with genomic DNA from mouse tissues using the DNeasy kit (QIAGEN), Ct values determined for MTCO2 gene encoded by mtDNA and 18S rRNA gene encoded by the nuclear DNA, and the relative mtDNA copy number calculated by normalizing to 18S rRNA gene copy number. Primer sequences for MTCO2 and 18S rRNA are provided below:

MTCO2,
F 5’-CCATAGGGCCACCAATGATACTG-3’,
R 5’-AGTCCGGCCAGGATGCACTG-3’;
18S rRNA,
F 5’-CTTAGAGGGACAAGTGGCGTTC-3’
R 5’-CGCTGAGCCGAGTAAGGTAATG-3’.

shRNA Sequences
Non-specific shRNA,
5’-ccggCAACAAGATGAGGACACCAActcagttggtgcctctctgcttgaatttt-3’,
TFAM-specific shRNA,
5’-ccggCGGAGACATCTCTGAGCATATcagtaatgctcagagtctctccgttttg-3’. 
ONLINE FIGURE LEGENDS

Online Figure I.
Muscle glycogen content is unaffected by p53 genotype. Glycogen was stained using the PAS method on p53+/+ and p53-/− soleus (SOL), tibialis anterior (TA), and gastrocnemius (GAS) with (+Diastase, right panel) or without (left panel) glycogen digestion using diastase. Magnification, 20X.

Online Figure II.
Morphology and body mass composition are similar in p53+/+ and p53-/− mice. A, Morphology of 10-week old age-matched male p53+/+ and p53-/− littermates (C57BL6/J background). B, Body mass (g) of p53+/+ and p53-/− mice, n = 5 each. C, Body mass composition (muscle, fat and free fluid) of p53+/+ (dark) and p53-/− (light) mice determined by NMR in non-anesthetized age- and sex-matched littermates, n = 5 each. P-values were non-significant; data shown as mean ± SEM.

Online Figure III.
Decreased mitochondrial density in p53-/− mouse soleus muscle. A, Representative images of transmission electron micrographs of intermyofibrillar mitochondria in p53+/+ and p53-/− mouse soleus muscles. Upper panel, 15000X; lower panel, 5000X. Scale bars, 1 µm. B, Estimated relative volume density of mitochondria. **P < 0.01; n=3. Data shown as mean ± SEM.

Online Figure IV.
p53R2, SCO2, FDXR and TIGAR expression levels are unchanged by p53 genotype. A, SCO2 mRNA (left panel) and protein (right panel) expression levels in soleus of p53+/+ and p53-/− mice. B, Relative p53R2 mRNA levels in soleus, heart and liver by RT-PCR. C, p53R2 protein expression in soleus, heart and liver tissues by western blotting. D, Relative FDXR (left panel) and TIGAR (right panel) mRNA levels. Tubulin levels are shown as protein loading control (A and C). p53+/+ (dark) and p53-/− (light) mice; data shown as mean ± SEM.

Online Figure V.
PGC-1α, other mitochondrial biogenesis regulators and mitochondrial respiratory subunit gene expression are not significantly affected by p53 genotype at baseline or after acute exercise. A, Basal PGC-1α protein levels detected by western blotting in soleus and gastrocnemius (Gastroc) of p53+/+ and p53-/− mice. The positive control mouse myoblast C2C12 cell protein lysate shows a strong band at 96 kDal as specified by the manufacturer. B, Time course of PGC-1α mRNA expression in response to acute exercise in p53+/+ and p53-/− soleus muscle. C, PGC-1β, other biogenesis regulators, and representative nuclear and mitochondrial genome-encoded component mRNA levels are not significantly affected by p53 genotype at baseline or after acute exercise. *P < 0.05 versus p53+/+ pre-exercise; n = 3. p53+/+ (dark) and p53-/− (light) mice; data shown as mean ± SEM.
REFERENCES


Online Figure I. Muscle glycogen content is unaffected by p53 genotype. Glycogen was stained using the PAS method on p53+/+ and p53−/− soleus (SOL), tibialis anterior (TA), and gastrocnemius (GAS) with (+Diastase, right panel) or without (left panel) glycogen digestion using diastase. Magnification, 20X.
Online Figure II. Morphology and body mass composition are similar in p53+/+ and p53/- mice. A, Morphology of 10-week old age-matched male p53+/+ and p53/- littermates (C57BL6/J background). B, Body mass (g) of p53+/+ and p53/- mice, n = 5 each. C, Body mass composition (muscle, fat and free fluid) of p53+/+ (dark) and p53/- (light) mice determined by NMR in non-anesthetized age- and sex-matched littermates, n = 5 each. P-values were non-significant; data shown as mean ± SEM.
Online Figure III. Decreased mitochondrial density in p53−/− mouse soleus muscle. A, Representative images of transmission electron micrographs of intermyofibrillar mitochondria in p53+/+ and p53−/− mouse soleus muscles. Upper panel, 15000X; lower panel, 5000X. Scale bars, 1 µm. B, Estimated relative volume density of mitochondria (%). **P < 0.01; n=3. Data shown as mean ± SEM.
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