The role of mRNA stability in mitochondrial biogenesis in skeletal muscle

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Abstract

Exercise promotes the elevated biosynthesis of mitochondria. To study the underlying adaptive response of mitochondria in skeletal muscle, we employed low frequency, chronic stimulation in the rat hindlimb. Chronic contractile activity (CCA) induced an enrichment of mitochondrial volume and an elevation of the important mitochondrial regulators, PGC-1α, c-myc and Tfam. The corresponding mRNA expression encoding these proteins, however, was unchanged. Interestingly, the mRNA turnover rates were differentially affected by CCA, which resulted in accelerated mRNA degradation of both PGC-1α and Tfam, but not c-myc. These variable sensitivities to CCA may be, in part, due to the co-upregulation of mRNA-stabilizing HuR and destabilizing total AUF1. Curiously, one of four AUF1 isoforms $p45^{AUF1}$, represented a greater proportion of total AUF1, suggesting that $p45^{AUF1}$ may be the most potent regulator in CCA-induced mRNA decay. Collectively, our study demonstrates that mRNA stability research in skeletal muscle is an exciting new continent awaiting excavation.

For curiosity and change.

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Movement never lies.
It is the magic of what I call the outer space of the imagination.
There is a great deal of outer space, distant from our daily lives,
where I feel our imagination wanders sometimes.
It will find a planet or it will not find a planet,
and that is what a dancer does.
- Martha Graham

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List of Abbreviations

AMPK	AMP-activated protein kinase
APRIL	acidic (leucine-rich) nuclear phosphoprotein 32 family memberB *Other aliases: ANP32B*
ANP32A	acid (lucine-rich) nuclear phosphorotein family, member A *Other aliases: pp32*
ANP32B	acid (lucine-rich) nuclear phosphorotein family, member B *Other aliases: APRIL*
ANT	adenine nucleotide translocase
AMD	ARE-mediated decay
ARE	AU-rich element
ARE-RBP	AU-rich element RNA-binding protein
AUF1	AU-rich element binding factor 1 *Other aliases: hnRNP D*
AU-rich	adenylate uridylate rich
CCA	chronic contractile activity
cdc25	cell diviion cycle 25
ChIP	chromatin immuniprecipitation
c-myc	cellular myelocytomatosis
COX	cytochrome c oxidase
CRM1	chromosome maintenance region 1
Dcp1	decapping protein 1
Dcp2	decapping protein 2
dpc	days past conception
EDL	extensor digitorum longus
Egr-1	early growth response protein 1
eIF4E	eukaryotic initiation factor 4 E
eIF4G	eukaryotic initiation factor 4 G
ELAV1	embryonic lethal, abnormal vision, Drosophila-like 1 *Other aliases: HuR*
ERRα	estrogen-related receptor, alpha
ERRγ	estrogen-related receptor, gamma
FRAP	fluorescence recovery after photobleaching
FTR	fast-twitch red
FTW	fast-twitch white
GFP	green fluorescence protein
HLH/LZ	helix-loop-helix leucine zipper
hnRNP D	heterogeneous nuclear ribonucleoprotein D *Other aliases: hnRNP D*
HNS	HuR nucleocytoplasmic shutting signal

HuB	human antigen B
HuC	human antigen C
HuD	human antigen D
HuR	human antigen R
	Other aliases: ELAV1
KSRP	KH-type splicing regulatory protein
Max	MYC associated factor X
MHC	myosin heavy chain
miRNA	microRNA
MnSOD	manganese superoxide dismutase
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
MyoD	myogenic differentiation 1
NES	nuclear export signals
NRF	nuclear respiratory factor
NUGEMPS	nuclear genes encoding mitochondrial proteins
p21	20 kDa accessory protein
p53	tumor suppressor p53
PABP	poly(A)-binding protein
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator-1, alpha
PGC-1β	peroxisome proliferator-activated receptor gamma coactivator-1, beta
POLG	polymerase gamma
Poly(A)	polyadenylate
pp32	*Other aliases: ANP32A*
PPARγ	peroxisome proliferator-activated receptors gamma
PRC	PGC-1-related coactivator
PKC	protein kinase C
RBP	RNA-binding protein
REMSA	RNA electromobility shift assay
RFP	red fluorescence protein
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNase	ribonuclease
RNP	Ribonucleoprotein
RRM	RNA recognition motif
SERCA	sarcoendoplasmic reticulum calcium ATPase
STR	slow-twitch red
TA	tibialis anterior
Tfam	mitochondrial transcription factor A
TFB1M	mitochondrial transcription factor B1
TFB2M	mitochondrial transcription factor B2
TIA-1	T-cell-restricted intracellular antigen 1

TIAR	TIA-1 related protein
TIM	inner-membrane translocase
TRN	transportin
TSAA	translation state array analysis
TTP	tristetraprolin
UTR	untranslated region
VEGF	vascular endothelial growth factor
Xrn1	5'-3' exoribonuclease 1

REVIEW OF LITERATURE

1.0 AU-rich element-mediated mRNA decay

1.1 Introduction

Many written accounts of eukaryotic gene expression describing messenger RNA (mRNA) might sound like this, "The primary role of the mRNA, as the name implies, is to serve as a subcarrier of genetic information, and to guide the synthesis of proteins. In eukaryotes, mRNAs are first synthesized in the nucleus as pre-mRNAs that are subject to 5′-end capping, splicing, 3′-end cleavage and polyadenylation. Once pre-mRNA processing is complete, mature mRNAs are exported to the cytoplasm, where they serve as the genetic blueprints for protein synthesis by ribosomes and then are degraded." Like an obituary found in the daily newspaper, this dull and dry biographical sketch illustrates nothing of the intricacies and vicissitudes defining the intriguing life history of even the most mundane mRNA. Across multiple cell types, the lives of eukaryotic mRNAs begin from their births in the nucleus. Throughout their short lifespan, many would have been trimmed and processed into a mature molecule, mingled with synergistic or hindering RNA-binding proteins, and quality-controlled by the nucleocytoplasmic surveillance coastguards. Finally, like the fuse carrying the sparks of life passing on to the ribosomes, their short existences are ended by a corps of ribonucleases.

The complex life cycle of an mRNA transcript from its genesis through its subsequent roles in gene expression is a tightly regulated biochemical process and a diverse array of regulatory activities essentially affects every aspect of their lives. mRNA, long regarded as a mere subcarrier of genetic information, is an essential linchpin

of regulation. The steady-state level of an mRNA is the product of both synthesis and degradation. Compared with other biological molecules, the distinguishing property of mRNA is its high rate of turnover, the regulation of which plays a key role in the control of gene expression. Each mRNA species differs in half-life. For example, the mRNA half-life of c-fos (6.6 min) is much less than β-globin (17.5 hours) (91). Furthermore, cytoplasmic stability is dependent on *trans*-acting factors that fluctuate in response to a wide variety of extracellular stimuli. Within the cell cycle, histone mRNA half-lives can vary between 10 and 110 minutes (74; 133; 151), and the half-lives of mammalian mRNAs may vary from a few minutes to more than 24 hours (151). Such variations can cause the steady-state of a particular mRNA to fluctuate by manyfold, without any change in transcription rate. Because changes in mRNA in the steady-state reflect the amount of protein produced, mRNA stability has emerged as a key step in post-transcriptional gene expression.

A set of highly complex and intricate mechanisms control the rate and timing of transcription, but these are of limited use without equally precise control over the rate of mRNA turnover, given that this determines the amount of mRNA that is available to direct protein production (81). Since mRNA expression is contributed by the processes of transcription and degradation, a fast decaying mRNA does not always correspond to a lower steady-state level. As a compensatory mechanism, transcription rate may multiply to match the rapid decaying rate, resulting in greater mRNA turnover. This is advantageous because it permits a cell to accelerate adaptation in response to its pattern

of protein synthesis, according to changing physiological demands. Perhaps not coincidentally, short-lived mRNAs usually encode proteins of regulatory significance. If the mRNA is unstable, the approach to a new steady state of the corresponding protein can tightly mirror transcription. It is this extraordinary feature of mRNA that permits a cell to perpetually adapt to its pattern of protein synthesis in response to changing metabolic demands. Whereas the intricacies of transcription have been well-characterized, research in mRNA stability controlling gene expression is relatively unexplored.

1.2 AU-rich *cis*-elements

Once exported from the nucleus, most mammalian mRNAs are relatively stable in the cytoplasm, with half-lives greater than 10 hours in cells. A subset of mRNAs, however, decays rapidly with half-lives ranging between 15 minutes and 2 hours (136). Specific destabilizing elements determine the rapid decay of these mRNAs. The most widespread *cis*-element is a heterogenous group of adenylate/uridylate-rich elements (AREs) located in the 3′-untranslated region (UTR) (Fig. 1). An estimated 8% of mRNAs bearing AREs (ARE-mRNAs) are transcribed in humans (12). Transcript degradation can occur in the form of ARE-mediated decay, which is determined by the physical interaction of AREs and specialized RNA-binding proteins (RBPs) (67; 130). The ARE is a destabilizing *cis*-element, as its deletion often results in mRNA stabilization (3; 128).

Cataloging of ARE-mRNAs is blossoming. To date, sequences of known AREs and the decay patterns of the mRNA in which they reside have led to three classes based on sequence and decay characteristics (18; 29). Class I AREs, such as the c-fos ARE, contain one to three scattered copies of the pentamer AUUUA embedded within U-rich regions (189). Class II AREs, like the granulocyte macrophage-colony stimulating factor (GM-CSF) ARE, harbour at least two overlapping copies of a critical nonamer UUAUUUA(U/A)(U/A), also in the context of a U-rich region (67; 98; 163). Class III AREs, an example of which is the c-jun ARE, lack the hallmark AUUUA pentamer, signal degradation with U-rich and other unclassified and unknown sequences (18). Typically, AREs range in size from 50 to 150 nucleotides (67). Despite the presence of AREs in many different mRNAs, there is no single evolutionarily conserved instability sequence (42). The ARE in the eukaryotic initiation factor 4E (eIF4E) mRNA, for example, does not harbor the hallmark sequences described in the three classifications (176). Together with *trans*-acting factors, the AREs, in part, determine mRNA decay, and hence the subsequent abundance of mRNA. Recently, ARE-mediated translation processes and protein expression have also been described (54; 176).

1.3 RNA-binding proteins as regulatory *trans*-acting factors

Numerous *trans*-acting factors such as RBPs regulate the differential interaction of *cis*-elements in the ARE-mRNAs (Fig. 1). Upon formation of messenger ribonucleoprotein complexes (RNPs), a processing factory that allows multiple

interconnections between regulatory events, mRNA degradation machinery recruitment efficiency and specificity is altered (132). In muscle, a pair of RBPs with reciprocal functions can act independently and synchronously to regulate the expression of labile genes (37). Hu antigen R (HuR) is the human homologue of the embryonic lethal abnormal vision gene (ELAV) class family, which comprises of the ubiquitous HuR along with the primarily neuronal proteins HuB, HuC and HuD, all of which are emerging as pivotal regulators of post-transcriptional regulation (114). Hu proteins possess two N-terminal RNA-recognition motifs (RRM1 and RRM2), a hinge region, and a C-terminal RRM3 (30; 51). Through the RRMs, HuR binds with high affinity and specificity to target labile ARE-bearing mRNAs, and modify their expression by altering stability, translation or both (114). The RRMs are conserved among all four Hu family proteins, whereas the hinge region between RRM2 and RRM3 differs (60). Each individual Hu protein, however, is highly conserved among vertebrates. HuR is 99.7% identical to that of mouse and 98.2% to chicken (51; 60; 139; 186).

Since over 90% of HuR is primarily localized within the nucleus under basal conditions, its export to the cytoplasm is a main prerequisite for its protective effects on cognate target mRNAs from rapid decay (46). Upon cellular stress, it translocates to the cytoplasm (18; 30) and associates with cytoplasmic mRNA transcripts (Fig. 1) (52). HuR is also involved in mRNA nucleocytoplasmic transport (55; 114). Since mRNA maturation and transport are tightly coupled, splicing, capping and polyadenylation all affect export (56; 180). These processing events are believed to be accompanied by the

deposition of adapter proteins on nascent transcripts. For example, HuR can act as an adapter protein by binding to both the c-fos mRNA as well as receptor proteins that interact with the nuclear pore complex (56).

Export is conducted through two pathways. In the hinge region between RRM2 and RRM 3 resides the HuR nucleocytoplasmic shutting (HNS) sequence (30; 51), which interacts with transportin 2 (TRN2) to facilitate mRNA export (56). In a separate export pathway, two nuclear phosphoprotein ligands of HuR, pp32 and acidic protein rich in leucine (APRIL), contain leucine-rich nuclear export signals (NES), and are recognized by the export receptor chromosome maintenance region 1 (CRM1) (55; 56). Thus, HuR accesses two alternative pathways for nuclear export. One pathway is CRM1-independent (using its endogenous HNS shuttling sequence) and the other is CRM1-dependent (involving its ligands pp32 and APRIL). Since post-transcriptional regulation of gene expression involves multiple checkpoints at which decisions are made concerning the fate of each mRNA species, the cytoplasmic presence of HuR is therefore intimately linked to its influence on target mRNA stabilization and translation (92).

HuR is particularly important during myogenic differentiation. Upon cytoplasmic accumulation, HuR interacts with the mRNAs of myogenic differentiation 1 (MyoD), myogenin and acetylcholinesterase (43; 44; 52; 181). In a human osteosarcoma cell line, up-regulation of HuR above the baseline level results in the increased stabilization of eIF4E mRNA and protein levels, and the subsequent malignant proliferation and tumor genesis in both cell culture and animal models (39; 176).

In contrast to HuR, AU-rich element RNA-binding factor 1 (AUF1) binds to the ARE of target mRNAs and promotes cytoplasmic ARE-mediated decay through a poorly understood mechanism (Fig. 1). The primary transcript of AUF1 contains ten exons that are alternatively spliced to produce four protein isoforms: $p37^{AUF1}$, $p40^{AUF1}$, $p42^{AUF1}$ and $p45^{AUF1}$ (185). In general, HuR and AUF1 target many common AU-rich mRNAs and influence mRNA decay in an opposing manner (130). Targets of AUF1 that are shared with HuR include c-fos, c-jun, early growth response protein 1 (egr-1), interleukins, 20 kDa accessory protein (p21), heat shock protein 70 (hsp70), manganese superoxide dismutase (MnSOD), catalase, cyclin D1, and cell division cycle 25 (cdc25) (99). Similar to HuR, the AUF1 proteins are primarily located in the nucleus (196). Upon nucleocytoplasmic shuttling, the AUF1 proteins associate with their target transcripts in the cytoplasm (31; 104; 157). Multiple studies have delineated that binding affinities and potencies of decay in the various isoforms differ. However, results remain inconclusive because each isoform behaves differently depending on cell type.

A number of studies have investigated the role of AUF1 in the regulation of the adrenergic nervous system in the heart. It has been known for many years that ligand-induced down-regulation of receptors correlates with the reduction of receptor mRNA. In conditions of heart failure, cardiac muscles are desensitized in response to the elevated concentrations of circulating and cardiac norepinephrine. One major component of the desensitization is the selective down-regulation of the dominant β_1-adrenergic receptor (β_1- AR) protein, as well as a corresponding down-regulation of β_1-AR mRNA (21; 179).

The decreased β_1-AR mRNA expression is, in part, due to the norepinephrine-induced up-regulation of AUF1. Specifically, purified recombinant $p37^{AUF1}$ destabilizes β_1-AR mRNA through its 3'UTR (143). Similarly, in hamster smooth muscle cells, exposure to β_2-agonist lead to a drastic increase in mRNA and protein expression of AUF1, and the down-regulation of steady state β_2-AR mRNA (70; 143). Thus, the mRNAs of both β_1- and β_2-AR undergo β-agonist-mediated destabilization (13).

In another example of the heart, hypertrophy and failure display abnormally slowed myocardial relaxation, which is associated with the down-regulation of sarcoendoplasmic reticulum calcium ATPase 2a (SERCA2a). In neonatal rat ventricular myocytes, protein kinase C (PKC) activation down-regulates SERCA2a mRNA by decreasing both promotor activity and mRNA stability. Specifically, PKC increases AUF1 phosphorylation and induces AUF1 binding and destabilization of SERCA2a mRNA via its 3'-UTR. Interestingly, contrary to the common subcellular compartment of AUF1 activity, this interaction occurs predominantly in the nucleus (17).

The dynamics of ARE-mediated decay can be illustrated when both HuR and AUF1 are antagonizing each other for a comment target. Homomultimerization of HuR and AUF1 can bind simultaneously and competitively to a single ARE (37). In conditions of high blood pressure, level of angiotensin II is elevated. Using human vascular smooth cells, Pende et al. (142) found that the majority of both HuR and AUF1 reside in the nucleus. Administration of angiotensin II mimicked the high adrenergic drive in the failing heart, and induces translocation of both AUF1 and HuR to the cytosol

and subsequent association with the angiotensin 1 receptor mRNA (142). In a separate study, Yasuda et al. (193) described that in metabolic bone disorders such as osteoporosis, calcitonin is widely used to inhibit osteoclastic bone resorption. Continuous treatment with calcitonin, however, causes a diminution of its inhibitory actions on bone resorption. This desensitization to calcitonin is closely associated with the down-regulation of the calcitonin receptor. In a mouse embryonic fibroblast cell line, both $p40^{AUF1}$ and HuR bind to the 3′UTR of the calcitonin receptor mRNA. Although treatment of osteoclasts with calcitonin decreased HuR expression, AUF1 expression was not affected. These findings suggest that the down-regulation of a stabilizing RBP may, in part, contribute to the decreased levels of calcitonin mRNA expression (193). Finally, the common targets of both HuR and AUF1 include genes involved in translation such as eIF4E, the rate-limiting factor for cap-dependent protein synthesis. Overexpression of $p42^{AUF1}$, which binds to the 3′UTR of eIF4E mRNA, in contrast to HuR, decreases the stability of eIF4E mRNA (176).

Regulation of AUF1 includes 14-3-3σ, which belongs to a subset of p53 transcriptional targets whose activation promotes cell cycle arrest (75). The 14-3-3σ protein interacts strongly with $p37^{AUF1}$ and to a lesser extent with $p40^{AUF1}$. Upon binding, 14-3-3σ facilitates AUF1 cytoplasmic transport and AU-rich mRNA decay (73). In cancer cells, the proto-oncogene c-myc transcribes genes regulating mitochondrial biogenesis, including peroxisome proliferator-activated receptor gamma coactivator-1β (PGC-1β) and mitochondrial transcription factor A (Tfam) (167). Transcription of PGC-

1β is c-myc-dependent and loss of PGC-1β expression results in reduced mitochondrial respiration (195). Although degradation of c-myc mRNA is accelerated by AUF1, HuR also targets c-myc mRNA (20; 37; 63; 195). Finally, AUF1 can also affect c-myc translation via the ARE without affecting mRNA abundance (104).

1.4 A complex tail

Virtually all aspects of mRNA metabolism such as conferring mRNA stability, nuclear export of processed mRNA, and promoting translational efficiency is influenced by the poly(A)tail (32; 151; 154). At the 3′ end of the nascent transcript, a large complex of multi-subunit proteins carries out the elaborate process of polyadenylation, which occurs through endonucleolytic cleavage and/or exonucleolytic digestion that is coupled to the addition of poly(A) tails to the upstream RNA fragment (23; 32). The polyadenylation element, a hexanucleotide AAUAAA, is situated approximately 10-30 nucleotides 5′ to the cleavage/polyadenylation site. Analogous to the transcription initiation TATA box in RNA polymerase II-dependent promoters (120), AAUAAA directs the assembly of a multicomponent protein complex (93; 120), one component of which, the poly(A) polymerase, synthesizes the poly(A) tail (1; 81; 119). As a consequence, the released mRNA has a tail ~70-90A and ~250A in yeast and in humans, respectively (81). The poly(A) tail is protected by the poly(A) binding protein (PABP), forming a poly(A)-PABP complex, which guards mRNA from degradation by the exosome.

Because removal of the poly(A) tail or deadenylation is the obligate step in the decay of many mRNAs, poly(A) protects the mRNAs from decay (151). Transcriptional pulse-chase experiments have almost invariably shown that mRNAs first lose their poly(A) tails by a process of continuous shortening from the 3′ end (41; 113; 129; 164; 173; 192). Only after the poly(A) tail has been degraded below a certain limit does the total amount of mRNA begin to decrease. This rapid destruction is catalyzed by several different ribonucleases (RNases) and is protected by the poly(A)-PABP complex (190). Conversely, depletion of PABP results in rapid degradation of polyadenylated mRNA, the half-life of which is rescued with replenishment of excess exogenous PABP. These results suggest a protective role of the poly(A)-PABP complex in delaying rapid or indiscriminate mRNA degradation.

ARE-mRNAs undergo rapid decay predominantly in the cytoplasm (29). In mammalian cells, ARE-mediated decay is triggered by deadenylation as first and necessary step. Mediated by the ARE, the PABP is released from the poly(A)-PABP complex, baring the poly(A) tail, which becomes vulnerable to rapid deadenylation by deadenylases. Following deadenylation, ARE-mRNAs are degraded primarily in the 3′-5′ direction by the exosome as demonstrated *in vitro* (19; 28).

In addition to disturbing the poly(A)-PABP complex, ARE-binding proteins may compete with PABP for binding to the poly(A) tail. Using polyadenylated RNA probes, Sagliocco et al. showed that all four recombinant AUF1 isoforms bind poly(A) sequences

as efficiently as PABP (154). Furthermore, the binding of AUF1 isoforms to poly(A) efficiently displaced PABP from the poly(A) tail (154).

ARE-mediated decay is further complicated by its link to translation. In many systems, degradation of ARE-mRNA is stimulated by active translation (4; 35; 158; 191). This mechanism may be mediated through the interaction between the AUF1 proteins and the poly(A)-PABP complex (115). Using recombinant proteins and a synthetic ARE, Lu et al. revealed that all four AUF1 proteins directly interact with eIF4G, independently of binding to the ARE-mRNA (115). Additionally, the AUF1 proteins also interacted strongly and directly with PABP, which was opposed by the binding of AUF1 to the ARE-mRNA (115). Interestingly, the AUF1-PABP complex also associates with eIF4G (115). The coordinated interactions between AUF1, ARE-mRNA, the component of the translation initiation apparatus, may regulate the rapid decay of ARE-mRNAs. Altogether, these results suggest that active translation of ARE-mRNA is linked to ARE-mediated decay.

1.5 Degradation machines in ARE-mediated decay

Understanding the mechanisms that regulate mRNA turnover requires the identification of the enzymatic machinery for mRNA degradation. Many enzymes involved in RNA degradation are multifunctional. Most genomes encode a plethora of ribonucleases (RNases), often with overlapping activities, making redundancy a general feature of RNA degradation systems. There are three major classes

of intracellular RNases. Endonucleases cleave RNA internally, 5′ exonucleases hydrolyze RNA from the 5′ end, and 3′ exonucleases degrade RNA from the 3′ end (81). The critical exoribonuclease in the 3′ to 5′ pathway in eukaryotes is the exosome, which is a large multi-subunit complex containing multiple 3′ to 5′ exonucleases (117). As a part of the RNA-processing machinery, it contributes to the processing, quality control and turnover of an extensive number of cellular RNAs in both the nucleus and the cytoplasm (80; 101; 117). The exosome is not only responsible for the exonucleolytic degradation of the mRNA body, but also has the ability to process and/or degrade many different RNA molecules independently of their sequence and structure in many types of organisms (22; 28).

Not surprisingly, the inherently unstable ARE-mRNAs are associated with the exosome. Chen et al. demonstrated that although purified human exosome does not exhibit ARE binding activity, it interacts with ARE-binding proteins, which recruit the exosome to degrade ARE-mRNAs (28). Through the recognition of AREs, ARE-binding proteins interact with the exosome (28). Specifically, addition of recombinant destabilizing ARE-binding proteins, KH-type splicing regulatory protein (KSRP) and tristetraprolin (TTP), but not HuR or AUF1, reconstituted degradation of c-fos ARE-mRNA (28).

ARE-mediated decay does not exclusively proceed in the 3′ to 5′ direction. At the 5′ end of the mRNA, the 7-methyl guanosine cap is removed by the decapping complex Dcp1/Dcp2, followed by degradation of the mRNA body by the 5′ to 3′ exonuclease Xrn1

(34). Although *in vitro* studies suggest that ARE-mediated decay primarily occurs in the 3′ to 5′ direction (28; 57; 116), the 5′ to 3′ pathway is required *in vivo* (166). Each of the two pathways was dissected using siRNAs in human fibrosarcoma cells. In the 5′ to 3′ direction, knockdown of Dcp1 inhibited ARE-mediated decay. Interestingly, down-regulation of Xrn1 strongly increased the half-life of β-globin-ARE mRNA from 2 to 5.2 hours. Conversely, knockdown of exosome-associated 3′ to 5′ exonucleases, Rrp40 and Rrp46, had a weak effect on ARE-mediated decay in the 3′ to 5′ direction (166).

Altogether, these studies suggest that ARE-mediated decay is a highly controlled process, involving the interaction of multiple protein complexes, such as RNases, RBPs, PABPs and the decapping complex, all of which have the ability to modulate their binding partners, thereby augmenting or delaying ARE-mediated decay. Not surprisingly, this process is coupled to translation, as components of the RNA surveillance system also associate with translation initiation factors. Like the hidden strings controlling all movements in a marionette, the silent mechanism of ARE-mediated decay regulating its protein expression is an exciting mystery awaiting excavation.

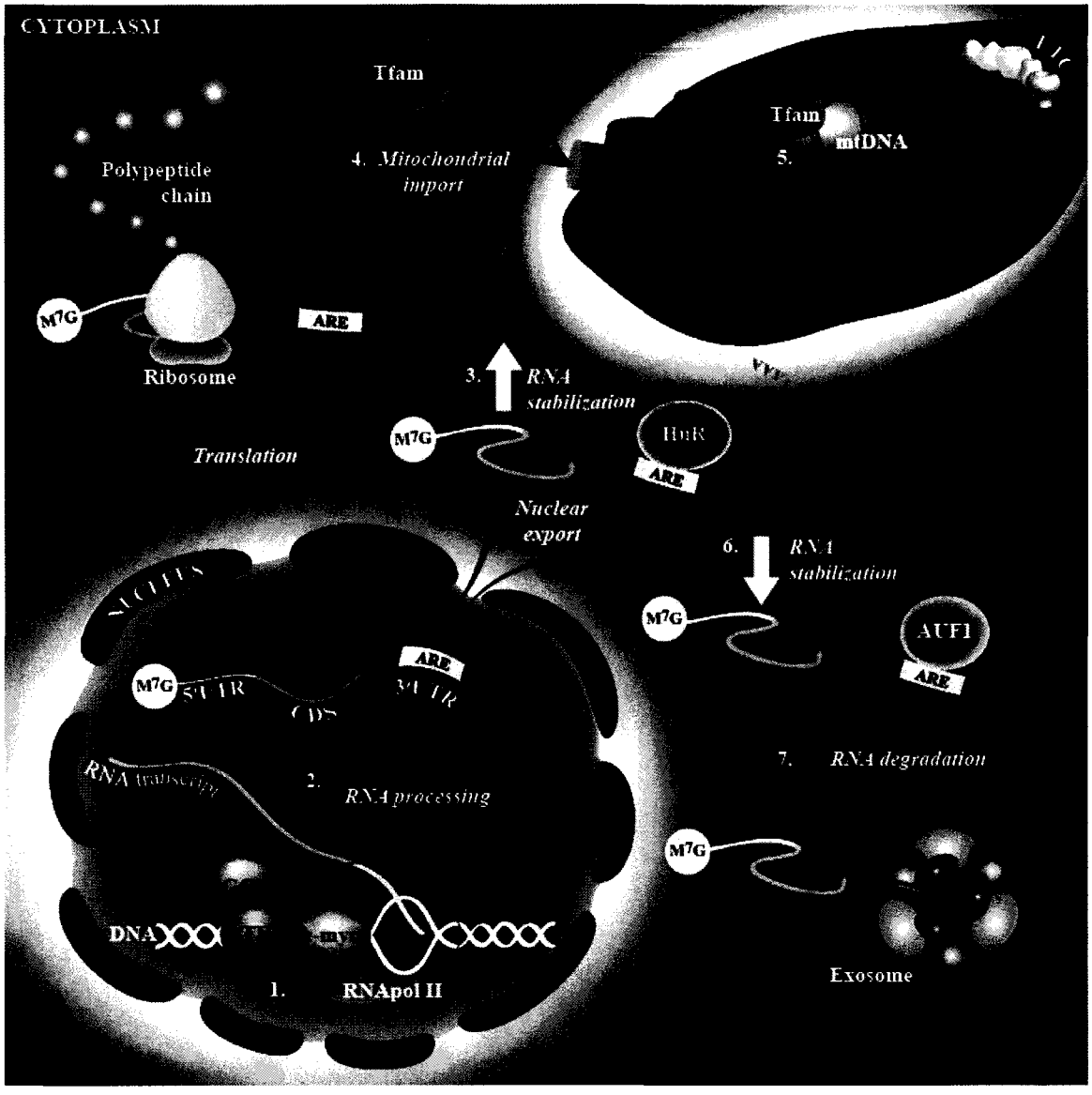

Figure 1. ARE-mediated decay in mitochondrial biogenesis. **(1)** PGC-1α coactivates transcription factors to up-regulate primary RNAs of NUGEMPs. C-myc also induces the expression of NUGEMPs by RNA polymerase II. **(2)** The primary transcript is spliced, capped and polyadenylated to form the mature mRNA, which undergoes nucleocytoplasmic export. **(3)** In the cytoplasm, mRNAs bearing an ARE can be bound by HuR, which promotes greater mRNA stability and/or translation. **(4)** Tfam is imported into the mitochondria via the protein import machinery. **(5)** Inside the matrix, Tfam promotes the transcription and replication of mtDNA. **(6)** ARE-bearing RNAs can also be destabilized by AUF1. **(7)** mRNAs are degraded by ribonucleases, the most prevalent of which in eukaryotic cells is the exosome.

2.0 Skeletal muscle

2.1 Oxidative properties in skeletal muscle fiber types

Skeletal muscle exhibits dynamic plasticity in response to functional demands. This unique phenotype is due, in part, to the nature of its fiber architecture that consists of highly organized heterogeneous arrangement. Methods such as histochemistry, immunohistochemistry and electrophoretic analysis have revealed four pure fiber types: slow type I, fast type IIA, fast type IIX and fast type IIB (144). Muscles can contain one single fiber type alone or as a hybrid that co-expresses specific pairs of myosin heavy chain (MHC) isoforms. In mammals, most muscles contain a mixture of fiber types, but some muscle beds are enriched in particular fiber types. For example, the soleus muscle is rich in type I and IIA MHCs and is characterized by an increased mitochondrial content and high oxidative phenotype. In contrast, quadriceps and gastrocnemius muscles contain more of the faster type IIB MHCs and lack rich mitochondrial networks and are more glycolytic.

In response to shifting metabolic stimuli, such as altered neuromuscular and contractile activity, mechanical loading or unloading, altered hormonal profiles and aging, muscles undergo fiber type transitions. This phenotypic adaptation is, in part, achieved at the transcriptional level by proteins that are additionally regulated by transcriptional coactivators (105-107; 146). For example, the sequential glycolytic-to-oxidative fiber type transition elicited in response to regular endurance exercise, undertaken over a number of weeks, is regulated by the well-characterized transcriptional

coactivator, PGC-1α (87; 107; 144; 146). Other members of the PGC-1 family of regulated coactivators are PGC-1α, PGC-1β and peroxisome proliferator-activated receptor gamma-related coactivator-1 (PRC), all of which orchestrate the expression of a multitude of proteins that are involved in muscle fiber type determination and is therefore a key factor regulating mitochondrial biogenesis (78; 86; 159-161).

2.2 Regulation of mitochondrial biogenesis

PGC-1α coactivates a number of transcription factors. Nuclear respiratory factor-1 (NRF-1), NRF-2, peroxisome proliferator-activated receptor gamma (PPARγ), estrogen-related receptor alpha (ERRα) and ERR gamma (ERRγ) (82; 83; 147; 184) all of which activate the promoters of nuclear genes encoding mitochondrial proteins (NUGEMPS), regulators and markers vital in oxidative phosphorylation. NRF-1 recognition sites, for example, are found in the promoters of a number of nuclear genes required for respiratory chain expression and function, such as cytochrome *c* (49; 50; 159). Furthermore, both NRF-1 and NRF-2 associate with the promoter regions of all ten nuclear-encoded cytochrome oxidase (COX) subunits (45; 141; 159), both mitochondrial transcription factor B1 (TFB1M) and TFB2M (59), as well as the essential mitochondrial transcription factor A (Tfam) (184). As one of the most important downstream gene targets of PGC-1α, Tfam is required for the transcription, replication and maintenance of mitochondrial DNA (mtDNA) (Fig. 1). Since the NRFs regulate Tfam, they also indirectly regulate the expressions of the three mitochondrial-encoded COX subunits (61;

78). Finally, other components of mitochondrial biogenesis such as the protein import and assembly machinery are also controlled by both NRF-1 and NRF-2 (14-16; 90).

Both PGC-1α mRNA and protein levels in the steady state have been studied in great detail and are increased in fibers enriched with mitochondria, for example in type I fibers and exercised muscles (58; 85; 137). Similarly, in response to chronic contractile activity (CCA), both Tfam mRNA and protein levels increase (61). Subsequent Tfam import and binding to the mtDNA promoter region are also elevated (61).

Regulation of mitochondrial biogenesis is not exclusive to PGC-1α. Cellular myelocytomatosis (c-myc), the cellular homolog of v-myc, is a member of a family of pro-oncogenes comprising c-myc, N-myc and L-myc (66), all of which encode transcriptional regulators involved in the control of cell proliferation, and are expressed in distinct patterns during embryogenesis (198). These genes are considered proto-oncogenes in the sense that alterations in their structure and expression have been linked to a wide variety of human and other animal cancers (135). One of the most compelling ideas about myc is that it functions to drive proliferation in response to a diverse set of signals. Through both transcriptional and post-transcriptional mechanisms, the increase in myc levels occurs as an immediate early response to most mitogenic factors, suggesting that the myc regulatory region is a nexus for multiple growth signal response pathways (66).

As a transcription factor, c-myc plays a central role in the regulation of cell size, cell proliferation and apoptosis (48; 103). Examining the primary sequence of the c-myc

protein suggests that it contains a transactivation domain within its N-terminal and a dimerization interface consisting of a helix-loop-helix leucine zipper (HLH/LZ) domain at its C-terminal. The c-myc protein forms a heterodimer with its partner protein MYC associated factor X (Max) and transactivates through the consensus E box element, 5′-CACGTG-3′ (6-8). Upon binding, the heterodimer activates transcription by recruitment of a histone acetyltransferase (127) and direct interaction of the c-myc transregulatory domain with the transcriptional machinery (36). The post-transcriptional mechanism of c-myc was first described in 1989 by Kindy and Sonenshein in smooth muscle cells (95). After serum addition, the mRNA expression of c-myc increased by 8-10-fold. Using nuclear runoff assays to measure transcription, no significant changes were observed. Therefore, transcription alone could not account for the enormous differential in mRNA expression. As a consequence, the authors investigated the stability of c-myc mRNA by the use of the transcriptional inhibitor actinomycin D. Following serum-stimulation, the half-life of c-myc mRNA increased, contributing to the elevated levels of steady-state mRNA. Therefore c-myc gene expression is regulated, in part, at the level of mRNA turnover.

Myc target genes are found in a variety of metabolic pathways, including amino acid, nucleotide, lipid and glucose metabolism (194). During embyogenesis, targeted homozygous deletions of the murine c-myc gene results in embryonic lethality at 10.5 dpc, suggesting that it is critical for development (38). Large-scale gene expression analysis suggest that overexpression of c-myc upregulates a number of NUGMEPS such

as adenine nucleotide transclocase (ANT), cytochrome *c*, mitochondrial inner-membrane translocase (TIM)23 and TIM9 (68; 138). Acute deletion of floxed murine Myc by Cre recombinase results in diminished mitochondrial mass (103). Furthermore, through homologous recombination, Myc null rat fibroblasts have diminished mitochondrial mass and decreased number of normal mitochondria (103). Also, using scanning chromatin immunoprecipitation (ChIP) assays, Li et al. found that Myc binds to the TFAM gene in human B lymphocytes (Fig. 1) (103). Finally, a combination of ChIP and promoter microarrays identified a total of 1469 Myc direct target genes (94). Among these targets, 107 were nuclear-encoded genes involved in mitochondrial biogenesis, some of which include DNA-directed polymerase gamma (POLG), POLG2 and NRF-1 (94). Together, these studies support a pivotal role for c-myc in the co-regulation of mitochondrial biogenesis.

2.3 Exercise-induced mitochondrial biogenesis

Skeletal muscle is unique in that it is a highly malleable tissue, capable of pronounced adaptations in response to contractile activity (78). For decades it has been known that exercise training enhances the oxidative capacity and metabolic efficiency of skeletal muscle (156). In rats, PGC-1α mRNA and protein levels are increased after a single bout of exercise as well as after several days of training (11; 62; 174). In humans, PGC-1α mRNA transiently increases after a single bout of exercise with low-volume intense interval exercise as well as with six weeks of endurance training (58; 145; 152).

Specifically, PGC-1α protein expression in type IIa fibers increases in greater amounts than in type I and IIx fibers (152). This suggests that PGC-1α plays an important role in the changes in mitochondrial content and oxidative phenotype induced by endurance exercise in skeletal muscle.

3.0 ARE-mediated decay in skeletal muscle

3.1 Introduction

Studies in ARE-mediated decay began in the late 1980s when researchers found that the removal of AU-rich sequences from the 3′UTRs of proto-oncogenes and cytokines resulted in diminished rate of decay (149; 150). These studies sculpted the founding steps for a new avenue of cancer research. It was not until approximately 20 years later that the research took a turn towards skeletal muscle-specific genes, those involved with myogenesis (52). Since then, a few laboratories have begun to delineate the role of ARE-mediated decay in mediating gene expression in skeletal muscle.

3.2 Adaptations in ARE-mediated decay with exercise training

Utrophin, a cytoskeletal protein is important for the proper formation of the neuromuscular junction where it participates in the full differentiation of the postsynaptic apparatus (25). Utrophin is more abundant in slow, versus fast muscles (24). A fiber type comparison using immunoflourescence revealed that slower fibers contain significantly more utrophin A compared with fast fibers (64). Furthermore, the rate of

transcription was similar between oxidative soleus and glycolytic extensor digitorum longus muscles, indicating that increased mRNA stability accounts for the higher level of utrophin in soleus muscles (64). Stimulated expression of a more oxidative phenotype by functional muscle overload induced an elevation of utrophin A mRNA, which was found to be mediated by calcineurin. The inhibition of calcineurin resulted in an 80% decrease in steady-state utrophin A mRNA (25), which was contributed, in part, by an accelerated rate of decay determined by the instability ARE in the 3′UTR (24). The different mRNA turnover rates between slow versus fast muscles suggest that post-transcriptional regulation differs in their myogenic programs.

Exercise induces angiogenesis, the process of which requires vascular endothelial growth factor (VEGF). Using Cre/loxP mice, selective deletion of VEGF in the gastrocnemius muscle within a small localized region results in capillary regression (171). Comparably, aerobic exercise training in muscle-specific VEGF knockout mice lead to reduced exercise capacity, maximal running speed and running capacity (140). This pathway is mediated by AMP-activated protein kinase (AMPK) through PGC-1α, as exercise-induced skeletal muscle VEGF expression was absent in PGC-1α knockout mice (102). Like many other genes of regulatory importance, VEGF is regulated by a post-transcriptional mechanism. During acute ischemia of the gastrocnemius muscle of the rat, HuR binds rapidly to the ARE in the VEGF mRNA 3′UTR (172). In skeletal muscle, this specific protein-RNA interaction may be an important posttranscriptional regulatory mechanism for increasing VEGF expression (172).

Like all growing research disciplines, mRNA stability in response to exercise is inconclusive. Using a low-frequency CCA as a model for endurance exercise, the increase of cytochrome *c* mRNA stability in the tibialis anterior (TA) muscle contributes to the elevated levels of steady-state mRNA expression in a time-dependent manner (53). Interestingly, endurance training on a treadmill led to decreased RNA-protein interaction in the 3′UTR of cytochrome *c* mRNA (53). Conversely, stimulation does not always result in prolonged mRNA half-life. In C2C12 muscle cells, for example, stimulation decreased the stability of Egr-1 mRNA (88). Thus, much more research in this area is required to understand the control of mRNA turnover as an important means of regulating both the level and timing of gene expression.

An extensive work has described the importance of PGC-1α, Tfam and c-myc in the regulation of gene expression. However, post-transcriptional regulation of each of these genes, along with putative RBPs in muscles of varying oxidative capacity has never been examined.

Research Objectives

Base on the review of literature, the objectives of my thesis were:

1. To characterize the mRNA stability of mitochondrial regulatory genes, namely, PGC-1α, c-myc and Tfam;
2. To investigate the presence of relevant AREs within the 3′-UTRs of PGC-1α, c-myc and Tfan regulatory genes;
3. To examine the effect of chronic contractile activity (CCA) on the mRNA stability of PGC-1α, Tfam and c-myc, in relation to steady state mRNA and protein levels; and
4. To determine the effect of CCA on the expression of mRNA destabilizing/destabilizing proteins along with coincident changes in mRNA half-lives.

Manuscript Author Contributions

Ruanne Y.J. Lai performed most of the experiments and assays, analyzed and interpreted the data, and wrote the manuscript.

Dr. Vladimir Ljubicic performed some of the COX activity assays and all of the chronic stimulation protocol.

Donna D'souza performed some of the polymerase chain reactions.

Dr. David A. Hood designed and supervised this project. He is the principle investigator.

The effect of chronic contractile activity on mRNA stability in skeletal muscle

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Abstract

Repeated bouts of exercise promote the biogenesis of mitochondria by multiple steps in the gene expression patterning. The role of mRNA stability in controlling the expression of mitochondrial proteins is relatively unexplored. To induce mitochondrial biogenesis, we chronically stimulated (10 Hz; 3 or 6 h/day) rat muscle for 7 days. Chronic contractile activity (CCA) increased the protein expression of PGC-1α, c-myc and Tfam by 1.6-, 1.7- and 2.0-fold, respectively. To determine mRNA stability, we incubated total RNA with cytosolic extracts using an *in vitro* cell-free system. We found that the intrinsic mRNA half-lives ($t_{½}$) were variable within control muscle. PGC-1α and Tfam mRNAs decayed more rapidly ($t_{½}$ = 22.7 min and 31.4 min) than c-myc mRNA ($t_{½}$ = 99.7 min). Furthermore, CCA resulted in a differential response in degradation kinetics. Following CCA, PGC-1α and Tfam mRNA half-lives decreased by 48% and 44%, respectively, whereas c-myc mRNA half-life was unchanged. CCA induced an elevation of cytosolic RNA-stabilizing HuR and destabilizing total AUF1 by 2.4- and 1.8-fold, respectively. Interestingly, the expression of each isoform was variable in EDL muscle. $p45^{AUF1}$ was highly abundant and $p42^{AUF1}$ isoform was barely detectable. Thus, these data indicate that CCA results in accelerated turnover rates of mRNAs encoding important mitochondrial biogenesis regulators in skeletal muscle. This adaptation is likely beneficial because in permitting more rapid phenotypic plasticity in response to subsequent contractile activity.

Introduction

Skeletal muscle exhibits dynamic plasticity in response to functional demands. In response to chronic contractile activity (CCA), muscles undergo a wide variety of biochemical and physiological adaptations (77; 112). For example, regular endurance exercise undertaken over a number of weeks leads to the biogenesis of mitochondria, eliciting a sequential phenotypic fiber type transition from glycolytic to oxidative metabolism (10; 72; 77). This process is largely orchestrated by the well-characterized key regulatory factor, peroxisome proliferator-activated receptor gamma, coactivator-1 alpha (PGC-1α) (9; 77; 178). Specifically, PGC-1α coactivates transcription factors to induce the expression of Tfam, a nuclear gene product that is involved in the transcription and replication of mitochondrial DNA (mtDNA) (159; 161).

In response to exercise, mRNA and protein levels of PGC-1α and Tfam increase (5; 61; 131; 134; 137; 165). An increase in the steady-state mRNA level as a result of a stimulus such as exercise is the product of both synthesis (transcription) and degradation (mRNA stability). Thus, the relative contribution of both of these processes plays an important role in phenotypic adaptations (53). However, the impact of changes in mRNA stability for muscle phenotypic adaptations has been relatively unexplored.

Compared with other biological molecules, the distinguishing property of mRNA is its high rate of turnover. Of particular interest is the class of mRNA bearing AU-rich elements (AREs) in the 3′-untranslated region (UTR) (18; 24; 40; 42). The physical interaction of AREs with specialized RNA-binding proteins (RBPs) is a strong

determinant of mRNA stability and/or translation, and hence the subsequent level of protein expression. In muscle, a pair of RBPs with reciprocal functions can act independently and synchronously to regulate the expression of labile genes (130; 142; 172; 181). Human antigen R (HuR) and the four isoforms of ARE-RNA binding factor 1 (AUF1) ($p37^{AUF1}$, $p40^{AUF1}$, $p42^{AUF1}$ and $p45^{AUF1}$) are primarily localized to the nucleus (37; 99; 185). Their export to the cytoplasm has been associated with the stabilization or destabilization of mRNA transcripts, respectively (37; 99). During muscle differentiation, HuR accumulates in the cytoplasm and interacts with mRNAs encoding MyoD, myogenin (181; 182) and acetylcholinesterase (43; 44). In contrast, the AUF1 proteins have been shown to bind to and destabilize the mRNAs encoding human angiotensin 1 receptor (142), sarcoendoplasmic reticulum calcium ATPase2a (17) and the calcitonin receptor (193). Furthermore, HuR and AUF1 have common targets, and growing evidence has demonstrated that homomultimerization of HuR and AUF1 can bind simultaneously and competitively to a single ARE (99; 142; 193). Interestingly, both HuR and the AUF1 proteins target the ARE in the c-myc 3′UTR (63; 97). C-myc is a transcription factor which has also been implicated in the regulation of mitochondrial biogenesis, via the control of Tfam expression (94; 103).

The effect of contractile activity on the stability of mRNAs which regulate mitochondrial biogenesis has never been investigated. Thus, the purposes of our study were 1) to characterize the mRNA stability of PGC-1α, c-myc and Tfam in relation to steady state mRNA and protein levels; 2) to investigate the presence of relevant AREs

within the 3′UTRs of PGC-1α, c-myc and Tfam regulatory genes; and 3) to determine the effect of CCA on the expression of mRNA destabilizing/destabilizing proteins along with coincident changes in mRNA half-lives.

Methods

Animals and in vivo chronic contractile activity protocol. All animals were housed in a temperature-controlled room (22.5 ± 0.5°C) with 12 h light (19:00-7:00) and 12 h dark (7:00-19:00) cycles and were allowed food and water *ad libitum*. In the first set of experiments, male Sprague-Dawley rats (320-462 g) were anaesthetized with a ketamine/xylazine combination dose via intraperitoneal injection (0.2 ml/100 g body weight), and the tibialis anterior (TA), muscles were removed.

In a separate set of experiments, male Sprague-Dawley rats (n = 18; Charles River, St. Constant, QC, Canada) weighing between 300-325 g were anaesthetized as described above. Under aseptic conditions, two stimulating electrodes (Medwire, Leico Industries, New York, NY) were passed subcutaneously from the thigh and exteriorized at the back of the neck. The electrodes were sutured to the underlying muscle ~1-2 mm on either side of the common peroneal nerve. The overlying hamstring muscle was sutured, the skin was stapled, and sterile ampicillin (Penbritin, Ayerst, Montreal, Canada) was injected to minimize risks of infection. In all animals, the contralateral limb was used as a non-stimulated internal control.

A portable stimulator unit was placed in a plastic housing connected to the exteriorized electrode wires at the back of the neck, and fastened to the back of the animal with cloth tape, as done previously (2; 108). Care was taken to ensure that the procedure did not restrict animal movement, cause discomfort, or restrict breathing. Stimulation was adjusted at the time of electrode implantation to result in palpable

contractions of the TA and extensor digitorum longus (EDL) muscles. When animals had regained ≥100% of their initial body weight and had recovered for a minimum of 1 wk, chronic stimulation (10 Hz, 0.1-ms duration) was begun for 3 h or 6 h/day, for 7 days. Animals were observed to move freely about their cages during the experimental periods. After the final recovery period (21 or 18 hours) on the eighth day, the EDL muscles were extracted and frozen in liquid nitrogen. All animals were sacrificed by removal of the heart. All samples were frozen at -80°C and pulverized at the temperature of liquid nitrogen for subsequent RNA and protein analyses. All procedures involving animals were approved by the York University Animal Care Committee, in accordance with the Canadian Council on Animal Care.

Cytochrome c oxidase (COX) activity. COX activity was measured as previously detailed (33). Briefly, protein extracts were added to the test solution containing fully reduced cytochrome *c*. Enzyme activity was determined as the maximal rate of oxidation of fully reduced cytochrome *c* measured by the change in absorbance at 550 nm in a Synergy HT microplate reader at 30°C.

In vitro RNA isolation. Total RNA was isolated using TRIzol (Invitrogen) as recommended by the manufacturer, with slight modifications (53). Frozen muscle powders (30 or 200 mg) and TRIzol were homogenized (Ultra Turrax 7-mm probe) at 30% power output for 30 s and subsequently transferred to a sterile tube. After 5 min of incubation at room temperature, 200 μl of chloroform was added to the homogenate that was then shaken vigorously for 15 s. A subsequent colour change was observed from

clear pink to milky pink, followed by centrifugation (Eppendorf, 5415 D) at 12,000 g at 4°C (15 min). After transferring the upper aqueous phase to a separate RNase-free tube, 500 µl of isopropanol was added, and the supernate was vigorously shaken. To precipitate total RNA, the supernate was incubated overnight at -20°C. The RNA pellet was collected by subjecting the supernate to centrifugation at 16,100 g at 4°C (10 min). The isopropanol was discarded and the pellet was washed with 75% ethanol. After a final spin at 16,100 g at 4° C (1 min), the RNA pellet was resuspended in sterile water. Using 995 µl of sterile water and 5 µl of RNA, total RNA concentration and purity were determined by ultraviolet photometry at 260 and 280 nm, respectively. RNA quality was verified by separation of the 28S and 18S rRNA on denaturing formaldehyde-1% agarose gels.

In vitro cytosolic protein extraction. EDL muscle powders (50 mg) were homogenized in sterile homogenization buffer (25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM HEPES pH 7.9, 0.5 mM 1,4-dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and RNase-free water) at 40% power output for 3 x 10s. The homogenates were centrifuged at 5,000 g at 4°C (15 min). The supernatant fractions were then subjected to further centrifugation at 15,000 g at 4°C (15 min). The resultant postmitochondrial supernatant (S15) was transferred to a sterile tube. Protein concentrations of the S15 fractions were determined by the Bradford colorimetric assay.

In vitro RNA decay assay. Analysis of RNA degradation was performed as previously described, with slight modifications (53). Total RNA (30 µg) from TA

muscles from control animals was incubated with S15 protein extract from EDL muscles (20 μg) in a 100-μl reaction volume at 37°C. Separate aliquots were removed at 5, 10, 20 and 30 mins. Total RNA was re-isolated with the phenol-chloroform extraction procedure and precipitated at -20°C overnight. Total RNA was subsequently washed, pelleted and resuspended in 20 μl of sterile ddH_2O. RNA concentration and purity were determined by measuring absorbance at 260 nm and 280 nm, respectively. The quality of total RNA was validated by separation of the 28S and 18S rRNA on denaturing formaldehyde-1% agarose gels.

Reverse transcription – polymerase chain reaction (RT-PCR). Total RNA (2 μg) was reverse-transcribed to cDNA using SuperScript™ III reverse transcriptase as recommended by the manufacturer (Invitrogen). cDNA was amplified by PCR using GoTaq™ Flexi DNA polymerase (Promega), using the manufacturer's recommendations. Sequence-specific primers listed in Table 1 for PGC-1α, c-myc, Tfam or S12 were added in a 50-μl volume PCR reaction containing 2 μl of cDNA. PCR products (40 μl each) were separated on 1.8% agarose gels and visualized by ethidium bromide (EtBr) staining.

Table 1. List of Primers.

Gene	Primer Sequences	Size (nt)
PGC-1α	5'-GAC CAC AAA CGA TGA CCC TCC-3' 5'-CCT GAG AGA GAC TTT GGA GGC-3'	635
c-myc	5'-TCA AGA GGC CAC AGC AAA C-3' 5'-AAA AGC TAC GCT TCA GCT CG-3'	274
Tfam	5'-ATG GCG CTG TTC CGG GGA ATG TGG G-3' 5'-TTA ATT CTC AGA GAT GTC TCC CGG G-3'	735
S12	5'-GGA AGG CAT AGC TGC TGG-3' 5'-CCT CGA TGA CAT CCT TGG-3'	638

Western blotting. Total cytosolic protein extracts were isolated from muscle powders as described above. Briefly, total protein (50 μg/lane) was electrophoresed through one-dimensional 12 or 15% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Blots were blocked (1 hr) at room temperature with 5% milk in 1 X TBS-Tween20 (Tris-buffered saline/Tween-20; 25 mM Tris HCl, ph7.5, 1mM NaCl and 0.1% Tween-20), followed by overnight incubation with antibodies diluted in 5% blocking buffer directed toward PGC-1α (1:500; Caymen Chemicals), Tfam (1:750; (61)), c-myc (1:500; Santa Cruz Biotechnology), HuR 3A2 (1:2,000; Santa Cruz Biotechnology), AUF1 (1:500; Upstate/Millipore), aciculin (1:200; produced in our laboratory) and GAPDH (1:40,000; Abcam). After 3 x 5 min washes with TBS-Tween20, blots were incubated at room temperature for 1 hr with the appropriate

secondary antibody conjugated to horseradish peroxidase. Blots were washed again 3 x 5 min with TBS-Tween20, developed with enhanced chemiluminescence, and quantified via densitometric analysis of the intensity of the signal with SigmaScanPro v.5 software (Jandel Scientific, San Rafael, CA).

Statistical analyses. Data were analyzed with GraphPad 4.0 software, and values are reported as means ± S.E.M. Where indicated, Students' paired t test, one- or two-way analyses of variance followed by Bonferroni post-hoc tests were used to determine individual differences between conditions. Results were considered to be statistically significant if $p<0.05$ was achieved. The nonlinear regression equation $50 = 100e^{(-kx)}$ was used to determine mRNA half-lives.

Computational analysis. To identify putative HuR and AUF1 binding motifs in PGC-1α 3'UTRs of mammals, we deployed sequence comparison of six mammalian species against human using the software ClustalW2 (100). A pairwise score was calculated as the number of identities in the best alignment divided by the number of residues compared. Gap positions were excluded. Both of these scores were initially calculated as percent identity scores and were converted to distances by dividing by 100 and subtracting from 1.0 to give number of differences per site (100).

Results

Effect of chronic contractile activity (CCA) on whole muscle mitochondrial biogenesis.

To evaluate the effect of CCA on mitochondrial content, we measured COX activity in stimulated and contralateral non-stimulated EDL muscle. COX activity in non-stimulated EDL muscle was 6.06 ± 0.56 U/g muscle. Seven days of stimulation resulted in an equal elevation in animals subjected to both 3 hrs/day and 6 hrs/day conditions (S. Fig. 1). Therefore the results were pooled to show a 1.5-fold elevation of COX activity (Fig. 1; $p<0.05$). Thus, our chronic stimulation protocol was effective in inducing mitochondrial biogenesis in whole muscle.

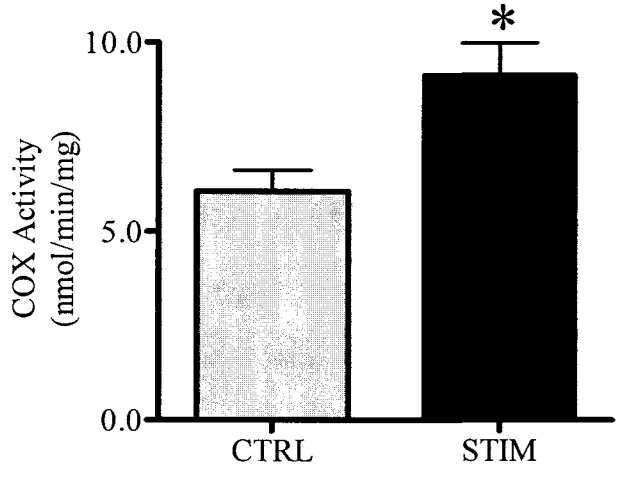


Figure 1. *Cytochrome c oxidase (COX) enzyme activity of whole muscle.* The EDL muscles of animals were subjected to CCA for 3 or 6 hrs/day, for 7 days (n=18; *$p<0.05$, CTRL vs. STIM).

Effect of chronic contractile activity on PGC-1α protein content, steady-state mRNA level and mRNA stability. Chronic contractile activity resulted in an increase of PGC-1α protein content by 1.6-fold, compared to non-stimulated control muscle (Fig. 2A; $p<0.05$). In contrast, the levels of PGC-1α mRNA were not affected by the chronic stimulation paradigm (Fig. 2B).

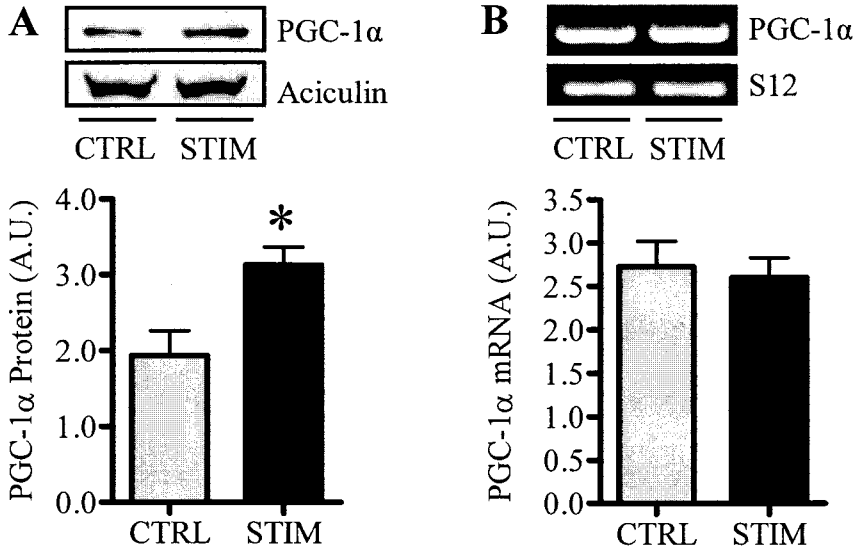


Figure 2. *Effect of CCA on PGC-1α protein and mRNA levels.* Animals were subjected to CCA for 7 days. (A) PGC-1α protein, along with a loading control (aciculin) were measured in extracts from CTRL or STIM EDL muscles (n=6; *$p<0.05$, CTRL vs. STIM). (B) PGC-1α and S12 mRNA transcripts were measured by RT-PCR from total RNA isolated from CTRL or STIM EDL muscles. A representative EtBr-stained agarose gel is illustrated along with a graphical representation of the data (n=10).

To evaluate PGC-1α mRNA stability, we employed a cell-free mRNA decay assay, as done previously (53). PGC-1α mRNA was degraded by cytosolic ribonucleases, as

incubation of total RNA in the absence of cytosolic proteins (Fig. 3A) and in the presence of a ribonuclease inhibitor (Fig. 3B) attenuated the mRNA decay.

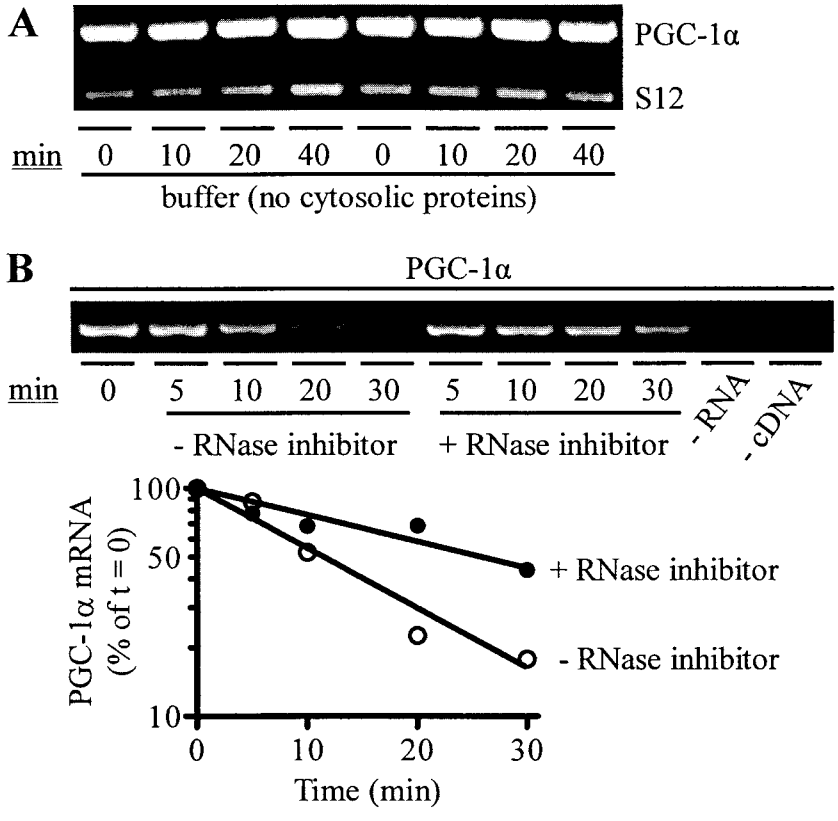


Figure 3. *PGC-1α mRNA stability in skeletal muscle.* **(A)** Total RNA (12 μg) from the TA muscle was incubated with RNase-free isolation buffer alone for 0, 10, 20 and 40 min. PGC-1α and S12 mRNA transcripts were examined by RT-PCR and EtBr-stained agarose gel electrophoresis. **(B)** Total RNA (30 μg) was incubated with 20 μg of cytosolic proteins for 0, 5, 10, 20 and 30 min in the presence or absence of a ribonuclease inhibitor. Reactions in the absence of RNA or cDNA are also shown as negative controls.

The half-life ($t_{½}$) of PGC-1α mRNA was 24.5 min (Fig. 4) in the presence of cytosolic proteins isolated from control muscle. In contrast, PGC-1α mRNA stability decreased by 48% in the cytosol isolated from chronically stimulated muscle (Fig. 4; $p<0.05$). S12

mRNA, used as an internal control, was more stable ($t_{½}$=58.6 min) than PGC-1α mRNA in the cytosol from control, non-stimulated muscle. No effect of chronic stimulation on S12 mRNA was observed (S. Fig. 4). Therefore, these data for S12 mRNA were combined (Fig. 4).

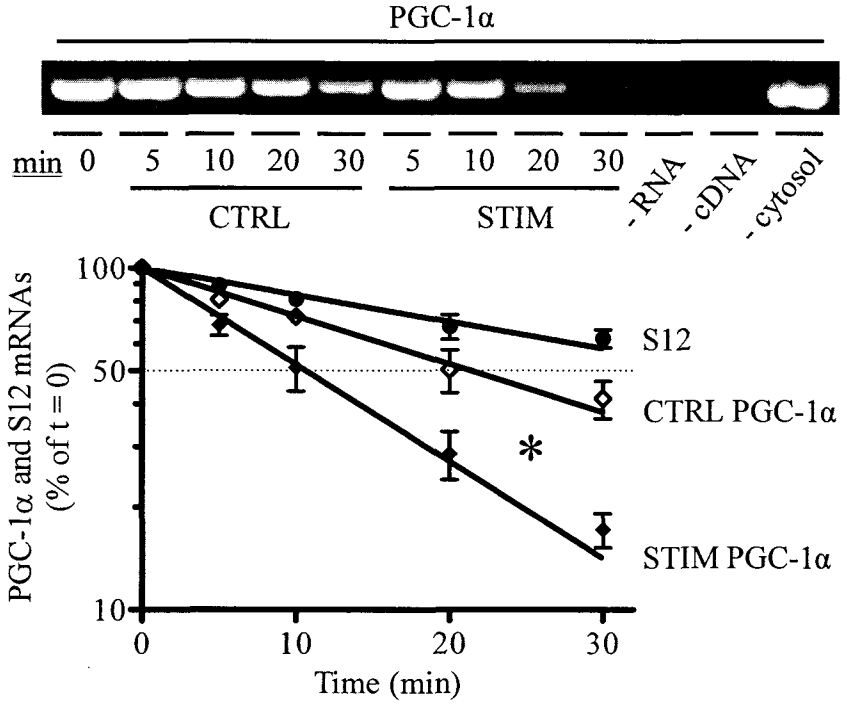


Figure 4. *Effect of PGC-1α mRNA stability in skeletal muscle.* Total RNA isolated from TA muscle was incubated with cytosolic proteins from CTRL or STIM EDL muscles of animals subjected to CCA for 7 days. After each time point, total RNA was re-isolated and PGC-1α (n=5; *$p<0.05$, CTRL vs. STIM) along with an internal control S12 (n=10) were examined by RT-PCR. A representative EtBr gel is illustrated along with a graphical representation of the data. Reactions in the absence of cytosolic proteins as well as the absence of RNA or cDNA, are shown as positive and negative controls, respectively.

Effect of chronic contractile activity on c-myc protein content, steady-state mRNA level and mRNA stability. Chronic contractile activity resulted in an increase of c-myc protein content by 1.7-fold (Fig. 5A; $p<0.05$). In contrast, both c-myc steady-state mRNA level and mRNA stability were unchanged in response to chronic stimulation (Figs. 5B, 6). The basal half-lives of c-myc (82.9 min) and S12 (52.6 min) mRNAs were not significantly different (S. Fig. 6).

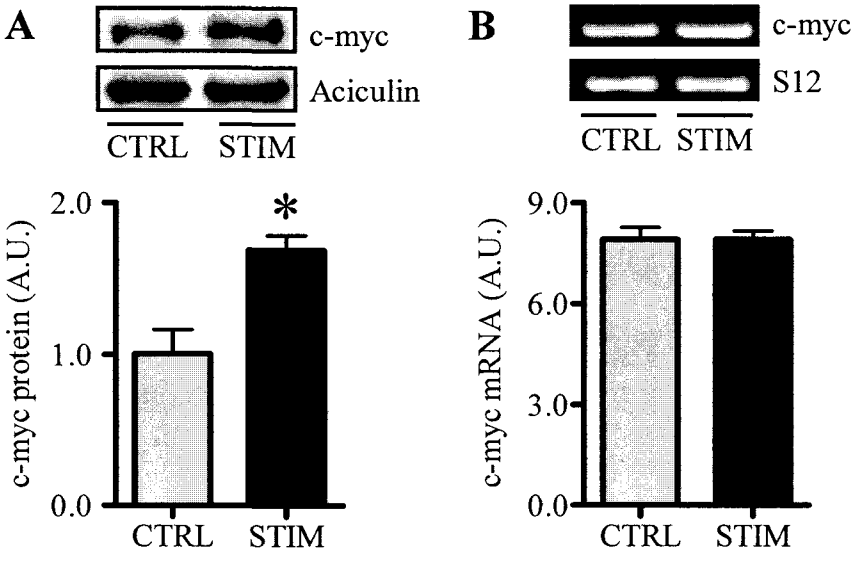


Figure 5. *Effect of CCA on c-myc protein and mRNA levels.* Animals were subjected to CCA for 7 days. **(A)** C-myc protein, along with a loading control (aciculin) were measured in extracts from CTRL or STIM EDL muscles (n=8; *$p<0.05$, CTRL vs. STIM). **(B)** C-myc and S12 mRNA transcripts were measured by RT-PCR from total RNA isolated from CTRL or STIM EDL muscles. A representative EtBr-stained agarose gel is illustrated along with a graphical representation of the data (n=10).

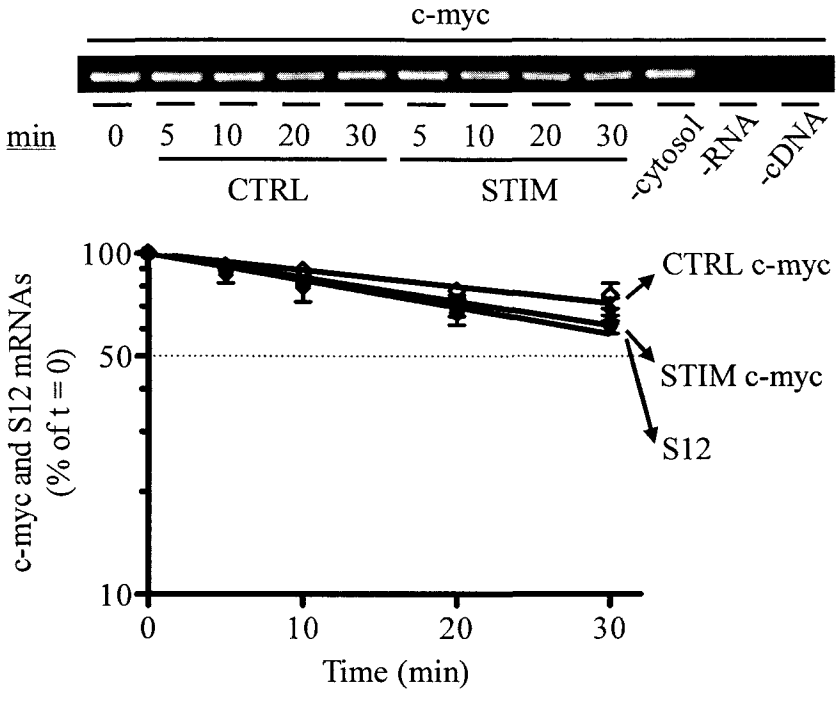


Figure 6. *Effect of CCA on c-myc mRNA stability in skeletal muscle.* Total RNA isolated from TA muscle was incubated with cytosolic proteins from CTRL or STIM EDL muscles of animals subjected to CCA for 7 days. After each time point, total RNA was re-isolated and c-myc (n=4) along with an internal control S12 (n=10) were examined by RT-PCR. A representative EtBr gel is illustrated along with a graphical representation of the data. Reactions in the absence of cytosolic proteins as well as the absence of RNA or cDNA, are shown as positive and negative controls, respectively.

Effect of chronic contractile activity on Tfam protein content, steady-state mRNA level and mRNA stability. Chronic contractile activity led to an increase of Tfam protein content by 2.0-fold (Fig. 7A; $p<0.05$), but not steady-state mRNA levels (Fig. 7B).

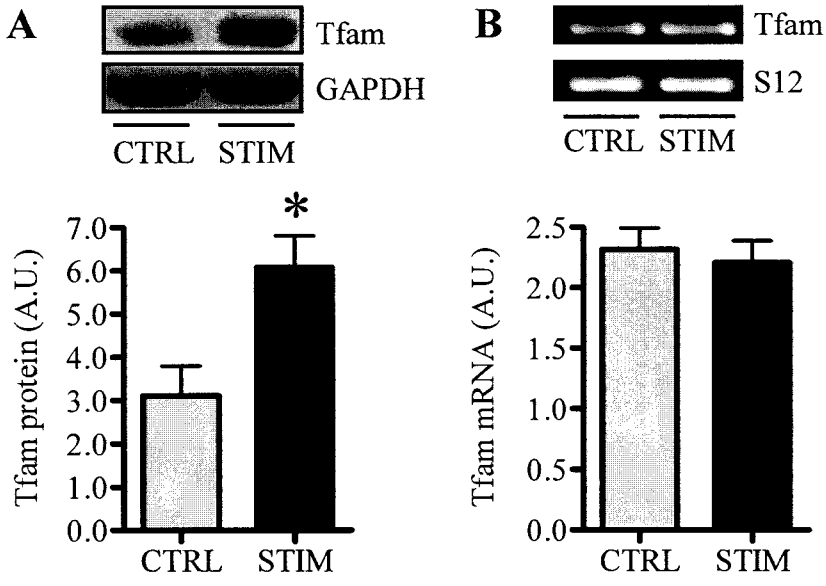


Figure 7. *Effect of CCA on Tfam protein and mRNA levels.* Animals were subjected to CCA for 7 days. **(A)** Tfam protein, along with a loading control (GAPDH) were measured in extracts from CTRL or STIM EDL muscles (n=8; *$p<0.05$, CTRL vs. STIM). **(B)** Tfam and S12 mRNA transcripts were measured by RT-PCR from total RNA isolated from CTRL or STIM EDL muscles. A representative EtBr-stained agarose gel is illustrated along with a graphical representation of the data (n=8).

In the presence of cytosolic proteins isolated from control muscle, Tfam mRNA half-life was 31.2 min, and this was reduced by 44% to 13.6 min in chronically stimulated muscle compared to the control condition (Fig. 8; $p<0.05$).

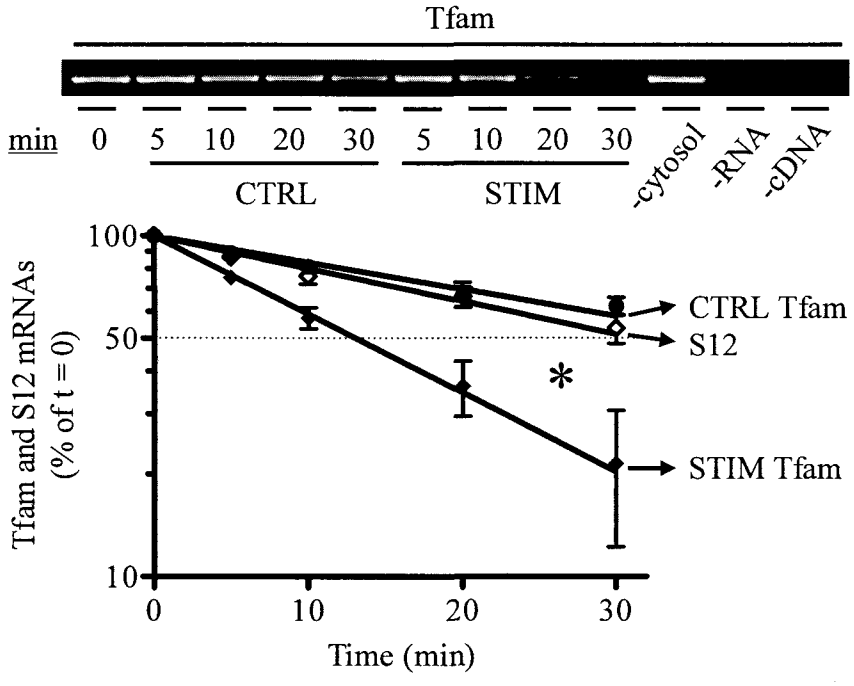
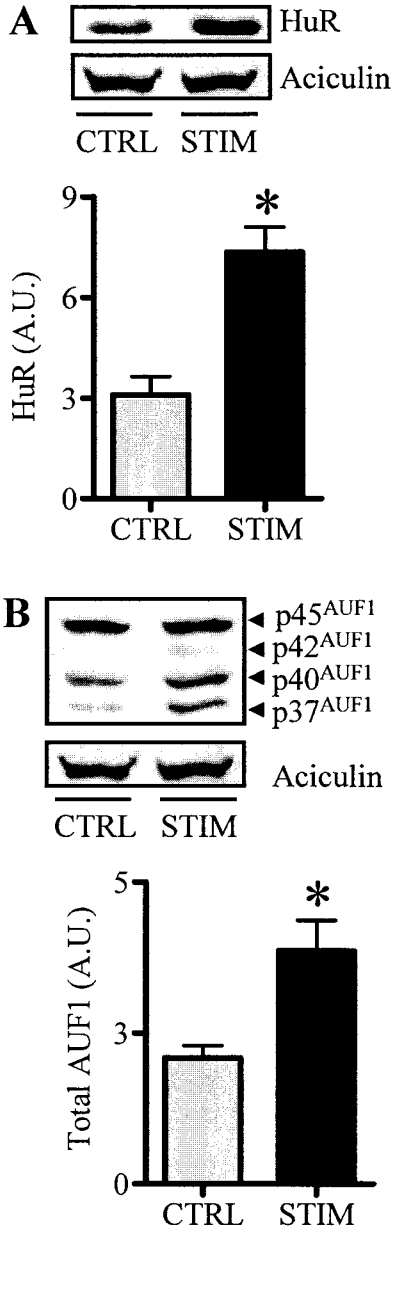


Figure 8. ***Effect of CCA on Tfam mRNA stability in skeletal muscle.*** Total RNA isolated from TA muscle was incubated with cytosolic proteins from CTRL or STIM EDL muscles of animals subjected to CCA for 7 days. After each time point, total RNA was re-isolated and Tfam (n=7, *$p<0.05$ CTRL vs STIM) along with an internal control S12 (n=10) were examined by RT-PCR. A representative EtBr gel is illustrated along with a graphical representation of the data. Reactions in the absence of cytosolic proteins as well as the absence of RNA or cDNA, are shown as positive and negative controls, respectively.

Expression of RNA-binding proteins HuR and AUF1. Analysis of each AUF1 isoform revealed that the most abundant isoform was $p45^{AUF1}$, whereas $p42^{AUF1}$ expression was low and barely detectable in skeletal muscle. CCA resulted in the increase of stabilizing HuR (Fig. 9A; $p<0.05$) as well as destabilizing total AUF1 proteins (Fig. 9B, left panel; $p<0.05$) by 2.4- and 1.9-fold, respectively. $p37^{AUF1}$, $p40^{AUF1}$, and $p45^{AUF1}$ increased in response to chronic stimulation by 2.3-, 1.7-, and 1.5-fold, respectively (Fig. 9B, right panel; $p<0.05$) compared to the contralateral control muscle.



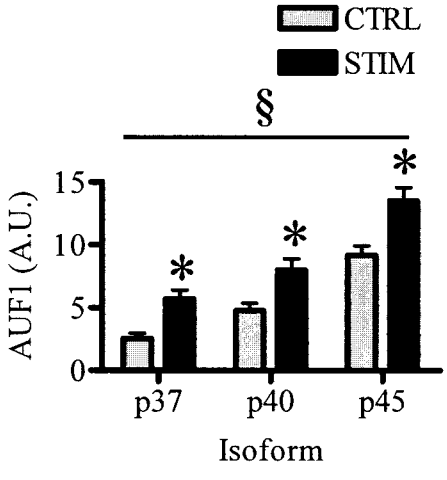


Figure 9. *Effect of CCA on the content of RNA-binding proteins, HuR and AUF1.* Animals were subjected to CCA for 7 days. **(A)** HuR, along with a loading control aciculin were measured in extracts from CTRL or STIM EDL muscles (n=8; *p<0.05, CTRL vs STIM). **(B)** Analyses of protein expression of $p37^{AUF1}$, $p40^{AUF1}$, $p42^{AUF1}$, $p45^{AUF1}$ and aciculin are shown and the positions of the different AUF1 isoforms are indicated. Graphical representations of total AUF1 (left panel) and the distribution of various isoforms (right panel) are shown. Symbols (*) and (§) denote statistical significance from respective CTRLs and a main effect for all CTRL isoforms, respectively).

Discussion

Repeated contractions of skeletal muscle over several weeks promote the biogenesis of mitochondria. The resulting increase in mitochondrial content in skeletal muscle leads to a preferential shift in energy utilization toward a greater dependence on oxidation phosphorylation for ATP provision. A consequence of this adaptation is that the muscle become more fatigue-resistant, a characteristic favorable for health in both young and old individuals (110; 112). Therefore, understanding the molecular basis of mitochondrial biogenesis is important for our comprehension of how exercise can improve the quality of life.

Mitochondrial biogenesis requires the coordinated regulation of two distinct genomes localized in separate cellular compartments. Three important biogenesis regulators investigated in our study were PGC-1α, c-myc and Tfam. PGC-1α coactivates genes encoding a number of transcription factors to induce the mRNA expression of NUGEMPs (77; 78; 89). One of the most important NUGEMPs is Tfam, which regulates the expression and copy number of mtDNA. The transcription factor, c-myc, is also involved in the regulation of Tfam expression (94; 103). Once Tfam is translated into protein, it is shuttled across the mitochondrial membranes to its functional destination. Inside the matrix, Tfam activates the transcription of the 13 mitochondrial proteins that are essential for oxidative phosphorylation (for review, see (159). There is an increasing body of evidence that describes the changes in protein and mRNA expression of both PGC-1α and Tfam in skeletal muscle. However, the regulation of these transcriptional

activators during exercise requires greater understanding. Studies in the past have focused largely on transcriptional mechanisms while the significance of mRNA stability as an important regulator of gene expression in skeletal muscle is just beginning to earn recognition (24; 181). Given the significance of mRNA stability in gene expression, we 1) compared the intrinsic mRNA stabilities of PGC-1α, c-myc and Tfam in skeletal muscle, and 2) examined effect of CCA on the stability of these mRNA species using an *in vitro* cell-free system.

First, we showed that the basal half-lives of these regulatory mRNAs were inherently different. PGC-1α and Tfam mRNAs were significantly more unstable than the mRNAs of c-myc and S12 (Fig. 10), supporting previous evidence that the intrinsic half-lives of transcripts in muscle can vary from minutes (Egr-1) to hours (utrophin) *in vivo* (65; 88; 162). Generally, the half-life of mRNA species negatively correlates with regulatory importance. For example, mRNAs encoding transcription factors are short-lived compared with mRNAs of genes related to metabolism and cell structure (162). C-myc mRNA is well-known for its short half-life (20-30 min) *in vivo* (148; 162). Our *in vitro* analyses of mRNA stability revealed that the turnover rates of PGC-1α and Tfam were greater than c-myc, suggesting that they are encoding proteins of greater regulatory importance, at least in skeletal muscle.

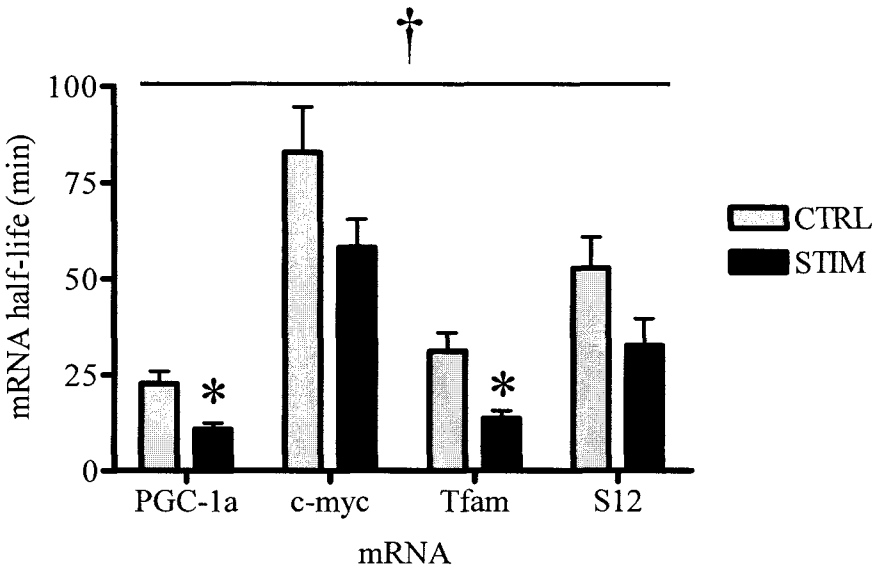


Figure 10. *Effect of CCA on mRNA stability in skeletal muscle.* Total RNA isolated from TA muscle was incubated with cytosolic proteins from CTRL or STIM EDL muscles of animals subjected to CCA for 7 days. After each time point, total RNA was re-isolated and PGC-1α, c-myc, Tfam along with an internal control S12 (n=4-10) were examined by RT-PCR. Symbols (*) and (†) denote statistical significance from respective CTRLs and a main effect for all CTRL mRNAs, respectively).

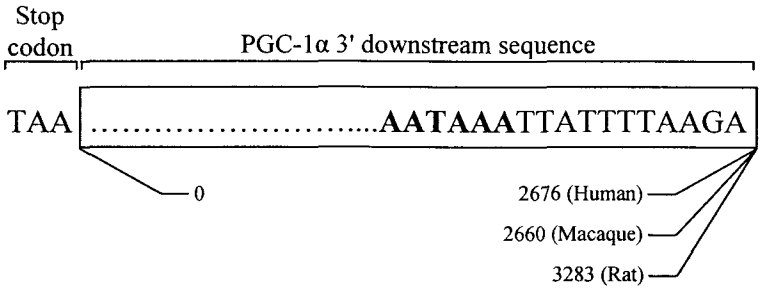
In an effort to understand the role of mRNA stability in exercise-induced mitochondrial biogenesis, we employed a chronic, low-frequency electrical stimulation model to simulate chronic exercise. This protocol provides a potent stimulus for the induction of mitochondrial biogenesis in skeletal muscle (2; 53; 108; 109; 170). Thus, this serves as a good experimental model to study the underlying biochemical and physiological adaptations involved in organelle synthesis. Our stimulation conditions were effective at inducing mitochondrial biogenesis, as COX activity increased, and the magnitude of up-regulation was similar to values reported previously (61; 108).

Contractile activity provoked increases in PGC-1α and Tfam protein expression, consistent with published findings from our laboratory (61; 87). In addition, we also showed that CCA induced an increase in c-myc protein expression in skeletal muscle. The extent of the induction of these proteins was typical of that observed with this chronic stimulation model (53; 79; 108; 169). However, the increases in protein expression were not accompanied by concomitant elevations in the corresponding steady-state mRNA levels. This is likely due to the transient nature of mRNA expression changes as a result of exercise. For example, using the identical CCA model as the present study, Gordon et al. found that Tfam mRNA expression peaked on day 4, but was reduced to resting muscle levels by day 14, while protein levels were elevated by approximately 1.5-fold (61). Other studies have reported that PGC-1a mRNA expression increases during, and immediately after, an acute bout of exercise (5; 131; 134; 137; 165), and returns to resting levels within hours post-exercise (5; 122). Similarly, c-myc mRNA expression also increases after an acute bout of exercise in mice (84) and in humans (118; 177). Together, these findings suggest that the mRNA responses of PGC-1α, c-myc and Tfam are time-dependent, post-exercise. They increase transiently after each period of contractile activity, contribute to the accumulation of protein, then decline thereafter.

The present study is the first to demonstrate a differential response in mRNA degradation kinetics as a result of CCA. In the presence of cytosolic proteins isolated from stimulated muscle, the half-lives of PGC-1α and Tfam mRNAs were significantly

reduced compared to the control, non-stimulated condition. In contrast, neither c-myc nor S12 mRNA stability was significantly altered following CCA. In a previous study employing the identical chronic stimulation protocol as the present study, Freyssenet et al. (53) showed that CCA resulted in the increase of cytochrome *c* mRNA abundance, which was attributed, in part, to an increased mRNA stability in the early stages (days 2, 3) of the CCA paradigm. Later, by day 7, mRNA stability had retuned to control levels (53). Conversely, Irrcher et al. showed that contractile activity resulted in reduced Egr-1 mRNA stability in cultured myotubes (88). These divergent data are clearly indicative of transcript-specific adaptations to CCA. This depends on the presence or absence of mRNA-specific sequences which support the interaction with stabilizing/destabilizing RBPs. We speculate that the short half-lives of PGC-1α and Tfam mRNAs are caused by one or more potent instability sequences, within the 3′UTR of each transcript. The 3′UTR of Tfam is known to contain AREs, which may serve as destabilizing *cis*-elements. Similarly, AREs may exist in the PGC-1α 3′UTR, the full-length of which has yet to be completely identified empirically. Multiple AREs covering a stretch of ~600 nucleotides are found within ~3 kb downstream of the PGC-1α coding sequence, followed by a consensus poly(A) signal (Fig. 11). Using ClustalW2 software (100), sequence alignment of the human PGC-1α sequences against six mammalian species, revealed scores $\geq$ 89 (Fig. 11), suggesting that they are evolutionary conserved and thus may dictate the mRNA turnover rate of this regulatory protein.

A.



B.

Sequence A		Sequence B		
Name	Length (nt)	Name	Length (nt)	Score
Human	2676	Macaque	2660	98
Human	2676	Horse	2600	95
Human	2676	Dog	2759	92
Human	2676	Cow	1832	93
Human	2676	Rat	3283	90
Human	2676	Mouse	2790	89

C.

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Human     2517  TAATCCGCCTAATTCTTGTTGTTCTGTAGGTTAAATGCAGGTATTTTAACT--GTGTGAA  2575
Macaque   2501  TAATCCGCCTAATTCTTGTTGTTCTGTAGGTTAAATGCAGGTATTTTAACT--GTGTGAA  2559
Horse     2436  TAATCCGCCTAATTCTTGTTGTTCTGTAGGTTAAATGCAGGTATTTTAACTCTGTGTGAA  2496
Dog       2595  TAATCCGCCTAATTCTTGTTGTTCTGTAGGTTAAATGCAGATATTTTAACTCTGTGTGAA  2655
Cow       1673  TAATCTGCCTAATTCTTGTT---CTGTAGGTTAAATGCAGGTATTTTAACTCTGTGTGGA  1730
Rat       3125  TAATCCGCCTAATTCTTGTT---CTGTAGGTTAAATGCAGGTATTTTAACTCTTTGTGAA  3182
Mouse     2632  TAATCCGCCTAATTCTTGTT---CTGTAGGATAAATGCAGGTATTTTAACTCTTTGTGAA  2689
                ***** ************** ******* ********* **********  **** *
                                                      ARE
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Figure 11. *PGC-1α 3' downstream sequences contain AU-rich elements.* **(A)** Schematic representation of the aligned sequence beginning from the stop codon (TAA) up to the consensus poly(A) signal (bold) and immediate downstream residues. For some species, this stretch of sequence is an amalgamation of the end and the beginning of two adjacent contigs, where bases may be missing. **(B)** Tabular result of PGC-1α sequence between the stop codon and the poly(A) signal comparing human (column A) against six mammals (column B) using the software ClustalW2. A pairwise score was calculated as the number of identities in the best alignment divided by the number of residues compared. Gap positions were excluded (100). **(C)** Sequence alignments of one of several putative HuR and AUF1 binding sites designated ARE (bold) within the PGC-1α 3′ downstream sequences suggest that this region is evolutionarily conserved.

Since mRNA stability is determined by the interaction between intrinsic *cis*-elements and extrinsic *trans*-regulatory factors, we investigated the expression of ARE-regulatory RBPs, stabilizing HuR and destabilizing AUF1. HuR and AUF1 are established regulators of c-myc mRNA and they may also be involved in PGC-1α and Tfam mRNA expression. We found that CCA resulted in elevated cytosolic levels of HuR, as well as, all four AUF1 isoforms. This suggests that the up-regulation, or the cytoplasmic re-distribution of HuR and AUF1 are in synchronization, supportive of previous evidence that shows a strong positive correlation between HuR and AUF1 expression in all tissues and during various stages of development (37; 63; 97; 121). However, the concomitant induction of both proteins does not necessarily indicate parallel cellular consequences, which depend on the starting concentration of each protein isoform within the cell, as well as the relative affinity of each isoform for the mRNA binding site. The affinities of HuR and AUF1 isoforms for the ARE are likely important in determining the final rate of mRNA decay, since these proteins compete with each other for binding in the 3′UTR (37; 99; 176). Affinity is determined, in part, by phosphorylation, and it has been shown that HuR is a target of p38 MAPK and AMPK (40; 96; 187; 188). Both of these kinases are activated by contractile activity (111). Thus, the alterations in mRNA stability observed with CCA may stem, in part, from kinase-induced phosphorylation of RBPs on the AREs of target mRNAs.

All four AUF1 isoforms were equally elevated in response to CCA. However, the content of each isoform was variable, with $p45^{AUF1}$ being the most abundant in EDL

muscle. This result suggests that $p45^{AUF1}$ may likely be the dominant AUF1 isoform for PGC-1α and Tfam ARE-mediated decay in skeletal muscle.

In summary, our data show that the half-lives of mRNAs encoding transcription factors are variable in skeletal muscle. With respect to PGC-1α, we have revealed that the 3′UTR may harbor functional regulatory sequences. Furthermore, the responsiveness of each mRNA species to extracellular stimuli was variable. CCA selectively increased the mRNA degradation rate of both PGC-1α and Tfam, two important regulatory proteins involved in mitochondrial biogenesis. We speculate that this characteristic is beneficial because, when combined with a parallel increase in transcription, this permits a higher turnover rate, and a more rapid adaptive response in the face of an imposed stress, such as acute contractile activity. In support of this, Pilegaard illustrated that PGC-1α transcription and mRNA expression reached higher levels following exercise in the trained condition, suggesting that the mechanisms regulating the exercise-induced activation of PGC-1α in muscle became more sensitive with chronic exercise (145). Furthermore, the dynamics of the mRNA reservoir may be fine-tuned by the induction of, and balance between, specific RBPs such as HuR and AUF1. Collectively, our study demonstrates that mRNA stability should spark interest for forthcoming studies which will revolutionize skeletal muscle research.

Acknowledgements

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SUMMARY AND FUTURE WORK

Summary and Future Work

The purpose of my thesis was to examine the role of mRNA stability in mitochondrial biogenesis. Given that mRNA stability has been shown to be a powerful regulatory mechanism, we investigated the role of mRNA stability in mitochondrial biogenesis in skeletal muscle. To test this, we used Sprague-Dawley rats and imposed a low frequency chronic stimulation model that simulates endurance exercise. To assess mRNA stability, we incubated total RNA with isolated cytosolic proteins in an *in vitro* system as done previously with slight modifications (53).

Our model was effective at inducing mitochondrial biogenesis as COX activity was elevated to levels observed in previous CCA studies (2; 53; 61; 108). To assess whether the elevation of protein expression was caused by a concomitant elevated abundance of mRNA, we measured both protein and mRNA expression at identical time-points. The magnitude of elevation of all mitochondrial regulatory proteins under investigation was typical to that observed in other CCA studies (2; 53; 61; 108). We observed that mRNA expression was unchanged in response to CCA. Since the steady-state level of mRNA is, in part, regulated by stability, we therefore investigated mRNA stability of these regulatory genes. First we characterized the intrinsic stability of each transcript and found that the basal half-lives of the transcripts were variable. Next, we observed that CCA induced a differential response in mRNA degradation kinetics. Specifically, CCA resulted in a greater instability of PGC-1α and Tfam mRNAs. This adaptive response may in part, be contributed by the greater affinity binding of

destabilizing RNA-binding proteins. Collectively, our study showed that mRNA stability is a fast responsive upstream mechanism that contributes to the regulation of mRNA abundance and translation.

Given these findings, future studies could focus on the following:

1. The steady-state mRNA level was unchanged in response to CCA (Figs 2B, 5B, 7B). However, the distinguishing property of mRNA is its fast rate of turnover, as observed with Tfam mRNA (61). Since our measurement of mRNA expression was taken at 21 hours after the final recovery period on the eighth day of stimulus, it is possible that any elevation of mRNA expression had dissipated. We could therefore examine mRNA expression along with stability in a temporal manner.
2. Since mRNA abundance is contributed by gene transcription and/or transcript degradation, it would be important to understand which of these two processes impose a greater impact on gene expression. To study transcription, a plasmid DNA can be directly injected into the muscle of interest with a reporter gene (e.g. chloramphenicol acetyltransferase (53) or firefly luciferase) linked to the PGC-1α, c-myc or Tfam promoter. Transcriptional activation is evaluated by measuring the activation of the reporter gene.
3. In the current study, mRNA stability was assessed *in vitro*. To reflect our finding in an *in vivo* model, we can employ the tetracycline (Tet)-controlled transactivation system. The Tet-controlled system consists of two vectors, a

regulator and a responder. The regulator is a fusion protein that contains a DNA-binding Tet represser joined to transcription activation domains. The response vector contains a Tet response element within the promoter of gene of interest gene linked to a reporter gene, such as luciferase. Once the regulator and response plasmids are stably integrated into the C2C12 muscle cell line, the expression of luciferase can be induced using doxycycline, a Tet derivative, to control transcription in a dose-dependent manner. In the Tet-on system, luciferase expression is repressed in the absence of doxycycline. In contrast, luciferase is expressed in the presence of doxycycline. Once the C2C12 cell-line-specific dose of doxycycline has been optimized, transcription can be precisely induced and turned off. This method allows for an uniform synthesis of mRNA, and avoiding the decay measurement of a mixture of "young" and "old" mRNAs, as would be observed with the use of transcriptional inhibitors such as actinomycin D.

4. The relevance of putative AREs in the mRNAs investigated in the current study can be verified by 3′UTR reporter constructs. Wild-type or mutant 3′UTR sequences can be subcloned into plasmids that contain a reporter gene such as luciferase. The effect of various 3′UTR constructs on the expression of reporter genes can be measured in C2C12 muscle cells.

5. In response to CCA, we observed elevated levels of protein expression which can be contributed by increased translation or reduced protein degradation or both.

To examine changes in the translation states of individual mRNAs at the genome-wide level, we can employ a translation state array analysis (TSAA) (197; 199), which identifies differentially translated genes based on the association of mRNAs with polysomes. Polysomes can be separated over a sucrose gradient and divide into low- and high- translated fractions. Each fraction can be hybridized to microarray chips and the ratio of RNA in high to low fractions can be expressed as a translation index of each mRNA.

6. We observed a parallel increase of both RNA-stabilizing HuR and RNA-destabilizing AUF1 proteins. To identify specific protein-RNA interactions we can use RNA pull-down assays. First we *in vitro* transcribe biotinylated RNA probes. These are incubated with whole-cell lysate that have been incubated with streptavidin-Sepharose beads. Beads can be collected by centrifugation and protein content in the eluted material can be monitored by Western blotting (176).
7. Once we have confirmed the binding of HuR or AUF1 to their target mRNAs encoding mitochondrial regulatory proteins, we can assess the binding affinities of HuR and AUF1 by construction and purification of recombinant His_6-HuR and His_6-AUF1 fusion proteins (42). The recombinant protein can be incubated with labelled RNA probes in an RNA electromobility shift assay (REMSA) with a parallel titration of the recombinant proteins. Using non-denaturing gel electrophoresis, we can observe binding activity at each and every protein concentration.

8. In the current study, we examined cytosolic HuR and AUF1, both of which are colocalized in the nucleus in the basal state. Under cellular stress, HuR and AUF1 undergo nucleocytoplasmic shuttling. We can assess the amount and the rate of colocalization by using fluorescence recovery after photobleaching (FRAP) (26). By bleaching the cells with high laser power, this resets the fluorscence emited by GFP-AUF1 or GSR-HuR. The recovery of fluorescence after bleaching measured at various time intervals will reveal the shuttling rate. Finally, the composition of co-regulatory proteins on mRNA binding proteins remains unknown in response to chronic stimulation. A number of these proteins (eIF4G, PABP, pp32, April, CRM1, TTP, transportin 2, 14-3-3σ, TIAR and components of RISC) have been described as interacting with either HuR, AUF1 or with the ARE directly (26; 69; 73; 104; 115; 124; 182). However, the order of these proteins in the overall mRNA-protein interacting complex is poorly understood. We can characterize the composition of these proteins in skeletal muscle as well as interaction by immunoprecipitation, immunodelpetion as well as RNA and/or protein pull-down assays.
9. Finally all of the above molecular and genetic techniques can be used in conjunction with other paradigms of mitochondrial biogenesis in skeletal muscle. We can study the different between fiber types, as well as with chronic disuse. Reporter constructs designed for cell culture can also be electroporated in tissue.

APPENDIX A:

SUPPLEMENTARY DISCUSSION

Supplementary Discussion

Another class of *trans*-regulatory factors, microRNAs (miRNAs), are highly responsive to extracellular stimuli and may play an additional role in CCA-induced down-regulation of mRNA stability in skeletal muscle (47; 125; 126). Out of 151 muscle-specific miRNAs, specific miRNA-23 expression correlates strongly with PGC-1α mRNA levels after an acute bout of endurance exercise in mice (126; 153). Thus, the elevated turnover rate of PGC-1α and Tfam mRNAs in response to CCA may be a result f changes in the expression of RBPs or miRNAs. This remains to be elucidated.

In contrast to PGC-1α and Tfam, the mRNA of c-myc has established functional AREs, and yet, the stability of c-myc mRNA was unresponsive to CCA. It is possible that in addition to primary sequence criteria, secondary structures also play a role in defining 3′UTR RNA-protein interaction and binding affinity, as observed with mRNA localization, which is dependent on unique secondary structures (27; 71; 76; 168).

Finally, the lack of correlation between the proteome and the transcriptome in response to CCA could be explained by ARE-mediated translation. This post-transcriptional mechanism has been described for p53, an important regulator of mitochondrial biogenesis (155). p53 protein can be enhanced without a concomitant elevation of p53 mRNA abundance through HuR binding on the ARE in the p53 3′UTR (123; 175; 183), suggesting that HuR is involved in ARE-mediated translation. In a separate study by Lu et al., using purified recombinant proteins and a synthetic ARE RNA oligonucleotide, all four AUF1 isoforms bound directly to components of the

translation apparatus (115). Additionally, Liao et al. demonstrated that AUF1 promotes the translation of c-myc mRNA in an ARE-dependent manner without affecting mRNA levels (115). Collectively, the current study and literature suggest that HuR and AUF1 may also be involved in ARE-mediated translation in skeletal muscle following CCA.

APPENDIX B:

DATA AND STATISTICAL ANALYSES

Table 1. Whole muscle COX activity (μmol/min/g tissue).

N	CTRL	STIM
1	3.84	5.70
2	3.76	4.73
3	7.21	8.70
4	6.48	7.97
5	5.64	8.89
6	4.35	7.69
7	2.58	4.87
8	3.55	4.31
9	4.76	6.69
10	4.69	8.12
11	9.34	15.55
12	7.93	10.75
13	5.09	7.92
14	11.79	18.00
15	9.00	11.63
16	7.71	12.29
17	5.64	10.03
18	5.64	10.22
Average	6.06	9.11
S.D.	2.38	3.64
S.E.M.	0.56	0.86

Student's paired t test	
P value	P < 0.0001
P value summary	***
Are means signifiantly different? (p < 0.05)	Yes

Table 2A. PGC-1α protein expression in EDL muscle.

N	CTRL	STIM
1	1.01	2.84
2	1.69	2.53
3	2.03	2.75
4	1.88	3.24
5	1.59	3.27
6	3.40	4.16
Average	1.93	3.13
S.D.	0.80	0.58
S.E.M.	0.33	0.24

Student's paired t test	
P value	0.0019
P value summary	**
Are means signifiantly different? ($p < 0.05$)	Yes

Table 2B. PGC-1α mRNA expression in EDL muscle.

N	CTRL	STIM
1	1.91	1.83
2	1.30	1.82
3	3.03	2.34
4	1.92	1.82
5	3.25	3.50
6	2.33	2.31
7	4.27	3.69
8	3.05	2.51
9	3.86	3.48
10	2.36	2.69
Average	2.73	2.60
S.D.	0.93	0.73
S.E.M.	0.29	0.23

Student's paired t test	
P value	0.3557
P value summary	ns
Are means signifiantly different? ($p < 0.05$)	No

Table 3B. PGC-1α mRNA (% of t = 0 min).

min	- Rnase inhibitor	+ Rnase inhibitor
0	100	100
5	87.36	77.68
10	52.18	68.33
20	22.60	68.44
30	17.80	43.74

Table 4. PGC-1α mRNA half-life (min).

N	CTRL	STIM
1	20.91	11.26
2	21.87	9.07
3	12.47	5.87
4	32.22	14.61
5	25.87	13.62
Average	22.67	10.89
S.D.	7.23	3.53
S.E.M.	3.24	1.58

Student's paired t test	
P value	0.003
P value summary	**
Are means signifiantly different? ($p < 0.05$)	Yes

Table 5A. C-myc protein expression in EDL muscle.

N	CTRL	STIM
1	1.48	1.58
2	1.04	1.48
3	0.50	1.70
4	1.72	1.78
5	0.95	1.27
6	0.44	2.09
7	0.77	1.55
8	1.15	2.04
Average	1.01	1.69
S.D.	0.45	0.28
S.E.M.	0.16	0.10

Student's paired t test	
P value	0.0105
P value summary	*
Are means signifiantly different? ($p < 0.05$)	Yes

Table 5B. C-myc mRNA expression in EDL muscle.

N	CTRL	STIM
1	10.10	6.50
2	7.89	7.04
3	7.42	7.66
4	7.96	7.60
5	6.82	8.82
6	7.41	7.79
7	7.61	7.94
8	6.27	8.57
9	8.36	8.88
10	9.22	8.32
Average	7.91	7.91
S.D.	1.12	0.77
S.E.M.	0.35	0.24

Student's paired t test	
P value	0.9924
P value summary	ns
Are means signifiantly different? ($p < 0.05$)	No

Table 6. C-myc mRNA half-life (min).

N	CTRL	STIM
1	111.13	70.19
2	53.90	49.12
3	81.33	71.17
4	85.28	42.39
Average	82.91	58.22
S.D.	23.43	14.65
S.E.M.	11.71	7.325

Student's paired t test	
P value	0.0904
P value summary	ns
Are means signifiantly different? ($p < 0.05$)	No

Table 7A. Tfam protein expression in EDL muscle.

N	CTRL	STIM
1	2.23	6.43
2	1.85	4.57
3	1.40	8.73
4	5.54	8.14
5	0.85	6.05
6	2.84	5.28
7	6.24	2.21
8	3.87	7.19
Average	3.10	6.07
S.D.	1.95	2.09
S.E.M.	0.69	0.74

Student's paired t test	
P value	0.0373
P value summary	*
Are means signifiantly different? ($p < 0.05$)	Yes

Table 7B. Tfam mRNA expression in EDL muscle.

N	CTRL	STIM
1	2.95	1.99
2	1.85	1.49
3	2.19	3.21
4	2.14	2.14
5	3.20	2.62
6	1.99	2.07
7	2.36	2.17
8	1.82	1.95
Average	2.31	2.21
S.D.	0.93	0.73
S.E.M.	0.29	0.23

Student's paired t test	
P value	0.6406
P value summary	ns
Are means signifiantly different? ($p < 0.05$)	No

Table 8. Tfam mRNA half-life (min).

N	CTRL	STIM
1	33.34	17.57
2	11.98	7.89
3	46.36	23.54
4	46.65	10.21
5	23.08	11.23
6	29.47	14.82
7	27.79	9.90
Average	31.24	13.60
S.D.	12.40	5.47
S.E.M.	4.69	2.07

Student's paired t test	
P value	0.0036
P value summary	**
Are means signifiantly different? ($p < 0.05$)	Yes

Table 9A. HuR protein expression in EDL muscle.

N	CTRL	STIM
1	1.98	8.46
2	2.57	7.20
3	2.18	9.59
4	3.64	3.47
5	3.45	6.08
6	3.47	8.36
7	1.23	6.06
8	6.28	9.61
Average	3.10	7.35
S.D.	1.54	2.10
S.E.M.	0.54	0.74

Student's paired t test	
P value	0.0014
P value summary	**
Are means signifiantly different? ($p < 0.05$)	Yes

Table 9B (Left Panel). Total AUF1 protein expression in EDL muscle.

N	CTRL	STIM
1	2.73	4.89
2	2.62	4.04
3	1.83	3.17
4	2.02	2.01
5	1.42	3.60
6	1.92	5.46
Average	2.09	3.86
S.D.	0.50	1.24
S.E.M.	0.20	0.50

Student's paired t test	
P value	0.0143
P value summary	*
Are means signifiantly different? ($p < 0.05$)	Yes

Table 9B (Right Panel). AUF1 isoform of total AUF1.

N	$p37^{AUF1}$	$p40^{AUF1}$	$p45^{AUF1}$
1	2.68	4.44	10.47
2	3.96	6.69	11.90
3	2.76	6.10	8.96
4	3.11	4.30	9.34
5	1.24	2.84	7.10
6	1.44	4.23	7.21
Average	2.53	4.77	9.16
S.D.	1.03	1.40	1.86
S.E.M.	0.42	0.57	0.76

One-way ANOVA*			
ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	136.6	2	68.3
Individual (between rows)	26.97	5	5.395
Residual (random)	5.469	10	0.5469
Total	169	17	

Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
p37 vs p40	-2.237	5.239	P < 0.01	-3.462 to -1.012
p37 vs p45	-6.632	15.53	P < 0.001	-7.857 to -5.406
p40 vs p45	-4.395	10.29	P < 0.001	-5.620 to -3.170

* One-way ANOVA was performed between all CTRL isoforms.

Table 9B (Left Panel). Effect of CCA on $p37^{AUF1}$, $p40^{AUF1}$, $p45^{AUF1}$

	$p37^{AUF1}$		$p40^{AUF1}$		$p45^{AUF1}$	
N	**CTRL**	**STIM**	**CTRL**	**STIM**	**CTRL**	**STIM**
1	2.68	6.18	4.44	9.61	10.47	12.80
2	3.96	7.07	6.69	10.06	11.90	16.49
3	2.76	5.76	6.10	6.66	8.96	15.57
4	3.11	3.81	4.30	5.48	9.34	10.70
5	1.24	3.52	2.84	6.01	7.10	10.60
6	1.44	7.78	4.23	10.14	7.21	14.92
Average	2.53	5.69	4.77	7.99	9.16	13.51
S.D.	1.03	1.72	1.40	2.17	1.86	2.53
S.E.M.	0.42	0.70	0.57	0.89	0.76	1.03

Student's paired t test			
Isoform	$p37^{AUF1}$	$p40^{AUF1}$	$p45^{AUF1}$
P value	0.0086	0.0134	0.0075
P value summary	**	*	**
Are means signifiantly different? ($p < 0.05$)	Yes	Yes	Yes

Table 10. mRNA stability in skeletal muscle.

	PGC-1α		c-myc		Tfam		S12	
N	CTRL	STIM	CTRL	STIM	CTRL	STIM	CTRL	STIM
1	20.91	11.258	111.13	70.185	33.34	17.57	25.37	21.04
2	21.87	9.069	53.90	49.125	11.98	7.8874	46.40	56.31
3	12.47	5.8741	81.33	71.165	46.36	23.544	66.58	31.87
4	32.22	14.608	85.28	42.394	46.65	10.211	54.92	37.19
5	25.87	13.618			23.08	11.232	70.56	16.91
6					29.47	14.82		
7					27.79	9.90		
Average	22.67	10.89	73.41	50.92	31.24	13.60	52.77	32.66
S.D.	7.23	3.53	29.37	20.67	12.40	5.47	18.05	15.52
S.E.M.	3.24	1.58	13.14	9.24	4.69	2.07	8.07	6.94

One-way ANOVA*			
ANOVA Table	SS	df	MS
Treatment (between columns)	10440	3	3479
Individual (between rows)	3009	3	1003
Residual (random)	360.7	9	40.08
Total	13810	15	

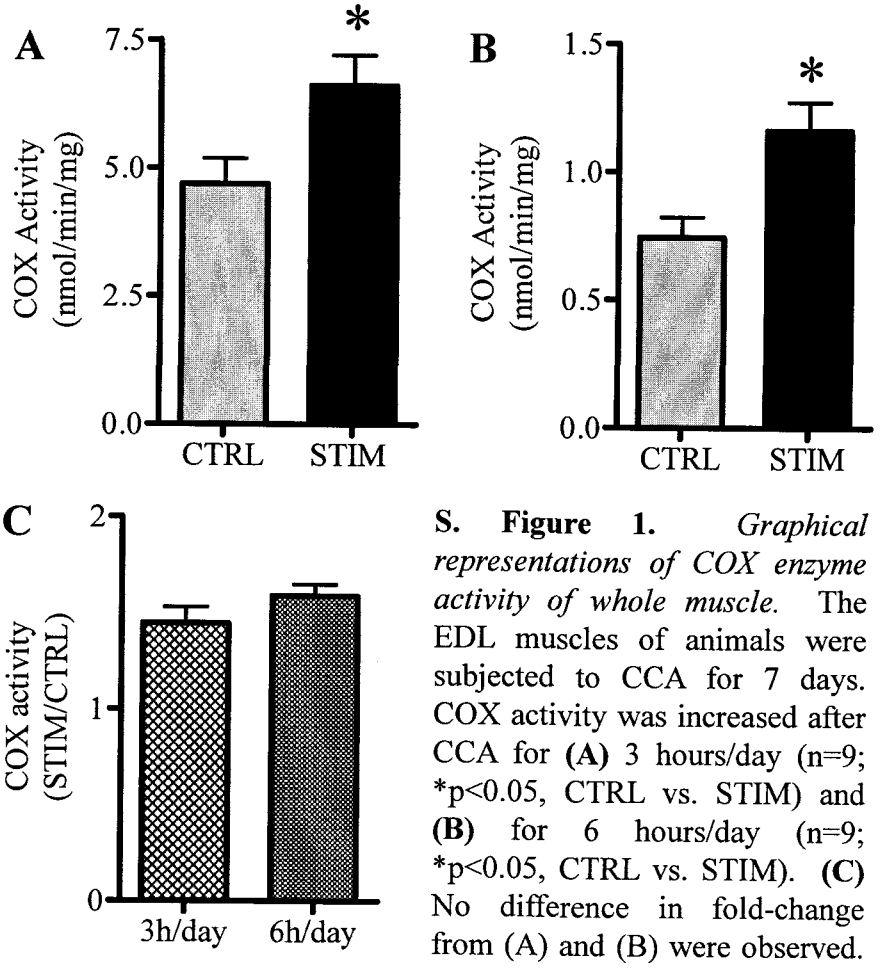
Bonferroni's Multiple Comparison Test	Mean Diff.	t	P value	95% CI of diff
PGC-1α vs c-myc	-60.24	15.05	P < 0.001	-73.71 to -46.77
PGC-1α vs Tfam	-8.569	2.14	P > 0.05	-22.04 to 4.900
PGC-1α vs S12	-30.1	7.517	P < 0.001	-43.57 to -16.63
c-myc vs Tfam	51.67	11.54	P < 0.001	36.61 to 66.73
c-myc vs S12	30.14	6.734	P < 0.001	15.08 to 45.20
Tfam vs S12	-21.53	6.362	P < 0.001	-32.91 to -10.14

* One-way ANOVA was performed between all CTRL mRNAs.

APPENDIX C:

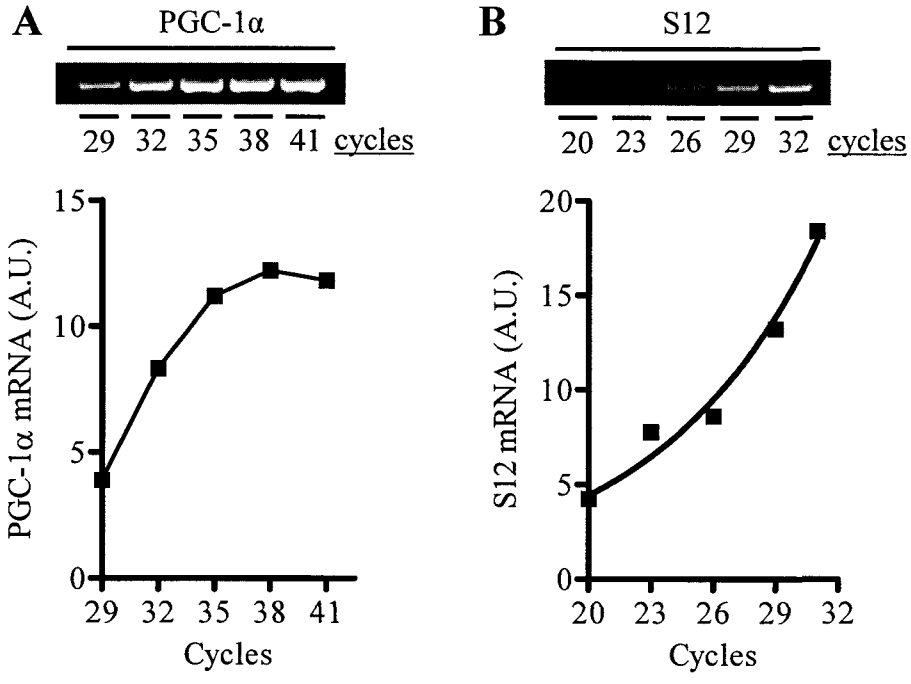
ADDITIONAL FIGURES

S. Figure 1



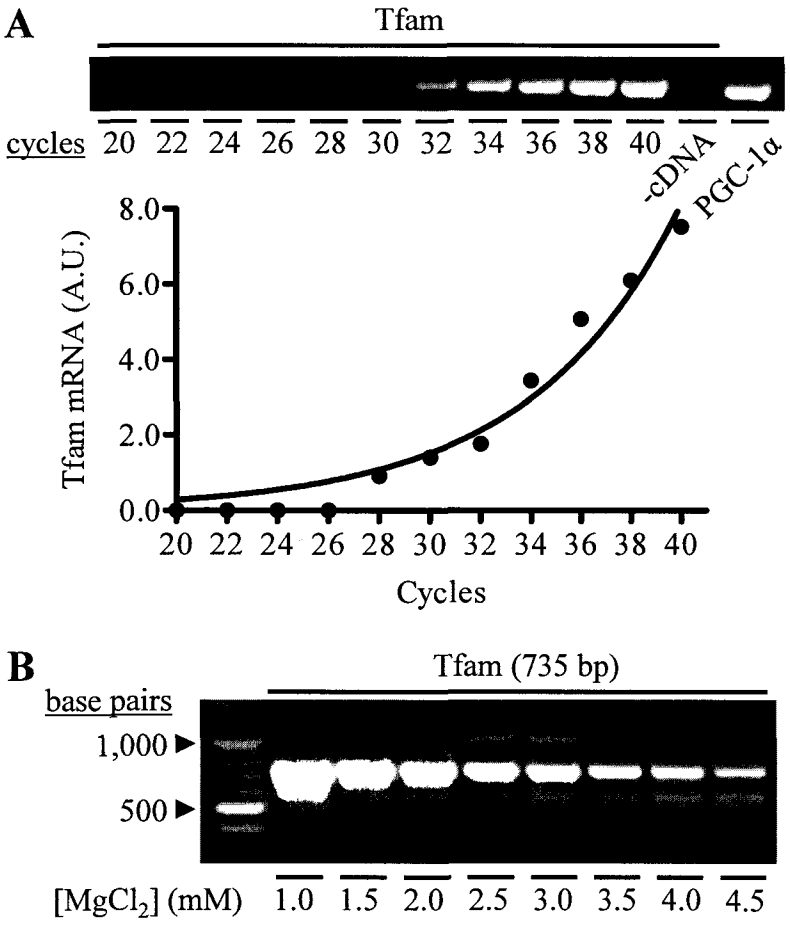
S. Figure 1. *Graphical representations of COX enzyme activity of whole muscle.* The EDL muscles of animals were subjected to CCA for 7 days. COX activity was increased after CCA for **(A)** 3 hours/day (n=9; *p<0.05, CTRL vs. STIM) and **(B)** for 6 hours/day (n=9; *p<0.05, CTRL vs. STIM). **(C)** No difference in fold-change from (A) and (B) were observed.

S. Figure 2



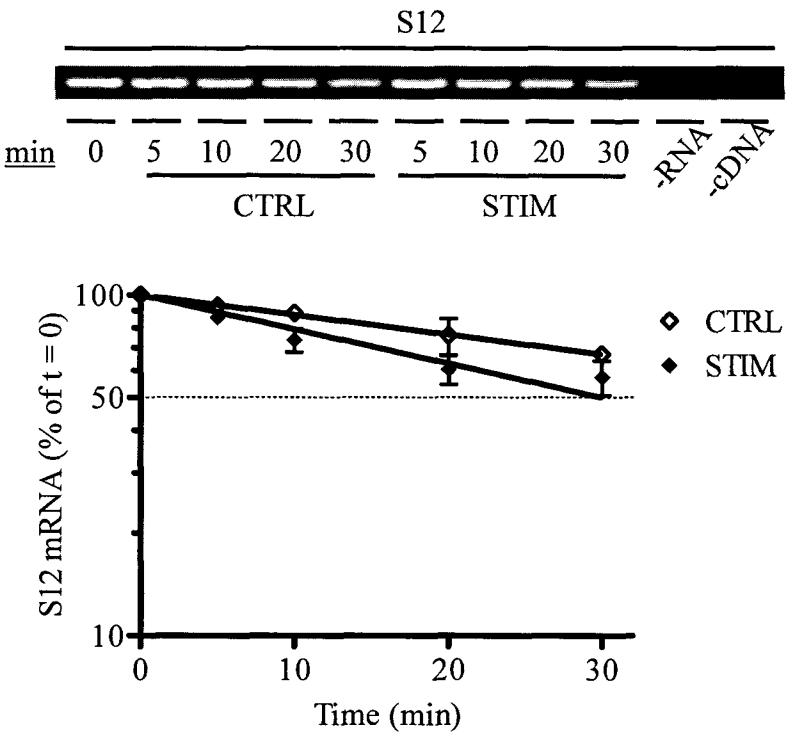
S. Figure 2. *PCR cycle curves.* Total RNA (1 μg) was reverse-transcribed to cDNA, 2 μl of which was used in PCR reaction along with primers probing for **(A)** PGC-1α and **(B)** S12. Each reaction was terminated after the number of cycles as indicated. For each reaction, 20 μl were run on an EtBr-stained agarose gel as shown.

S. Figure 3



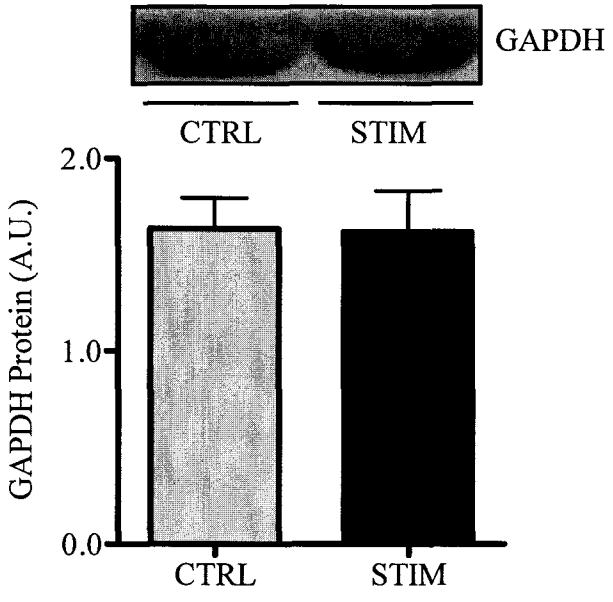
S. Figure 3. *PCR cycle curve and $MgCl_2$ titration for Tfam mRNA.* Total RNA (2 μg) was reverse-transcribed to cDNA, 2 μl of which was added into each PCR reaction along with primers probing for Tfam. For each reaction, 20 μl of amplicon were loaded and run on an EtBr-stained agarose gel as shown. **(A)** Each reaction was terminated after the number of cycles as indicated using 1.5 mM of $MgCl_2$. **(B)** $MgCl_2$ was titrated from 1.0 to 4.5 mM. Each reaction was run for 34 cycles.

S. Figure 4



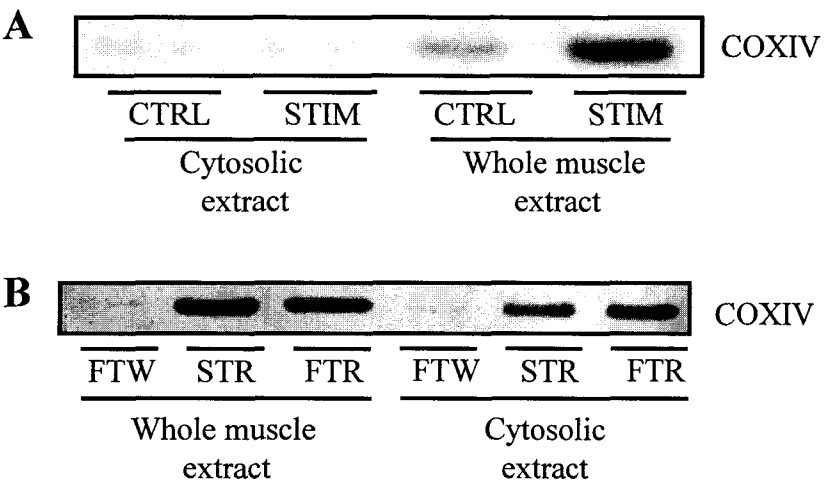
S. Figure 4. *S12 mRNA stability in skeletal muscle.* Total RNA isolated from TA muscle was incubated with cytosolic proteins from CTRL or STIM EDL muscles of animals subjected to CCA for 7 days. After each time point, total RNA was re-isolated S12 (n=10) was examined by RT-PCR. A representative EtBr gel is illustrated along with a graphical representation of the data. Reactions in the absence of RNA or cDNA are shown as negative controls.

S. Figure 5



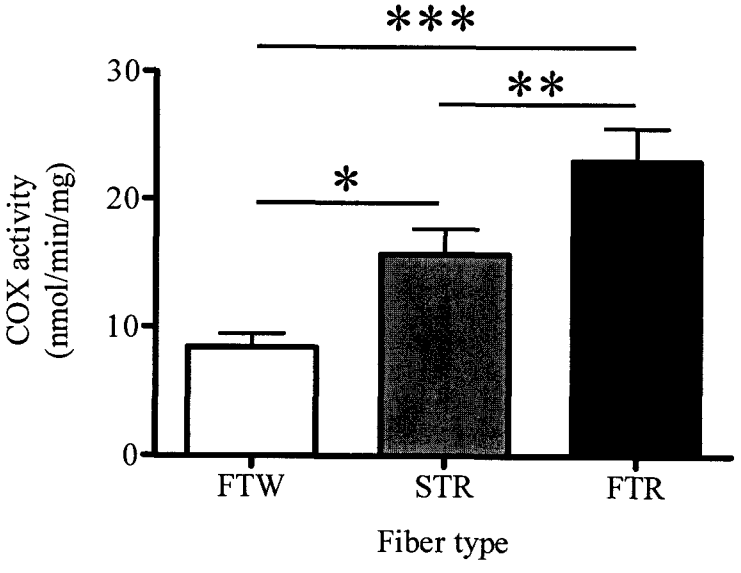
S. Figure 5. GAPDH protein expression in whole muscle. GAPDH was measured in extracts from CTRL or STIM EDL whole muscles (n=8; CTRL vs. STIM).

S. Figure 6



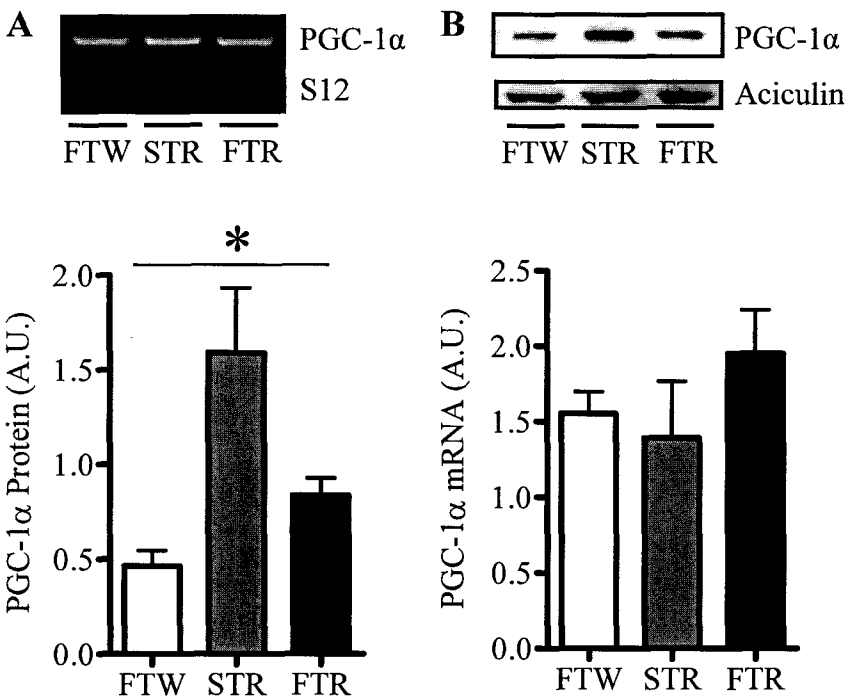
S. Figure 6. *Purity of cytosolic fraction.* Cysotolic and whole muscle COXIV protein expression in **(A)** CTRL or STIM muscles, and **(B)** FTW (fast-twitch white), STR (slow-twitch red), and FTR (fast-twitch red) muscles.

S. Figure 7



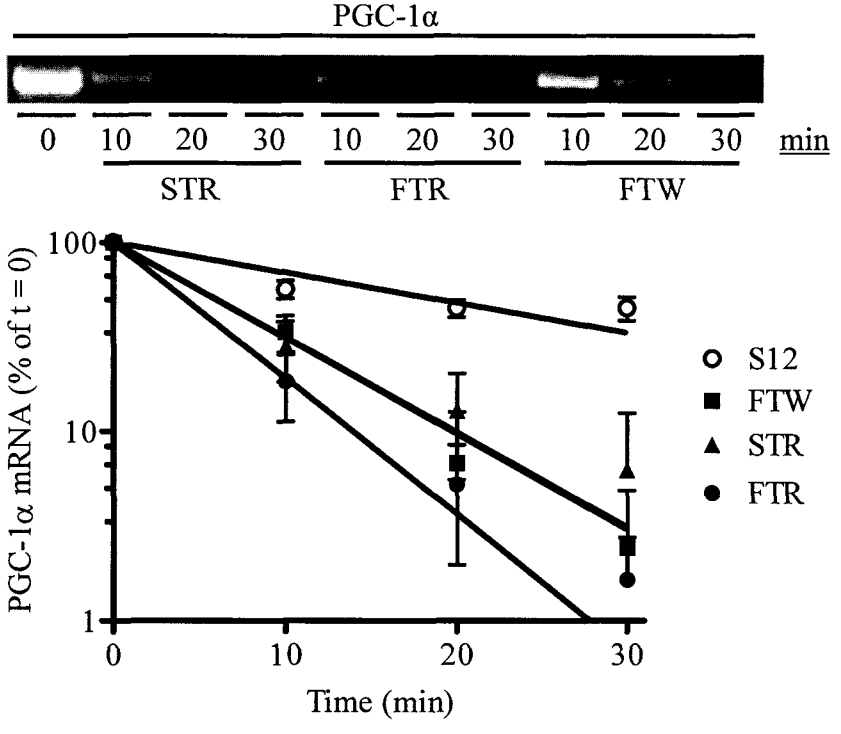
S. Figure 7. *Graphical representations of cytochrome c oxidase (COX) enzyme activity of whole muscle.* FTW, STR and FTR muscles were excised from CTRL animals (n=9-10; *p<0.05, FTW vs STR, FTW vs FTR, STR vs FTR).

S. Figure 8



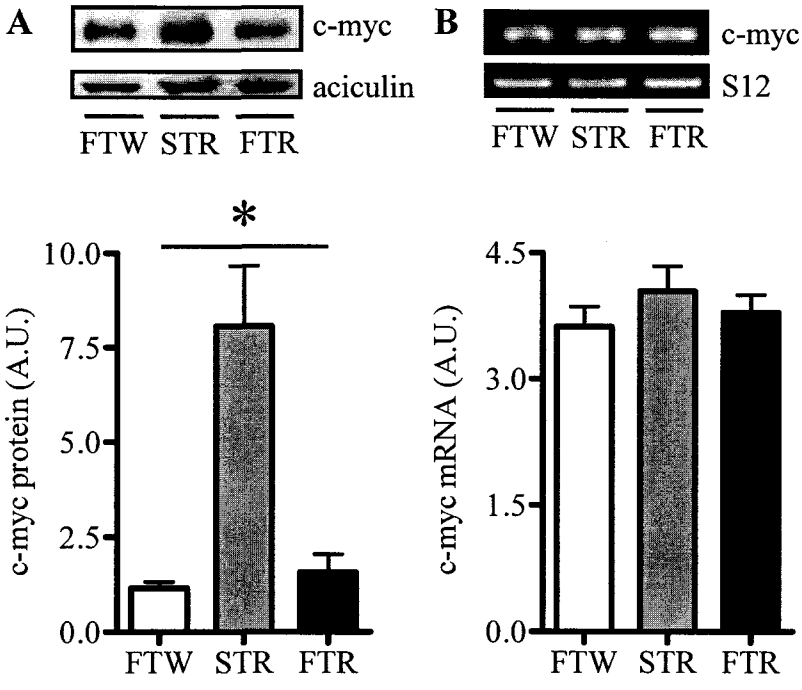
S. Figure 8. *PGC-1α protein and mRNA expression in various fiber types.* FTW, STR and FTR muscles were harvested from CTRL animals. **(A)** PGC-1α protein, along with a loading control (aciculin) were measured in extracts from muscles of varying oxidative capacity (n=7; *$p<0.05$). **(B)** PGC-1α and S12 mRNA transcripts were measured by RT-PCR from total RNA isolated from muscles of varying fiber types. A representative EtBr-stained agarose gel is illustrated along with a graphical representation of the data (n=7).

S. Figure 9



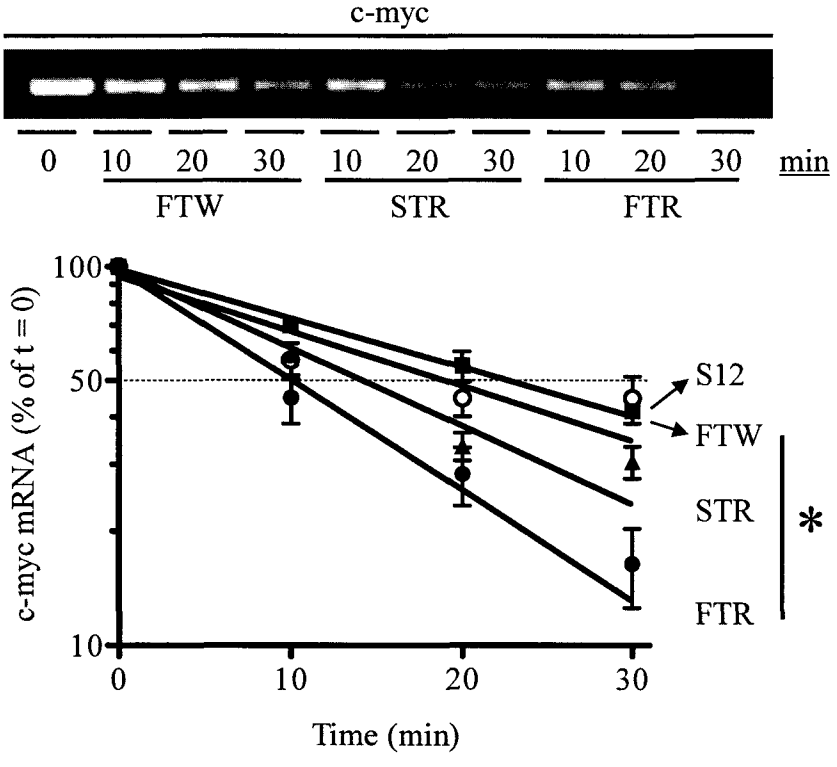
S. Figure 9. *PGC-1α mRNA stability in various fiber types.* Total RNA isolated from TA muscle was incubated with cytosolic proteins extracted from STR, FTR and FTW muscles of CTRL animals. After each time point, total RNA was re-isolated and PGC-1α (n=2-9) along with an internal control S12 (n=9) were examined by RT-PCR. A representative EtBr gel is illustrated along with a graphical representation of the data.

S. Figure 10



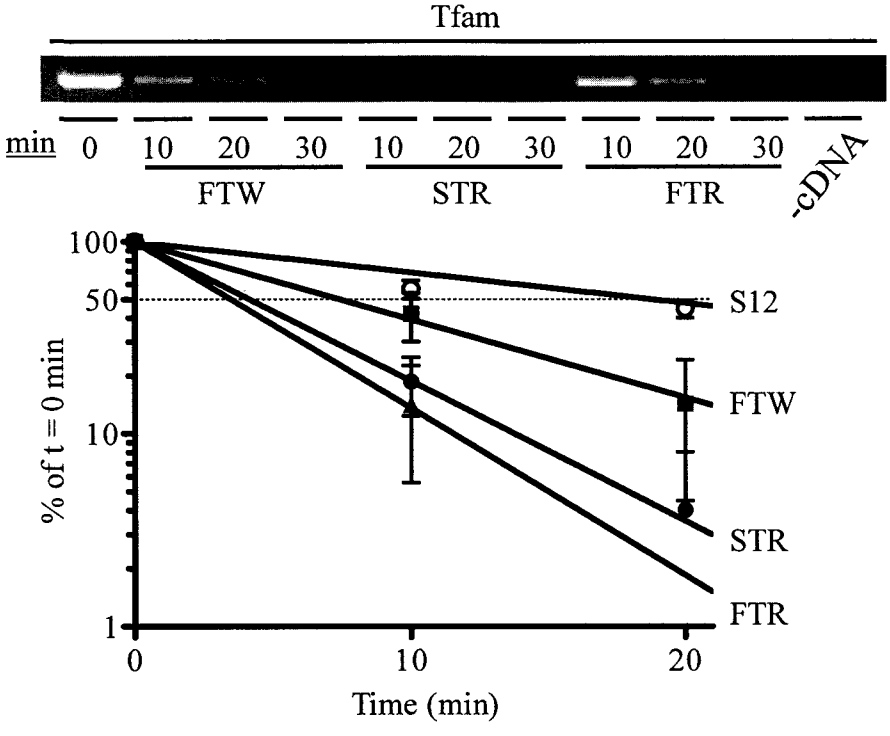
S. Figure 10. *C-myc protein and mRNA expression in various fiber types.* FTW, STR and FTR were extracted from CTRL animals. **(A)** C-myc protein, along with a loading control (aciculin) were measured in extracts from muscles of varying oxidative capacity (n=4; *$p<0.05$). **(B)** C-myc and S12 mRNA transcripts were measured by RT-PCR from total RNA isolated from muscles of varying fiber types. A representative EtBr-stained agarose gel is illustrated along with a graphical representation of the data (n=7).

S. Figure 11



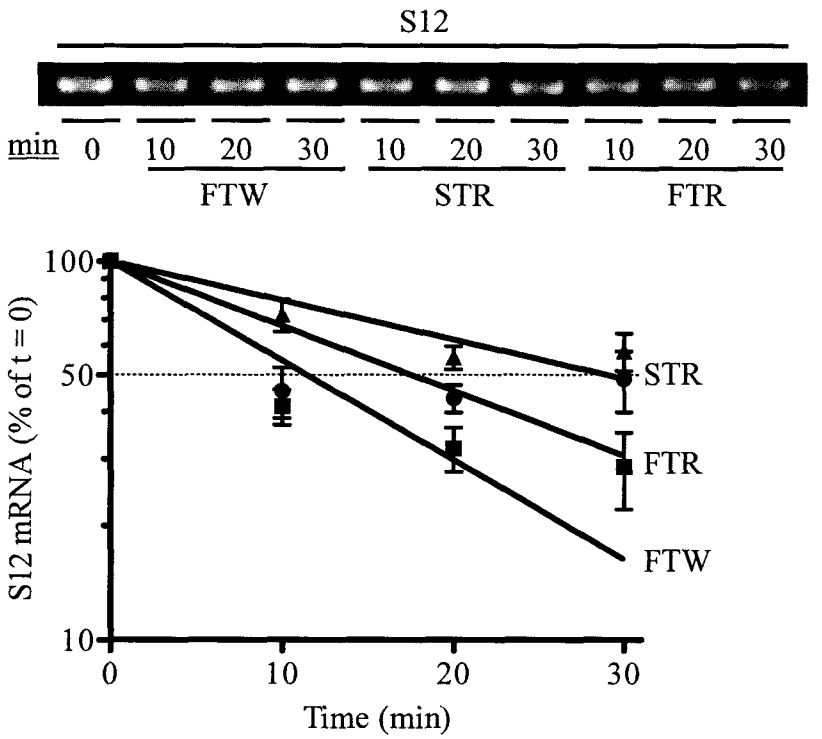
S. Figure 11. *C-myc mRNA stability in various fiber types.* Totall RNA isolated from TA muscle was incubated with cytosolic proteins from FTW, STR or FTR muscles of CTRL animals. After each time point, total RNA was re-isolated and c-myc (n=4) along with an internal control S12 (n=) were examined by RT-PCR. A representative EtBr gel is illustrated along with a graphical representation of the data.

S. Figure 12



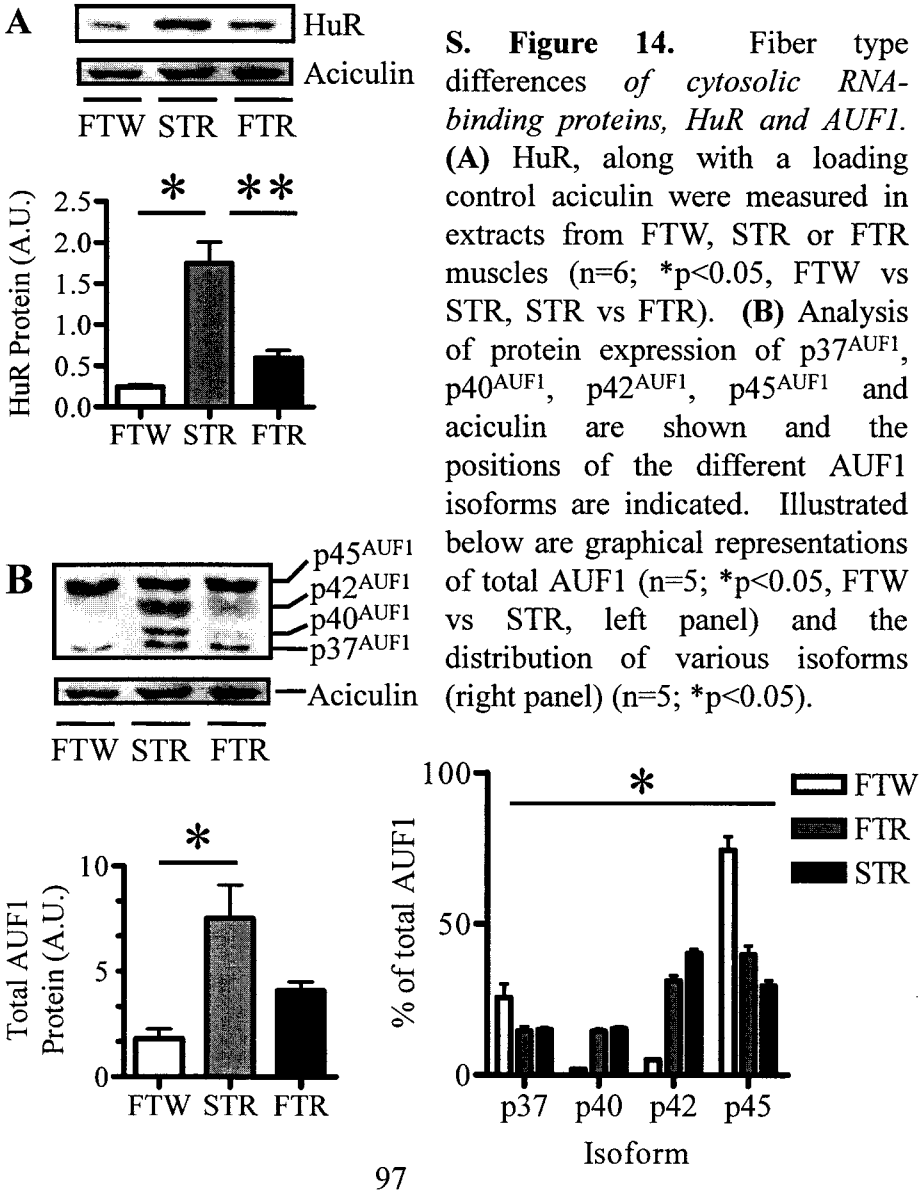
S. Figure 12. *Tfam mRNA stability in various fiber types.* Total RNA isolated from TA muscle was incubated with cytosolic proteins from FTW, STR or FTR muscles of CTRL animals. After each time point, total RNA was re-isolated and Tfam (n=5) along with an internal control S12 (n=9) were examined by RT-PCR. A representative EtBr gel is illustrated along with a graphical representation of the data.

S. Figure 13



S. Figure 13. *S12 mRNA stability in various fiber types.* Total RNA isolated from TA muscle was incubated with cytosolic proteins from FTW, STR or FTR muscles of CTRL animals. After each time point, total RNA was re-isolated and S12 (n=4) was examined by RT-PCR. A representative EtBr gel is illustrated along with a graphical representation of the data.

S. Figure 14



S. Figure 14. Fiber type differences *of cytosolic RNA-binding proteins, HuR and AUF1.* **(A)** HuR, along with a loading control aciculin were measured in extracts from FTW, STR or FTR muscles (n=6; *p<0.05, FTW vs STR, STR vs FTR). **(B)** Analysis of protein expression of $p37^{AUF1}$, $p40^{AUF1}$, $p42^{AUF1}$, $p45^{AUF1}$ and aciculin are shown and the positions of the different AUF1 isoforms are indicated. Illustrated below are graphical representations of total AUF1 (n=5; *p<0.05, FTW vs STR, left panel) and the distribution of various isoforms (right panel) (n=5; *p<0.05).

APPENDIX D:

DATA AND STATISTICAL ANALYSES FOR ADDITIONAL FIGURES

Table 1A. Whole muscle COX activity.

N	Condition	CTRL	STIM
1	3 hrs/day, 7 days	3.84	5.70
2		3.76	4.73
3		7.21	8.70
4		6.48	7.97
5		5.64	8.89
6		4.35	7.69
7		2.58	4.87
8		3.55	4.31
9		4.76	6.69
Average		4.69	6.62
S.D.		1.50	1.78
S.E.M.		0.50	0.59

Student's paired t test	
P value	0.0002
P value summary	***
Are means signifiantly different? ($p < 0.05$)	Yes

Table 1B. Whole muscle COX activity.

N	Condition	CTRL	STIM
1	6 hrs/day, 7 days	4.69	8.12
2		9.34	15.55
3		7.93	10.75
4		5.09	7.92
5		11.79	18.00
6		9.00	11.63
7		7.71	12.29
8		5.64	10.03
9		5.64	10.22
Average		7.43	11.61
S.D.		2.37	3.31
S.E.M.		0.79	1.10

Student's paired t test	
P value	P<0.0001
P value summary	***
Are means signifiantly different? (p < 0.05)	Yes

Table 1C. Fold change (STIM/CTRL) of whole muscle COX activity.

N	3h/d, 7d	6h/d, 7d
1	1.48	1.73
2	1.26	1.66
3	1.21	1.36
4	1.23	1.55
5	1.58	1.53
6	1.77	1.29
7	1.88	1.59
8	1.21	1.78
9	1.41	1.81
Average	1.45	1.59
S.D.	0.25	0.18
S.E.M.	0.08	0.06

Student's unpaired t test	
P value	0.1895
P value summary	ns
Are means signifiantly different? ($p < 0.05$)	No

Table 2A. PCR cycle curve of PGC-1α mRNA.

Cycle Number	Total Intensity (A.U.)
29	3.887616
32	8.316672
35	11.189760
38	12.204290
41	11.814910

Table 2B. PCR cycle curve of S12 mRNA.

Cycle Number	Total Intensity (A.U.)
20	4.234096
23	7.765220
26	8.592074
29	13.198050
31	18.399190

Table 3A. PCR cycle curve of Tfam mRNA.

Cycle Number	Total Intensity (A.U.)
20	0.0000
22	0.0000
24	0.0000
26	0.0000
28	0.9175
30	1.4118
32	1.7640
34	3.4461
36	5.0768
38	6.1049
40	7.5261

Table 4. S12 mRNA half-life.

N	CTRL	STIM
1	25.37	21.04
2	46.40	56.31
3	66.58	31.87
4	54.92	37.19
5	70.56	16.91
Average	52.77	32.66
S.D.	18.05	15.52
S.E.M.	8.07	6.94

Student's paired t test	
P value	0.1462
P value summary	ns
Are means signifiantly different? ($p < 0.05$)	No

Table 5. GAPDH protein expression.

N	CTRL	STIM
1	2.28	1.75
2	1.73	1.68
3	1.41	1.10
4	1.23	1.16
5	1.89	1.37
6	2.55	2.77
7	1.07	1.64
8	1.83	1.50
Average	1.75	1.62
S.D.	0.51	0.52
S.E.M.	0.18	0.18

Student's paired t test	
P value	0.3716
P value summary	ns
Are means signifiantly different? ($p < 0.05$)	No

APPENDIX E:

PROTOCOLS AND EXTENDED METHODS

Calculation of mRNA half-life

Example

$50 = 100\ e^{(-0.02835x)}$	Divide both sides by 100 to isolate the exponential portion.
$0.5 = e^{(-0.02835x)}$	Take *ln* of both sides (use *ln* beause the base is *e*).
$\ln 0.5 = \ln e^{(-0.02835x)}$	Bring the power into coefficient position using the law of *logs*.
$\ln 0.5 = (-0.02835x) \ln e$	ln *e* is equivalent to 1.
$\ln 0.5 = -0.02835x$	Solve for x using algebra.
$x = 24.45$ min	

In vitro mRNA decay assay

Preparation of Cytosolic Extracts

1. In a 13 ml Sarstedt tube homogenize skeletal muscle powders (40 – 100 mg) 3 x 10 sec (10 mm probe; 40% maximum) in 0.5 – 1 ml of homogenization buffer.

2. Centrifuge the homgenates for 15 min at 5,000 g (4°C). Transfer the supernate to a sterile eppendorf tube.

3. Centrifuge the supernate at 15,000 g (4°C) for 15 min and transfer the resulting post-mitochondrial supernate (S15) to a sterile eppendorf tube.

4. Determine the protein concentrations of the S15 fractions using the Bradford total protein assay.

***In vitro* Decay Reaction**

1. Incubate total RNA (30 μg) and S15 extract (20 μg) in a sterile eppendorf tube. Volume the reaction up to 100 μl with sterile RNAse-free water and incubate at 37°C.

2. Remove aliquots (100 μl) after the desired time periods and transfer them to a sterile eppendorf tube containing 100 μl of phenol. Mix vigorously.

3. Spin in a microcentrifuge for 30 sec.

4. Transfer the aqueous phase to a sterile eppendorf tube. Add 100 μl of phenol/chloroform/isoamyl alcohol (25:24:1) and mix vigorously.

5. Spin in a microcentrifuge for 30 sec.

6. Transfer the aqueous phase to a sterile eppendorf tube. Add 100 μl of chloroform/isoamyl alcohol (24:1) and mix vigorously.

7. Spin in a microcentrifuge for 30 sec.

8. Transfer the aqueous phase to a sterile eppendorf tube. Add 100 μl of 3 M Na Acetate (pH5.2) and 300 μl of ice-cold anhydrous ethyl alcohol. Mix vigorously and then precipitate at -20° overnight.

9. Spin in a microcentrifuge for 15 min at 4°C.

10. Wash the pellet with 75% ethanol and spin in a microcentrifuge for 5 min at 4°C. Pipette away the supernate.

11. Air-dry the pellet.

12. Resuspend the pellet in 15 µl of sterile water.

13. Add 2.25 µl of 0.5 mg/ml EtBr and 2 µg of RNA and run on an EtBr-stained 1% formaldehyde-agarose gel.

First-Strand cDNA Synthesis

The following procedure is designed to convert 1 pg to 5 μg of total RNA or 1 pg to 500 ng of poly(A)+ RNA into first-strand cDNA:

1. Mix and briefly centrifuge each component before use.

2. Combine the following in a 0.2- or 0.5-ml tube:

Component	**Amount**
2 μg total RNA	*n* μl
50 μM oligo(dT)$_{20}$ primer	1 μl
10 mM dNTP mix	1 μl
Sterile water to	13 μl

3. Incubate at 65°C for 5 min, then place on ice for at least 1 min.

4. Prepare the following cDNA Synthesis Mix.

Component 1 Rxn

5X RT buffer 4 μl
0.1 M DTT 1 μl
RNaseOUT™ (40 U/μl) 1 μl
SuperScript™ III RT (200 U/μl) 1 μl

5. Mix by pipetting up and down. Incubate for 50 min at 50°C.

6. Terminate the reactions at 70°C for 15 min. Chill on ice.

7. cDNA synthesis reaction can be stored at -20°C or used for PCR immediately.

Polymerase Chain Reaction

1. In a sterile, nuclease-free microcentrifuge tube, combine the following on ice:

5X Green GoTaq® Flexi Buffer 10µl
$MgCl_2$ Solution, 25mM 2–8µl 1.0–4.0mM
PCR Nucleotide Mix, 10mM each 1µl 0.2mM each dNTP
upstream primer 1 µl 10mM
downstream primer 1 µl 10mM
GoTaq® DNA Polymerase (5u/µl) 1µl
template DNA 2µl <0.5µg/50µl
Nuclease-Free Water to 50µl

2. Overlay the reaction mix with 2 drops (approximately 50µl) of mineral oil to prevent evaporation during thermal cycling. Centrifuge the reactions in a microcentrifuge for 5 seconds.

3. Perform PCR using standard thermal cycling parameters.
GoTaq® Flexi Buffer, load the reaction onto the gel directly after amplification. Reactions containing the 5X Colorless GoTaq® Flexi Buffer also can be loaded directly into the wells of an agarose gel, but a tracking dye will need to be added to monitor the progress of electrophoresis.

SOLUTIONS

Homogenization Solution	**For 100 ml**
25% glycerol	25 ml of sterile 100% solution
0.42 M NaCl	10 ml of a 4.2 M stock solution
1.5 mM $MgCl_2$	1 ml of a 150 mM stock solution
0.2 mM EDTA	80 µl of a 0.25 M stock solution
20 mM HEPES (pH 7.9)	5 ml of a 400 mM stock solution
0.5 mM DTT	50 µl of a 1M stock solution
0.5 mM PMSF	500 µl of a 100 mM stock solution
Sterile water	58.76 ml

APPENDIX F:

OTHER CONTRIBUTIONS TO THE LITERATURE

Other Contributions to the Literature

Refereed Publications

1. Ljubicic V, Joseph A-M, Saleem A, Uguccioni G, Collu-Marchese M, **Lai RY**, Nguyen LM, Hood DA. Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: effects of exercise and aging. *Biochim Biophys Acta, accepted and in press.*

2. **Lai RYJ**, Krasnow D, Thomas M. Communication between medical practitioners and dancers. *J Dance Med & Sci 12*, 2008.

Manuscript in Preparation

1. **Lai RYJ**, Ljubicic V, D'souza D, Hood DA. Effect of chronic contractile activity on mRNA stability in skeletal muscle. 2009.
   (Submitted to Am J Physiol Cell Physiol).

Conference Proceedings and Presentations

1. **Lai RYJ**, Ljubicic V, Hood DA. mRNA turnover is accelerated in oxidative muscle.
   14th International Biochemistry of Exercise Conference.
   Guelph, Ontario, Canada. June 3, 2009.

2. **Lai RYJ**, Hood DA. The role of mRNA stability in regulating PGC-1α in skeletal muscle fiber types. *Ontario Exercise Physiology Conference.*
   Barrie, Ontario, Canada. January 25, 2009.

3. **Lai RYJ**, Krasnow D, Thomas M. Communication between medical practitioners and dancers. *16th Annual Meeting of the International Association of Dance Medicine & Science.*
   West Palm Beach, Florida, USA. October 21, 2006.

Conference Proceedings and Presentation in Preparation

1. D'souza D, **Lai RYJ**, Hood DA. The role of Tfam mRNA stability in various fiber types.
   Experimental Biology.
   Anaheim, California. April 24-28, 2010.

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