Identification of bovel mechanisms mediating skeletal muscle atrophy

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University of Iowa

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IDENTIFICATION OF NOVEL MECHANISMS MEDIATING
SKELETAL MUSCLE ATROPHY

by

Daniel Kenneth Fox

A thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Molecular Physiology and Biophysics
in the Graduate College of
The University of Iowa

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This is to certify that the Ph.D. thesis of

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has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Molecular Physiology and Biophysics at the May 2016 graduation.

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To my family, friends, and colleagues.
Any intelligent fool can make things bigger, more complex, and more violent. It takes a touch of genius -- and a lot of courage -- to move in the opposite direction.

E.F. Schumacher
Small is Beautiful
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ABSTRACT

Skeletal muscle atrophy is a common, debilitating consequence of muscle disuse, malnutrition, critical illness, musculoskeletal conditions, neurological disease, cancer, and organ failure. Despite its prevalence, little is known about the molecular pathogenesis of this devastating condition due in large part to an incomplete understanding of the molecular mechanisms that drive the atrophy process.

In previous studies, we identified the transcription factor ATF4 as a critical mediator of skeletal muscle atrophy. We found that ATF4 is necessary and sufficient for skeletal muscle atrophy during limb immobilization. However, ATF4 mKO mice were only partially protected from skeletal muscle atrophy during limb immobilization, indicating the existence of another pro-atrophy factor that acts independently of the ATF4 pathway.

Using mouse models, we identify p53 as this ATF4-independent factor. We show that skeletal muscle atrophy increases p53 expression in skeletal muscle fibers. In addition, overexpression of p53 causes skeletal muscle atrophy. Further, p53 mKO mice are partially resistant to muscle atrophy during limb immobilization. Taken together, these data indicate that like ATF4, p53 is sufficient and required for skeletal muscle atrophy during limb immobilization.

Importantly, overexpression of p53 induces muscle atrophy in the absence of ATF4, whereas ATF4-mediated muscle atrophy does not require p53. Furthermore, overexpression of p53 and ATF4 induces greater muscle atrophy than p53 or ATF4 alone. Moreover, skeletal muscle lacking both p53 and ATF4 is more resistant to skeletal
muscle atrophy than muscle lacking either p53 or ATF4 alone. Taken together, these data indicate that p53 and ATF4 mediate distinct and additive mechanisms to skeletal muscle atrophy.

However, the precise mechanism by which p53 and ATF4 cause skeletal muscle atrophy remained unclear. Using genome-wide expression arrays, we identify p21 as a skeletal muscle mRNA that is highly induced by p53 and ATF4 during limb immobilization. Further, overexpression of p21 causes skeletal muscle atrophy. In addition, p21 is required for muscle atrophy due to limb immobilization, p53, and ATF4. Collectively, these results identify p53 and ATF4 as critical and complementary mediators of skeletal muscle atrophy during limb immobilization, and discover p21 as an essential downstream mediator of the p53 and ATF4 pathways.
Skeletal muscle atrophy is a very common condition that accompanies malnutrition, critical illness, aging, cancer, heart failure, diabetes, neurological disease, and musculoskeletal disorders. Skeletal muscle atrophy also occurs as a side effect of medicines such as high-dose steroids or anti-androgen prostate cancer regimens. Skeletal muscle atrophy leads to weakness, which limits activity, decreases quality of life and leads to subsequent falls, fractures, and loss of independent living. Skeletal muscle atrophy places tremendous burdens on patients, their families, and society in general. Despite its broad implications for health and human disease, skeletal muscle atrophy is poorly understood and lacks an effective therapy.

Our current study therefore focuses on identifying novel mechanisms of skeletal muscle atrophy. In previous work we identified a cellular protein called ATF4, which we found served as a critical regulator of the atrophy process. However, in subsequent studies, it became clear that other factors must exist that act independently of ATF4. Therefore, the focus of this study was to identify this novel factor that regulates skeletal muscle mass. We identified p53 as this ATF4-independent factor inasmuch as p53 serves as an essential and causative factor in the development of skeletal muscle atrophy. Further studies identified the cellular protein p21 as a critical downstream regulator of the p53 and ATF4 pathways. Understanding these mechanisms at a deeper level may help us identify novel therapeutic approaches, a critical step towards our long-term goal of identifying a therapy to prevent or reverse muscle atrophy in those who are ill or aged.
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LIST OF ABBREVIATIONS

Ad  Adenovirus
ATF4  Activating Transcription Factor 4
ATF4 mKO  Skeletal Muscle-Specific ATF4 Knockout Mice
ATF4/p53 mKO  Skeletal Muscle-Specific ATF4/p53 Knockout Mice
ATPase  Adenosine Triphosphatase
bZIP  Basic Leucine Zipper
CMV  Cytomegalovirus
Cre  Cre Recombinase
eGFP  Enhanced Green Fluorescent Protein
Flox  Flanked by LoxP Restriction Sites
Gadd45a  Growth Arrest and DNA Damage-Inducible 45a
GFP  Green Fluorescent Protein
H&E  Hematoxylin and Eosin
i.p.  Intraperitoneal Injection
miRNA  MicroRNA
mKO  Muscle-Specific Knock-Out
p21  Cyclin-Dependent Kinase Inhibitor 1
p53  Tumor Suppressor p53
p53 mKO  Skeletal Muscle-Specific p53 Knockout Mice
qPCR  Quantitative Real-Time RT-PCR
RNAi  RNA Interference
TA  Tibialis Anterior
CHAPTER I: INTRODUCTION

Skeletal Muscle

Skeletal muscle is a dynamic tissue that is responsible for support of the skeleton, force generation, locomotion, and amino acid and glucose homeostasis (1,2). Skeletal muscles are composed of multi-nucleated muscle fibers (myofibers) that are formed from the fusion of myoblasts during development in a process termed myogenesis (1,3). By mass, skeletal muscle is the largest non-contiguous tissue in the human body (3,4). Striated skeletal muscle is controlled by the somatic nervous system and is therefore voluntary in nature.

Skeletal myofibers contain myofibrils, which in turn are composed of thick (myosin) and thin (actin) filaments. Along with other structural proteins, actin and myosin make up the basic structural unit of skeletal muscle known as the sarcomere, which is responsible for muscle contraction (3). The visible striations in skeletal muscle are due to repetitive sarcomere units (5). There exist two general types of myofibers in mature skeletal muscle: type I or slow twitch muscle fibers and type II or fast twitch muscle fibers. Using basic staining protocols such as myosin ATPase or immunohistochemistry for myosin ATPase sub-types, one can easily identify type I vs. type II muscle fibers (5). Type I fibers are primarily slow contractile, highly resistant to fatigue, oxidative due to high mitochondria content, and red in color due to high myoglobin content. By contrast, type II fibers are primarily fast contractile, highly fatigable, glycolytic, and white in color due to low myoglobin content. Given their
distinct properties, muscles with high type I content are primarily responsible for sustained contraction (e.g. posture, endurance training), while those with a high type II content are responsible for shorter periods of contraction (e.g. sprint training) (1,3).

While the total number of cells within a given muscle does not appear to change in an adult (e.g. hyperplasia or hypoplasia), the muscle fibers within a given muscle do undergo atrophy and hypertrophy in response to different stimuli (1,4). Known atrophy stimuli include aging, muscle disuse, malnutrition, organ failure, and acute and chronic illness, resulting in generalized muscle weakness (6,7). Conversely, muscle hypertrophy is due to strength training and anabolic factors (e.g. human growth hormone, androgens, insulin, insulin-like growth factor-1, etc.), resulting in an increase in muscle size and concomitant strength (7-9). The balance between hypertrophy and atrophy is largely controlled by the rate of skeletal muscle protein synthesis and proteolysis (7,10,11).

While much is known regarding the role of skeletal muscle in force generation and locomotion, comparatively little is known regarding its role in metabolism. Skeletal muscle functions as a critical energy reservoir during periods of fasting or malnutrition. Specifically, muscle functions to regulate whole body amino acid levels as free amino acids released by muscle are used by the liver during gluconeogenesis (12,13). This process is vital during periods of prolonged hypoglycemia such as fasting or malnutrition (12,13). In addition to its role in amino acid balance, skeletal muscle is critical for glucose homeostasis given its role as the primary reservoir for glucose in the human body.
Insulin resistance is therefore a complex condition as it is both a cause and effect of muscle atrophy (11,12,15).

**Skeletal Muscle Atrophy**

Skeletal muscle atrophy is a very common condition that frequently accompanies diabetes, cancer, heart failure, COPD, renal failure, cirrhosis, neurological diseases (e.g., diabetic neuropathy, stroke, multiple sclerosis, amyotrophic lateral sclerosis, spinal cord injury), musculoskeletal disorders (e.g., fractures, casting, immobilization, arthritis), chronic infectious diseases (e.g., HIV/AIDS), critical illness, malnutrition, aging, bed rest, and spaceflight (7,8,16). Skeletal muscle atrophy also occurs as an unavoidable side effect of medicines (e.g. high-dose glucocorticoids and anti-androgen prostate cancer regimens) (17,18). Skeletal muscle atrophy is characterized by a decrease in the size and strength of muscle fibers, but does not result in a decrease in the absolute number of skeletal muscle fibers (7).

In addition to being very common, muscle atrophy places tremendous burdens on patients, their families and society in general. Weakness from muscle atrophy limits activity, impairs quality of life, and leads to falls and fractures. In later stages, muscle atrophy causes debilitation, insulin resistance, and loss of independent living (7,15). Muscle atrophy also affects the respiratory muscles, and in the ICU setting can prevent or delay recovery from mechanical ventilation (19,20). Despite its broad implications for health and disease, muscle atrophy is poorly understood and lacks a pharmacological therapy.
While little is known about its molecular pathogenesis, some aspects of the condition have been well described. For example, it has been shown that in muscles undergoing atrophy, the ratio of protein synthesis to protein degradation decreases (10,18). This results in a decrease in the size and strength of muscle fibers. Known molecular pathways responsible for this flux in protein balance are the autophagy and ubiquitin-proteasome pathways (6,8). Additionally, a lack of anabolic signaling (e.g. IGF-1, insulin, testosterone and other androgens, human growth hormone, thyroid hormone, beta agonists such as clenbuterol, etc.) or excess of catabolic signaling (e.g. glucocorticoids, TGF-β receptor agonists, etc.) results in muscle atrophy (6-9). It has also been shown recently that conserved changes in skeletal muscle gene expression initiate the atrophy process regardless of stimulus (e.g. fasting, sciatic nerve transection, cancer cachexia, etc.) (21-23). For the vast majority of these changes, the individual role of specific mRNAs is unclear. However, in some cases, mRNA expression changes have been shown to mediate functional changes in muscle fiber size (e.g. induction of genes that promote protein degradation or decrease protein synthesis, as well as induction of genes that decrease mitochondrial function (21,22,24-26).

Our current research therefore focuses on discovering novel molecular pathways that sense stress and drive muscle atrophy. Understanding these pathways will help to identify novel mechanisms of muscle atrophy and may identify novel therapeutic approaches, a critical step towards our long-term goal of identifying a therapy to prevent or reverse muscle atrophy in those who are ill or aged. This thesis focuses on the

**Tumor Suppressor p53 (p53)**

p53 (tumor antigen p53, phosphoprotein `p53, transformation-related protein 53) is a well-described stress-response transcription factor that mediates a number of cellular responses to stress such as DNA damage, osmotic shock, oxidative stress, oncogene activation, heat shock, membrane damage, among others (27-29). p53 functions to regulate the cell cycle, apoptosis, DNA repair following genotoxic stress, as well as induce widespread changes in gene expression (13).

p53 retains two N-terminal transactivation domains (TAD), a proline-rich N-terminal domain that is critical for apoptosis regulation, a highly conserved DNA-binding domain (DBD), an oligomerization domain, and a regulatory domain at the C-terminus (30-32). The N-terminus is the primary site for regulation by upstream kinases, as there are a number of critical sites required for p53 activation (32). The DNA binding domain is crucial for p53 function and in many cancers, recessive loss-of-function p53 mutations result from single amino acid substitutions within this region of the protein (30,31). Li-Fraumeni syndrome is a rare autosomal dominant genetic disease that results from a single missense mutation within the DNA binding domain leading to a large spectrum of malignancies (33,34). p53 functions as a homotetramer and therefore relies on its tetramerization domain for its ability to bind DNA and affect gene expression (35,36).
In mammalian cells, p53 is regulated by a number of upstream kinases that respond to varying cellular stressors. In response to genotoxic stressors (e.g. UV, IR, hydrogen peroxide), activation of kinases such as ATM, ATR, CHK1, CHK2, DNA-PK, CAK leads to subsequent phosphorylation of p53 at the N-terminus (37). This phosphorylation leads to stabilization and accumulation of p53 protein. Similarly, cellular stressors such as osmotic or heat shock, result in activation of p38 MAP Kinase, ERK1/2, JNK1-JNK3 resulting in p53 accumulation (37,38). Lastly, p53 can be activated by p14ARF in the presence of oncogenes such as Myc or Ras (39). In all cases, p53 levels rise following degradation of the ubiquitin ligase Mdm2, which acts a negative regulator of p53 levels in many cell types (40).

Following activation, p53 can initiate a number of downstream effects. In response to DNA damage, p53 is activated and results in cell cycle arrest at the G1/S transition (via p21) (41). This cell cycle inactivation event allows for DNA repair and subsequent reentry into the cell cycle (42). In the event of severe DNA damage, p53 activation results in programmed cell death to avoid pathologic genomic instability (42,43). Lastly, p53 activation can result in widespread changes in gene expression that are independent of its cell cycle and apoptosis effects (44).

While p53 remains one of the most studied genes in the human genome, its role in skeletal muscle fibers remains unclear. However, there is some evidence that p53 may play a role in the pathogenesis of muscle fiber atrophy. For example, a number of canonical p53 gene targets (e.g. p21, Gadd45a, etc.) are highly induced by various muscle
atrophy stimuli (45-49). In addition, it has been previously shown that generation of
global p53 knockout (KO) mice results in partial protection from cancer-induced muscle
fiber atrophy (50). Conversely, transgenic mice with global expression of an activated
p53 construct display early onset aging and sarcopenia (age-related muscle atrophy) (51).
However, the role of p53 in skeletal muscle atrophy remains unclear. In chapters II and II
of this thesis, we investigate the role of p53 in the pathogenesis of skeletal muscle
atrophy.

**Activating Transcription Factor 4 (ATF4)**

ATF4 is a basic leucine zipper (bZIP) transcription factor with an evolutionarily
ancient role in cellular stress signaling (52,53). Under basal conditions, ATF4 levels
remain low, but in the presence of metabolic stress, ATF4 levels rise due to the actions of
the eukaryotic initiation factor 2α (eIF2α) kinase family (53-55). Phosphorylation of
eIF2α results in an increase in ATF4 expression, and a subsequent decrease in translation
of other mRNAs (55). Four eIF2α kinases exist in mammalian cells and each responds to
distinct cellular stresses such as essential amino acid deprivation (GCN2), heme
deficiency (HRI), dsRNA (PKR), and ER stress (PERK) (55).

Studies of the role of ATF4 in skeletal muscle have been hindered by a lack of
 genetic mouse models currently available. Global ATF4 knockout mice display an
 abnormally high death rate shortly after birth (56). Additionally, mice that are born
display a profoundly decreased body habitus, anemia, and blindness (56-58). These
effects help to explain the critical role of ATF4 in development and in a number of adult
tissues and organ systems. However, the pleiotropic effects of global ATF4 knockout mice have limited our understanding of the role of ATF4 in adult skeletal muscle.

However, work by our group and others has demonstrated a critical role for ATF4 in the pathogenesis of skeletal muscle atrophy (59,60). It has been previously shown that diverse stresses (including fasting, muscle disuse, insulin deficiency, renal failure, and cancer cachexia) increase skeletal muscle ATF4 expression (23). In addition, we have previously shown that forced expression of ATF4 in mouse skeletal muscle results in muscle fiber atrophy (60). To that end, we generated a transcriptionally inactive ATF4 construct, which was unable to induce muscle fiber atrophy (60). Additionally, generation of muscle-specific ATF4 knockout mice resulted in no observable phenotype under basal conditions (59). However, in the presence of skeletal muscle stress (e.g. fasting, muscle immobilization), muscle-specific ATF4 knockout mice were partially resistant to muscle fiber atrophy (59). Collectively, these data indicate that ATF4 is both necessary and sufficient for skeletal muscle atrophy.

However, it is also clear that ATF4 likely works in parallel or in concert to other factors that induce muscle atrophy. This conclusion is based on the finding that muscle-specific ATF4 knockout mice are only partially protected from skeletal muscle fiber atrophy (59). The resultant atrophy that does occur in the absence of ATF4 expression indicates the existence of another factor that promotes muscle atrophy. In Chapters II and III we investigate this ATF4-independent factor that promotes skeletal muscle fiber atrophy.
Cyclin-Dependent Kinase Inhibitor 1 (p21, CIP1, WAF1, Cdkn1a)

p21 (CIP1/WAF1, Cdkn1a) is a cyclin-dependent kinase inhibitor that functions to regulate cell cycle progression, cellular senescence, DNA repair, apoptosis, and cellular gene expression. p21 functions largely by inhibiting CDK complexes (e.g. CDK1, CDK2, CDK4, CDK6) and preventing progression from G1 to S phase. p53 is the primary upstream regulator of p21 expression and resultant cell cycle arrest functions. However, p53 is not required for all p21-mediated gene expression effects (61).

A diverse array of cellular stressors induce p21 expression in mammalian cells, of which some require p53 expression while others do not (61). DNA damage, oxidative stress, cytokines, mitogens, tumor viruses, and certain chemotherapeutic agents increase p21 expression (62). This induction can occur in a p53-dependent or -independent manner depending on cell type and cellular stressor. p53-dependent activation of p21 expression occurs by a direct mechanism (61,63-65). There are two p53 response elements in the p21 promoter that have been shown to mediate p53-dependent p21 induction (61). p53-independent induction of p21 expression occurs via Sp1, Sp3, E2F1, E2F3 as well as p53 homologues p63 and p73 (66). Induction of p21 results in an inhibition of CDKs that ultimately arrests the cell in the G1 phase (62).

While little is known regarding the role of p21 in skeletal muscle atrophy, it has been previously shown that p21 mRNA is one of the most highly induced skeletal muscle mRNAs during aging (45,46,48,67), muscle denervation (22,68), fasting (60), hindlimb unloading (47), amyotrophic lateral sclerosis (ALS) (69), and critical illness (49,70). In
addition, it has been shown that during muscle atrophy, p21 specifically increases in the nuclei of skeletal muscle fibers, but not in satellite cells (71). However, the functional role of p21 in skeletal muscle remains unclear. In Chapter III of this thesis, we investigate the role of p21 in the pathogenesis of skeletal muscle atrophy.

**Overall Significance**

Skeletal muscle atrophy is a common and debilitating consequence of malnutrition, muscle disuse, and severe systemic diseases such as uncontrolled diabetes (72,73). Muscle atrophy also plays a role in the insulin resistance that accompanies systemic disease and aging (15,74). However, the pathogenesis of muscle atrophy is poorly understood, and medical therapies that specifically target muscle atrophy do not exist. Therefore, this thesis is focused on defining molecular mediators of the atrophy process in the hopes of identifying novel targets for pharmacotherapy for patients that suffer from this debilitating condition.
CHAPTER II: p53 AND ATF4 MEDIATE DISTINCT AND ADDITIVE PATHWAYS TO SKELETAL MUSCLE ATROPHY DURING LIMB IMMOBILIZATION

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Abstract

Immobilization causes skeletal muscle atrophy via complex molecular mechanisms that are not well understood. To better understand these mechanisms, we investigated and compared the roles of p53 and ATF4, two transcription factors that mediate adaptations to a wide variety of cellular stresses. Previous studies established that muscle immobilization increases skeletal muscle ATF4 expression, which is sufficient to induce muscle fiber atrophy. However, skeletal muscles lacking ATF4 are only partially resistant to muscle atrophy during limb immobilization, indicating the existence of an ATF4-independent pathway. Here, we demonstrate that p53 mediates an ATF4-independent pathway to muscle atrophy during limb immobilization. Using mouse models, we show that limb immobilization increases p53 expression in skeletal muscle, and forced expression of p53 in skeletal muscle fibers is sufficient to induce muscle fiber atrophy. Conversely, mice lacking p53 expression in skeletal muscle fibers are partially resistant to immobilization-induced skeletal muscle atrophy, similar to mice lacking ATF4 expression in skeletal muscle fibers. Importantly, p53 promotes muscle fiber
atrophy in the absence of ATF4, whereas ATF4 promotes muscle fiber atrophy in the absence of p53. Furthermore, forced expression of both p53 and ATF4 induces more muscle fiber atrophy than either p53 alone or ATF4 alone. In addition, skeletal muscle lacking both p53 and ATF4 is more resistant to immobilization-induced muscle atrophy than muscle lacking only p53 or ATF4. Collectively, these results demonstrate that p53 and ATF4 mediate distinct and additive pathways to skeletal muscle atrophy during limb immobilization.

**Introduction**

Injuries and illnesses typically involve a period of immobility, which may be generalized (as in bed rest) or localized (as in limb casting). Immobility can have therapeutic effects; for example, it can allow healing of a fracture. However, immobility also has a deleterious consequence, skeletal muscle atrophy. When skeletal muscles are immobilized, they undergo disuse muscle atrophy. This causes weakness, which in turn delays recovery (75,76). Physical therapy is often employed to enhance recovery of muscle mass and strength, but is frequently insufficient for complete recovery (77-80). Nutritional or pharmacologic therapies to prevent muscle atrophy during immobilization would be ideal, but do not exist. Moreover, the development of therapies is hindered by a lack of knowledge. Although immobilization-induced skeletal muscle atrophy is a common and serious problem in medicine and surgery, and a significant unmet medical need, it is scarcely understood at the molecular level.
To better understand molecular mechanisms of immobilization-induced skeletal muscle atrophy, we recently investigated the role of ATF4, an evolutionarily ancient basic leucine zipper transcription factor that mediates a wide variety of cellular stress responses (53-55). In skeletal muscle, muscle disuse increases ATF4 expression (23), and increased skeletal muscle ATF4 expression is sufficient to induce skeletal muscle fiber atrophy (60). Furthermore, when mice lack ATF4 expression in skeletal muscle fibers, they undergo less skeletal muscle atrophy during limb immobilization (59). Taken together, these findings indicate that ATF4 plays a key role in immobilization-induced muscle atrophy.

However, it is also clear that ATF4 is not the only mediator of immobilization-induced muscle atrophy. This conclusion is based on the finding that muscles lacking ATF4 are not completely resistant to immobilization-induced muscle atrophy (59). The atrophy that occurs in the absence of ATF4 indicates the existence of another essential pro-atrophy factor, which works in concert with ATF4 to promote muscle atrophy during immobilization. The identity of this other factor remains unknown.

In the current study, we sought to identify an ATF4-independent factor that promotes immobilization-induced skeletal muscle atrophy. Because ATF4 is a fundamental mediator of cellular stress responses (53-55), we reasoned that its counterpart might also be a fundamental mediator of cellular stress responses. From this perspective, the tumor suppressor p53 seemed an attractive candidate. Like ATF4, p53 is a stress-induced transcription factor that mediates cellular adaptations to stress (27-29).
Although the role of p53 in skeletal muscle fibers is unknown, there is some evidence that p53 may promote skeletal muscle atrophy. For example, transgenic mice with global expression of an activated p53 construct age prematurely, and this is accompanied by severe muscle atrophy (51). Conversely, mice with a global loss of p53 expression are prone to cancer but resistant to cancer-induced skeletal muscle atrophy (50). Based on these considerations, we hypothesized that p53 might mediate the ATF4-independent pathway to skeletal muscle atrophy during limb immobilization.

**Materials and Methods**

*Mouse Strains*

C57BL/6 mice were obtained from the National Cancer Institute. All transgenic and knockout mice were on a C57BL/6 background. \textit{p53}\textsuperscript{f/f} mice were obtained from Jackson Laboratories, and are homozygous for a floxed \textit{p53} allele (exons 2-10 of the \textit{p53} gene are flanked by \textit{LoxP} restriction sites (81)). \textit{p53} mKO (muscle knockout) mice were generated by crossing \textit{p53}\textsuperscript{f/f} mice to transgenic mice expressing Cre recombinase under control of the \textit{muscle creatine kinase} (\textit{MCK}) promoter (82). In studies of \textit{p53} mKO mice, control mice were \textit{p53}\textsuperscript{f/f} littermates that lacked the \textit{MCK-Cre} transgene. \textit{ATF4} mKO mice were described previously (59) and were generated by crossing mice homozygous for a floxed \textit{ATF4} allele (\textit{ATF4}\textsuperscript{f/f}) to \textit{MCK-Cre} mice. In studies of \textit{ATF4} mKO mice, control mice were \textit{ATF4}\textsuperscript{f/f} littermates that lacked the \textit{MCK-Cre} transgene. \textit{p53}/\textit{ATF4} double mKO mice were generated by crossing \textit{p53}\textsuperscript{f/f} and \textit{ATF4}\textsuperscript{f/f} mice to generate mice homozygous for floxed \textit{p53} and \textit{ATF4} alleles (\textit{p53}\textsuperscript{f/f}\textit{ATF4}\textsuperscript{f/f}), and then
crossing $p53^{f/f}$/$ATF4^{f/f}$ mice to $MCK-Cre$ mice. In studies of $p53$/$ATF4$ double mKO mice, control mice were $p53^{f/f}$/$ATF4^{f/f}$ littermates that lacked the $MCK-Cre$ transgene.

Mouse Protocols

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Iowa. All mice were 8-12 week old males. Mice were housed at 21 °C in colony cages with 12 h light/12 h dark cycles and provided ad libitum access to standard chow (Harlan Teklad formula 7913) and water. Unilateral hindlimb immobilization was performed under isoflurane anesthesia using an Autosuture Royal 35W skinstapler (Tyco Healthcare), as described previously (59,83,84). Forelimb grip strength was determined using a grip strength meter equipped with a triangular pull bar (Columbus Instruments), as described previously (59,85,86). Transfection of mouse skeletal muscle with plasmid DNA was performed as described previously (59,60,87); mice were anesthetized with an i.p. injection of 91 mg/kg ketamine and 9.1 mg/kg xylazine, hindlimbs were shaved, and the tibialis anterior muscles (TAs) were injected with 30 µl of 0.4 U/µl bovine placental hyaluronidase (Sigma) resuspended in sterile 0.9% saline. Two hours later, mice were re-anesthetized, and TAs were injected with 30 µl plasmid DNA in sterile saline, coated with ultrasound jelly, and subjected to ten, 20-msec pulses of 175 V/cm using an ECM-830 electroporator (BTX Harvard Apparatus).

C2C12 Myotube Culture and Infection

Mouse C2C12 myoblasts were obtained from ATCC (CRL-1772), and maintained at 37°C and 5% CO2 in Dulbecco’s Modified Eagle’s Medium (DMEM) (ATCC #30-
2002) containing antibiotics (100 units/ml penicillin, 100 mg/ml streptomycin sulfate) and 10% (v/v) fetal bovine serum (FBS). Myoblasts were set up for experiments on day 0 in 6-well plates at a density of 2.5 X 10^5 cells / well. On day 2, differentiation was induced by replacing 10% FBS with 2% horse serum. On day 7, cells were rinsed once with PBS, and then 1 ml DMEM containing adenovirus (MOI 125) was added to each well. Two hours later, 1 ml DMEM containing 1% horse serum plus antibiotics was added to each well. On day 8, cells were rinsed twice with PBS, and then 2 ml DMEM containing 2% horse serum and antibiotics were added to each well. Infection efficiency was > 90%. All assays except protein degradation (described below) were performed on day 9, 48 h post-infection.

Adenoviruses and Plasmids

*p-wt-p53* encodes wild-type mouse p53 (NM_011640.3) under control of the cytomegalovirus (CMV) promoter. *p-mut-p53* encodes mouse p53 with two point mutations (R270H and P275S) that disrupt DNA binding (88), under control of the CMV promoter. *p-ATF4-FLAG* was described previously (60) and encodes wild-type mouse ATF4 (NM_009716) with three copies of the FLAG epitope tag at the NH3-terminus, under control of the CMV promoter. *p-eGFP* encodes eGFP under control of the CMV promoter.

Ad-p53 and Ad-p53 R175H were generated by subcloning each cDNA into the pacAd5 K-N pA shuttle plasmid (89), after which replication-deficient (E1, E3 deleted) recombinant adenoviruses co-expressing eGFP were generated by the University of Iowa
Gene Transfer Vector Core (90). Ad-GFP control virus has been described previously (89). Adenovirus titers were determined by plaque assays on HEK 293 cells. Viruses were stored in phosphate-buffered saline (PBS) with 3% sucrose at –80°C.

*Histological Analysis of Mouse Skeletal Muscle and Mouse Skeletal Myotubes.*

Hematoxylin and eosin (H&E) stains and myosin ATPase stains were performed by embedding and freezing skeletal muscles in Tissue Freezing Medium (Triangle Biomedical), and then preparing 10-µm sections from the midbelly of the muscle with a Microm HM 505E cryostat. For H&E stains, muscle sections were fixed in ice cold zinc formalin for 15 min before staining with a DRS-601 automatic slide stainer (Sakura). Myosin ATPase stains were performed as previously described (5,91). Briefly, muscle sections were incubated for 5 min in buffer A (50 mM sodium acetate, 30 mM sodium barbital, pH 4.3) to stain type I muscle fibers; or incubated for 15 min in buffer B (20 mM sodium barbital, 18 mM CaCl₂, pH 9.4) to stain type II muscle fibers. Sections were then incubated for 30 min in buffer C (20 mM sodium barbital, 9 mM CaCl₂, 2.7 mM ATP, pH 9.4), followed by three 3 min washes with 1% (w/v) CaCl₂. Slides were then incubated for 3 min in 2% (w/v) CoCl₂, followed by three 1 min washes in deionized water. Slides were then incubated for 20 sec in 1% (v/v) (NH₄)₂S, followed by a 5 min wash in water. Slides were then dehydrated with ethanol, cleared with xylene, and mounted with solvent 100 (IMEB Inc.). H&E- and myosin ATPase-stained sections were imaged using an Olympus BX-61 automated upright microscope, and image analysis was performed with ImageJ. For visualization of GFP fluorescence, muscles were fixed in 4% paraformaldehyde (w/v) for 16 h, incubated in 30% sucrose for 24 h, embedded in
Tissue Freezing Medium, and then snap frozen using a Stand-Alone Gentle Jane. A Microm HM 505E cryostat was then used to prepare 10-µm sections from the midbelly of the muscle. Sections were then washed 3X with PBS and mounted with Vectashield (Vector Laboratories). Sections were imaged on an Olympus IX-71 microscope equipped with a DP-70 camera and epifluorescence filters, and image analysis was performed with ImageJ. Transfected fibers were defined as fibers having a mean fluorescence ≥ 25 arbitrary units above background, as described previously (60). For all muscle fiber measurements, the diameters of ≥ 200 muscle fibers per muscle were measured using the lesser diameter (minimal Feret diameter) method, as recommended elsewhere (5).

All myotube imaging was performed on an Olympus IX-71 microscope equipped with a DP-70 camera and epifluorescence filters. Image analysis was performed using ImageJ software. To analyze myotube size, we averaged three width measurements per GFP-positive myofiber. To localize p53, myotubes were washed 3X with ice-cold PBS, fixed in 4% paraformaldehyde for 10 min, and permeabilized with PBS (7.4 pH) containing 0.5% Triton X-100 for 15 min. Permeabilized myotubes were blocked with PBS containing 1% bovine serum albumin (BSA) and 5% normal goat serum (NGS) for 1 h, followed by an overnight incubation with a 1:100 dilution of mouse monoclonal anti-p53 antibody (Cell Signaling Technologies, #2524) in PBS containing 5% NGS at 4°C. After incubation, the myotubes were washed 3X with PBS and then incubated with Alexa 568-conjugated secondary antibody (1:400) for 1 h at room temperature. Myotubes were then washed 3X with PBS and then covered with Vectashield mounting medium. For trypan blue staining, myotubes were rinsed 3X with PBS, stained with 0.2% trypan blue
in PBS for 5 min at room temperature, and then rinsed 3X with PBS. As a positive control for cell death, myotubes were treated with 80% ethanol for 20 min prior to staining with 0.2 % trypan blue.

*Immunoblot Analysis of Mouse Skeletal Muscle and C2C12 Myotubes*

Skeletal muscle protein extracts were prepared as described previously (59,60,87). Briefly, skeletal muscles were snap-frozen in liquid nitrogen and homogenized in 1 ml ice-cold homogenization buffer (50 mM HEPES, 4 mM EGTA, 10 mM EDTA, 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, cOmplete Mini protease inhibitor mixture (Roche Applied Science), 25 mM sodium fluoride, 1% (vol/vol) Triton X-100, and 1:100 dilution of phosphatase inhibitor cocktails 2 and 3 (Sigma)) using a Tissue Master 240 (Omni International) for 1 min on setting #10. The muscle homogenate was rotated for 1 h at 4 °C, and then centrifuged at 16,000 x g for 20 min at 4 °C. An aliquot of the supernatant was used to determine protein concentration by the BCA method (Pierce), and another aliquot was mixed with 0.25 volume of sample buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 25% glycerol, 0.2% (w/v) bromophenol blue, and 5% (w/v) 2-mercaptoethanol) and heated at 95 °C for 5 min. An equal amount of protein from each sample was subjected to SDS-PAGE, and then transferred to Hybond-C extra nitrocellulose filters (Millipore).

Myotube protein extracts were prepared by scraping PBS-washed myotubes into cold lysis buffer (10 mM Tris-HCl, pH 7.6, 100 mM NaCl, and 1% (w/v) SDS, cOmplete Mini protease inhibitor cocktail (Roche), and a 1:100 dilution of phosphatase inhibitor
cocktails 2 and 3 (Sigma)), and then lysing with 10 passes through a 22-gauge needle.

Aliquots of muscle and myotube protein extracts were mixed with 0.25 volume of sample buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 25% glycerol, 0.2% (w/v) bromophenol blue, and 5% (w/v) 2-mercaptoethanol) and heated for 5 min at 95°C. A separate aliquot of each extract was used to determine protein concentration by the BCA kit (Pierce), after which an equal amount of protein from each sample was subjected to SDS-PAGE, and then transferred to Hybond-C extra nitrocellulose filters (Millipore). Immunoblots were performed at 4 °C for 16 h using a 1:1000 dilution of monoclonal anti-mouse p53 (Cell Signaling Technologies, #2524) or a 1:100,000 dilution of polyclonal anti-actin antiserum (Sigma #A2103).

**Quantitative Real-time RT-qPCR (qPCR)**

Extraction of skeletal muscle RNA was performed using TRIzol solution (Invitrogen) and purified with Turbo DNAfree kit (Ambion) as described previously (59,60). Quantitative real-time RT-PCR (qPCR) was performed as previously described (59,60) using a High Capacity cDNA reverse transcription kit (Applied Biosystems). qPCR studies were performed with a 7500 Fast Real-time PCR System (Applied Biosystems) using p53 and ATF4 Taqman Gene Expression Assays (Applied Biosystems). All qPCR samples were run in triplicate and the cycle threshold (Ct) values were averaged. For data analysis, the ΔΔCt method was utilized, with 36B4 mRNA serving as the invariant control.
Statistical Analysis

For comparisons between two groups, paired $t$ tests were used for within-subject samples and unpaired $t$ tests were used for independent samples. Comparisons involving multiple groups were analyzed by one-way ANOVA with Sidak’s post hoc test.

Results

Limb immobilization increases skeletal muscle p53 expression and induces skeletal muscle atrophy.

Because the molecular mechanisms of immobilization-induced muscle atrophy are highly complex, we focused on a relatively short period of limb immobilization (three days) in order to identify early events that initiate the atrophy process. We previously found that three days of limb immobilization induces skeletal muscle atrophy partly through induction of ATF4 (59). To begin to investigate the potential role of p53, we tested the hypothesis that three days of limb immobilization might increase skeletal muscle p53 expression. To test this, we immobilized one hindlimb in mice, as illustrated in Fig. 2-1. In each mouse, the contralateral hindlimb remained mobile and served as an intrasubject control. After three days of unilateral hindlimb immobilization, we examined bilateral tibialis anterior (TA) muscles. We found that immobilization decreased the size of TA muscle fibers, indicating muscle fiber atrophy (Figs. 2-2), and significantly increased the level of p53 (Fig. 2-3). Furthermore, a time course study showed that p53 expression significantly increased within one day of immobilization and preceded muscle atrophy (Fig. 2-3). Because p53 activity can also be increased by phosphorylation of serine 15 (92-94), we examined whether immobilization increases


serine 15 phosphorylation, but found that it did not (data not shown). These results identified p53 expression as an early effect of skeletal muscle immobilization and suggested that p53 might contribute to immobilization-induced skeletal muscle atrophy.

*Increased skeletal muscle p53 expression induces skeletal muscle atrophy.*

To test the hypothesis that p53 promotes skeletal muscle atrophy, we used an electroporation-based method to transflect mouse TA muscle with a plasmid encoding p53. Importantly, this method transfects terminally differentiated muscle fibers, but not satellite cells or connective tissue cells (95). In each mouse, the contralateral TA was transfected with empty plasmid, and served as a negative control. Both TA muscles remained mobile throughout the experiment. We found that transfection of p53 plasmid increased p53 protein and induced muscle fiber atrophy (Fig. 2-4). As an additional control, we tested a full-length, transcriptionally inactive p53 construct (p53(R270H/P275S); (88)). Although p53(R270H/P275S) was highly expressed, it did not induce muscle fiber atrophy (Figs. 2-5). Interestingly, infection of C2C12 skeletal myotubes with adenovirus co-expressing p53 and GFP (Ad-p53) induced myotube atrophy (Fig. 2-6). We generated a full-length transcriptionally inactive p53 construct ((Ad-p53-R175H; (96)), which did not induce myotube atrophy (Fig. 2-6). Immunoblot analysis confirmed p53 overexpression (Fig. 2-6). To begin to determine how p53 promotes atrophy, we used immunohistochemistry to localize p53 in myotubes and muscle fibers. Consistent with previous findings in non-muscle cells (97,98), p53 was predominantly localized to myotube nuclei (Fig. 2-7), suggesting that p53 promotes muscle atrophy by altering nuclear processes. In addition, forced expression of p53 did
not induce myotube death (Fig. 2-8). Collectively, these data indicate that increased expression of p53 in muscle fibers is sufficient to induce atrophy.

*p53 is partially required for skeletal muscle atrophy during limb immobilization.*

To test the hypothesis that p53 is required for muscle atrophy during limb immobilization, we generated and studied mice lacking p53 expression in differentiated skeletal muscle fibers (p53 mKO mice). We generated p53 mKO mice by crossing mice homozygous for a floxed p53 allele (p53<sup>f/f</sup> mice; (81)) to transgenic MCK-Cre mice, which express Cre recombinase in skeletal muscle fibers and heart, but not satellite cells (82,99). As expected, the MCK-Cre transgene excised the floxed p53 allele in skeletal muscle and heart of p53 mKO mice and eliminated skeletal muscle p53 expression (Fig. 2-9).

Importantly, p53 mKO mice were born at the expected Mendelian frequency and lacked any overt phenotype up to 15 months of age, the longest period of observation. Careful comparisons of p53 mKO mice and littermate control mice revealed no baseline differences in total body weight, skeletal muscle weight, skeletal muscle histology, grip strength, heart weight, liver weight, kidney weight, or fat pad weight (Figs. 2-10). Thus, p53 expression in fully differentiated (post-mitotic) muscle fibers is not required for skeletal muscle development or the maintenance of postnatal skeletal muscle mass during early and middle adulthood. Furthermore, the absence of p53 expression in muscle fibers does not induce muscle hypertrophy.
To test the hypothesis that p53 is necessary for immobilization-induced muscle atrophy, we subjected p53 mKO mice and littermate control mice to 3 days of unilateral limb immobilization. We found that immobilization significantly reduced muscle fiber diameter in both littermate control mice and p53 mKO mice (Figs. 2-11). However, p53 mKO muscle fibers underwent significantly less atrophy than control muscle fibers (Figs. 2-11). These results indicate that p53 is partially required for immobilization-induced skeletal muscle atrophy.

*p53 and ATF4 mediate distinct pathways to skeletal muscle atrophy.*

In skeletal muscle, p53 appeared to resemble ATF4 in three important ways. First, p53 expression was increased by muscle disuse (Fig. 2-3), similar to ATF4 expression (23). Second, p53 was sufficient to induce skeletal muscle fiber atrophy (Figs. 2-4, 2-5, 2-6), similar to ATF4 (60). Third, p53 mKO muscles appeared normal under basal conditions (Fig. 2-10) but were partially resistant to immobilization-muscle atrophy (Fig. 2-11), similar to muscles that lack ATF4 (59). These similarities suggested that p53 and ATF4 might be key components of the same molecular pathway to muscle atrophy.

To test this possibility, we performed complementation studies. We first tested whether ATF4 might be an essential downstream mediator of p53. To test this, we overexpressed p53 in muscles of ATF4 mKO mice, which lack skeletal muscle ATF4 expression secondary to *MCK-Cre*-mediated excision of a homozygous floxed *ATF4* allele (59). However, in ATF4 mKO muscle, p53 caused muscle fiber atrophy (Figs. 2-
12), indicating that ATF4 is not required for p53-mediated muscle atrophy. We next overexpressed ATF4 in p53 mKO muscles to determine whether p53 might be an essential downstream mediator of ATF4. However, in p53 mKO muscles, ATF4 caused muscle fiber atrophy (Figs. 2-13), indicating that p53 is not required for ATF4-mediated muscle atrophy. These data suggest that p53 and ATF4 induce muscle atrophy by independent and potentially additive mechanisms.

*p53 and mediate additive pathways to skeletal muscle atrophy.*

As an initial test of the hypothesis that p53 and ATF4 have additive effects on muscle fiber size, we compared the effects of p53 expression, ATF4 expression, and co-expression of p53 and ATF4. Individually, p53 and ATF4 reduced muscle fiber diameter by similar amounts (Figs. 2-14). However, the combination of p53 and ATF4 generated greater muscle fiber atrophy than either transcription factor alone, and the effect was close to additive (Figs. 2-14).

As a second test of the hypothesis that p53 and ATF4 induce muscle atrophy by additive mechanisms, we compared p53 mKO mice and ATF4 mKO mice to double knockout mice that lack both p53 and ATF4 in skeletal muscle (p53/ATF4 mKO mice). Relative to immobilized control muscles, immobilized p53 mKO muscles contained reduced *p53* mRNA and a normal level of *ATF4* mRNA; immobilized ATF4 mKO muscles contained reduced *ATF4* mRNA and a normal level of *p53* mRNA; and immobilized p53/ATF4 mKO muscles contained reduced levels of both *ATF4* and *p53* mRNAs (Fig. 2-15). Of note, residual *p53* mRNA in p53 mKO and p53/ATF4 mKO
muscles is likely from satellite cells and non-muscle cells, which do not express MCK-Cre (99).

Similar to the single knockout mice (p53 mKO and ATF4 mKO), double knockout mice (p53/ATF4 mKO) were born at the expected Mendelian frequency, appeared healthy and grossly indistinguishable from their wild-type littermates up to 15 months of age, the longest period of observation. Furthermore, p53/ATF4 mKO mice lacked detectable baseline changes in body weight, muscle weight, muscle histology, grip strength and heart, liver and fat pad weight (Figs. 2-16).

To determine whether p53 and ATF4 have additive effects on muscle fiber size during immobilization, we subjected the single and double knockout mice to three days of unilateral hindlimb immobilization. Consistent with previous results, p53 mKO muscles and ATF4 mKO muscles were partially resistant to immobilization-induced muscle fiber atrophy (Fig. 2-17) and immobilization-induced muscle loss (Fig. 2-18). Moreover, in the double knockout mice (p53/ATF4 mKO) mice, there was an additive reduction in immobilization-induced muscle fiber atrophy (Fig. 2-17) and immobilization-induced muscle loss (Fig. 2-18). Further examination of the muscle fibers revealed that immobilization induced atrophy in type II fibers, but not type I fibers (Figs. 2-19, 2-20, 2-21). In addition, loss of either p53 or ATF4 partially protected type II fibers from immobilization-induced atrophy, and the combined loss of p53 and ATF4 provided complete protection of type II fibers from immobilization-induced atrophy (Figs. 2-19, 2-20, 2-21). Taken together, these results indicate that the combined actions
of p53 and ATF4 are required for type II muscle fiber atrophy during immobilization. Collectively, our results identify p53 and ATF4 as distinct and additive mechanisms that mediate immobilization-induced muscle atrophy (Fig. 2-22).

**Discussion**

Immobilization-induced skeletal muscle atrophy is a common and serious condition, but its molecular mechanisms are not well understood. Previous work showed that ATF4 contributes to immobilization-induced muscle atrophy (59,60). However, it was also clear that ATF4 is not the only factor involved, inasmuch as ATF4-deficient muscles are only partly resistant to immobilization-induced atrophy (59 and Fig. 6D-E). Thus, in the current study, we sought to identify an ATF4-independent factor that promotes skeletal muscle atrophy during immobilization.

Our results identify p53 as a key ATF4-independent mediator of immobilization-induced muscle atrophy. This conclusion is supported by our findings that: 1) immobilization increases p53 expression; 2) p53 induces muscle fiber atrophy, even in the absence of ATF4; 3) combined expression of p53 and ATF4 induces more atrophy than either transcription factor alone; 4) loss of p53 expression in muscle fibers provides partial protection against immobilization-induced muscle atrophy; and 5) when coupled with loss of ATF4, loss of p53 provides nearly complete protection against immobilization-induced muscle atrophy. These results, in conjunction with previous work, indicate that immobilization increases expression of both p53 and ATF4, which function in parallel to promote muscle atrophy.
Interestingly, loss of p53 and/or ATF4 expression in muscle fibers appears to have little if any effect on muscle development or the maintenance of muscle mass during early to middle adulthood. This finding is consistent with the notion that ATF4 and p53 are transcription factors that must be induced by stress. In the absence of stress (e.g. muscle immobilization), ATF4 and p53 are not induced, and thus, lack of ATF4 and/or p53 has no effect.

The way in which immobilization increases p53 remains to be determined. Our results suggest that immobilization increases p53 synthesis and/or decreases p53 turnover in muscle fibers. In other cell types, p53 synthesis can be augmented by several mechanisms that activate the p53 gene (100-102), stabilize p53 mRNA (103-105) and/or enhance translation of p53 mRNA (106-109); and p53 turnover can be reduced by several mechanisms that decrease expression or activity of Mdm2 and related E3 ubiquitin ligases (37,38,110). We speculate that muscle immobilization stimulates one or more of these mechanisms in muscle fibers, leading to increased p53 expression. However, other possibilities exist, and this is an important area for future investigation.

Another important and challenging area for future work lies downstream of p53. Like most transcription factors, p53 stimulates a multitude of positive and negative changes in gene expression (111-113). Presumably, p53 promotes muscle atrophy by activating genes that promote muscle atrophy and/or repressing genes that inhibit muscle atrophy. However, the genes that are controlled by p53 in skeletal muscle fibers have not
been fully defined. Thus, it will be important to determine which genes are controlled by p53 in muscle fibers, and which p53-mediated changes in gene expression promote muscle fiber atrophy. To understand the distinct and additive roles of p53 and ATF4 in immobilization-induced muscle atrophy, it will be important to compare the genetic programs that are regulated by p53 and ATF4, alone and in combination. We speculate that p53 and ATF4 may induce certain pro-atrophy genes and/or repress certain anti-atrophy genes in an additive manner. In addition, p53 may have some pro-atrophy effects that are not shared by ATF4, and vice versa.

In the current study, we focused on a relatively short period of immobilization (three days) in order to identify key triggers of the atrophy process. However, it will also be important to investigate the relative contributions of p53 and ATF4 to muscle atrophy during longer periods of muscle immobilization. In a previous study, we found that ATF4 mKO mice undergo a normal amount of muscle atrophy with a longer period of immobilization (seven days) (59), presumably due to compensatory mechanisms that are not yet known. The role of p53 during longer periods of immobilization is also not yet known, although a previous study in quail muscle showed that p53 expression is induced after seven days of immobilization, and returns to baseline levels after fourteen days of immobilization (114).

Because the current study focused entirely on the TA muscle as a model system, it will also interesting to explore the relative contributions of p53 and ATF4 to immobilization-induced atrophy in other muscle types. There may be differences, as
suggested by our finding that p53 and ATF4 promote atrophy of type II but not type I fibers, and the previous finding that immobilization does not increase p53 and ATF4 in soleus muscle, which is primarily composed of type I fibers (115).

In summary, the current study demonstrates that two fundamental mediators of cellular stress responses, p53 and ATF4, work in parallel to promote muscle loss during immobilization. The study helps explain how immobilization-induced muscle atrophy occurs, and it opens several new avenues of investigation. In addition, the current study has potential implications for patients with immobilization-induced muscle atrophy due to injury or illness: optimal prevention and treatment of this condition may require interventions that inhibit the expression and/or key downstream effects of both p53 and ATF4 in skeletal muscle fibers.
Figure 2-1. Schematic illustrating the hindlimb immobilization model.
Figure 2-2. Hindlimb immobilization induces muscle fiber atrophy. **A-B:** C57BL/6 mice were subjected to unilateral hindlimb immobilization for three days before bilateral tibialis anterior (TA) muscles were harvested for further analysis. **A:** Representative H&E stains of muscle fibers. **B:** Quantification of muscle fiber size. **Left,** mean fiber diameter. Each data point represents the mean diameter of ≥ 250 fibers from one muscle. Horizontal bars denote means ± SEM. **P-value** was determined with a paired *t*-test. **Right,** fiber size distributions; each distribution represents ≥ 1100 fibers from 4 muscles.
Figure 2-3. Limb immobilization increases skeletal muscle p53 expression and induces skeletal muscle atrophy. A: C57BL/6 mice were subjected to unilateral hindlimb immobilization for three days before bilateral tibialis anterior (TA) muscles were harvested for further analysis. An equal amount of protein from each muscle (100 µg) was subjected to SDS-PAGE and immunoblot analysis with anti-p53 polyclonal IgG. Membranes were stained with Ponceau S to confirm equal loading. B: C57BL/6 mice were subjected to unilateral hindlimb immobilization for the indicated times before bilateral TA muscles were harvested for analysis of muscle weight (upper) and p53 protein level (lower). In each mouse, values from the immobile muscle were normalized to values from the mobile muscle. Data are means ± SEM from 5-6 mice per time point. Some error bars are too small to see. At each time point, paired t-tests were used to compare values from mobile and immobile TAs (*P ≤ 0.05).
**Figure 2-4.** Increased p53 expression causes skeletal muscle fiber atrophy.  

**A-C:** In C57BL/6 mice, one TA muscle was transfected with 20 µg p-wt-p53 plus 2 µg p-eGFP, and the contralateral TA ("Control") was transfected with 20 µg empty plasmid (pcDNA3) plus 2 µg p-eGFP. eGFP served as a transfection marker and does not alter muscle fiber size (60). Bilateral TAs were harvested seven days post-transfection for further analysis. 

**A:** An equal amount of protein from each muscle (100 µg) was subjected to SDS-PAGE and immunoblot analysis with anti-p53 polyclonal IgG. Membranes were stained with Ponceau S to confirm equal loading.  

**B-C:** Histological analysis of muscles. 

**B:** Representative images.  

**C:** Quantification of muscle fiber size.  

*Left,* fiber size distributions; each distribution represents ≥1600 fibers from 4 muscles.  

*Right,* mean fiber diameters; each data point represents the mean diameter of ≥400 fibers from one muscle. Horizontal bars denote means ± SEM.  

*P*-value was determined with a paired *t*-test.
Figure 2-5. A transcriptionally inactive p53 constructs fails to induce muscle fiber atrophy. 

A-C: In C57BL/6 mice, one TA muscle was transfected with 20 µg *p-mut-p53* plus 2 µg *p-eGFP*, and the contralateral TA ("Control") was transfected with 20 µg empty plasmid (*pcDNA3*) plus 2 µg *p-eGFP*. Bilateral TAs were harvested seven days post-transfection for further analysis. 

A: An equal amount of protein from each muscle (50 µg) was subjected to SDS-PAGE and immunoblot analysis with anti-p53 polyclonal IgG. Membranes were stained with Ponceau S to confirm equal loading. 

B-C: Histological analysis of muscles. 

B: Representative images. 

C: Quantification of muscle fiber size. 

Left, fiber size distributions; each distribution represents ≥ 2300 fibers from 6 muscles. 

Right, mean fiber diameters; each data point represents the mean diameter of ≥ 450 fibers from one muscle. Horizontal bars denote means ± SEM. 

P-value was determined with a paired *t*-test.
Figure 2-6. Increased p53 expression induces skeletal myotube atrophy. C2C12 myotubes were infected for 48 h with Ad-Control, Ad-p53, or Ad-p53 R175H (MOI 125 for all adenoviruses), and then harvested 48 h after infection for further analysis. A: An equal amount of protein from each lysate (50 µg) was subjected to SDS-PAGE and immunoblot analysis with anti-p53 polyclonal IgG or anti-actin polyclonal IgG. B-C: Histological analysis of muscles. B: Representative fluorescence microscopy images of myotubes. C: Quantification of muscle fiber size. Each data point represents the mean diameter of ≥ 200 fibers from three separate experiments. Horizontal bars denote means ± SEM. Statistical analysis used the one-way ANOVA with Sidak’s post hoc test; different letters are statistically different (P ≤ 0.05).
**Figure 2-7.** Immunohistochemical detection of p53 in skeletal myotube nuclei. C2C12 myotubes were infected for 48 h with Ad-p53 (MOI 125), before immunohistochemical analysis.
Figure 2-8. p53 does not induce skeletal myotube death. C2C12 myotubes were infected for 48 h with Ad-p53 (MOI 125), and then harvested 48 h after infection and stained with 0.2% trypan blue. As a positive control for cell death, myotubes were treated with 80% ethanol for 20 min before trypan blue staining.
Figure 2-9. Baseline analysis of control and p53 mKO mice. **A-B:** p53 mKO mice are homozygous for a floxed p53 allele (p53\(^{f/f}\)) and possess the MCK-Cre transgene, which directs Cre recombinase expression to skeletal muscle fibers and heart. Control mice were littermates of p53 mKO mice that lacked the MCK-Cre transgene. **A:** PCR confirmation that MCK-Cre directs excision of the floxed p53 allele (p53\(^{f}\)) in striated muscle of p53 mKO mice. TA: tibialis anterior. Gastroc: gastrocnemius. Quad: quadriceps. **B:** Control and p53 mKO mice were subjected to unilateral hindlimb immobilization for three days before bilateral TA muscles were harvested. An equal amount of protein from each muscle (100 µg) was subjected to SDS-PAGE and immunoblot analysis with anti-p53 polyclonal IgG. Membranes were stained with Ponceau S to confirm equal loading.
### Figure 2-10. p53 mKO phenotypic analysis under basal conditions. Data are means ± SEM from 8 mice per genotype. Muscle weights are combined weight of bilateral muscles. Fat pad weights are combined weights of bilateral epididymal, retroperitoneal and scapular fat pads. *P*-values were determined with unpaired *t*-tests.

<table>
<thead>
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<th>Parameters</th>
<th>Control</th>
<th>p53 mKO</th>
<th><em>P</em></th>
</tr>
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<tbody>
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<td>Body Weight (g)</td>
<td>22.0 ± 0.5</td>
<td>21.4 ± 0.6</td>
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<td>Gastrocnemius (mg)</td>
<td>261.0 ± 11.5</td>
<td>249.0 ± 14.9</td>
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<tr>
<td>Soleus (mg)</td>
<td>14.3 ± 0.7</td>
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<td>Quadriceps (mg)</td>
<td>243.4 ± 13.0</td>
<td>244.1 ± 11.5</td>
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<td>Triceps (mg)</td>
<td>162.6 ± 4.8</td>
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<td>Biceps (mg)</td>
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<td>% Type II</td>
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<td>94.6 ± 0.9</td>
<td>0.36</td>
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<td>Grip Strength (g)</td>
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<tr>
<td>Liver (mg)</td>
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<td>1036.4 ± 27.2</td>
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</tr>
<tr>
<td>Fat Pads (mg)</td>
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<td>499.1 ± 28.2</td>
<td>0.46</td>
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Figure 2-11. p53 is partially required for immobilization-induced muscle atrophy. **A-B:** Control and p53 mKO mice were subjected to unilateral hindlimb immobilization for three days before bilateral TA muscles were harvested for histological analysis. **A:** Representative H&E images. **B:** Quantification of TA muscle fiber size. Each data point represents the mean diameter of ≥ 250 fibers from one muscle. Horizontal bars denote means ± SEM. Statistical analysis used the one-way ANOVA with Sidak’s post hoc test; different letters are statistically different (P ≤ 0.05).
Figure 2-12. p53 is sufficient to induce muscle fiber atrophy in an ATF4-independent manner. A-C: The experiments utilized ATF4 mKO mice, which lack ATF4 expression in skeletal muscle fibers (59). In each mouse, one TA muscle was transfected with 20 µg p-wt-p53 plus 2 µg p-eGFP, and the contralateral TA ("Control") was transfected with 20 µg empty plasmid (pcDNA3) plus 2 µg p-eGFP. Bilateral TAs were harvested 10 days post-transfection for further analysis. A: An equal amount of protein from each muscle (100 µg) was subjected to SDS-PAGE and immunoblot analysis with anti-p53 polyclonal IgG. Membranes were stained with Ponceau S to confirm equal loading. B: Representative images. C: Quantification of muscle fiber size. Left, fiber size distributions; each distribution represents ≥ 800 fibers from 4 muscles. Right, mean fiber diameters; each data point represents the mean diameter of ≥ 200 fibers from one muscle. Horizontal bars denote means ± SEM. P-values were determined with paired t-tests.
Figure 2-13. ATF4 is sufficient to induce muscle fiber atrophy in a p53-independent manner. A-C: The experiments utilized p53 mKO mice. In each mouse, one TA muscle was transfected with 20 µg p-ATF4-FLAG plus 2 µg p-eGFP, and the contralateral TA ("Control") was transfected with 20 µg empty plasmid (pcDNA3) plus 2 µg p-eGFP. Bilateral TAs were harvested 10 days post-transfection for further analysis. A: An equal amount of protein from each muscle (40 µg) was subjected to SDS-PAGE and immunoblot analysis with anti-FLAG monoclonal IgG. Membranes were stained with Ponceau S to confirm equal loading. B: Representative images. C: Quantification of muscle fiber size. Left, fiber size distributions; each distribution represents ≥ 1200 fibers from 5 muscles. Right, mean fiber diameters; each data point represents the mean diameter of ≥ 200 fibers from one muscle. Horizontal bars denote means ± SEM. P-values were determined with paired t-tests.
Figure 2-14. Co-expression of p53 and ATF4 induces more muscle fiber atrophy than either transcription factor alone.  

**A-B:** TA muscles of C57BL/6 mice were transfected with 2 µg p-eGFP plus either 20 µg pcDNA3 ("Control"); 10 µg p-wt-p53 +10 µg pcDNA3 ("p53"); 10 µg p-ATF4-FLAG +10 µg pcDNA3 ("ATF4"); or 10 µg p-wt-p53 + 10 µg p-ATF4-FLAG ("p53 + ATF4"). Muscles were harvested seven days post-transfection.  

**A:** Representative images.  

**B:** Mean fiber diameters; each data point represents the mean diameter of ≥ 200 fibers from one muscle. Horizontal bars denote means ± SEM. Statistical analysis used the one-way ANOVA with Sidak’s post hoc test; different letters are statistically different (P ≤ 0.05).
Figure 2-15. Baseline analysis of p53 mKO, ATF4 mKO, p53/ATF4 mKO, and littermate control mice. p53/ATF4 mKO mice are homozygous for a floxed p53 allele \((p53^{f/f})\) and a floxed \(ATF4\) allele \((ATF4^{f/f})\) and possess the \(MCK-Cre\) transgene. Control mice for p53/ATF4 mKO were littermates of p53/ATF4 mKO mice that lacked the \(MCK-Cre\) transgene. Control mice for p53 mKO were littermates of p53 mKO mice that lacked the \(MCK-Cre\) transgene. Control mice for ATF4 mKO were littermates of ATF4 mKO mice that lacked the \(MCK-Cre\) transgene. A: Mice from the indicated genotypes were subjected to three days of hindlimb immobilization, and then immobilized TA muscles were subjected to qPCR analysis of \(p53\) and \(ATF4\) mRNA levels. Data are means ± SEM from \(\geq 6\) mice per genotype. \(P\)-values were determined with unpaired \(t\)-tests. \(*P \leq 0.05\). N.S.: \(P \geq 0.05\).
Figure 2-16. **A**: Baseline analysis of p53/ATF4 mKO and littermate control mice. Data are means ± SEM from 8 mice per genotype. *P*-values were determined with unpaired *t*-tests. **B**: Representative H&E images from TA muscles of p53/ATF4 mKO and littermate control mice under basal conditions.
Figure 2-17. Combined loss of p53 and ATF4 prevents immobilization-induced muscle fiber atrophy. Bilateral TA muscles were collected from the indicated genotypes before and after three days of unilateral hindlimb immobilization. In each mouse, values from the immobile muscle were normalized to values from the mobile muscle. Data are means ± SEM from ≥ 4 mice per time point (≥ 250 fibers measured per muscle). Some error bars are too small to see. At each time point, *P*-values were determined by comparing values from knockout mice to values from their corresponding littermate control mice with an unpaired *t*-test (*P ≤ 0.05).
Figure 2-18. Combined loss of p53 and ATF4 prevents muscle atrophy during limb immobilization. Bilateral TA muscles were collected from the indicated genotypes before and after three days of unilateral hindlimb immobilization. In each mouse, values from the immobile muscle were normalized to values from the mobile muscle. Data are means ± SEM from ≥ 10 mice per time point. Some error bars are too small to see. At each time point, P-values were determined by comparing values from knockout mice to values from their corresponding littermate control mice with an unpaired t-test (*P ≤ 0.05).
**Figure 2-19.** Loss of p53 protects type II muscle fibers from immobilization-induced muscle fiber atrophy. p53 mKO and control mice were subjected to three days of unilateral hindlimb immobilization, and then bilateral TA muscles were sectioned and stained for myosin ATPase activity to distinguish type I and type II muscle fibers. Data are means ± SEM from ≥ 4 mice per genotype (≥ 250 fibers analyzed per muscle). *P*-values were determined with an unpaired *t*-test.

<table>
<thead>
<tr>
<th></th>
<th>Mobile</th>
<th>Immobile</th>
<th>Immobile / Mobile</th>
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<tbody>
<tr>
<td></td>
<td>p53 mKO Control</td>
<td>p53 mKO</td>
<td><em>P</em></td>
</tr>
<tr>
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<td>22.3 ± 1.2</td>
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<tr>
<td>Type II Diameter (μm)</td>
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<td>29.4 ± 0.5</td>
<td>0.48</td>
</tr>
<tr>
<td>% Type I</td>
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<td>0.18</td>
</tr>
<tr>
<td>% Type II</td>
<td>95.7 ± 0.5</td>
<td>95.0 ± 0.5</td>
<td>0.18</td>
</tr>
</tbody>
</table>
Figure 2-20. Loss of ATF4 protects type II muscle fibers from immobilization-induced muscle fiber atrophy. ATF4 mKO and control mice were subjected to three days of unilateral hindlimb immobilization, and then bilateral TA muscles were sectioned and stained for myosin ATPase activity to distinguish type I and type II muscle fibers. Data are means ± SEM from ≥4 mice per genotype (≥250 fibers analyzed per muscle). *P*-values were determined with an unpaired *t*-test.

<table>
<thead>
<tr>
<th></th>
<th>Mobile</th>
<th>Immobile</th>
<th>Immobile / Mobile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>ATF4 mKO</td>
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</tr>
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<tr>
<td>% Type I</td>
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<td>0.21</td>
</tr>
<tr>
<td>% Type II</td>
<td>95.6 ± 0.2</td>
<td>95.3 ± 0.3</td>
<td>0.21</td>
</tr>
</tbody>
</table>
Figure 2-21. Immobilization induces atrophy of type II muscle fibers via the combined actions of p53 and ATF4. p53/ATF4 mKO and control mice were subjected to three days of unilateral hindlimb immobilization, and then bilateral TA muscles were sectioned and stained for myosin ATPase activity to distinguish type I and type II muscle fibers. Data are means ± SEM from ≥ 4 mice per genotype (≥ 250 fibers analyzed per muscle). P-values were determined with an unpaired t-test.
**Figure 2-22.** Proposed relationship between limb immobilization, p53 expression, ATF4 expression, and skeletal muscle atrophy.
CHAPTER III: p53 AND ATF4 JOINTLY INDUCE p21 EXPRESSION DURING IMMOBILIZATION, LEADING TO MUSCLE ATROPHY.

This work was adapted from: Fox, D. K., Ebert, S. M., Bongers, K. S., Dyle, M. C., Bullard, S. A., Dierdorff, J. M., Kunkel, S. D., and Adams, C. M. (2014) p53 and ATF4 mediate distinct and additive pathways to skeletal muscle atrophy during limb immobilization. Am J Physiol Endocrinol Metab 307, E245-261

Abstract

Skeletal muscle atrophy is caused by complex signaling mechanisms that are incompletely understood at the molecular level. In previous studies, we identified the transcription factor ATF4 as an essential mediator of skeletal muscle atrophy. Using mouse models, we showed that ATF4 is necessary and sufficient for skeletal muscle atrophy during limb immobilization. However, loss of ATF4 in skeletal muscle did not completely abrogate skeletal muscle atrophy indicating the existence of another pro-atrophy factor. We identified p53 as this ATF4-independent factor. We found that like ATF4, p53 is necessary and sufficient for skeletal muscle atrophy. In addition, we found that p53 and ATF4 mediated distinct and additive pathways to skeletal muscle atrophy. However, the precise mechanism by which p53 and ATF4 cause skeletal muscle atrophy remained undetermined. Using genome-wide mRNA expression arrays, we identified p21 mRNA as a skeletal muscle transcript that is highly induced in immobilized muscle via the combined actions of p53 and ATF4. Additionally, in mouse muscle, p21 induces atrophy in a manner that does not require immobilization, p53 or ATF4; and p21 is
required for atrophy induced by immobilization, p53 and ATF4. Collectively, these results identify p53 and ATF4 as essential and complementary mediators of immobilization-induced muscle atrophy, and discover p21 as a critical downstream effector of the p53 and ATF4 pathways.

Introduction

Skeletal muscle atrophy is a common and debilitating consequence of critical illness, aging, muscle disuse, musculoskeletal and neurological disease, cancer, organ failure, chronic disease, and malnutrition (72,73). Skeletal muscle atrophy causes weakness, falls, fractures, and loss of independent living (7). While skeletal muscle atrophy is very common, it currently lacks a medical therapy. This is largely due to an incomplete understanding of the molecular mechanisms that drive the process.

To better understand the pathogenesis of this devastating condition, we recently investigated the role of the canonical stress response transcription factor ATF4. We found that ATF4 is sufficient to induce muscle fiber atrophy in vivo and that loss of ATF4 expression in skeletal muscle partially protects against muscle atrophy (59,60). While it appears that ATF4 plays a critical role in the setting of skeletal muscle atrophy, it was also clear that it was not the only pro-atrophy mediator of muscle fiber atrophy. This conclusion was based on the finding that muscle-specific ATF4 knockout mice were only partially resistant to skeletal muscle atrophy (59). We hypothesized that an ATF4-independent factor must exist and that this mediator likely works in parallel to ATF4 to promote muscle fiber atrophy.
We hypothesized that this atrophy-promoting factor might be the tumor suppressor p53 given that like ATF4, p53 functions as a canonical stress-induced transcription factor (27-29,53-55). In addition, previous work by others demonstrated that p53 is partially required for skeletal muscle atrophy during cancer cachexia (cancer-induced muscle atrophy) (50). Using mouse models, we found that p53 is both necessary and sufficient for skeletal muscle atrophy during limb immobilization (116). In addition, we found that p53-mediated muscle atrophy does not require ATF4 expression in skeletal muscle (116). Further, p53 is not required for ATF4-dependent muscle atrophy (116). Thus, p53 and ATF4 mediate distinct pathways to skeletal muscle atrophy. To determine whether these independent pathways might also be additive, we performed two experiments. First, we tested whether coexpression of p53 and ATF4 in mouse skeletal muscle might induce greater atrophy than overexpression of either transcription factor alone. We found that forced expression of p53 and ATF4 caused greater muscle atrophy than with either p53 or ATF4 alone (116). Second, we generated and phenotyped p53/ATF4 double knockout mice. During limb immobilization, p53/ATF4 mKO mice exhibit near complete resistance to skeletal muscle atrophy (116). Collectively, these data indicate that p53 and ATF4 mediate distinct and additive mechanisms to muscle atrophy during limb immobilization.

However, the precise mechanisms by which p53 and ATF4 induce muscle fiber atrophy remain unclear. Therefore, the focus of this study is to determine the downstream mechanisms that drive p53- and ATF4-dependent muscle atrophy. We hypothesize that
this unknown factor might also be highly induced by skeletal muscle atrophy. In addition, like p53 and ATF4, we hypothesize that this pro-atrophy mediator might also be a canonical stress-response protein. Thus, the goal of the current study is to identify how p53 and ATF4 drive skeletal muscle fiber atrophy.

**Materials And Methods**

*Mouse Strains*

C57BL/6 mice were obtained from the National Cancer Institute. All transgenic and knockout mice were on a C57BL/6 background. \textit{p53}^f/f mice were obtained from Jackson Laboratories, and are homozygous for a floxed \textit{p53} allele (exons 2-10 of the \textit{p53} gene are flanked by \textit{LoxP} restriction sites (81)). \textit{p53} mKO (muscle knockout) mice were generated by crossing \textit{p53}^f/f mice to transgenic mice expressing Cre recombinase under control of the \textit{muscle creatine kinase} (MCK) promoter (82,116). In studies of \textit{p53} mKO mice, control mice were \textit{p53}^f/f littermates that lacked the \textit{MCK-Cre} transgene (116). \textit{ATF4} mKO mice were described previously (59) and were generated by crossing mice homozygous for a floxed \textit{ATF4} allele (\textit{ATF4}^f/f) to \textit{MCK-Cre} mice. In studies of \textit{ATF4} mKO mice, control mice were \textit{ATF4}^f/f littermates that lacked the \textit{MCK-Cre} transgene. \textit{p53}/\textit{ATF4} double mKO mice were generated by crossing \textit{p53}^f/f and \textit{ATF4}^f/f mice to generate mice homozygous for floxed \textit{p53} and \textit{ATF4} alleles (\textit{p53}^f/f\textit{ATF4}^f/f), and then crossing \textit{p53}^f/f\textit{ATF4}^f/f mice to \textit{MCK-Cre} mice (116). In studies of \textit{p53}/\textit{ATF4} double mKO mice, control mice were \textit{p53}^f/f\textit{ATF4}^f/f littermates that lacked the \textit{MCK-Cre} transgene.
Mouse Protocols

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Iowa. All mice were 8-12 week old males. Mice were housed at 21 °C in colony cages with 12 h light/12 h dark cycles and provided ad libitum access to standard chow (Harlan Teklad formula 7913) and water. Unilateral hindlimb immobilization was performed under isoflurane anesthesia using an Autosuture Royal 35W skinstapler (Tyco Healthcare). Briefly, the ankle joint and tibialis anterior muscle were immobilized by fixing the ventral side of the foot to the distal portion of the calf; the knee and hip joints remained mobile, and ambulation was impaired but not entirely prevented, as described previously (59,83,116). Transfection of mouse skeletal muscle with plasmid DNA was performed as described previously (60,116).

Histological Analysis of Mouse Skeletal Muscle

Histologic analysis of mouse skeletal muscle was performed as described previously (59,60,85-87,116). Briefly, hematoxylin and eosin (H&E) stains were performed by embedding and freezing skeletal muscles in Tissue Freezing Medium (Triangle Biomedical), and then preparing 10-µm sections from the midbelly of the muscle with a Microm HM 505E cryostat. Muscle sections were then fixed in ice cold zinc formalin for 15 min before staining with a DRS-601 automatic slide stainer (Sakura). Sections were imaged using an Olympus BX-61 automated upright microscope, and image analysis was performed with ImageJ. For visualization of GFP fluorescence, muscles were fixed in 4% paraformaldehyde (w/v) for 16 h, incubated in 30% sucrose for 24 h, embedded in Tissue Freezing Medium, and then snap frozen using a Stand-Alone
Gentle Jane (Instrumedics). A Microm HM 505E cryostat was then used to prepare 10-µm sections from the midbelly of the muscle. Sections were then washed 3X with PBS and mounted with Vectashield (Vector Laboratories). Sections were imaged on an Olympus IX-71 microscope, and image analysis was performed with ImageJ. Transfected fibers were defined as fibers having a mean fluorescence ≥ 25 arbitrary units above background, as described previously (59,60,87). For all muscle fiber measurements, the diameters of ≥ 200 muscle fibers per muscle were measured using the lesser diameter (minimal Feret diameter) method, as recommended elsewhere (5).

**Plasmids**

*p-wt-p53* encodes wild-type mouse p53 (NM_011640.3) under control of the cytomegalovirus (CMV) promoter. *p-eGFP* encodes eGFP under control of the CMV promoter. *p-p21-FLAG* was generated by amplifying the coding region of mouse p21 (NM_007669) from mouse muscle cDNA, and then cloning into p3XFLAG-CMV10 (Sigma) to place three copies of the FLAG epitope tag at the NH3-terminus. *p-miR-Control* encodes a non-targeting pre-miRNA and EmGFP, both under control of the CMV promoter (59). *p-miR-p21 #1* and *p-miR-p21 #2* were generated by ligating Mmi506259 and Mmi506257 oligonucleotide duplexes (Invitrogen), respectively, into the pcDNA6.2GW/EmGFP miR plasmid (Invitrogen), which contains a CMV promoter driving co-cistronic expression of engineered pre-miRNAs and EmGFP (Invitrogen).
**Immunoblot Analysis of Mouse Skeletal Muscle**

Skeletal muscles were snap-frozen in liquid nitrogen and homogenized in 1 ml ice cold homogenization buffer (50 mM HEPES, 4 mM EGTA, 10 mM EDTA, 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, cOmplete Mini protease inhibitor mixture (Roche Applied Science), 25 mM sodium fluoride, 1% (vol/vol) Triton X-100, and 1:100 dilution of phosphatase inhibitor cocktails 2 and 3 (Sigma)) (87) using a Tissue Master 240 (Omni International) for 1 min on setting #10. The muscle homogenate was rotated for 1 h at 4 °C, and then centrifuged at 16,000 x g for 20 min at 4 °C. An aliquot of the supernatant was used to determine protein concentration by the BCA method (Pierce), and another aliquot was mixed with 0.25 volume of sample buffer (250 mM Tris-HCl, pH 6.8, 10% SDS, 25% glycerol, 0.2% (w/v) bromophenol blue, and 5% (w/v) 2-mercaptoethanol) and heated at 95 °C for 5 min. An equal amount of protein from each sample was subjected to SDS-PAGE, and then transferred to Hybond-C extra nitrocellulose filters (Millipore). Immunoblots were performed at 4 °C for 16 h using a 1:4,000 dilution of monoclonal anti-mouse FLAG (Sigma #F1804), or a 1:1,000 dilution of monoclonal anti-mouse p21 (Santa Cruz Biotechnology SC-6246: F-5).

**Quantitative Real-time RT-qPCR (qPCR)**

Extraction of skeletal muscle RNA was performed using TRIzol solution (Invitrogen) and purified with Turbo DNAfree kit (Ambion) as described previously (60). Quantitative real-time RT-PCR (qPCR) was performed as previously described (60) using a High Capacity cDNA reverse transcription kit (Applied Biosystems). qPCR studies were performed with a 7500 Fast Real-time PCR System (Applied Biosystems).
using p21 Taqman Gene Expression Assays (Applied Biosystems). All qPCR samples were run in triplicate and the cycle threshold (C<sub>t</sub>) values were averaged. For data analysis, the ΔΔC<sub>t</sub> method was utilized, with 36B4 mRNA serving as the invariant control.

**mRNA Expression Arrays**

Skeletal muscle RNA was extracted using TRIzol solution (Invitrogen), and then purified using the RNeasy kit and RNase Free DNase Set (Qiagen). RNA hybridization to Mouse Ref-8 v2.0 BeadChip arrays (Illumina) was performed by the Southern California Genotyping Consortium (University of California, Los Angeles). Briefly, purified RNA was quantified using a Ribogreen fluorescent assay and normalized to 10 ng/ul prior to amplification. RNA was then amplified and labeled (Ambion TotalPrep Kit) and cDNA was synthesized using robotic-assisted magnetic capture. Using the cDNA template, biotinylated cRNA was produced via an in vitro transcription reaction. Amplified and labeled cRNA was quantified (Ribogreen assay) and hybridized to Mouse Ref-8 v2.0 BeadChip Arrays (Illumina) overnight at 58 °C. Following hybridization, arrays were washed, blocked, stained, and dried (Little Dipper Processor). Arrays were scanned with an iScan reader and data were extracted and analyzed with BeadStudio Software (Illumina).
**Statistical Analysis**

For comparisons between two groups, paired *t*-tests were used for within-subject samples and unpaired *t*-tests were used for independent samples. Comparisons involving multiple groups were analyzed by one-way ANOVA with Sidak’s post hoc test.

**Results**

*Identification of p21 as a skeletal muscle mRNA that is highly induced by immobilization via the combined actions of p53 and ATF4.*

Since p53 and ATF4 are transcription factors, we reasoned that they might promote muscle atrophy by altering skeletal muscle gene expression. In previous studies, we used genome-wide mRNA expression arrays to determine the effects of ATF4 on muscle mRNA expression (59,60). To determine the effects of p53 on muscle mRNA expression, we used mRNA expression arrays that quantitated levels of 17,192 mRNAs. We began by searching for TA muscle mRNAs that were strongly upregulated (≥ 2-fold) by three days of hindlimb immobilization in C57BL/6 mice. We identified 33 mRNAs (represented by 37 probes) that met these criteria (Fig. 3-1). We then performed additional mRNA expression arrays to determine which of these 33 mRNAs were also: 1) decreased in immobilized p53 mKO TA muscle relative to immobilized control TA muscle; and 2) increased by p53 overexpression in mobile C57BL/6 TA muscle relative to mobile C57BL/6 TA muscle that was transfected with a control plasmid.

As expected, immobilization strongly induced mRNAs encoding several known mediators of muscle atrophy, including Gadd45a, HDAC4, MuRF1 and MAFbx/atrogen-
1 (Fig. 3-1). However, none of these mRNAs were decreased by loss of p53, and none were increased by p53 overexpression (Fig. 3-1). In contrast, two skeletal muscle mRNAs were highly induced by hindlimb immobilization, and also decreased by loss of p53 in immobilized muscle, and increased by p53 overexpression. These two mRNAs encode p21 (also known as Waf1, Cip1, and cyclin-dependent kinase inhibitor 1a (Cdkn1a), and the α1 subunit of the nicotinic acetylcholine receptor (Fig. 3-1).

Interestingly, p21 is a well-established p53 target gene (63,117), and p21 mRNA is highly induced during muscle atrophy (22,45-49,60,67-70). In addition, p21 mRNA is regulated by ATF4, which is sufficient to increase p21 mRNA and p21 protein in mouse TA muscle (60). Based on these considerations, we chose to investigate p21 as a potential downstream mediator of p53 and ATF4 in immobilized muscle.

To identify cellular processes that are induced or repressed by hindlimb immobilization and p53 overexpression and regulated in the opposite direction by genetic deletion of the p53 in p53 mKO mice, we performed Gene Set Enrichment Analysis (118). Using the KEGG database, we identified seven gene sets that were induced by muscle immobilization and p53 overexpression and reduced in p53 mKO skeletal muscle (FDR ≤ 0.25 and P ≤ 0.05; (Fig. 3-3)). Interestingly, the majority of these gene sets represent molecular processes that are known to be involved in muscle atrophy, including inflammation, altered protein homeostasis, and maintenance of the extracellular matrix. Conversely, six gene sets were reduced by muscle immobilization and p53 overexpression and induced in p53 mKO skeletal muscle (Fig. 3-3). All of these gene sets represent cellular processes that regulate the biogenesis and function of mitochondria in a
number of different cell types. Importantly, in skeletal muscle, mitochondrial function is critical for the maintenance of muscle fiber size and mass.

Using qPCR, we confirmed that hindlimb immobilization increased p21 mRNA (Fig. 3-4). As expected, this increase in p21 mRNA was accompanied by an increase in p21 protein (Fig. 3-5). Likewise, p53 overexpression in non-immobilized muscle increased p21 mRNA (Fig. 3-6) and p21 protein (Fig. 3-7), similar to the effects of ATF4 overexpression in non-immobilized muscle (59). qPCR analysis of p53 mKO muscle also confirmed that p53 was partially required for the induction of p21 mRNA during immobilization (Fig. 3-8).

Importantly, qPCR analysis of ATF4 mKO muscle showed that ATF4, like p53, was partially required for the induction of p21 mRNA during immobilization (Fig. 3-8). Furthermore, the combined loss of p53 and ATF4 in p53/ATF4 mKO muscle decreased p21 mRNA more than loss of either transcription factor alone, and the effect was nearly additive (Fig. 3-8). Collectively, these results indicate that immobilization increases p21 mRNA through the combined actions of p53 and ATF4. This suggested the hypothesis that p53 and ATF4 might promote muscle atrophy by increasing p21 expression.

*Increased skeletal muscle p21 expression induces skeletal muscle atrophy.*

To test the hypothesis that p21 promotes muscle atrophy, we transfected TA muscles of C57BL/6 mice with a plasmid encoding p21. The contralateral TA in each mouse was transfected with empty plasmid, and both TA muscles remained mobile
throughout the experiment. We found that transfection of p21 plasmid increased p21 expression (Fig. 3-9). Like p53 and ATF4, forced expression of p21 induced muscle fiber atrophy in C57BL/6 mice. These data indicate that p21 is sufficient to induce muscle fiber atrophy.

We next hypothesized that p21 might induce muscle atrophy in a p53- and ATF4-independent manner since p21 likely lies downstream of both transcription factors. We therefore transfected p53/ATF4 mKO muscles with plasmid encoding p21. Like in C57BL/6 mice, we found that p21 causes muscle fiber atrophy even in the absence of p53 and ATF4 expression. Taken together, p21 induces muscle atrophy in a manner that does not require immobilization, p53, or ATF4.

*p21 is required for muscle fiber atrophy induced by immobilization*

Because loss of p53 and ATF4 expression in muscle provides protection from immobilization-induced muscle fiber atrophy, we hypothesized that p21 might also be required for muscle atrophy as well. We therefore generated two artificial microRNA constructs (miR-p21 #1 and miR-p21 #2) that target distinct regions of the p21 mRNA and specifically reduce p21 protein (Fig. 3-11). We then transfected the TA muscles of C57BL/6 mice with plasmids encoding either miR-p21 #1, miR-p21 #2, or a nontargeting control miRNA (miR-Control). Following transfection, we immobilized one TA in each mouse for three days before comparing muscle fibers from bilateral TAs. Neither miR-p21 construct altered fiber size in mobile muscles (Figs. 3-12), consistent with the finding that p21 mRNA is low under basal conditions. However, both miR-p21 constructs
reduced muscle fiber atrophy in immobilized muscles (Figs. 3-12), indicating that p21 is required for immobilization-induced muscle atrophy.

*p21 is required for p53- and ATF4-mediated muscle fiber atrophy.*

To test the hypothesis that p21 is a critical downstream mediator of p53 and ATF4, we co-transfected mouse TA muscle with plasmids encoding p53 plus plasmids encoding miR-Control, miR-p21 #1, or miR-p21 #2. Both miR-p21 constructs significantly decreased muscle fiber atrophy caused by p53 (Figs. 3-13, 3-14). These data indicate that p21 is required for p53-mediated muscle atrophy.

We next tested whether p21 might also be required for ATF4-mediated muscle atrophy, we transfected C57BL/6 mice with plasmids encoding ATF4 plus plasmids encoding miR-Control, miR-p21 #1, or miR-p21 #2. Both miR-p21 constructs decreased ATF4-mediated muscle fiber atrophy (Figs. 3-15, 3-16). Collectively, these data indicate that p21 is required for atrophy due to immobilization, p53, and ATF4. Our data indicate that limb immobilization jointly increases expression of the transcription factors p53 and ATF4 leading to induction of p21 expression and skeletal muscle fiber atrophy (Fig. 3-17).

**Discussion**

Skeletal muscle atrophy is a highly prevalent, debilitating condition that results from aging, muscle disuse, cancer, malnutrition, chronic disease and organ failure (72, 73). Skeletal muscle atrophy often leads to falls, fractures, and loss of independent
living (7). Despite its prevalence, little is known regarding the pathogenesis of this devastating condition. As such, few therapies exist to treat the patients that suffer from this condition.

In order to determine the molecular mechanisms that mediate the atrophy process, we recently investigated the role of the basic leucine zipper transcription factor ATF4. We found that ATF4 is highly induced by cellular stress and that forced expression of ATF4 causes muscle fiber atrophy (60). In addition, we found that muscle-specific ATF4 knockout mice were partially protected from skeletal muscle atrophy (59). While ATF4 appears to play an essential role in the pathogenesis of skeletal muscle atrophy, it became clear that another pro-atrophy factor must exist that drives muscle atrophy even in the absence of ATF4 expression. Using mouse models, we identified the tumor suppressor p53 as an ATF4-independent factor to skeletal muscle atrophy. We found that like ATF4, p53 is both necessary and sufficient for skeletal muscle atrophy during limb immobilization (116). In addition, we found that p53 and ATF4 mediate distinct and additive pathways to skeletal muscle atrophy (116). However, the exact mechanism by which p53 and ATF4 cause muscle fiber atrophy remained undetermined.

Therefore, the goal of the current study was to decipher the downstream mechanisms of p53- and ATF4-mediated muscle atrophy. Our results identify p21 as a key factor that is regulated by p53 and ATF4 during skeletal muscle atrophy. Using an unbiased approach, we found that immobilization strongly induces p21 mRNA in a manner that is dependent upon both p53 and ATF4. In addition, we found that p53, like
ATF4 (60), is sufficient to increase p21 mRNA and p21 protein in skeletal muscle. Furthermore, our data demonstrate that p21 is required for muscle fiber atrophy induced by immobilization, p53, and ATF4; and p21 is sufficient to induce muscle fiber atrophy in the absence of immobilization, p53, and ATF4. Thus, p53 and ATF4 jointly induce p21 expression during immobilization, leading to muscle atrophy.

The mechanisms by which p53 and ATF4 increase p21 mRNA require further investigation. It seems likely that p53 directly activates the p21 gene, given that p53 binds and activates the p21 promoter in other cell types (61,63-65). However, ATF4 is not known to directly regulate the p21 gene. Thus, it remains unknown if ATF4 increases p21 expression by a direct or indirect mechanism. It is also important to note that the combined loss of p53 or ATF4 did not completely eliminate p21 mRNA in immobilized muscle. This may reflect the existence of other factors that contribute to p21 expression in immobilized skeletal muscle fibers. Alternatively, the residual p21 mRNA in immobilized p53/ATF4 mKO muscles may have arisen from satellite cells and non-muscle cells, which do not express MCK-Cre, and thus, maintain p53 and ATF4 expression. Additional studies are needed to resolve this issue.

Understanding how p21 causes muscle atrophy is another important area for future study. Although p21 is a well-known cell cycle inhibitor (62), it seems unlikely that p21 promotes muscle fiber atrophy by inhibiting the cell cycle. During muscle atrophy, p21 specifically increases in the nuclei of skeletal muscle fibers (which are post-mitotic), but not in satellite cells (which retain mitotic potential) (71). Furthermore, the
interventions we used in this study specifically targeted skeletal muscle fibers (95). Thus, it seems probable that p21 promotes muscle atrophy via cell cycle-independent mechanisms in skeletal muscle fibers. We speculate that these mechanisms may lead to cellular changes that are known to promote muscle atrophy, including reduced anabolic signaling, increased proteolysis, decreased protein synthesis, and impaired mitochondrial function. We also speculate that p21 may play an essential role in other types of skeletal muscle atrophy, since p21 mRNA is one of the most highly induced skeletal muscle mRNAs during aging (45,46,48,67), muscle denervation (22,68), fasting (60), hindlimb unloading (47), amyotrophic lateral sclerosis (ALS) (69), and critical illness (49,70).

Although p21 is a key downstream mediator of p53 and ATF4, it is also clear that p53 and ATF4 regulate many genes and promote muscle atrophy by additional mechanisms. For example, ATF4 also promotes muscle atrophy by activating the Gadd45a gene, and Gadd45a is sufficient to induce muscle fiber atrophy and partially required for muscle fiber atrophy during limb immobilization (59). In the current study, we found that Gadd45a mRNA was the most highly induced mRNA in immobilized skeletal muscle, presumably due to the actions of ATF4. However, we also found that p53 is neither sufficient to increase Gadd45a mRNA nor required for immobilization-induced Gadd45a expression - a somewhat surprising result since p53 directly activates the Gadd45a gene in many other cell types (119-121). In the setting of Huntington disease, p53 increases skeletal muscle expression of caspase-6, which is thought to contribute to muscle atrophy (122). Furthermore, in other cell types, p53 induces genes that could potentially play a role in skeletal muscle atrophy, such as the genes encoding
sestrin 1 and sestrin 2 (123). Thus, in addition to *p21*, other genes contribute to the pro-
atrophy effects of p53 and ATF4.

In summary, the current study helps explain how p53 and ATF4 causes muscle
atrophy during limb immobilization, and it opens several new avenues of investigation.
In addition, the current study has potential implications for patients with immobilization-
induced muscle atrophy due to injury or illness: optimal prevention and treatment of this
condition may require interventions that effectively inhibit the p53, ATF4, and/or p21 in
skeletal muscle.
Figure 3-1. Skeletal muscle mRNA transcripts that are ≥ 2-fold induced by immobilization, and the effects of p53 overexpression and p53 gene deletion on those transcripts. C57BL/6 mice were subjected to three days of unilateral hindlimb immobilization, and then mRNA levels in bilateral TA muscles were analyzed with Mouse Ref-8 v2.0 BeadChip arrays. In each mouse, mRNA levels in the immobilized TA were normalized to mRNA levels in the contralateral (mobile) TA, and P-values were determined with paired t-tests (n = 4 arrays per condition). Statistical significance was arbitrarily defined as P ≤ 0.05. The figure shows all mRNAs that were at least 2-fold increased by hindlimb immobilization (33 mRNAs represented by 37 probes). To determine the effects of p53 gene deletion on these mRNAs, p53 mKO mice and littermate control mice were subjected to three days of hindlimb immobilization, and then immobilized TA muscles were harvested and analyzed with Mouse Ref-8 v2.0 BeadChip arrays. mRNA levels in immobilized p53 mKO muscles were normalized to mRNA levels in immobilized control muscles, and P-values were determined with unpaired t-
Figure 3-1 continued. To determine the effects of p53 overexpression on these mRNAs, one TA muscle in C57BL/6 mice was transfected with 20 µg p-wt-p53, and the contralateral TA was transfected with 20 µg empty plasmid (pcDNA3). Three days later, bilateral TAs were harvested and analyzed with Mouse Ref-8 v2.0 BeadChip arrays. mRNA levels in muscles transfected with p53 plasmid were normalized to mRNA levels in muscles transfected with control plasmid, and P-values were determined with paired t-tests (n = 4 arrays per condition). Shading indicates mRNAs that were ≥ 2-fold increased by immobilization, reduced by loss of p53, and increased by p53 overexpression.
Figure 3-2. Comparison of transcripts increased by muscle immobilization, p53 overexpression and decreased in p53 mKO skeletal muscle. Illumina Mouse Ref-8 v2.0 BeadChip arrays were used to identify mRNAs that were increased by hindlimb immobilization (C57BL/6 TA immobile versus mobile) and p53 overexpression (p53 plasmid versus control plasmid) and decreased by p53 gene deletion (immobile p53 mKO mice versus immobile littermate control mice). n ≥ 3 arrays per condition and statistical significance was arbitrarily defined as p ≤ 0.04 by t-test with a fold change of 1.3. Numbers indicate the number of transcripts in each category.
Table 3.1. KEGG gene sets similarly affected by hindlimb immobilization, forced expression of p53, and p53 gene deletion (p53 mKO mice), as assessed by Gene Set Enrichment Analysis (GSEA). Data used for this analysis is presented in Figures 3-1, 3-2. FDR ≤ 0.25 and P ≤ 0.05 for all gene sets shown, as previously described (24).

<table>
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<tr>
<th>Gene Sets</th>
<th>Effect of Immobilization</th>
<th>Effect of p53 Overexpression</th>
<th>Effect of p53 mKO</th>
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<td>Alzheimers Disease</td>
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**Figure 3-3.** KEGG gene sets similarly affected by hindlimb immobilization, forced expression of p53, and p53 gene deletion (p53 mKO mice), as assessed by Gene Set Enrichment Analysis (GSEA). Data used for this analysis is presented in Figures 3-1, 3-2. FDR ≤ 0.25 and P ≤ 0.05 for all gene sets shown, as previously described (24).
Figure 3-4. Hindlimb immobilization increases \( p21 \) expression in skeletal muscle. C57BL/6 were subjected to three days of unilateral hindlimb immobilization, and then bilateral TA muscles were harvested for qPCR analysis of \( p21 \) mRNA levels. Data are means ± SEM from 4 mice. \( P \)-values were determined with paired \( t \)-tests (*\( P \leq 0.05 \)).
Figure 3-5. Limb immobilization increases skeletal muscle p21 expression. C57BL/6 were subjected to three days of unilateral hindlimb immobilization, and then bilateral TA muscles were harvested for further analysis. An equal amount of protein (100 µg) from each muscle was subjected to immunoblot analysis with anti-p21 monoclonal IgG. Membranes were stained with Ponceau S to confirm equal loading. **Left**, representative immunoblots. **Right**, quantification. Data are means ± SEM from 4 mice. *P*-values were determined with paired *t*-tests (*P* ≤ 0.05).
Figure 3-6. p53 increases p21 expression in mouse skeletal muscle. In C57BL/6 mice, one TA muscle was transfected with 20 µg p-wt-p53, and the contralateral TA ("Control") was transfected with 20 µg empty plasmid (pcDNA3). Three days later, bilateral TAs were harvested for qPCR analysis of p21 mRNA levels. Data are means ± SEM from 4 mice. *P-values were determined with paired t-tests (*P ≤ 0.05).
Figure 3-7. p53 induces expression of p21 in mouse skeletal muscle fibers. In C57BL/6 mice, one TA muscle was transfected with 20 µg p-wt-p53, and the contralateral TA ("Control") was transfected with 20 µg empty plasmid (pcDNA3). Three days later, bilateral TAs were harvested for further analysis. An equal amount of protein (100 µg) from each muscle was subjected to immunoblot analysis with anti-p21 monoclonal IgG. Membranes were stained with Ponceau S to confirm equal loading. Left, representative immunoblots. Right, quantification. Data are means ± SEM from 4 mice. P-values were determined with paired t-tests (*P ≤ 0.05).
Figure 3-8. Immobilization increases skeletal muscle $p21$ mRNA in a manner that is dependent on both p53 and ATF4. Mice with the indicated genotypes were subjected to three days of unilateral hindlimb immobilization, and then qPCR was used to quantitate $p21$ mRNA levels in bilateral TA muscles. Data are means ± SEM from ≥ 10 mice per genotype. For each knockout line and its corresponding littermate control line, $p21$ mRNA levels were normalized to the level in the control line, and $P$-values were determined with unpaired $t$-tests (*$P \leq 0.05$).
**Figure 3-9.** p21 is sufficient to induce skeletal muscle fiber atrophy. **A-C:** In C57BL/6 mice, one TA muscle was transfected with 15 µg p-p21-FLAG plus 2 µg p-eGFP, and the contralateral TA ("Control") was transfected with 15 µg empty plasmid (pcDNA3) plus 2 µg p-eGFP. Bilateral TAs were harvested seven days later for further analysis. **A:** An equal amount of protein from each muscle (40 µg) was subjected to immunoblot analysis with anti-FLAG monoclonal IgG. Membranes were stained with Ponceau S to confirm equal loading. **B-C:** Histological analysis. **B:** Representative images. **C:** Quantification of muscle fiber size. Left, fiber size distributions; each distribution represents ≥ 2500 fibers from 5 muscles. Right, mean fiber diameters; each data point represents the mean diameter of ≥ 400 fibers from one muscle. Horizontal bars denote means ± SEM. *P*-values were determined with a paired *t*-test.
Figure 3-10. p21 induces muscle fiber atrophy in a manner that does not require p53 or ATF4. A-B: TA muscles of p53/ATF4 mKO mice were transfected with 15 µg p-p21-FLAG plus 2 µg p-eGFP, and the contralateral TA ("Control") was transfected with 15 µg empty plasmid (pcDNA3) plus 2 µg p-eGFP. Bilateral TAs were harvested seven days later for further analysis. A: Representative images. B: Quantification of muscle fiber size. Left, fiber size distributions; each distribution represents ≥1200 fibers from 4 muscles. Right, mean fiber diameters; each data point represents the mean diameter of ≥250 fibers from one muscle. Horizontal bars denote means ± SEM. P-values were determined with a paired t-test.
RNA interference targeting p21 mRNA reduces p21 expression in mouse skeletal muscle. TA muscles in C57BL/6 mice were transfected with 10 µg p-p21-FLAG plus either 20 µg p-miR-Control, 20 µg p-miR-p21#1, or 20 µg p-miR-p21#2, then harvested three days later for immunoblot analysis with anti-FLAG monoclonal IgG (40 µg protein loaded per sample). Membranes were stained with Ponceau S to confirm equal loading.

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Figure 3-12. p21 is required for immobilization-induced muscle fiber atrophy. **A-B:** On day 0, bilateral TA muscles in C57BL/6 mice were transfected with 20 µg p-miR-Control, 20 µg p-miR-p21#1, or 20 µg p-miR-p21#2. On day 3, mice were subjected to unilateral hindlimb immobilization. On day 6, bilateral TA muscles were harvested for histological analysis. **A:** Representative images. **B:** Quantification of muscle fiber size. Each data point represents the mean diameter of ≥ 250 fibers from one muscle. Horizontal bars denote means ± SEM. Statistical analysis used the one-way ANOVA with Sidak’s post hoc test; different letters are statistically different (P ≤ 0.05).
Figure 3-13. p21 is required for p53-mediated muscle fiber atrophy. **A-B**: In C57BL/6 mice, one TA muscle was transfected with 10 µg p-wt-p53 plus 20 µg p-miR-p21#1; the contralateral TA ("Control") was transfected with 10 µg p-wt-p53 plus 20 µg p-miR-Control. Bilateral TAs were harvested seven days later for histological analysis. **A**: Representative images. **B**: Quantification of muscle fiber size. Left, fiber size distributions; each distribution represents ≥ 1500 fibers from 6 muscles. Right, mean fiber diameters; each data point represents the mean diameter of ≥ 250 fibers from one muscle. Horizontal bars denote means ± SEM. $P$-values were determined with a paired $t$-test.
Figure 3-14. RNAi Targeting p21 expression reduces p53-mediated muscle fiber atrophy. **A-B:** In C57BL/6 mice, one TA muscle was transfected with 10 µg p-wt-p53 plus 20 µg p-miR-p21#2; the contralateral TA ("Control") was transfected with 10 µg p-wt-p53 plus 20 µg p-miR-Control. Bilateral TAs were harvested seven days later for histological analysis. **A:** Representative images. **B:** Quantification of muscle fiber size. **Left,** fiber size distributions; each distribution represents ≥ 1500 fibers from 6 muscles. **Right,** mean fiber diameters; each data point represents the mean diameter of ≥ 250 fibers from one muscle. Horizontal bars denote means ± SEM. *P*-values were determined with a paired *t*-test.
**Figure 3-15.** p21 is required for ATF4-mediated muscle fiber atrophy. **A-B:** In C57BL/6 mice, one TA muscle was transfected with 10 µg p-ATF4-FLAG plus 20 µg p-miR-p21#1, as indicated; the contralateral TA ("Control") was transfected with 10 µg p-ATF4-FLAG plus 20 µg p-miR-Control. Bilateral TAs were harvested seven days later for histological analysis. **A:** Representative images. **B:** Quantification of muscle fiber size. Left, fiber size distributions; each distribution represents ≥ 1600 fibers from 6 muscles. Right, mean fiber diameters; each data point represents the mean diameter of ≥ 250 fibers from one muscle. Horizontal bars denote means ± SEM. *P*-values were determined with a paired *t*-test.
Figure 3-16. RNAi Targeting p21 expression reduces ATF4-mediated muscle fiber atrophy. 

**A-B:** In C57BL/6 mice, one TA muscle was transfected with 10 µg p-ATF4-FLAG plus 20 µg p-miR-p21#2, as indicated; the contralateral TA ("Control") was transfected with 10 µg p-ATF4-FLAG plus 20 µg p-miR-Control. Bilateral TAs were harvested seven days later for histological analysis.  

**A:** Representative images.  

**B:** Quantification of muscle fiber size.  
- Left, fiber size distributions; each distribution represents ≥1600 fibers from 6 muscles.  
- Right, mean fiber diameters; each data point represents the mean diameter of ≥250 fibers from one muscle.  

Horizontal bars denote means ± SEM. *P*-values were determined with a paired *t*-test.
Figure 3-17. Proposed relationship between hindlimb immobilization, p53 expression, ATF4 expression, p21 expression, and skeletal muscle fiber atrophy.
Conclusions

Skeletal muscle atrophy is a highly prevalent condition that occurs in the setting of critical illness, malnutrition, aging, bed rest, musculoskeletal disorders (e.g. immobilization, arthritis), neurological disease (e.g. multiple sclerosis, spinal cord injury, amyotrophic lateral sclerosis, stroke, diabetic neuropathy), renal failure, cirrhosis, COPD, heart failure, and cancer (7,8,16). Skeletal muscle atrophy results in a decrease in muscle fiber size and strength, leading to weakness, falls and fractures (7). In addition, those affected suffer from debilitation, loss of independent living, and often insulin resistance (7,15). Despite its prevalence, no pharmacotherapies exist to treat this devastating condition. This is likely the result of an incomplete understanding of its pathogenesis at the molecular level.

The goal of this thesis is therefore to improve upon our understanding of the basic mechanisms that occur at the cellular level to drive muscle fiber atrophy. Using genetic models, we identified p53 and ATF4 as novel regulators of skeletal muscle atrophy during limb immobilization. Additionally, we identified p21 as an essential downstream regulator of p53- and ATF4-mediated muscle atrophy using an unbiased gene expression microarray approach. These results greatly advance our understanding of the pathogenesis of skeletal muscle atrophy, and open several important new areas for future studies. Moreover, the current study has potential implications for patients with skeletal muscle atrophy: optimal treatment of this debilitating condition may require therapeutic interventions that decrease expression or activity of p53, ATF4, and/or p21 in skeletal
Identification of p53 and ATF4 as Novel Mediators of Immobilization-Induced Muscle Atrophy

In Chapter II, we describe the roles of the stress-induced transcription factors p53 and ATF4 in the setting of immobilization-induced muscle atrophy. In a previous study, we identified ATF4 as an essential mediator of skeletal muscle fiber atrophy (60). We found that skeletal muscle ATF4 expression was induced by muscle disuse and fasting (23,59,60). In addition, we found that forced expression of ATF4 was sufficient to induce muscle atrophy in mouse skeletal muscle (60). Furthermore, mice that lack ATF4 expression in skeletal muscle fibers undergo less muscle atrophy when compared with littermate controls (59). However, it was also clear that ATF4 was not the sole mediator of skeletal muscle atrophy given that muscle-specific ATF4 knockout (ATF4 mKO) mice were not completely resistant to muscle atrophy (59). The muscle fiber atrophy that occurred in the absence of ATF4 expression indicated that there might be another mediator of muscle atrophy that works in parallel to or in concert with ATF4 to induce muscle fiber atrophy.

We therefore focused our studies on the identification of this ATF4-independent factor. Because ATF4 functions as a canonical stress response transcription factor (53-55), we hypothesized that its counterpart might also mediate cellular responses to stress. The tumor suppressor p53 represented an attractive candidate given its role as a classical stress-response transcription factor (27-29). In addition, previous studies demonstrated
that global p53 knockout mice are partially resistant to cancer-induced muscle fiber atrophy (50). In addition, others have shown that global expression of an activated p53 construct in mice leads to severe age-related muscle atrophy (sarcopenia) (51). We therefore hypothesized that p53 might function as the ATF4-independent factor to skeletal muscle atrophy.

We first studied whether p53 expression was induced in the presence of muscle atrophy. We therefore assessed p53 expression levels in the presence or absence of limb immobilization. We found that limb immobilization increased p53 expression and induced muscle atrophy. We next tested whether forced expression of p53 might be sufficient to induce muscle fiber atrophy similar to ATF4. We found that p53 overexpression caused muscle fiber atrophy in mice. In addition, we generated a transcriptionally inactive mutant p53 construct with two well-described amino acid substitutions in the DNA-binding domain (R270H, P275S). Interestingly, this mutant p53 construct was unable to induce muscle fiber atrophy (unlike wild type p53). Taken together, these data indicated that p53 is sufficient to induce muscle atrophy and that this effect requires an intact DNA-binding domain.

We next tested whether p53, like ATF4, was required for skeletal muscle atrophy. To that end, we generated muscle-specific p53 knockout mice (p53 mKO), which lack p53 expression in skeletal muscle. Like ATF4 mKO mice, p53 mKO mice lacked an observable phenotype under basal conditions. However, in the presence of limb immobilization, p53 mKO mice displayed partial protection from skeletal muscle fiber
atrophy. These data indicated that p53, like ATF4, is both necessary and sufficient to muscle fiber atrophy in vivo.

In skeletal muscle fibers, p53 appeared to resemble ATF4 in that both p53 and ATF4 expression were increased by muscle atrophy stimuli. In addition, p53 and ATF4 were both sufficient to induce skeletal muscle atrophy. Furthermore, p53 mKO and ATF4 mKO mice were partial protected from skeletal muscle fiber atrophy. We therefore hypothesized that p53 and ATF4 might function in concert to promote muscle atrophy.

To test this hypothesis, we first assessed whether ATF4 might function as a critical downstream mediator of p53-dependent muscle fiber atrophy. We therefore overexpressed p53 in ATF4 mKO skeletal muscle fibers. Interestingly, like in wild type mice, forced expression of p53 induced muscle fiber atrophy, even in the absence of ATF4 expression. This indicated that ATF4 was not required for p53-mediated muscle atrophy.

We next tested whether ATF4 might be sufficient to induce muscle fiber atrophy in a p53-independent manner. We therefore overexpressed ATF4 in p53 mKO skeletal muscle fibers to determine whether p53 might function as a downstream mediator of ATF4-dependent muscle atrophy. However, like in wild type skeletal muscle fibers, ATF4 caused muscle atrophy indicating that p53 expression was not required for ATF4 to induce muscle atrophy. Collectively, these data indicated that p53 and ATF4 induce muscle atrophy by independent mechanisms.
To test whether p53 and ATF4 might mediate independent and additive pathways to skeletal muscle atrophy, we performed an experiment comparing the effects of overexpression of p53 or ATF4 alone, or both transcription factors together. We found that forced expression of p53 and ATF4 induced comparable levels of muscle fiber atrophy. However, forced expression of both p53 and ATF4 induced greater muscle fiber atrophy than either transcription factor alone. Interestingly, the effect of both transcription factors compared to either transcription factor alone, was nearly additive.

As a subsequent test of the potential additive mechanisms of p53- and ATF4-dependent muscle atrophy, we generated double knockout mice (p53/ATF4 mKO mice). Like their single knockout counterparts (e.g. p53 mKO, ATF4 mKO), p53/ATF4 mKO mice lacked an overt phenotype under basal conditions. As expected, we observed reduced p53 expression levels in p53 mKO and p53/ATF4 mKO. In addition, ATF4 mKO and p53/ATF4 mKO mice displayed reduced ATF4 expression levels. Furthermore, ATF4 levels remained unchanged in p53 mKO mice and similarly p53 levels were unchanged in ATF4 mKO mice.

To test further whether p53 and ATF4 might mediate additive mechanisms to skeletal muscle atrophy, we compared p53 mKO, ATF4 mKO, and p53/ATF4 mKO mice during limb immobilization. We found that p53 mKO and ATF4 mKO skeletal muscles were partially resistant to muscle atrophy due to limb immobilization. However, p53/ATF4 mKO mice displayed near complete resistance to skeletal muscle atrophy.
during limb immobilization. In addition, there was an additive reduction in immobilization-induced muscle fiber atrophy when compared with p53 mKO and ATF4 mKO mice. Collectively, these results identify p53 and ATF4 as distinct and additive pathways to skeletal muscle atrophy during limb immobilization.

Further examination of muscle fibers of each knockout strain revealed that limb immobilization induced atrophy of type II muscle fibers (but not type I fibers). Additionally, genetic deletion of $p53$ or $ATF4$ provided partial protection from muscle atrophy of type II muscle fibers. Interestingly, loss of both p53 and ATF4 provided complete protection from type II muscle fiber atrophy during limb immobilization. These results indicate that type II muscle fiber atrophy due to muscle immobilization requires both p53 and ATF4.

Taken together, these studies were important as they 1.) identified p53 as a novel regulator of skeletal muscle atrophy that is necessary and sufficient for muscle atrophy during limb immobilization; 2.) demonstrated p53 and ATF4 as independent mechanisms to skeletal muscle atrophy; 3.) indicated that p53 and ATF4 mediate additive mechanisms to immobilization-induced skeletal muscle atrophy. Importantly, these studies opened up several new areas for future investigation.

$p53$ and $ATF4$ Jointly Regulate $p21$ Expression During Immobilization-Induced Skeletal Muscle Atrophy.

In Chapter III, we continued our studies of p53 and ATF4 in the setting of limb
immobilization. In our previous work, we used gene expression microarrays to determine the effects of ATF4 on skeletal muscle gene expression in the setting of muscle atrophy (59). We utilized a similar approach to ascertain the role of p53 on skeletal muscle gene expression using mRNA expression arrays. We first examined the effects of muscle immobilization and identified 33 unique mRNAs that were highly induced. We performed subsequent genome-wide microarray studies to ascertain which of the mRNA changes that we observed during muscle immobilization were also decreased in skeletal muscle fibers of p53 mKO mice, and increased by p53 overexpression in wild type mice. Only two mRNAs met these criteria: *p21* (Waf1/Cip1/Cdkn1a) and *Chrna1*. We found *p21* to be particularly intriguing given that it is a canonical p53 gene target (63,117) and *p21* is one of the most highly induced mRNAs during many types of skeletal muscle atrophy (22,45-49,60,67-70). Additionally, in previous work we identified *p21* as an ATF4-inducible gene (60). We therefore focused our studies on p21 as a potential mechanism of p53- and ATF4-dependent muscle atrophy during limb immobilization.

We first examined whether p21 expression was increased by limb immobilization or forced expression of p53. We found that *p21* mRNA and p21 protein were increased by muscle immobilization as well as p53 overexpression (similar to ATF4 overexpression). Interestingly, analysis of *p21* mRNA levels in p53 mKO skeletal muscle fibers indicated that p53 was partially required for maximal induction of p21 during immobilization.

Furthermore, analysis of *p21* mRNA levels in ATF4 mKO revealed a similar
dependence, as p21 expression was reduced in ATF4 mKO muscles relative to immobilized littermate control muscles. Additionally, loss of p53 and ATF4 in p53/ATF4 mKO mice resulted in a nearly additive decrease in p21 expression relative to loss of either p53 or ATF4 alone. These results suggested that immobilization increases p21 expression in a manner that requires p53 and ATF4.

To test whether p21 might have a functional effect on skeletal muscle fiber size, we overexpressed p21 in wild type mouse muscle. We found that forced expression of p21 resulted in muscle fiber atrophy similar to p53 and ATF4. We next tested whether p21-mediated muscle fiber atrophy might require p53 and ATF4 expression. We therefore overexpressed p21 in p53/ATF4 mKO skeletal muscle. We found that similar to wild type mouse muscle, p21 induces muscle fiber atrophy even in the absence of both transcription factors. Taken together, these data indicated that p21 is sufficient to induce skeletal muscle atrophy in a manner that does not require p53 or ATF4 expression.

Because we had previously shown that loss of p53 and ATF4 provides partial protection from skeletal muscle atrophy, we next tested whether loss of p21 expression would also protect skeletal muscle fibers from atrophy. Therefore, we generated microRNA constructs specifically targeting p21 mRNA (e.g. miR-p21 #1, miR-p21 #2) and decrease p21 expression. In the absence of immobilization, we observed no differences between the muscle fibers of each microRNA construct targeting p21 mRNA when compared with a non-targeting microRNA construct. However, in the presence of immobilization, each miR-p21 construct reduced skeletal muscle fiber atrophy. These
results indicated that p21 is required for muscle atrophy during limb immobilization.

Since p21 is necessary for immobilization-induced muscle atrophy, we hypothesized that it might also be required for muscle atrophy due to p53 overexpression. We therefore co-transfected wild type skeletal muscle fibers with p53 in the absence or presence of each miR-p21 construct. We found that both microRNA constructs reduced p53-mediated muscle fiber atrophy. These data suggested that p21 is required for muscle atrophy due to muscle immobilization and forced expression of p53.

Since p53 and ATF4 appear to act in a similar manner to promote muscle fiber atrophy, we tested the hypothesis that p21 might function as a downstream mediator of ATF4 as well. To that end, we transfected wild type mice with ATF4 in the presence or absence of each miR-p21 construct and found that knockdown of p21 expression reduced ATF4-mediated muscle fiber atrophy. Collectively, these data demonstrate that p21 is required for immobilization-, p53- and ATF4-dependent muscle fiber atrophy.

In summary, these studies were essential in helping to determine the downstream mechanisms of p53 and ATF4-mediated muscle atrophy. First, they identified p21 mRNA as a key transcript that is induced by muscle immobilization, decreased by loss of p53 expression in skeletal muscle, and increased by p53 overexpression. Second, they demonstrated that immobilization induces p21 expression in a manner that requires p53 and ATF4. Third, they indicated that p21 is sufficient to induce muscle fiber atrophy in a p53- and ATF4-independent manner. Lastly, they demonstrated that p21 is required for
muscle atrophy due to limb immobilization, p53, and ATF4. These studies point to the p53 and ATF4 pathways as important therapeutic intervention points given their role in regulating p21 expression and skeletal muscle atrophy.

In conclusion, the studies described in this thesis have been instrumental in broadening our understanding of the molecular mechanisms that mediate skeletal muscle atrophy. In our previous work, we identified the bZIP transcription factor ATF4 as an essential mediator of muscle fiber atrophy (59,60). However, it became clear that another pro-atrophy factor must exist that acts in parallel to the ATF4 pathway. The current work identifies p53 as an ATF4-independent factor that is sufficient to induce muscle atrophy and is partially required for immobilization-induced muscle atrophy. Additionally, we identify p21 as a critical downstream effector of p53 and ATF4 that is also necessary and sufficient for muscle atrophy during limb immobilization. Identification of these skeletal muscle atrophy mediators may aid in the discovery of novel pharmacotherapies that function to decrease activity or expression of skeletal muscle p53, ATF4, and/or p21 during muscle atrophy. Collectively, these findings help to deepen our understanding of the molecular pathogenesis of skeletal muscle atrophy and open several new areas for future investigation of the causes and potential treatments of this debilitating condition.

**Future Directions**

The studies presented in this thesis have broadened our understanding of the pathways that sense muscle stress and drive muscle atrophy. However, it is clear that more work needs to be done to deepen our understanding of the molecular mechanisms...
that cause muscle atrophy. Therefore, in this section, we will discuss the implications and future directions of the studies presented in the hopes of advancing our understanding of the processes that induce skeletal muscle atrophy.

In Chapter II, we identify p53 and ATF4 as independent and additive mechanisms to skeletal muscle atrophy during limb immobilization. In this study, we identify muscle immobilization as a key regulator of p53 expression. However, the upstream mechanisms that increase p53 expression remain to be determined. The results of our study suggest that muscle immobilization likely increases p53 synthesis and/or decreases turnover of p53 in skeletal muscle. p53 levels can be increased through mechanisms that regulate mRNA stability, gene activation, and/or translation (100-105,107-109,124); additionally, p53 levels can be regulated by modulating levels of Mdm2 and related E3 ubiquitin ligases (37,38,110). We hypothesize that one or more of these mechanisms increase p53 expression during limb immobilization. Future studies in this area are needed to help decipher the critical upstream regulation events that increase p53 expression during limb immobilization.

In addition, the current study focused primarily on a short period of muscle immobilization (three days) in order to identify early molecular events that initiate the atrophy process. However, it will also be essential to determine the role of p53 and ATF4 during longer periods of immobilization-induced muscle atrophy. In previous work, we found that genetic deletion of ATF4 during longer periods of muscle immobilization (seven days), does not protect against skeletal muscle atrophy. This may be due to
compensatory mechanisms that remain unclear. In future studies, we will examine the role of p53 during longer periods of muscle immobilization, since it is unclear whether loss of p53 in skeletal muscle protects against longer periods of immobilization.

The current study focuses on the effect of hindlimb immobilization on the tibialis anterior muscle. However, in future studies, it will be interesting to study the roles of p53 and ATF4 in other muscle types. This may reveal differences in fiber type effects as suggested by the finding that loss of p53 and ATF4 protects type II muscle fibers from immobilization-induced atrophy, but does not protect type I muscle fibers. These studies may aid in the identification of fiber type-specific roles for p53 and ATF4 in skeletal muscle.

In Chapter II, we identify p21 as a key downstream regulator of p53- and ATF4-mediated muscle fiber atrophy. However, the mechanisms by which p53 and ATF4 increase p21 expression remain unclear. Based on previous work, it seems likely that p53 activates p21 expression in a direct manner, given that p53 has been shown to bind the p21 promoter and increase p21 transcription in non-muscle cells (61,63-65). By contrast, ATF4 has not been shown to directly activate p21 transcription. Thus, there may be other factors that increase p21 expression in skeletal muscle given that loss of p53 and ATF4 did not fully decrease p21 mRNA levels. Therefore, it is unclear whether p53 and/or ATF4 directly regulate p21 expression in skeletal muscle and if so whether other factors are required for this effect. Understanding how p21 expression is regulated by p53 and ATF4 is an important area for future studies.
Deciphering the mechanisms by which p21 induces muscle fiber atrophy is another important area for future investigation. While p21 classically functions as an inhibitor of cell cycle progression following DNA damage (62), it seems unlikely that p21 would function in this capacity given that adult skeletal muscle fibers lack mitotic potential. To that end, p21 has been shown previously to be present in the nuclei of skeletal myofibers and not in satellite cells (which are mitotically active) (71). In addition, genetic interventions to modulate p21 expression presented in our studies specifically target muscle fibers (95). Therefore, we hypothesize that p21 causes muscle fiber atrophy in a manner that is independent of its role in cell cycle regulation. These mechanisms may lead to changes in skeletal muscle protein synthesis, protein degradation, mitochondrial function, and anabolic signaling. Given that p21 is highly induced by many types of muscle atrophy (e.g. aging, malnutrition, critical illness, denervation, amyotrophic lateral sclerosis), we speculate that p21-mediated muscle atrophy regulates a number of these downstream mechanisms that have been shown to induce muscle atrophy (22,45-49,60,67-70).

Lastly, while p21 is an essential downstream regulator of p53- and ATF4-dependent muscle fiber atrophy, it is also likely that these two pathways regulate other genes that cause muscle atrophy by different mechanisms. We have previously shown that ATF4 increases expression of Gadd45a (59). Upon induction, Gadd45a drives muscle fiber atrophy; conversely, RNAi knockdown of Gadd45a decreases muscle fiber atrophy (59). In Chapter III of this thesis, we show that limb immobilization increases
Gadd45a expression more so than any other mRNA likely due to ATF4. However, Gadd45a does not appear to be regulated by p53 since it was neither required nor sufficient for Gadd45a induction during limb immobilization. Because p53 has been shown to directly activate Gadd45a transcription in other cell types (119-121), we found this be an unexpected result. While Gadd45a does not appear to be a key convergence point for p53 and ATF4, there are likely a number of pro-atrophy genes that are regulated by both transcription factors that mediate skeletal muscle atrophy during limb immobilization.
REFERENCES


