# Elucidation of the Function and Regulation of STAT3 during IFNγ- and TNFα-induced Muscle Wasting

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2017

Thesis submitted to McGill University in partial fulfillment of the

requirements of the degree of Doctor of Philosophy

# Preface

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## List of Abbreviations

| | |
|---|---|
| AEE | Activity energy expenditure |
| ActRIIB | Activin receptor type 2 B |
| AP-1 | Activator protein 1 |
| APRIL | Acidic protein rich in leucine |
| Arid5a | AT-rich interactive domain 5a protein |
| ATP | Adenosine triphosphate |
| $Apc^{Min/+}$ | Adenomatosis polyposis coli, Multiple intestinal neoplasia |
| DEE | Diet-induced energy expenditure |
| DMD | Duchenne Muscular Dystrophy |
| d.n. | dominant negative |
| eIF | Eukaryotic initiation factor |
| ELAV | Embryonic lethal abnormal vision |
| FFA | Free fatty-acids |
| HuR | Human antigen R |
| HNS | HuR nucleocytoplasmic shuttling domain |
| GC | Glucocorticoid |
| GR | Glucocorticoid receptor |
| IFNγ | Interferon gamma / type 2 interferon |
| IKK | Inhibitor of kappa B kinase |
| IF | Immunofluorescence |
| IL-6 | Interleukin-6 |
| IRES | Internal ribosomal entry site |
| IRF | Interferon regulatory factor |

| | |
|---|---|
| JAK | Janus kinase |
| LPS | lipopolysaccharide |
| mTOR | Mammalian target of rapamycin |
| mdx | Mouse model for the human Duchenne muscular dystrophy |
| MyHC | Myosin heavy chain |
| NSCLC | Non-small cell lung carcinoma |
| NF-κB | Nuclear factor kappa B |
| NO | Nitric oxide |
| p | phospho |
| PABP | Poly(A)-binding protein |
| PIAS | Protein inhibitor of activated STATs |
| PI3K | Phosphoinositide 3-kinase |
| REE | Resting energy expenditure |
| RRM | RNA recognition motif |
| S | Serine |
| STAT | Signal transducer and activator of transcription |
| TEE | Total energy expenditure |
| TGF-β | Transforming growth factor beta |
| iNOS | Inducible nitric oxide synthase |
| qPCR | Quantitative polymerase chain reaction |
| RBP | RNA-binding protein |
| RIP-Chip | RNA immunoprecipitation coupled to microarray |
| RIP-Seq | RNA immunoprecipitation coupled to sequencing |
| RISC | RNA-induced silencing complex |
| SHP-1 | SH2 domain-containing phosphatase-1 |

| | |
|---|---|
| SMA | Spinal muscle atrophy |
| SMN | Survival motor neuron |
| sIBM | Sporadic inclusion body myositis |
| TAD | Transactivation domain |
| TNF$\alpha$ | Tumor necrosis factor alpha / cachectin |
| UTR | Untranslated region |
| Y | Tyrosine |
| ZAG | Zinc alpha 2 - glycoprotein; Lipid mobilizing factor |

## Abstract

Skeletal muscle is a highly adaptable tissue capable of responding to stimuli. In the late-stages of chronic disease such as kidney disease and cancer, a condition called cachexia often arises. Cachexia is characterized by rapid weight loss due to the involuntary wasting of muscle and adipose tissue. During muscle wasting, increased protein degradation of key muscle proteins and decreased protein synthesis leads to leads to muscle deterioration. Moreover, the development of cachexia reduces the quality of life and contributes to patient mortality. Chronic inflammation associated with these diseases is believed to be the main trigger of muscle wasting, although pharmacological inhibition of individual cytokines as proven largely ineffective at treating cachexia. In this thesis I have explored the role of STAT3 in promoting muscle wasting triggered by IFNγ and TNFα and a posttranscriptional regulatory mechanism involved in promoting STAT3 expression in muscle.

In chapter 2 I have uncovered a novel mechanism through which the transcription factor STAT3 is contributing to muscle wasting independently of its known downstream function of IL-6. I show that the rapid wasting induced by IFNγ plus TNFα relies on the phosphorylation of STAT3 on its key $Tyr^{705}$ residue through JAKs and that this phosphorylation occurs independently of IL-6, another known pro-inflammatory cytokine known to induce wasting through STAT3. Furthermore, STAT3 collaborates with NF-κB to translocate to the nucleus and activates the expression of the pro-cachectic gene iNOS. Importantly, this identifies how IFNγ/TNFα treatment are able to

activate downstream factors capable of collaborating to activate gene expression. Together, this work identifies how STAT3 is central in promoting muscle wasting and highlights STAT3 as a likely key therapeutic target for treating muscle wasting.

Given that STAT3 expression was observed to increase during cytokine treatment and that changes in STAT3 are associated with enhanced activity, in chapter 3, I investigated how STAT3 expression is regulated at the posttranscriptional level. First, I demonstrate that HuR associates with *Stat3* mRNA and regulates STAT3 expression, likely at the level of translation. Within the STAT3 3'UTR, I identify multiple direct HuR binding sites. In addition, I identify miR-330-5p as a negative modulator of STAT3 expression, suggesting that STAT3 expression relies on an interplay between HuR and miR-330-5p for its appropriate expression. Together, this work identifies STAT3 as a target for posttranscriptional regulation to maintain its expression.

Overall, this thesis attempts to elucidate molecular mechanisms involving STAT3 that occur during muscle wasting induced by the pro-inflammatory cytokines IFN$\gamma$ and TNF$\alpha$. In doing so, I have highlighted a novel mechanism of STAT3-mediated muscle wasting and its HuR-mediated regulation that may lead to novel therapeutic options.

**Résumé**

Le muscle squelettique, un tissu hautement adaptable, est capable de répondre aux stimuli. Dans les derniers stades des maladies chroniques tels que la maladie rénale et le cancer, les patients développent une condition appelée cachexie. La cachexie est caractérisée par une perte de poids causé par un gaspillage involontaire du muscle et du tissu adipeux. Durant la perte musculaire, la dégradation accrue des protéines musculaires clés et la diminution de la synthèse des protéines entraînent une détérioration musculaire. De plus, le développement de la cachexie réduit la qualité de vie et contribue à la mortalité des patients. L'inflammation chronique associée à ces maladies est considéré comme étant le principal déclencheur de la perte musculaire. Malheureusement, l'inhibition pharmacologique des cytokines est largement inefficace dans le traitement de la cachexie. Dans cette thèse, j'ai exploré le rôle de STAT3 dans la promotion de la perte musculaire déclenché par IFN$\gamma$ et TNF$\alpha$, un mécanisme de réglementation post-transcriptionnel impliqué dans la promotion de l'expression STAT3 dans le muscle.

Dans le chapitre II, j'ai découvert un nouveau mécanisme par lequel le facteur de transcription STAT3 contribue à la perte musculaire indépendamment de sa fonction avale connue d'IL-6. Je démontre que la perte musculaire rapide induit par IFN$\gamma$, puis par TNF$\alpha$ repose sur la phosphorylation du résidu Tyr705 de STAT3 par JAK. Cette phosphorylation se produit indépendamment de l'IL-6, une autre cytokine pro-inflammatoire connue pour induire la perte musculaire par l'intermédiaire de STAT3. De

plus, STAT3 collabore avec NF-κB pour se translocaliser au noyau et pour activer l'expression du gène pro-cachectique iNOS. Il est important de noter que cela permet d'identifier comment le traitement par IFNγ/ TNFα peut activer des facteurs en aval capables de collaborer pour activer l'expression génique. L'ensemble de ce travail identifie comment STAT3 est central dans la promotion de la perte musculaire et met en évidence STAT3 comme une cible thérapeutique clé potentielle pour le traitement de perte musculaire.

Étant donné que l'expression de STAT3 augmente pendant le traitement de cytokine et que les changements associés à STAT3 sont due à une amélioration de l'activité enzymatique, dans le chapitre III, j'ai étudié comment l'expression de STAT3 est contrôlé au niveau post transcriptionnel. Tout d'abord, je démontre que HuR s'associe à l'ARNm de STAT3 et régule son expression, probablement au niveau de la traduction. Dans l'UTR-3' de STAT3, j'identifie plusieurs sites de liaisons directes d'HuR. En outre, j'identifie le micro-ARN, miR-330-5p, comme un modulateur négatif de l'expression de STAT3, ce qui suggère que l'expression génique appropriée de STAT3 repose l'interaction entre HuR et miR-330-5p. L'ensemble de cet ouvrage identifie STAT3 comme une cible pour la régulation post transcriptionnelle permettant ainsi de maintenir son expression.

Conjointement, cette thèse tente d'élucider les mécanismes moléculaires impliquant STAT3 qui se produisent au cours de la perte musculaire induite par les cytokines pro-inflammatoires IFNγ et TNFα. En faisant cela, j'ai mis en évidence un

nouveau mécanisme de perte musculaire médiée par STAT3 et puis de sa régulation par HuR qui peut-être une cible thérapeutique potentielle.

## Acknowledgements

First, I would like to thank Imed for accepting me into his lab and for the opportunity to work on this project. I am grateful for your advice, guidance, time, and enthusiasm for science throughout my degree. I have the deepest gratitude to the many lab members who have made my time in the lab a much more pleasant experience. To Sergio, for the many years of assistance with experiments and exchanges of ideas and everyday life things. To Virginie, for taking me under your wing and your invaluable insight during the early years of my time in the lab. To Anne, for your unfailing helpfulness and loud music to sing to at the end of the day. The both of you demonstrate extraordinary drive for experimenting that I continue to strive for. To Xian, for your reliability, kindness and patience every day. To Erzebet, for your kindness, willingness to teach, and invaluable advice on animals experiments. Thank you a million times!!

I would like to express my appreciation of Michel Tremblay and Simon Wing for many years of committee meetings with helpful feedback and suggestions. I am grateful to the McGill Integrated Cancer Research Training Program for stipend and travel funding during my degree.

To friends from the lab and from other labs, it has been a privilege to cross paths with all of you, whether it be a few semesters or several years. In particular, to Lauren and Katie for being friends that I can rely on and reminding me to get out of the lab and have fun. To Derek and Brenda, for many good times and talks in and out of the lab. My

sanity remains intact because of you. To the many other students in the lab, both past and present, for always being welcoming and making the long days in the lab much easier to get through.

Lastly, to my family, especially Mom and Dad. Know that your love, support, and words of encouragement have helped get me through many obstacles I've encountered, especially that one last experiment.

## Contribution of Authors

The specific contributions of co-authors are outlined here for the manuscripts presented as chapter 2 and 3, and for the general discussion (chapter 4).

**Chapter 2:** I was responsible for the experimental design and execution of experiments outlined in this chapter, including the data analysis and preparation of all the figures and manuscript, in consultation with Dr. Gallouzi. Brenda Sanchez assisted with performing intra-muscular injections for animal experiments, sacrifice of animals, and assistance with sectioning of muscle. Derek Hall assisted with performing intra-muscular injections for animals and with the sacrifice of animals. Anne-Marie Tremblay performed experiments treated with individual cytokines used for immunofluorescence and took images as well as sectioned and counted all the muscles for the IL-6 KO animal experiment. Dr. Sergio Di Marco assisted with preparation of samples used for confocal microscopy and was consulted during manuscript preparation.

**Chapter 3:** I was responsible for the experimental design and execution of experiments outlined in this chapter, including the data analysis, and preparation of all the figures and manuscript, in consultation with Dr. Gallouzi. Xian Lian assisted with many of the RNA experiments, including the immunoprecipitation, RT-qPCR, FISH, and the polysome experiments. Samantha Robinson assisted with performing polysome experiments and cloning of STAT3 3'UTR into a plasmid. Dr. Anne Cammas prepared samples for and analysed the miRNA-Seq experiment. Dr. Virginie Dormoy-Raclet prepared samples for and analysed the microarray data of the HuR-immunoprecipitated

RNAs and assisted with Actinomycin D experiments. Dr. Sergio Di Marco assisted with planning and performing several HuR knockdown experiments, and was consulted during manuscript preparation.

**Chapter 4:** I designed and performed the ChIP-Seq experiment in consultation with Dr. Gallouzi. The analysis of the ChIP-Seq data was performed as part of a service at Institute for Research in Immunology and Cancer (IRIC). I am responsible for all the ideas and other experiments presented in this chapter.

# CHAPTER 1:

# General Introduction

### 1.1: Skeletal muscle, disease, and its impact on health – Brief Overview

Healthy muscle requires a balance of protein synthesis and degradation to ensure muscle maintenance and integrity. The loss of skeletal muscle mass, strength, and function can be induced under various conditions including immobilization/disuse, denervation, starvation, ageing, and disease. Muscle atrophy induced by diseases such as cancer, AIDS, kidney disease, and chronic obstructive pulmonary disorders is called cachexia. A common hallmark of these diseases is chronic inflammation, which is well accepted as the main trigger of muscle wasting. The presence of chronic inflammation is also recognized as the main trigger of sarcopenia, muscle wasting that occurs during ageing. The loss of skeletal muscle that occurs during cachexia and sarcopenia negatively correlates with quality of life and survival. Despite this, there are still no therapies approved for treatment of cachexia or sarcopenia, highlighting the need for a better understanding of the molecular mechanisms of how muscle wasting arises. The complexity of cachexia has hindered efforts in counteracting its symptoms, likely due to the numerous factors linked to the onset of cachexia (Fearon et al, 2012). In this thesis, I have identified a key signaling pathway event that is required for cytokine-induced muscle wasting involving the collaboration of the transcription factors STAT3 and NF-κB. I have also investigated the mechanism through which skeletal muscle cells promote the expression of STAT3 through posttranscriptional regulation by miRNA-330 and the RNA-binding protein HuR.

## 1.2. Clinical Cachexia

In the clinic, the classical presentation of cachexia is of an extremely thin and wasted individual (Fearon et al, 2012). Cachexia is a complex metabolic syndrome characterized by rapid loss of skeletal muscle mass with or without loss of fat mass, decreased food intake, loss of strength, and fatigue (Argiles et al, 2014a; Evans et al, 2008). It is estimated that up to 50% of cancer patients suffer from cachexia (Argiles et al, 2014a; Tisdale, 2009). However, the incidence of cachexia varies between different types of cancer, from 40% of breast cancer patients or leukemias, to 80% in pancreatic or gastric cancers (Ali & Garcia, 2014; Teunissen et al, 2007). Those who develop cachexia are less likely to respond to chemotherapy and are more prone to toxic side-effect (Fearon et al, 2013). The loss of skeletal muscle is a powerful prognostic factor in predicting patient survival and estimated to be the direct cause of death in at least 20% of cachectic patients, likely due to compromised diaphragm or heart function (Argiles et al, 2014a; Fearon et al, 2013). The management of cachexia is a major challenge, requiring significant healthcare resources with palliative care most often occurring as the primary goal of therapy (Tsoli & Robertson, 2013). The limited treatment options for cachectic patients represent an important unmet need (Fearon et al, 2011).

There are numerous attempts to create a classification and staging of cachexia to improve its identification and diagnosis (Argiles et al, 2011; Blum et al, 2011; Blum et al, 2010; Blum et al, 2014; Evans et al, 2008; Fearon et al, 2011; Vigano et al, 2014). The stages are based on increasing severity of cachexia and include criteria such as

the loss of body mass and strength, inflammation, metabolic disturbances/immunosuppression, physical performance, anorexia, and quality of life (Argiles et al, 2011). There have been a few recent reports supporting the use of a classification system to identify the presence of cachexia. A retrospective study of a stage III non-small-cell lung carcinoma cohort found that pre-cachexia and cachexia was prevalent and associated with reduced quality of life and shorter survival (van der Meij et al, 2013). Other studies, one with a pancreatic cancer cohort and another with a mixed group of cancer patients, also found a good overall agreement in defining which patients were cachectic and were associated with poorer survival (Blum et al, 2014; Wesseltoft-Rao et al, 2015). The recognition of the different stages of cachexia is promising to be a useful avenue in providing guidance for earlier and more effective treatment (Fearon et al, 2013).

### 1.3: General Mechanisms of Cachexia

Cachexia is a multi-organ syndrome associated with cancer and other systemic diseases (Argiles et al, 2015). Recent evidence has suggested that other organs and not just skeletal muscle are involved in promoting cachexia, including the brain, gut, and liver (Figure 1.1). The involvement of multiple organs emphasizes the complex nature of cachexia. The chronic inflammation caused by tumor- and host-derived factors cause systemic inflammation, leading to abnormal metabolism of carbohydrate, lipid, proteins, and an overall hyper-catabolic state (Argiles et al, 2014a; Fearon et al, 2012; Tsoli &

Robertson, 2013). The contributions of other non-muscle tissues/organs to key characteristics of cachexia are briefly described here.

### 1.3.1: Anorexia

Anorexia is the loss of desire to eat and is a common symptom in patients with advanced cancer, including those no longer receiving chemotherapy treatment (Tisdale, 2009). Cachexia-associated anorexia is accompanied by a decrease in appetite and early satiation, as well as changes in body composition due to equal loss of fat and muscle (Gordon et al, 2005; Tisdale, 2009). Numerous clinical studies evaluating appetite stimulants have demonstrated limited success to prevent body weight loss during cachexia (Cohen et al, 2015). More recently, ghrelin, a hormone produced mainly in the stomach, was identified as a possible target for therapy to improve appetite (Argiles et al, 2014a). Ghrelin increases food intake by stimulating the hypothalamus in the brain (Argiles et al, 2014a). Animal studies have demonstrated that targeting ghrelin may be a useful in reversing body weight loss (Fujitsuka et al, 2012; Fujitsuka et al, 2011). However, more studies will be needed to prove its efficacy in the clinic to strengthen initial positive results (Ali et al, 2013; Mansson et al, 2016).

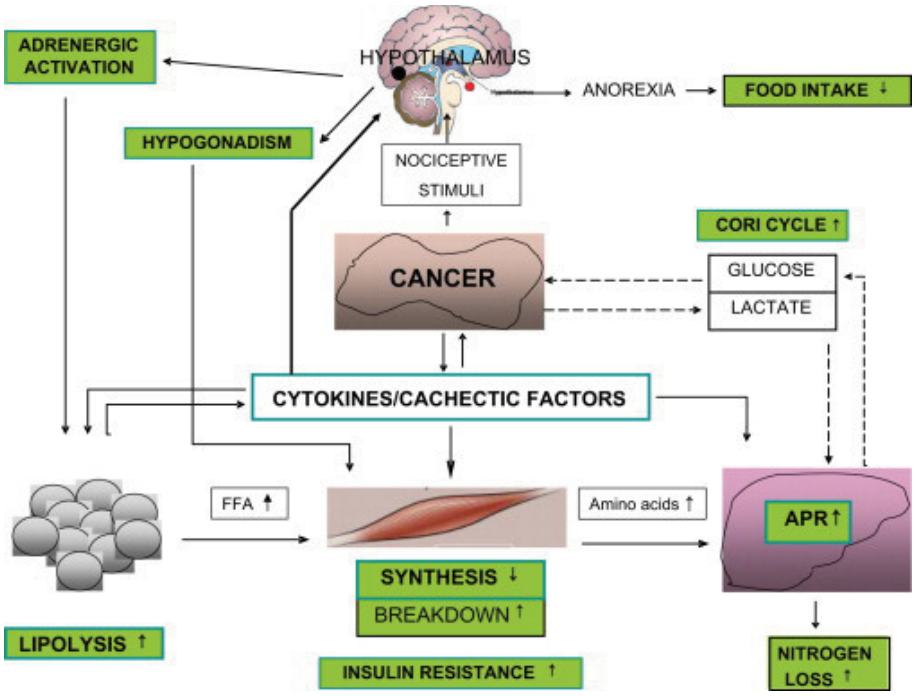



**Figure 1.1: Integrative Physiology of Cancer Cachexia.** The tumor is associated with activation of proinflammatory and neuroendocrine responses. These result in reduced food intake and metabolic change. Adrenergic activation and tumor-related lipolytic factors lead to enhanced lipolysis. The effects of hypogonadism, insulin resistance, adrenergic activation, and systemic inflammation coupled with semistarvation lead to muscle atrophy. Liver export protein synthesis is stimulated as part of the acute phase response. In addition, futile substrate cycles (such as the Cori cycle) contribute to hypermetabolism. *Reprinted with permission from Fearon KCH, Glass DJ, and Guttridge DC, "Cancer cachexia: Mediators, signalling, and metabolic pathways" Cell Metabolism, Vol 16 (2) 153-166, copyright (2012) with permission from Elsevier.*

### 1.3.2: Energy Expenditure

Cachexia is considered an energy-wasting syndrome, where energy intake is decreased and energy expenditure is increased, resulting in an energy deficit (Argiles et al, 2014a). The total energy expenditure (TEE) is the sum of resting energy expenditure (REE), diet-induced energy expenditure (DEE), and the energy cost of physical activity (AEE) (Fearon et al, 2012). The REE is strongly determined by the tumor type and elevated in lung and pancreatic cancers while not increased in gastric or colorectal cancer (Tisdale, 2009). A reason for increased REE may be due to the conversion of white adipose tissue to brown adipose tissue, resulting in increased thermogenesis instead of the production of ATP, the energy currency of the cell (Argiles et al, 2014a). Another reason is the development of the Cori cycle, a process that converts lactate produced by tumors back to glucose in the liver (Argiles et al, 2014a). This futile energy cycle is fueled by the tumor's need to use glucose as its primary source to produce ATP instead of oxidative phosphorylation in the mitochondria, wasting an estimated 300 kcal per day (Tisdale, 2001) (Friesen et al, 2015). The emergence of insulin resistance has also been suggested as a way to divert nutrients away from muscle and fat towards energy-demanding processes in other tissues (Tsoli & Robertson, 2013). These metabolic abnormalities represent important components of cachexia that contribute to an overall catabolic state of wasting.

### 1.3.3: Adipose tissue

The loss of skeletal muscle and adipose tissue represent the key hallmarks of cachexia in the clinic. Chronic inflammation is known to trigger the loss of adipose tissue, mainly due to increased lipolysis of triglycerides into free fatty acids (FFA) and glycerol that are released into circulation (Fearon et al, 2012). Evidence from a mouse model using MAC16 colon adenocarcinoma tumors shows that wasting of adipose tissue occurs due to impaired formation of new adipocytes and decreased ability to store lipid (Bing et al, 2006). In addition, the lipid-mobilizing factor zinc-$\alpha$2-glycoprotein (ZAG) has also been shown to promote lipolysis in mouse models and in human patients (Fearon et al, 2012; Mracek et al, 2011). Interestingly, preventing lipolysis by deleting adipose triglyceride lipase also unexpectedly preserving skeletal muscle mass, demonstrating that cross-talk between adipose tissue and skeletal muscle likely plays an important role in the progression of cachexia (Das et al, 2011; Fearon, 2011). Early detection and prevention of adipose tissue loss may be a way to prevent the progression of cachexia.

In addition, studies have indicated that adipose tissue can undergo “browning” during cachexia, which can significantly contribute to energy expenditure through thermogenesis (Tsoli et al, 2012). One study found that the browning of white adipose tissue was dependent on IL-6-producing tumors in mice and was ameliorated by blocking inflammation (Petruzzelli et al, 2014). Furthermore, tumor-induced systemic inflammation can also lead to increased BAT heat production and dysregulation of lipid

metabolism genes (Tsoli et al, 2012). These studies highlight how adipose tissue contributes to a hypermetabolic state during cachexia.

### 1.3.4: Liver

The liver plays an important role in regulating systemic energy demands by regulating glucose and lipid metabolism (Porporato, 2016). The liver is known to convert the lactate produced by tumor cells back to glucose, which can subsequently return to the tumor, in a process called the Cori cycle (Argiles et al, 2014a). Liver mass substantially increases as cachexia progresses and has been shown to correlate with increased energy expenditure and with the loss of glycogen content (Lieffers et al, 2009; Narsale et al, 2015). Furthermore, the liver worsens inflammation by secreting acute phase proteins, such as albumin and C-reactive protein, which are often used as inflammatory markers (Tisdale, 2009). Additional studies are needed to fully define how the liver contributes to the cachectic process.

## 1.4: Mediators of Cachexia

Chronic inflammation is recognized as the main trigger of cachexia and muscle wasting. Pro-inflammatory cytokines have been shown to mimic the metabolic abnormalities seen in cancer patients (Argiles et al, 2009). In addition to pro-inflammatory cytokines, there are tumor derived factors and the muscle-specific factor

myostatin, a negative regulator of muscle mass, that have been extensively studied to assess their role in cachexia (Toledo et al, 2016; Tsoli & Robertson, 2013). Below is a brief description of pro-cachectic factors.

### 1.4.1: Tumor Necrosis Factor alpha

The role of tumor necrosis factor alpha (TNFα), also called “cachectin” has long been associated with wasting associated with chronic disease (Fearon et al, 2012). Evidence from rodent studies has provided considerable support for a causative role for TNFα in wasting. Mice with TNFα -secreting tumors developed cachexia and have severe weight loss (Oliff et al, 1987). Rats bearing the Yoshida AH-130 ascites hepatoma showed enhanced fractional rates of protein degradation in the gastrocnemius muscle and administration of anti-TNFα antibodies effectively decreased the observed effects (Costelli et al, 1993). In addition, treatment of cultured myotubes promoted apoptosis and induced time and concentration-dependent loss of total protein content including the muscle-specific protein myosin heavy chain (Tolosa et al, 2005 {Li, 1998 #7429). Despite the numerous animal studies supporting TNFα, the involvement of TNFα in cancer patients is less clear. Although detectable, TNFα was found to be inversely correlated with body weight or body mass index or not correlated at all (Karayiannakis et al, 2001; Maltoni et al, 1997). Furthermore, administration of anti-TNFα therapies has had no beneficial effect on appetite or body weight (Goldberg et al,

1995; Jatoi et al, 2010b; Wiedenmann et al, 2008). It is believed that despite its direct catabolic effect on skeletal muscle, TNFα likely acts with other cachectic factors and not alone (Argiles et al, 2009; Fearon et al, 2012).

### 1.4.2: Interferon gamma

Interferon gamma (IFNγ) is produced mainly by activated T and natural killer cells (Cowdery et al, 1996; Okamura et al, 1995). Innoculation of nude mice with IFNγ-expression CHO cells can induce severe cachexia (Matthys et al, 1991). Using the Lewis lung carcinoma model to induce cachexia in mice, early or late treatment with anti-IFNγ monoclonal antibodies was able to inhibit wasting, while only early treatment was able to prevent tumor growth (Matthys et al, 1991). IFNγ is also known to synergize with TNFα and promote rapid atrophy of muscle by decreasing MyoD mRNA levels (Guttridge et al, 2000). Treatment of C2C12 muscle cells with IFNγ alone was found to inhibit proliferation and reduce the expression of myogenin, a key factor involved in the early stages of differentiation, but not MyoD (Villalta et al, 2011). In mdx mice, a mouse model for the human disease Duchenne muscular dystrophy (DMD), a null mutation of IFNγ reduced muscle damage and improved muscle function (Villalta et al, 2011). Although IFNγ is often overlooked, it is clear that IFNγ has a key role in various models of inflammation-induced muscle loss.

### 1.4.3: Interleukin 6

Interleukin-6 (IL-6) is a cytokine elevated in many types of cancers that induce cachexia (reviewed in (Carson & Baltgalvis, 2010)). Importantly, the overproduction of IL-6 has been correlated with weight loss and reduced survival in pancreatic cancer patients (Moses et al, 2009). Evidence from numerous mouse models, including the C26 adenocarcinoma model and the $Apc^{Min/+}$ model for colorectal cancer, supports the notion that the tumor itself is an important source of IL-6 (Baltgalvis et al, 2008; Bonetto et al, 2011; Fujimoto-Ouchi et al, 1995). Angiotensin II (AngII)-mediated muscle wasting also depends on IL-6, which mediates SOCS3 upregulation and leads to impaired IGF-1 signaling (Zhang et al, 2009a). Attempts to block IL-6 with neutralizing antibodies or inhibitors can decrease the extent of muscle wasting, further indicating a key role for IL-6 (Bonetto et al, 2012; Strassmann et al, 1992; Zaki et al, 2004). In NSCLC patients, serum IL-6 was correlated with malnutrition and survival (Songur et al, 2004). Phase I and II clinical trials of ALD518, a humanized monoclonal antibody against IL-6, have shown that ALD518 is well tolerated with the potential to improve cachexia, though further studies are needed to demonstrate improvement in survival (Bayliss et al, 2011). These studies demonstrate that IL-6 is a prime candidate for therapeutic intervention.

### 1.4.4: Proteolysis-inducing factor

Proteolysis-inducing factor (PIF) is a tumor-derived factor produced by both several rodent and human tumours known to increase protein degradation and inhibit total protein synthesis (Hussey et al, 2000; Mirza & Tisdale, 2014; Tisdale, 2008). PIF contains N-linked and O-linked sulphated oligosaccharides attached to the core peptide, which are essential for binding to its receptor (Tisdale, 2008). The receptor for PIF was found only on the surface of skeletal muscle and the liver but absent on adipose tissue, while blocking PIF receptor prevents muscle loss (Mirza & Tisdale, 2014; Smith et al, 2004). Additionally, PIF is detectable in urine samples of approximately 50% of cachectic cancer patients, suggesting it could be used as a biomarker for muscle wasting (Tisdale, 2008). Further studies are needed to determine its use in assessing and treating cachexia.

### 1.4.5: Myostatin

Myostatin is a member of the TGF-β superfamily of growth factors and expressed almost exclusively in cells of the skeletal muscle lineage (Elkina et al, 2011; Zimmers et al, 2002). Myostatin is a potent negative regulator of skeletal muscle mass and is upregulated during many catabolic conditions associated with cachexia. Mice overexpressing myostatin exhibit profound skeletal muscle and fat atrophy that resembles cachexia (Zimmers et al, 2002). Inhibition of myostatin resulted in an equally

profound increase in muscle mass and myofiber size (Schakman et al, 2008). As a result, the development of therapies that target myostatin signaling to prevent muscle wasting has been of great interest. Preventing myostatin signaling by genetic ablation or by using an antibody that targets either myostatin or the myostatin receptor ActRIIB-Fc has also prevented loss of skeletal muscle in rodent models on wasting (Busquets et al, 2012; Gallot et al, 2014; Hatakeyama et al, 2016; Murphy et al, 2011). The ActRII ligands activin A and GDF11 also limit skeletal muscle growth and may be important in human ageing and disease (Cohen et al, 2015; Lach-Trifilieff et al, 2014; Loumaye et al, 2015). There are numerous clinical trials evaluating ActRIIB-specific monoclonal antibody BYM338 for the treatment of muscle wasting (Cohen et al, 2015).

### 1.4.6: Glucocorticoids

Glucocorticoids (GCs) are a class of steroid hormones involved in regulating glucose metabolism and synthesized in the adrenal cortex (Cain & Cidlowski, 2017). GCs are involved in promoting muscle wasting under numerous conditions including cancer, sepsis, fasting, diabetes, and uremia (Schakman et al, 2013). Studies have demonstrated that GCs upregulate ubiquitin-proteasome proteolysis, calcium-dependent degradation, and diminish protein synthesis (Hasselgren, 1999; Kayali et al, 1987; Wang et al, 1998; Wing & Goldberg, 1993). However, in mice bearing C26 adenocarcinoma tumors, adrenalectomy could not prevent muscle atrophy (Svaninger et al, 1987). Genetic ablation of muscle-specific glucocorticoid receptor (GR) and

treatment with the GR antagonist RU-486 protects muscle from atrophy induced by glucocorticoids (Braun et al, 2013; Schakman et al, 2013; Watson et al, 2012). Interestingly, one recent study showed that chemotherapy itself can induce inflammation and subsequent muscle wasting that was dependent on the endogenous GC corticosterone (Braun et al, 2014).

### 1.4.7: Nitric oxide

Nitric oxide (NO) is a gaseous signaling molecule generated by the three isoforms of nitric oxide synthase (NOS), neuronal NOS (nNOS/NOS1), inducible NOS (iNOS/NOS2), and endothelial NOS (eNOS/NOS3) (Aktan, 2004). The expression of inducible NOS (iNOS) can be detected in a range of cells and tissues in response to inflammatory signals (Aktan, 2004). High levels of NO produced by iNOS have beneficial microbicidal, antiviral, and antiparasital effects (Pautz et al, 2010). The only known receptor for NO is soluble guanylate cyclase (sGC), which transduces signal by producing the secondary messenger cyclic GMP (cGMP) and is notably involved the regulation of vascular tone, platelet function, and neurotransmission (reviewed in (Denninger & Marletta, 1999)). Additionally, NO can react with oxygen radicals to form peroxynitrite ($ONO_2^-$), a toxic reactive nitrogen species shown to be necessary for the iNOS/NO pathway to promote muscle wasting (Di Marco et al, 2005). In fact, there are multiple reports that have linked iNOS/NO pathway to muscle wasting, though no

conclusive mechanism has been elucidated (Bae et al, 2012; Buck & Chojkier, 1996; Di Marco et al, 2012; Yu et al, 2008).

### 1.4.8: Insulin-like growth factor 1

One of the best-characterized factors involved in promoting skeletal muscle hypertrophy is the insulin-like growth factor 1 (IGF-1). Skeletal muscle is a source of IGF-1 during resistance exercise and its autocrine effects is a major mechanism through which muscle growth is controlled (Schiaffino et al, 2013; Schiaffino & Mammucari, 2011). Reduced levels of circulating IGF-1 have been reported in experimental cancer cachexia rodent models (Costelli et al, 2006). However, attempts to improve muscle mass observed in these rodent models by either hyper-expression of IGF-1 or exogenous administration were unable to prevent cachexia (Costelli et al, 2006; Penna et al, 2010). In contrast, a similar study showed a low dose of exogenous IGF-1 was able to attenuate loss of body weight, muscle mass, and reduce mortality (Schmidt et al, 2011). Further studies are needed to clarify how IGF-1 is involved in regulating muscle wasting.

## 1.5: Molecular Mechanisms of Skeletal Muscle Atrophy

Skeletal muscle wasting is caused by a decrease in protein synthesis and an increase in protein degradation. The factors discussed above are able to impact either

the initiation of translation or upregulate downstream factors that mediate protein degradation. This section will provide an overview of the molecular mechanisms involved in regulating skeletal muscle mass and how these pathways contribute to wasting of skeletal muscle.

### 1.5.1: Protein Synthesis and Translation

Protein synthesis is accomplished through a process called translation. The initiation of translation is a highly regulated complex process that has been extensively studied and requires the activation of key factors to allow for the recruitment of ribosomes to mRNA (Bhat et al, 2015; Tisdale, 2009). The serine/threonine kinase Akt is a critical regulator of skeletal muscle mass. Importantly, decreased levels of total Akt, total mTOR, and p-mTOR levels were observed in biopsies from cachectic patients (Schmitt et al, 2007). Decreased levels of p-Akt in muscle and in adipose tissue were also observed in C26-tumour bearing mice (Asp et al, 2010). The Akt-mTOR pathway can be influenced by various factors described in section 1.4, including inflammatory cytokines, IGF-1, myostatin, and GCs (Figures 1.2) (Amirouche et al, 2009; Dehoux et al, 2007; Gilson et al, 2007; Trendelenburg et al, 2009; White et al, 2013).

Akt controls translation through the kinases mTOR and glycogen synthase kinase 3$\beta$ (GSK3$\beta$) (Fig 1.2) (Schiaffino & Mammucari, 2011). One point of control is the phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2$\alpha$) via GSK3$\beta$

(Fig 1.2). eIF2 mediates the binding of the initiator methionyl-tRNA to the small ribosomal subunit (40S) to promote translation initiation (Bhat et al, 2015; Tisdale, 2009). The phosphorylation of eIF2$\alpha$ inhibits protein synthesis and can also be mediated by other upstream kinases under stress or starvation, such as PKR. Increased levels of p-PKR and p-eIF2$\alpha$ were detected in rectus abdominus muscle of weight-losing cancer patients compared to healthy controls as well as MAC16-tumor bearing mice (Eley et al, 2007; Eley et al, 2008b; Eley & Tisdale, 2007). In addition, the proinflammatory cytokines TNF$\alpha$ and IFN$\gamma$ together can also increase p-eIF2$\alpha$ via PKR resulting in a decrease in global translation (Di Marco et al, 2012; Eley et al, 2008a).

Another point of control is through the phosphorylation of the downstream mTOR targets eIF4E binding protein-1 (4E-BP1) and p70 S6 Kinase (S6K) (Figure 1.2). First, the phosphorylation of 4E-BP1 prevents association with eIF4E with eIF4G, the rate-limiting factor in cap-dependent translation initiation (Bhat et al, 2015; Tisdale, 2009). Second, the phosphorylation and activation of S6K leads to hyper-phosphorylation of ribosomal protein S6 and enhanced translation (Bhat et al, 2015). In myotubes, PIF inhibited protein synthesis by 50% and was accompanied by increased phosphorylation of mTOR, S6K, 4E-BP1, and eIF2$\alpha$ (Eley et al, 2007). Similarly in rats bearing AH-130 tumors, phosphorylation of mTOR, S6K, 4E-BP1, and eIF2$\alpha$ were increased (Aversa et al, 2011). It is clear that modulation of protein synthesis can contribute to muscle wasting.

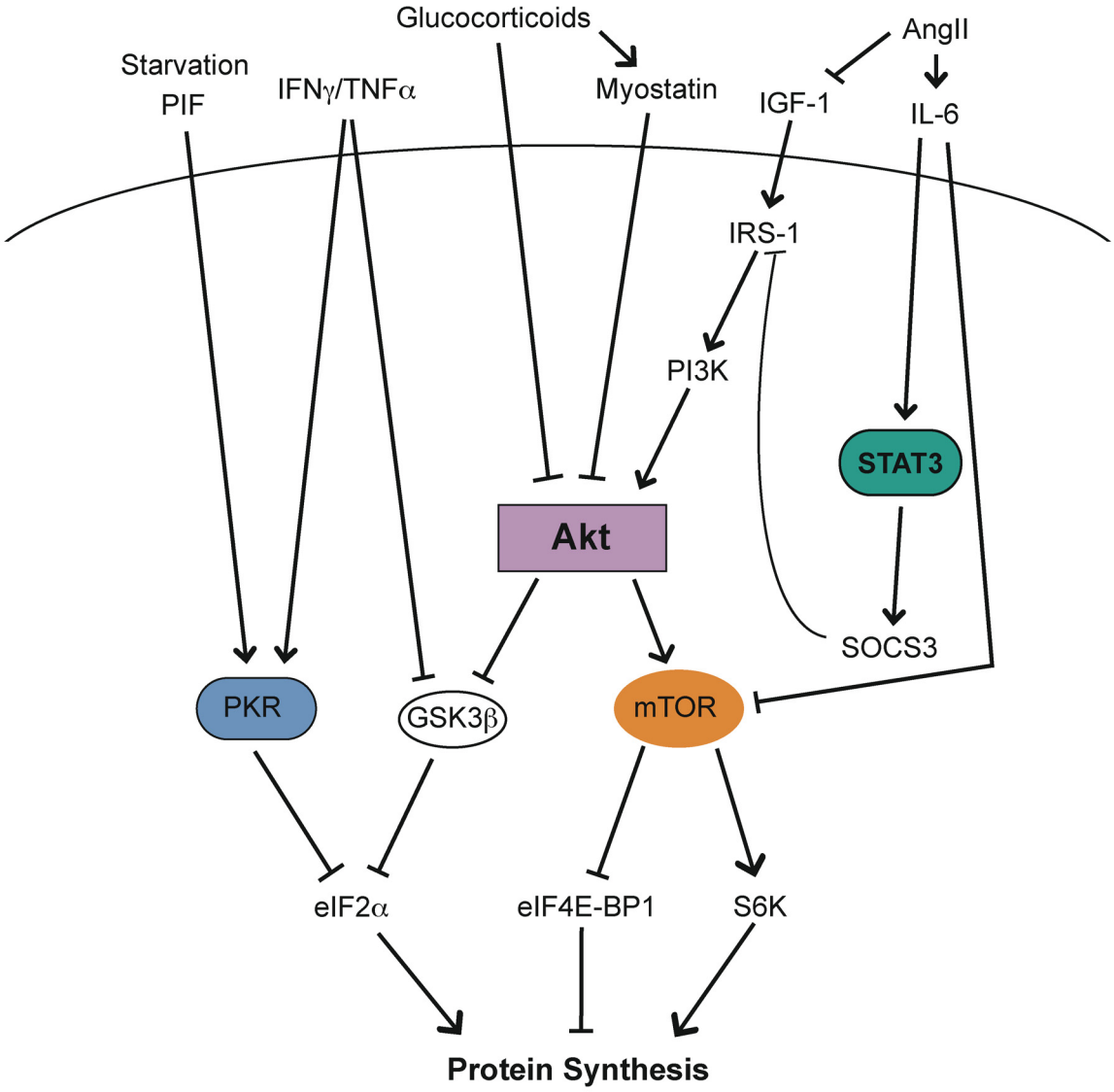



**Figure 1.2: Pathways involved in regulating protein synthesis in skeletal muscle.** Various factors involved in regulating muscle mass lead to changes in protein synthesis through several mediators. The kinase Akt is an important signaling molecule that is affected by glucocorticoids, myostatin, as well as insulin-like growth factor 1 (IGF1), a known hypertrophic signal. PKR is another kinase that can inhibit protein synthesis during muscle wasting triggered by PIF, IFNγ/TNFα, and starvation. AngII is dependent on IL-6 for mediating increases in SOCS3 and impaired IGF-1 signaling.

### 1.5.2: Protein Degradation

Protein degradation in skeletal muscle is mediated by the ubiquitin-proteasome pathway (UPP), the autophagy-lysosomal system, and the calcium-activated systems. Activation of the UPP in muscle atrophy is most commonly associated with the expression of the muscle-specific E3 Ub-ligases atrogin-1/MAFbx and MuRF-1, (Bodine & Baehr, 2014). These E3 enzymes are highly induced during various models of muscle atrophy including fasting and cancer cachexia and recognized as key markers of muscle atrophy (Bodine et al, 2001; Gomes et al, 2001)}(Lecker et al, 2004). In fact, a common set of transcriptional adaptations termed “atrogenes” occurs under these different muscle wasting conditions (Lecker et al, 2004). Genetic and pharmacologic inhibition of atrogin-1, MuRF1, or their upstream transcriptional activators FoxO or NF-κB leads to inhibition of muscle atrophy (Bodine & Baehr, 2014; Cai et al, 2004; Sandri et al, 2004). Identification of a limited number of targets of MuRF1 and atrogin-1 have been identified and include myosin heavy chain, MyoD, and eIF3f (Acharyya et al, 2004; Bodine & Baehr, 2014). The degradation of myofibril proteins via the UPP plays a critical role in promoting muscle wasting.

The autophagy-lysosomal system is a self-degradative process that removes misfolded or aggregated proteins and damaged organelles such as mitochondria, therefore playing a crucial role in the degradation and recycling of cell components (Glick et al, 2010). In general, autophagy is a multi-step process where cytoplasmic material is sequestered by a double-membraned vesicle (called “autophagosome”),

which fuses with a lysosome for degradation (Kroemer et al, 2010). The knock-out of various autophagy genes in mice displays decreased muscle strength (Bonaldo & Sandri, 2013). In fact, the accumulation of autophagosomes is a major feature of a group of muscle disorders called autophagic vacuolar myopathies and characterized by mutations in lysosomal proteins (Bonaldo & Sandri, 2013). Autophagy was shown to be induced in murine models of cancer cachexia and in glucocorticoid-induced atrophy (Penna et al, 2013; Sandri, 2013). Additional studies to further clarify the role of autophagy during cancer-related muscle wasting are needed.

Many studies have investigated the signaling mechanisms involved in triggering muscle wasting. The transcription factors implicated in changes in protein degradation and mediating muscle wasting are briefly discussed below.

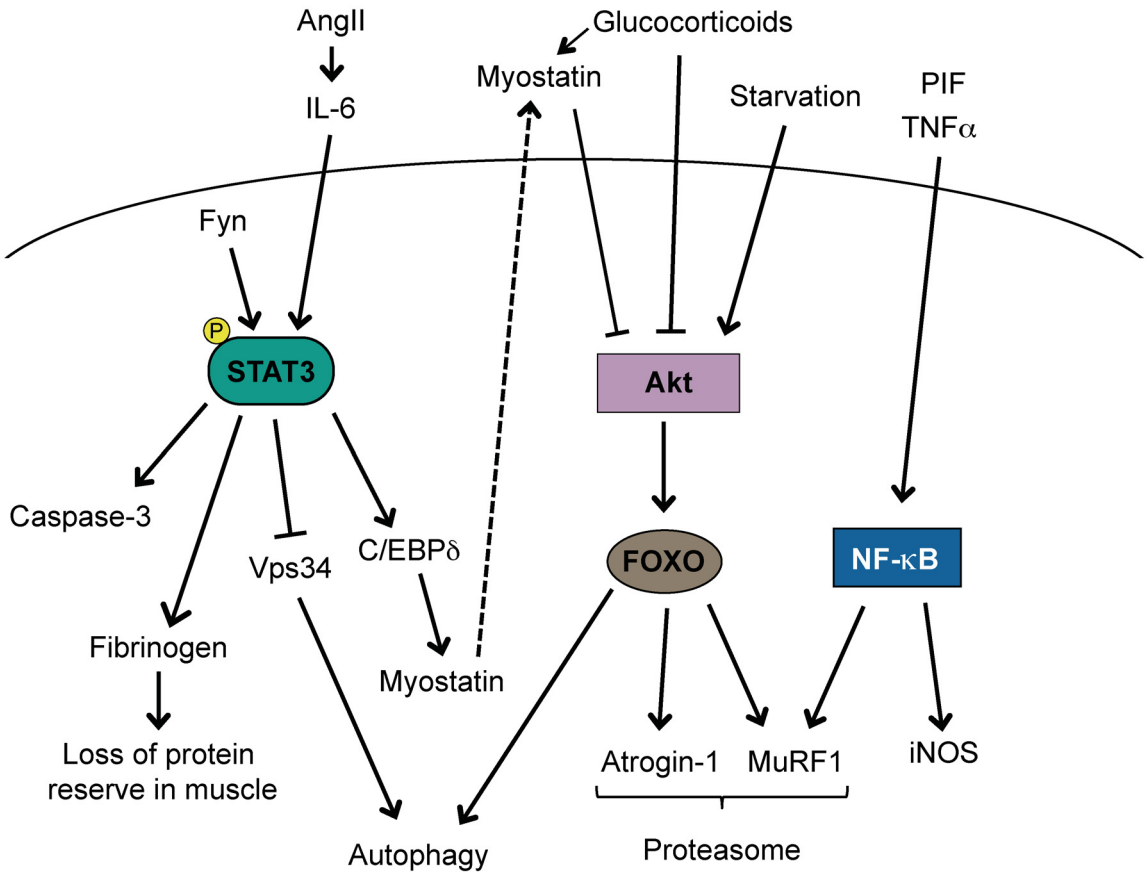



**Figure 1.3: Pathways involved in regulating protein degradation during skeletal muscle atrophy.** Various factors involved in protein degradation activate several downstream effectors. FOXO transcription factors can be activated by starvation or upon Akt suppression by glucocorticoids and myostatin, and upregulate atrogin-1, MuRF1, and autophagy. NF-κB also induces MuRF1, but also iNOS to promote protein degradation. STAT3 can activate caspase-3 to promote proteolysis and inhibit Vps34, a core component required for macroautophagy. STAT3 activation can lead to loss of protein reserves in skeletal muscle by increased transcription and secretion of acute phase proteins such as fibrinogen.

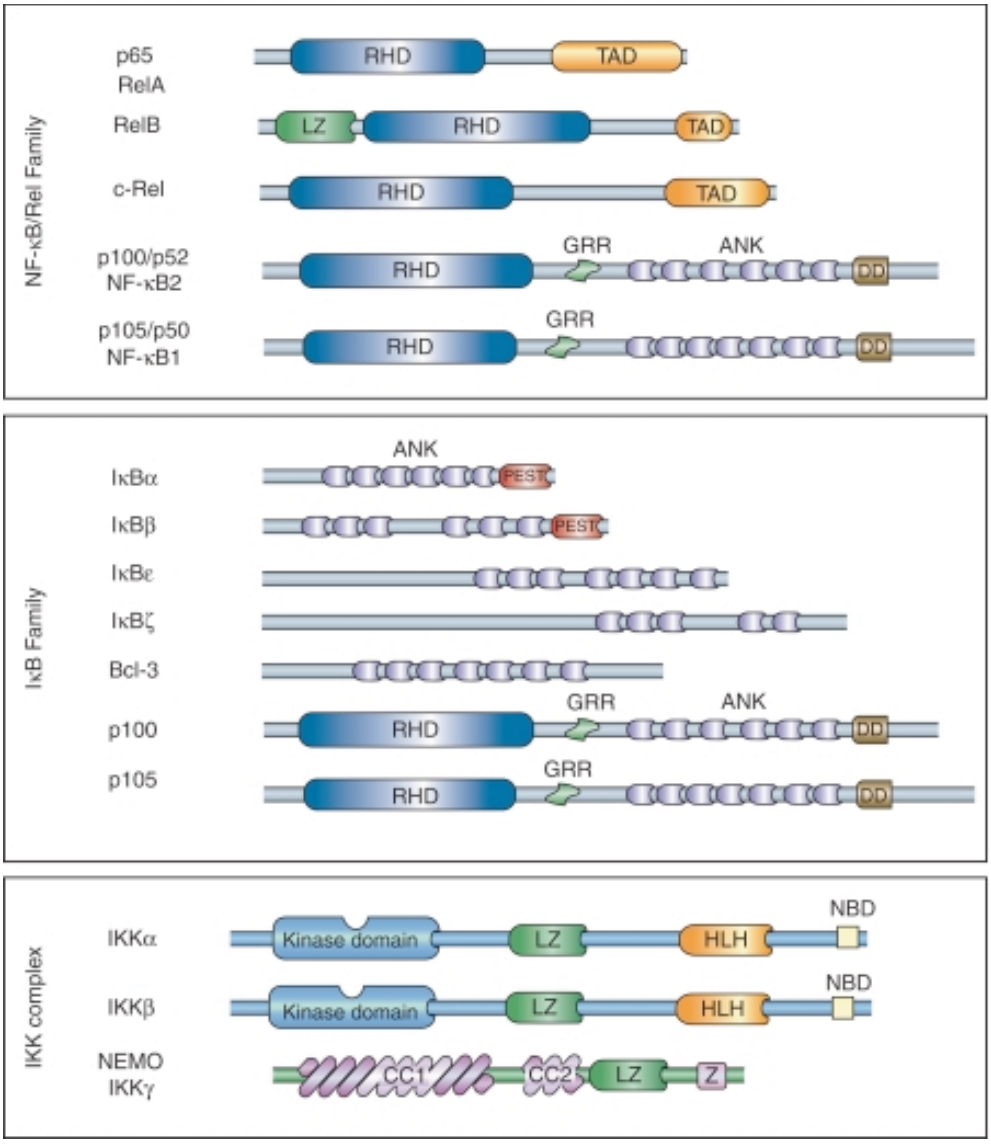
#### 1.5.2.1: Forkhead box protein O transcription factors

The Forkhead box protein O (FOXO) family of transcription factors contains three predominant genes in mammals: FOXO1, FOXO3, and FOXO4 (Arden, 2008). A wide range to stimuli including GCs, IGF-1, and starvation leads to suppression of Akt and activation of FoxO, further leading to FoxO nuclear localization (Stitt et al, 2004). FoxO1 and FoxO3a were both found to be upregulated in muscle biopsies of cancer patients (Schmitt et al, 2007). Animal studies have demonstrated the involvement of FoxO1 and FoxO3 in promoting skeletal muscle atrophy (Kamei et al, 2004; Reed et al, 2011; Sandri et al, 2004). In particular, FoxO3 was found to promote increase protein degradation by activating genes involved in both autophagy and the UPP (Zhao et al, 2007). Together, these studies suggest a central role for FoxO family of proteins in regulating protein homeostasis under catabolic conditions.

#### 1.5.2.4: Nuclear factor kappa B (NF-κB)

NF-κB is a ubiquitous transcription factor regulated by an array of factors including pro-inflammatory cytokines, such as TNFα (Hoesel & Schmid, 2013). Skeletal muscle expresses all five subunits coding for RelA/p65, RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) (Hoesel & Schmid, 2013). Each subunit contains a Rel homology domain (RHD) that is essential for subunit dimerization, though only p65,

**Figure 1.4: Members of the NF-κB, IκB, and IKK protein families.** The Rel homology domain (RHD) is characteristic for the NF-κB proteins, whereas IκB proteins contain ankyrin repeats (ANK) typical for this protein family. The precursor proteins p100 and p105 can therefore be assigned to and fulfill the functions of both the NF-κB and IκB protein families. The domains that typify each protein are indicated schematically. CC, coiled-coil; DD, death domain; GRR, glycine-rich region; HLH, helix-loop-helix; IKK, IκB kinase; LZ, leucine-zipper; NBD, NEMO binding domain; PEST, proline-, glutamic acid-, serine-, and threonine-rich region; TAD, transactivation domain; ZF, zinc finger. *Reprinted from Oeckinghaus and Ghosh, "The NF-kB Family of transcription factors", Cold Spring Harb. Perspect. Biol. 1(4): a000034, doi: 10.1101/cshperspect.a000034, copyright (2009), with permission from Cold Spring Harbor.*


NF-κB/Rel Family
p65
RelA
RHD
TAD
RelB
LZ
RHD
TAD
c-Rel
RHD
TAD
p100/p52
NF-κB2
RHD
GRR
ANK
DD
p105/p50
NF-κB1
RHD
GRR
DD
IκB Family
IκBα
ANK
PEST
IκBβ
PEST
IκBε
IκBζ
Bcl-3
p100
RHD
GRR
ANK
DD
p105
RHD
GRR
DD
IKK complex
IKKα
Kinase domain
LZ
HLH
NBD
IKKβ
Kinase domain
LZ
HLH
NBD
NEMO
IKKγ
CC1
CC2
LZ
Z

RelB, and c-Rel possess a carboxy-terminal TAD and are able to activate transcription (Fig. 1.4) (Hayden & Ghosh, 2004). NF-κB is sequestered to the cytoplasm through its association with IκBα (Hoesel & Schmid, 2013). Upon phosphorylation, IκBα is degraded and releases NF-κB, allowing its translocation to the nucleus where it can bind to DNA to stimulate gene expression (Hoesel & Schmid, 2013). The classical NF-κB signaling cascade is activated by cytokines such as TNFα and IL-1β, triggering receptor-mediated assembly of a multi-protein complex that leads to the phosphorylation and activation of IKK, then IκBα phosphorylation and degradation, releasing NF-κB (Fig. 1.5) (Peterson et al, 2011).

Transgenic mice expressing members of the IKK/NF-κB pathway have been developed to elucidate the role of specific members during muscle atrophy. Activation of NF-κB by the overexpression of its upstream kinase IKKβ induced severe muscle wasting in mice, while genetic and pharmalogical inhibition of IκBα and MuRF1 substantially reduced muscle loss induced by denervation and LLC tumors (Cai et al, 2004). Furthermore, In addition, studies with cultured myotubes have demonstrated the induction of the NF-κB pathway by TNFα and PIF (Di Marco et al, 2005; Guttridge et al, 2000; Li & Reid, 2000; Wyke & Tisdale, 2005). Similarly, patients with gastric tumors exhibited higher p-p65 and decreased expression of IκBα indicating the activation of NF-κB pathway (Rhoads et al, 2010). It is clear that the IKK/NF-κB pathway plays a role in promoting muscle wasting.

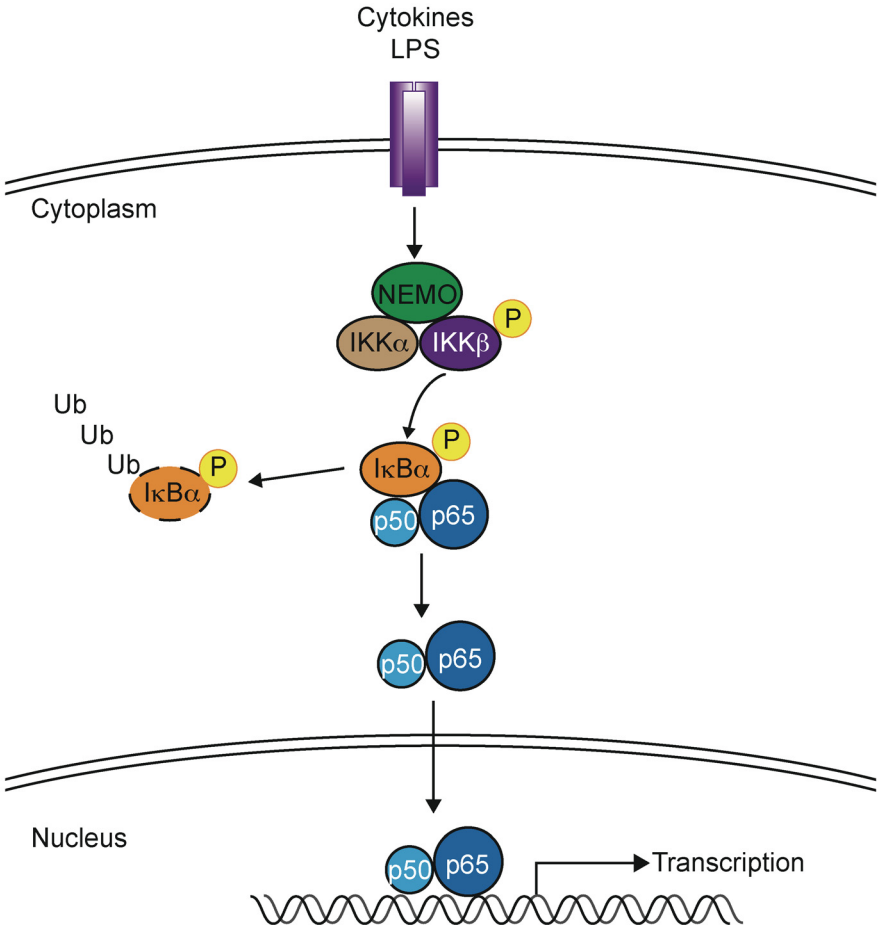



**Figure 1.5: Canonical NF-κB signaling pathway.** NF-κB proteins are inactive in the cytoplasm as a result of their association with inhibitor of κB (IκB) proteins. Extracellular signals induce phosphorylation of IκB via IκB kinase (IKK) complex. Phosphorylated IκB is subsequently ubiquitylated and degradated by the proteasome, releasing NF-κB and allowing nuclear translocation to occur.

### 1.5.2.3: Signal Transducer and Activator of Transcription 3

The Signal Transducer and Activator of Transcription (STAT) 3 protein is one of seven members of the STAT family of transcription factors in mammals (Reich, 2013). STAT proteins play an essential role in regulating development, proliferation, and immune response (Reich, 2013). However, hyperactive STAT signaling can lead to disease, such as cancer and autoimmune disorders (Johnston & Grandis, 2011; Reich, 2013). Importantly, STAT3 has been shown in several murine models including IL-6-dependent cancer cachexia and chronic kidney disease to promote muscle wasting (Bonetto et al, 2012; Bonetto et al, 2011; Silva et al, 2015; Zhang et al, 2013). One clinical study has demonstrated that inhibition of IL-6 has a limited effect at blocking muscle wasting (Bayliss et al, 2011). However, it is known that STAT3 can be activated by extracellular ligands other than IL-6, which may be contributing to STAT3 activation during muscle wasting. In Chapter 2, I further explore the involvement of STAT3 during muscle wasting independently of IL-6. This section is a description of STAT3, how it is regulated, and what is known about STAT3 during muscle wasting.

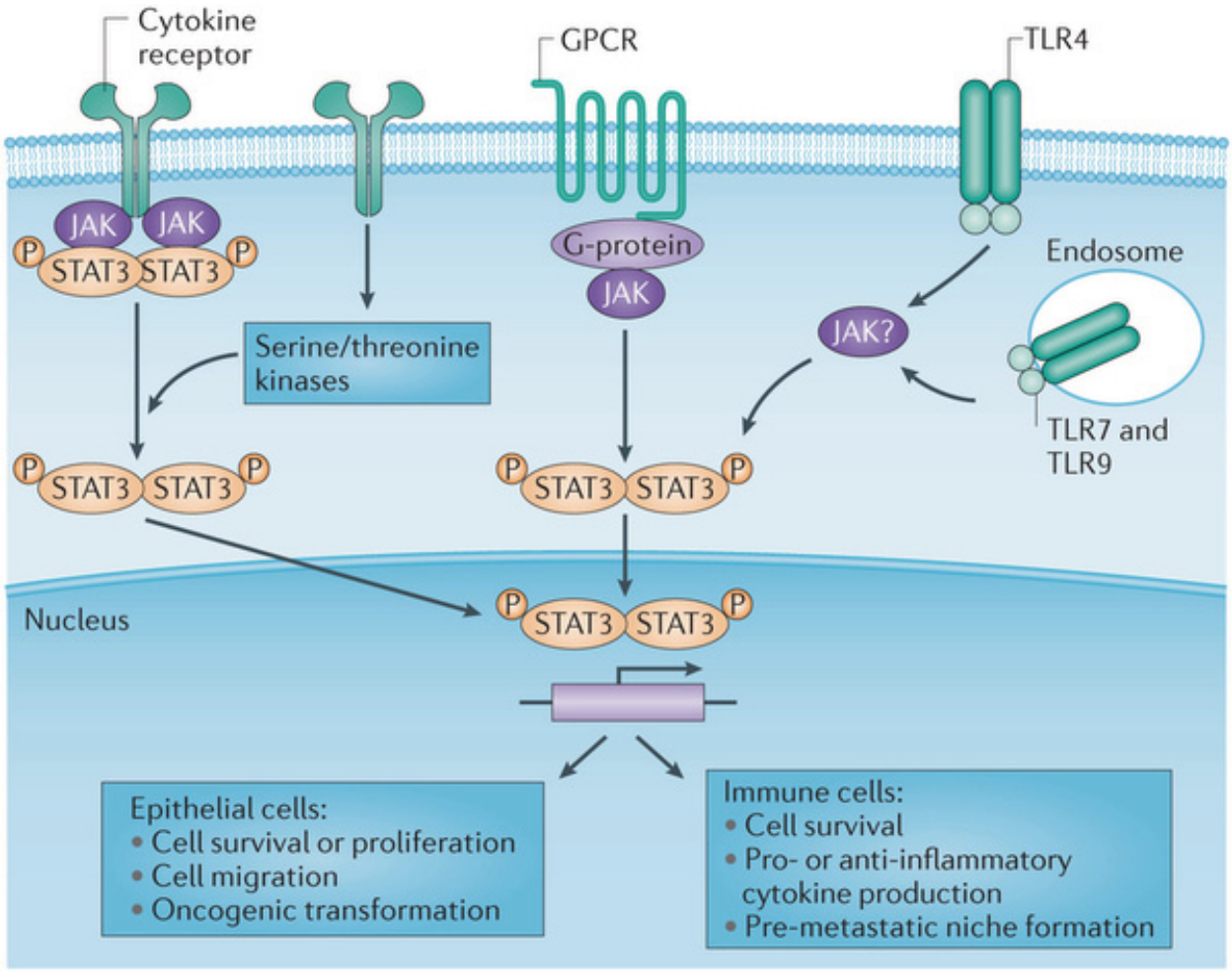
#### *1.5.2.3.1: STAT3 function and activity*

All STAT proteins contain seven domains, which includes N-terminal, coiled-coil, DNA-binding, linker, a Src homology 2 (SH2), and a C-terminal trans-activation domain (TAD) (Reich & Liu, 2006). STAT3 has two key phosphorylation sites found in the TAD,

a tyrosine$^{705}$ residue crucial for activation and dimerization and a serine$^{727}$ residue shown to enhance transcriptional activity (Reich & Liu, 2006). Dimer formation occurs through reciprocal interaction between phospho-tyrosine residue of one STAT protein and the SH2 domain of another (Reich, 2013). Dimers of pY-STAT proteins can then bind to DNA and promote transcription, a key feature of STAT proteins (Johnston & Grandis, 2011). After phosphorylation and nuclear localization, STAT3 homodimers bind to a palindromic sequence (5'-TTC$(N)_{2-4}$GAA-3') of DNA to activate transcription (Ehret et al, 2001; Hutchins et al, 2013b; Hutchins et al, 2012; Seidel et al, 1995; Vallania et al, 2009). Additionally, acetylation of STAT3 at lysine$^{685}$ by the co-activator CBP/p300 has also been found to stimulate its DNA-binding and transcriptional activity (Wang et al, 2005).

STAT3 was initially described as the acute-phase response factor (APRF) activated by IL-6 and epidermal growth factor (EGF) (Kim et al, 2016). Since then, STAT3 has been found to mediate signal transduction for many other stimuli including the IL-6 family (IL-11, IL-31, LIF, CNTF, OSM), the IL-10 family (IL-10, IL-19, IL-20, -22, -24, -26), interferon's, and G-CSF (Schindler & Plumlee, 2008; Yu et al, 2014) (Fig.1.6). G-protein-coupled receptors (GPCRs) and Toll-like receptors have also been shown to activate STAT3 (Eyking et al, 2011; Xin et al, 2013).

**Figure 1.6: Pathways activating JAK–STAT3 signaling in cancer.** Biological processes that are crucial for cancer progression are mediated by Janus kinase (JAK)–signal transducer and activator of transcription 3 (STAT3) signaling. The JAK–STAT3 pathway is activated by diverse receptors, including those for interleukin-6 (IL-6) and IL-6 family cytokines, as well as G-protein-coupled receptors (GPCRs) and Toll-like receptors (TLRs). Unlike receptor tyrosine kinases (RTKs), these receptors lack intrinsic kinase activity. Instead, upon binding to their cognate ligands, these receptors undergo conformational changes and form interacting sites for adaptor proteins to propagate signals. For example, IL-6 receptors rely on the tyrosine kinases JAK1 or JAK2, which associate with the cytoplasmic tail of gp130 and directly phosphorylate (P) STAT3. The gp130 subunit of the IL-6 receptor is the signaling component that is shared with other cytokine receptors. While cytokine receptors and TLRs function as dimers after activation by ligands, G-protein-bound GPCRs catalyse the conversion of guanine nucleotides and activate a sequence of downstream signaling effectors and kinases, including JAK2. In addition, JAK2 can act as a direct mediator of certain GPCR signals to activate STAT3. Activated JAKs, such as JAK1 and JAK2, phosphorylate STAT3 at Tyr705, resulting in translocation of activated STAT3 dimers to the nucleus. Meanwhile, serine/threonine kinases mediate Ser727 phosphorylation of STAT3 that enhances its transcriptional activity. In the nucleus, STAT3 binds to the promoters of genes and induces a genetic program that promotes various cellular processes that are required for cancer progression. *Reprinted from Yu et al., "Revisiting STAT3 singaling in cancer: new and unexpected biological functions", Nature Reviews, 14: 736-746, copyright (2014), with permission from Elsevier.*

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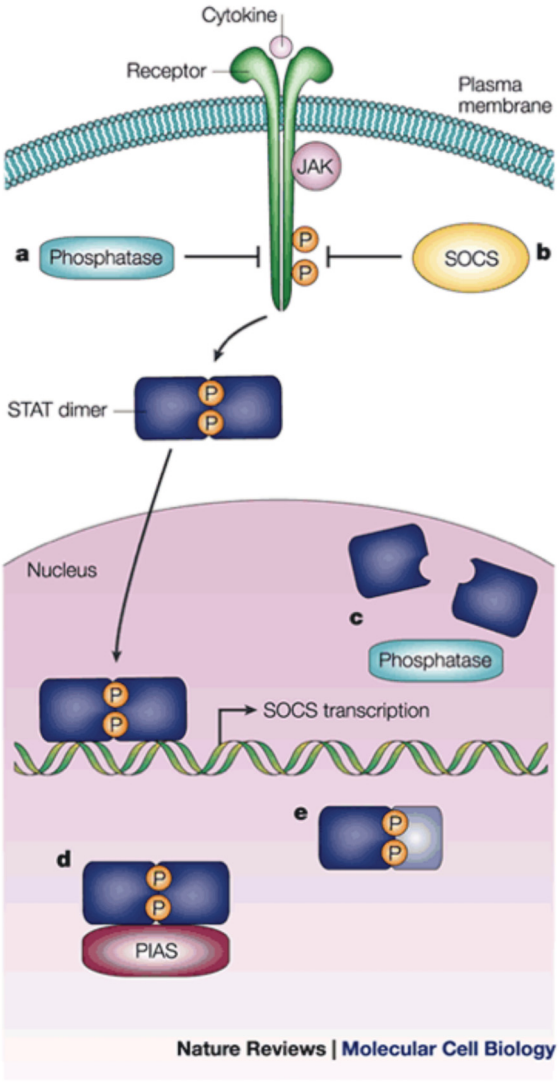
Stimulation by one of these factors recruits STAT3 to the corresponding cell-surface receptors where the receptor-associated tyrosine kinase Janus Kinase (JAK) can phosphorylate STAT3 on its key tyrosine residue (Kim et al, 2016). STAT3 can also be activated by the non-receptor kinases Src and Bcr-Abl (Johnston & Grandis, 2011). The nuclear localization of STAT3 is independent of its Tyr phosphorylation, unlike STAT1, which requires phosphorylation to enter the nucleus (Reich, 2013). A constitutive nuclear import domain of STAT3 is recognized mainly by importin-$\alpha$3 and enters the nucleus via the Ran nuclear import pathway (Cimica et al, 2011; Liu et al, 2005).

Furthermore, STAT3 is essential for early development, as indicated by a study that showed STAT3 deficient embryos have an early embryonic lethal phenotype at E6.5 - 7.5 (Takeda et al, 1997). Expression of only STAT3$\alpha$ causes death perinatally 16h after birth with no gross abnormalities (Maritano et al, 2004). Interestingly, expression of only STAT3$\beta$ was found to rescue embryonic lethality (Maritano et al, 2004). Despite having a truncated TAD, STAT3$\beta$ can activate a unique subset of genes relative to the genes activated by STAT$\alpha$, indicating that it is not just a dominant-negative regulator of transcription (Maritano et al, 2004; Ng et al, 2012).

**Figure 1.7: The negative regulators of STAT proteins.** Phosphatases (a) and suppressors of cytokine signalling (SOCS proteins) (b) block further STAT activation in the cell cytoplasm. In the nucleus, nuclear phosphatases (c) can mediate STAT dephosphorylation, and interactions with proteins that inhibit activated STAT proteins (PIAS) (d) can also occur. In addition, naturally occurring short forms of STATs can potentially act as dominant-negative proteins by occupying DNA as non-functional protein or by binding to a wild-type STAT protein (e). JAK, Janus kinase; STAT, signal transducers and activators of transcription. *Reprinted from Levy and Darnell, "STATs: transcriptional control and biological impact", Nature Reviews Mol Cell Bio, 3: 651-662, copyright (2002), with permission from Elsevier.*


Cytokine
Receptor
Plasma membrane
JAK
a
Phosphatase
P
P
SOCS
b
STAT dimer
P
P
Nucleus
c
Phosphatase
P
P
SOCS transcription
e
P
P
d
P
P
PIAS
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*1.5.2.3.2: Regulation of STAT3 Activity*

Under normal conditions, STAT3 signaling is negatively regulated by inhibitory factors including protein inhibitor of activated STAT 3 (PIAS3), suppressor of cytokine signaling 3 (SOCS3), SH2 domain-containing phosphatase (SHP)-1 and SHP-2, and protein tyrosine phosphatases (PTPs), such as TCPTP (Figure 1.7) (Bohmer & Friedrich, 2014). The best studied are the SOCS proteins, which are transcriptionally activated in response to cytokine stimulation and act on active receptor complexes to inhibit continued signaling (Yoshimura et al, 2012). SOCS3 is upregulated in response to IL-6 and can bind to gp130 receptor to block further IL-6 signaling. PTP's prevent further signaling by dephosphorylating active receptor complexes (Rawlings et al, 2004). Of the approximately 107 identified PTPs, TCPTP, PTPRD, and PTPRT have been shown to directly target STAT3 for de-phosphorylation (Bohmer & Friedrich, 2014). In the nucleus, PIAS3 can block DNA binding by interacting with STAT3 dimers directly and thereby preventing STAT3-dependent transcription (Rawlings et al, 2004). Control of STAT-mediated signaling is critical since aberrant STAT3 signaling is associated with disease (Bohmer & Friedrich, 2014).

*1.5.2.3.3: The role of STAT3 during muscle wasting*

Studies have indicated that STAT3 is a major regulator of muscle wasting (Bonetto et al, 2012; Bonetto et al, 2011; Silva et al, 2015; Yamada et al, 2012; Zhang

et al, 2013). STAT3 was characterized as a key downstream mediator of muscle wasting activated by the IL-6-dependent C26 adenocarcinoma tumor model of muscle wasting (Bonetto et al, 2011). Under these conditions, gene expression analysis revealed an upregulation of pathway components including IL-6, STAT3, and coagulation. Furthermore, this study suggested that activated STAT3 (pY-STAT3) influences muscle wasting by altering gene expression to increase production of acute phase proteins for secretion into the blood stream (Bonetto et al, 2011). In addition, activation of STAT3 was necessary and sufficient for muscle wasting. Various models of muscle wasting including sepsis, streptozotocin-induced diabetes, and chronic kidney disease (CKD) also reveal that STAT3 is activated, indicating that pY-STAT3 may be a common feature of muscle wasting (Bonetto et al, 2012; Silva et al, 2015; Zhang et al, 2013). Studies further indicate that pY-STAT3 can activate proteolytic mechanisms that lead to atrophy (Silva et al, 2015; Zhang et al, 2013). C26 and LLC tumors were found to activate STAT3 leading to increased caspase-3 transcription and activity to promote protein degradation (Silva et al, 2015). pY-STAT3 also activated C/EBPδ expression, which further led to increased MuRF-1 and Atrogin-1 expression promoting proteolysis during muscle wasting induced by C26 tumors, LLC tumors, and CKD (Zhang et al, 2013).

In addition, autophagy is activated during numerous catabolic conditions including cancer, ageing, fasting, and critical illness (Sandri, 2013). One study has demonstrated a key role for STAT3 in the regulation of autophagy during starvation-induced muscle wasting has also been demonstrated (Yamada et al, 2012). STAT3 was

shown to be phosphorylated by the Src kinase family member Fyn, which led to decreased protein levels of Vps34, a key component required for autophagic flux (Yamada et al, 2012). In addition, the Fyn-STAT3-Vps34 pathway induced wasting specifically in glycolytic fibers, providing evidence for fiber-type specific regulation of muscle wasting (Yamada et al, 2012). Interestingly, IL-6 was found to inhibit starvation-induced autophagy through the STAT3-Bcl2 signaling pathway in the human cancer cells, suggesting that IL-6-STAT3 may also be involved in regulating autophagy-mediated muscle loss (Qin et al, 2015). STAT3 has also been suggested to regulate autophagy through its interaction with proteins such as PKR/EIF2AK2, FOXO1/3 or localization to the mitochondria (You et al, 2015). In this thesis, I uncover a mechanism through which STAT3 is activated during muscle wasting by TNF$\alpha$ and IFN$\gamma$ but independently of IL-6. Furthermore, my work has uncovered a novel role for STAT3 in promoting muscle wasting in collaboration with NF-$\kappa$B in order to activate the pro-cachectic gene iNOS.

### 1.6: Post-transcriptional control of gene expression

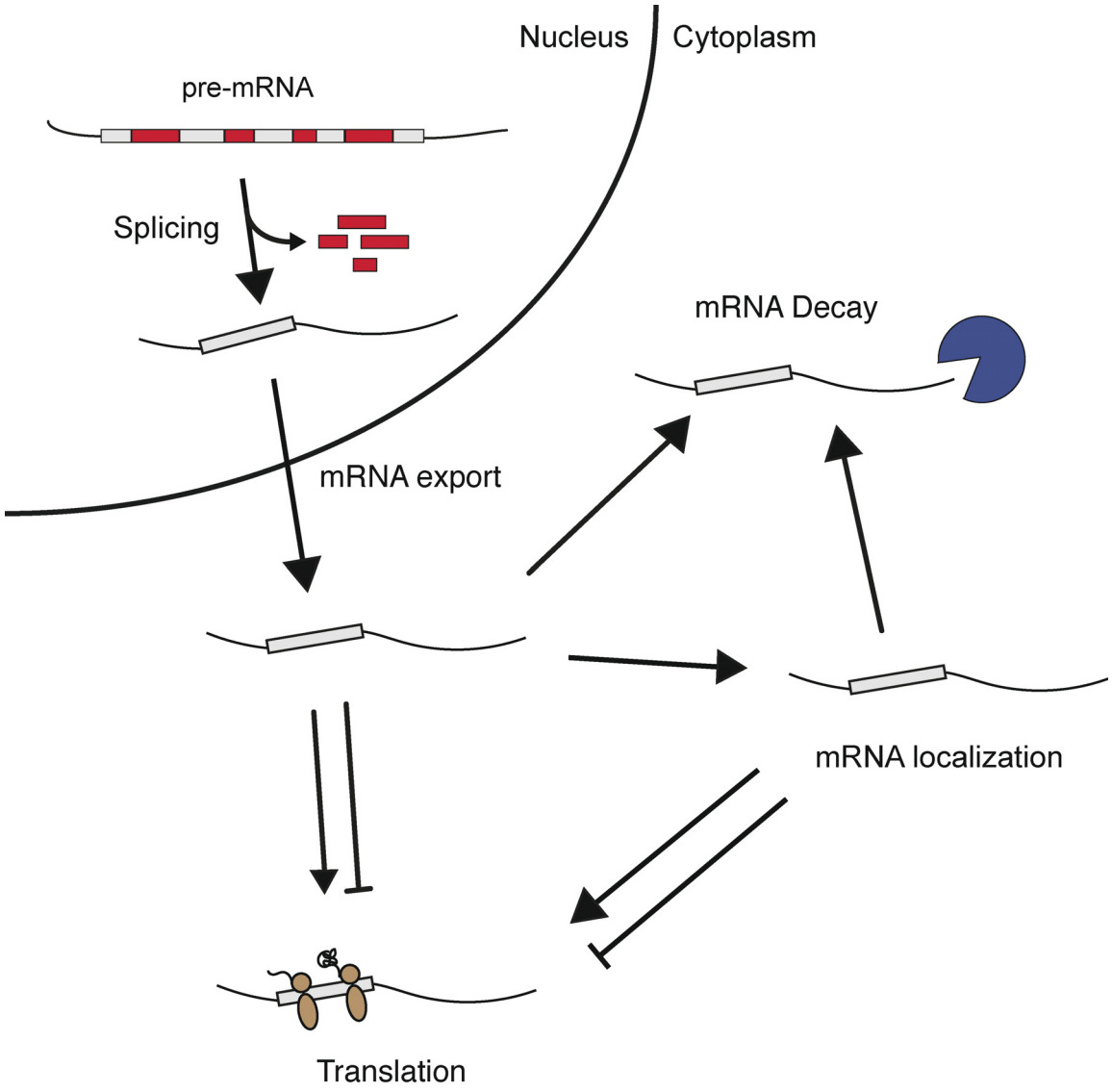
STAT3 has a crucial role in important cellular process including survival, proliferation, differentiation, and immunity (Yu et al, 2014). The expression of STAT3 is an important aspect of STAT3 signaling since increased levels of STAT3 can lead to aberrant signaling (Shukla et al, 2010). The material covered in this section pertains to my work in chapter 3 where I investigated the regulation of STAT3 at the

posttranscriptional level involving the RNA-binding protein Human antigen R (“HuR”) and the small non-coding RNA miR-330-5p.

Transcription has a key role in promoting the expression of certain genes involved in muscle wasting (Argiles et al, 2014a). However, it is known that transcription alone does not explain the increase and maintenance of gene expression (Grammatikakis et al, 2017). Evidence has implicated the involvement of posttranscriptional events at the level of mRNA stability and mRNA translation as crucial steps that ensures proper gene expression (Srikantan & Gorospe, 2012; von Roretz et al, 2011b). Over the years, the dysregulation of post-transcriptional regulatory events have emerged as important steps in physiological and pathological processes including various muscle wasting disorders (Hurt & Silver, 2008).

An mRNA undergoes several layers of posttranscriptional regulation before the final protein is produced (Figure 1.8). As transcription occurs, the pre-mRNA immediately begins to fold and RNA-binding proteins immediately begin to associate. Splicing removes introns from the pre-mRNA and can create splice variants that result in different protein products. Next, transport of mRNA out of the nucleus to the cytoplasm occurs. Then, the mRNA assembles with one or more ribosomes for protein synthesis to occur. The stability of an mRNA influences how long it remains intact for translation to occur. The steps involved in the posttranscriptional regulation of genes are briefly described below.

**Figure 1.8: General posttranscriptional regulatory mechanisms.** Gene expression is regulated at several levels after transcription and addition of the 5' cap and poly(A) tail. Prior to export, pre-mRNA undergoes splicing to remove introns and ligate exons to form the mature mRNA. Next, mature mRNA is exported into the cytoplasm and can be further localized to a specific compartment where translation or repression of translation can occur. Localization of mRNA can also lead to its decay. The translation of mRNA can be either cap-dependent or cap-independent, and also subject to regulation. Lastly, gene expression can also be regulated by mRNA stability or decay, also referred to as mRNA turnover, which is influenced by various factors.


Nucleus
Cytoplasm
pre-mRNA
Splicing
mRNA Decay
mRNA export
mRNA localization
Translation

### 1.6.1: Splicing

Alternative splicing (AS) is important for increasing the diversity of mRNA's that are expressed (Kelemen et al, 2013). An estimated 90% of human genes encode at least two mRNA isoforms (Havens & Hastings, 2016). The splicing of a pre-mRNA involves precisely excising intervening sequences called introns and ligating exons by the spliceosome (Kelemen et al, 2013; Wahl et al, 2009). Regulation of AS may involve other factors including heteronuclear RNP's (hnRNP's) and serine-arginine-rich (SR) proteins (Kelemen et al, 2013). Aberrant regulation of AS caused by point mutations is known to lead to human diseases including cancer and muscular dystrophies (Graveley, 2009). STAT3 has two different isoforms generated by AS, the full-length STAT3$\alpha$ (92kDa) and the shorter isoform STAT3$\beta$ (84 kDa) (Ng et al, 2012) (described in section 1.5.2.3.1). Interestingly, there are studies that have used specific redirection of AS by using chemically modified antisense oligonucleotides called splice-switching oligonucleotides (SSOs) to induce the expression of different isoforms rather than degrading the target mRNA (Bauman et al, 2009). One study has shown that use of SSOs to induce a splicing switch from the STAT$\alpha$ isoform to the STAT3$\beta$ isoform could induce tumor regression (Zammarchi et al, 2011). The use of SSOs is an ongoing therapy being tested for Duchenne Muscular Dystrophy, a disorder where approximately 70% of mutations are deletions of exons that disrupt the mRNA reading frame and create premature termination codons (Havens & Hastings, 2016). Targeting the dystrophin pre-mRNA with SSOs to restore the mRNA reading frame has yielded

promising results in animal models and clinical testing is ongoing (Havens & Hastings, 2016).

### 1.6.2: mRNA turnover

The rate of decay, or "turnover" of an mRNA is an important process involved in controlling its abundance (Wu & Brewer, 2012). The major pathway of mRNA decay involves removing the 3' poly(A) tail (deadenylation) then the 5' cap structure, which are crucial mRNA-stabilizing structures, followed by degradation by mRNA decay machinery (Wu & Brewer, 2012). Several enzymes have been described to deadenylate mRNAs including the Ccr4-Not complex, PARN, and Pan2-Pan3, while decapping requires the decapping proteins Dcp1-Dcp2 (Wu & Brewer, 2012). The control of mRNA turnover is regulated by *cis*-acting elements usually found in the 3' UTR and the trans-acting factor recruited to these elements (Wu & Brewer, 2012). The interaction between *cis* and *trans* acting factors can leads to decay, stabilization, or enhanced or reduced translation, resulting in mRNA turnover that differs considerably (Schoenberg & Maquat, 2012; von Roretz et al, 2011b). Both RBPs and miRNAs are *trans*-acting factors that have a well-studied impact on skeletal muscle development (Guller & Russell, 2010; Kirby et al, 2015), but less is known about their influence on skeletal muscle under catabolic conditions such as muscle wasting.

#### *1.6.2.1 Destabilizing elements*

The adenylate- and uridylate-(AU) rich element (ARE) is a common and well-studied *cis* element found in the 3' untranslated region (UTR) of many labile mRNAs, such as ones encoding for growth factors, proto-oncogenes, nuclear transcription factors and cytokines (Chen et al, 1995; Vlasova-St Louis & Bohjanen, 2016). Initially, three different classes of AREs (Class I, II, III) grouped based on the sequence of adenine and uracil nucleotides was proposed (Chen & Shyu, 1995). More recently, five clusters of ARE sequences was proposed based on the number of AUUUA repeats and the surrounding sequences (Vlasova-St Louis & Bohjanen, 2016). Similar to the AU-rich element, the GU-rich element (GRE) is also involved in mediating the decay of mRNAs that encode for factors involved in cell cycle, metabolism, and transcriptional regulation (Vlasova-St Louis & Bohjanen, 2016). A similar classification for GREs was also proposed and also has five subclasses based on the number of GUUUG repeats. This conserved *cis* element controls mRNA turnover by interacting exclusively with the protein CUGBP1 (Vlasova-St Louis & Bohjanen, 2014). Studies assessing the effect of RBP's on ARE-mediated decay are dominant, however, there are also miRNA that have been identified to bind to ARE's as well.

*1.6.2.2: Regulation of mRNA Turnover by RNA-binding proteins during inflammation*

RNA-binding proteins (RBPs) are key players in regulating the turnover of mRNA containing AREs (Pullmann & Rabb, 2014; von Roretz et al, 2011b). There are a number of ARE-binding proteins (ARE-BPs) that have been identified to mediate mRNA decay including AUF1, tristetraprolin (TTP), and KH-type slicing regulatory protein (KSRP) (Pullmann & Rabb, 2014). These ARE-BPs promote rapid deadenylation followed by degradation by the exosome (Wu & Brewer, 2012). In addition, ARE-BPs can promote mRNA stability, such as the ubiquitously expressed Human antigen R protein (HuR) (Schoenberg & Maquat, 2012).

Systemic inflammation is a hallmark of chronic diseases believed to trigger muscle wasting (Fearon et al, 2012). RBPs play a key role in regulating the expression of inflammatory cytokines. In fact, the mRNAs for TNF$\alpha$ and IL-6, both known to induce muscle wasting, are posttranscriptionally regulated due to AU-rich elements (AREs) found in their 3' UTRs (Chen & Shyu, 1995). Numerous ARE-BPs including HuR, AUF-1, TTP, and CUGBP1 are known to regulate TNF$\alpha$ mRNA stability (Deleault et al, 2008; Di Marco et al, 2001; Zhang et al, 2008). Similarly, HuR, TTP, and AUF1 regulate IL-6 mRNA (Paschoud et al, 2006; Raineri et al, 2004; Zhao et al, 2011). The AT-rich interactive domain 5a (Arid5a) protein was recently described to associate with mRNA and regulate the IL-6 and STAT3 mRNAs, but not the TNF$\alpha$ mRNA (Masuda et al, 2013; Masuda et al, 2016). While RBPs are known to bind to and regulate mRNAs, the activation of signaling pathways can influence RBP function by changing expression

level or by affecting phosphorylation status (Schoenberg & Maquat, 2012). Examples of RBPs that are regulated by phosphorylation include HuR, TTP, and KSRP (Schoenberg & Maquat, 2012). For example, p38-dependent KSRP phosphorylation prevents the binding of KSRP to ARE-containing mRNAs such as myogenin, preventing mRNA decay (Briata et al, 2005). Together these observations indicate that the regulation of gene expression during inflammation by RBP is intricate and may be important during muscle wasting.

*1.6.2.3: The role of HuR in regulating mRNA stabilization*

HuR is best known as a stabilizer of its target mRNA. In addition to HuR's role in stabilizing numerous cell cycle, cell growth, and inflammatory genes, important genes required for muscle development (myogenesis) and a variety of muscular atrophy diseases and disorders have been linked to HuR (Srikantan & Gorospe, 2012). In skeletal muscle, HuR was found to stabilize p21, MyoD and myogenin mRNA, key factors that promote the fusion of muscle cells to form myotubes (Figueroa et al, 2003; van der Giessen et al, 2003; van der Giessen & Gallouzi, 2007). Curiously, under the same conditions, HuR was also found to destabilize nucleophosmin (NPM) mRNA during the early steps of myogenesis by associating with the decay factor KSRP and promote myogenesis (Cammas et al, 2014). Together, these data show that HuR is a key factor in stabilizing factors that are involved in muscle formation and maintenance.

In addition to promoting muscle differentiation, HuR has been implicated in sporadic inclusion body myositis (sIBM), a slow on-set inflammatory muscular atrophy of unclear causes (Dalakas, 2006; Nakano et al, 2005). IFN$\gamma$/TNF$\alpha$-induced iNOS mRNA requires HuR-mediated mRNA stabilization for proper expression and leads to muscle atrophy (Di Marco et al, 2005). These studies suggest that HuR may also have a role in regulating muscle wasting and have yet to be uncovered.

*1.6.2.4: Regulation of mRNA turnover by miRNA*

A class of small, non-coding RNA called microRNA (miRNA) plays a key role in regulating gene expression. miRNA are ~22 nucleotide single-stranded RNA that act as a guide for the RNA-induced silencing complex (miRISC) through its association with Argonaute (Ago) proteins (Pasquinelli, 2012). In animals, most miRNA bind to the 3'UTR of target mRNA through partial complementarity (Pasquinelli, 2012). The most common motif for miRNA binding is perfect pairing between nucleotides 2 though 7 at the 5' end of the miRNA, called the seed region, and the target mRNA (Pasquinelli, 2012). Partial complementarity between one miRNA and its target means that one miRNA could regulate hundreds of mRNAs (Fabian et al, 2010a; Filipowicz et al, 2008). Furthermore, one mRNA can be targeted by multiple miRNA at the same time, which can lead to a greater effect on gene expression (Bartel, 2009; Fabian et al, 2010a; Filipowicz et al, 2008).

Evidence has suggested that binding of miRNA to its target mRNA generally results in either mRNA destabilization or translational repression (Fig. 1.9) (Pasquinelli, 2012). In animal cells, miRNAs rarely induce endonuclear cleavage of the mRNA by an active Ago2 protein (Fig. 1.9a) (Macfarlane & Murphy, 2010). An example of this rare cleavage event was observed between miR-196 and the *HOXB8* mRNA in mouse embryos (Yekta et al, 2004). More commonly, miRNA recruit the CCR4-NOT complex to promote deadenylation and subsequent mRNA decay (Weill et al, 2012) (Fig. 1.9b). In colorectal cancer cells and tumors, miR-16 was found to promote the rapid decay of *COX-2* mRNA (Young et al, 2012). In contrast, the miR-3134 was found to stabilize *SOX9*, *VEGFA*, and *EGFR* mRNAs by increasing each mRNAs half-life (Sharma et al, 2013).

*1.6.2.5: Global studies in miRNA expression during muscle atrophy*

There are numerous studies that have investigated the global changes in miRNA expression during muscle atrophy. One study compared four different models of muscle atrophy including denervation, starvation, diabetes, and cancer cachexia but identified no common miRNA profile between the different conditions (Soares et al, 2014). They found that most miRNA were inhibited in cachectic muscles, which was the opposite miRNA expression profile compared to profiles from the other models of muscle wasting (Soares et al, 2014). Another study comparing ten major muscular disorders reported five miRNA (miR-146b, -221, -155, -214, -222) consistently regulated in almost all

samples within an otherwise distinctive miRNA expression profiles (Eisenberg et al, 2007). Interestingly, a common miRNA signature in skeletal muscle was reported between DMD and acute ischemic models of muscle atrophy (Greco et al, 2009). The occurrence of distinct miRNA profiles suggests that miRNA likely offer subtle control of underlying signaling pathways and highlights the complexity of different types of muscle wasting.

*1.6.2.6: STAT3 - miRNA regulatory network*

Recently, studies have identified miRNA that regulate STAT3 expression in various tissues. In acute myeloid lymphoma cells, miR-17 and miR-20a both directly bind to the STAT3 3'UTR, repressing growth arrest and differentiation (He et al, 2013). In another study, decreased levels of miR-124 resulted in increased STAT3 expression and pY-STAT3 levels and suggested to promote the pathogenesis of pediatric ulcerative colitis (Koukos et al, 2013). These studies suggest that the regulation of STAT3 by miRNA is an important mechanism that contributes to disease.

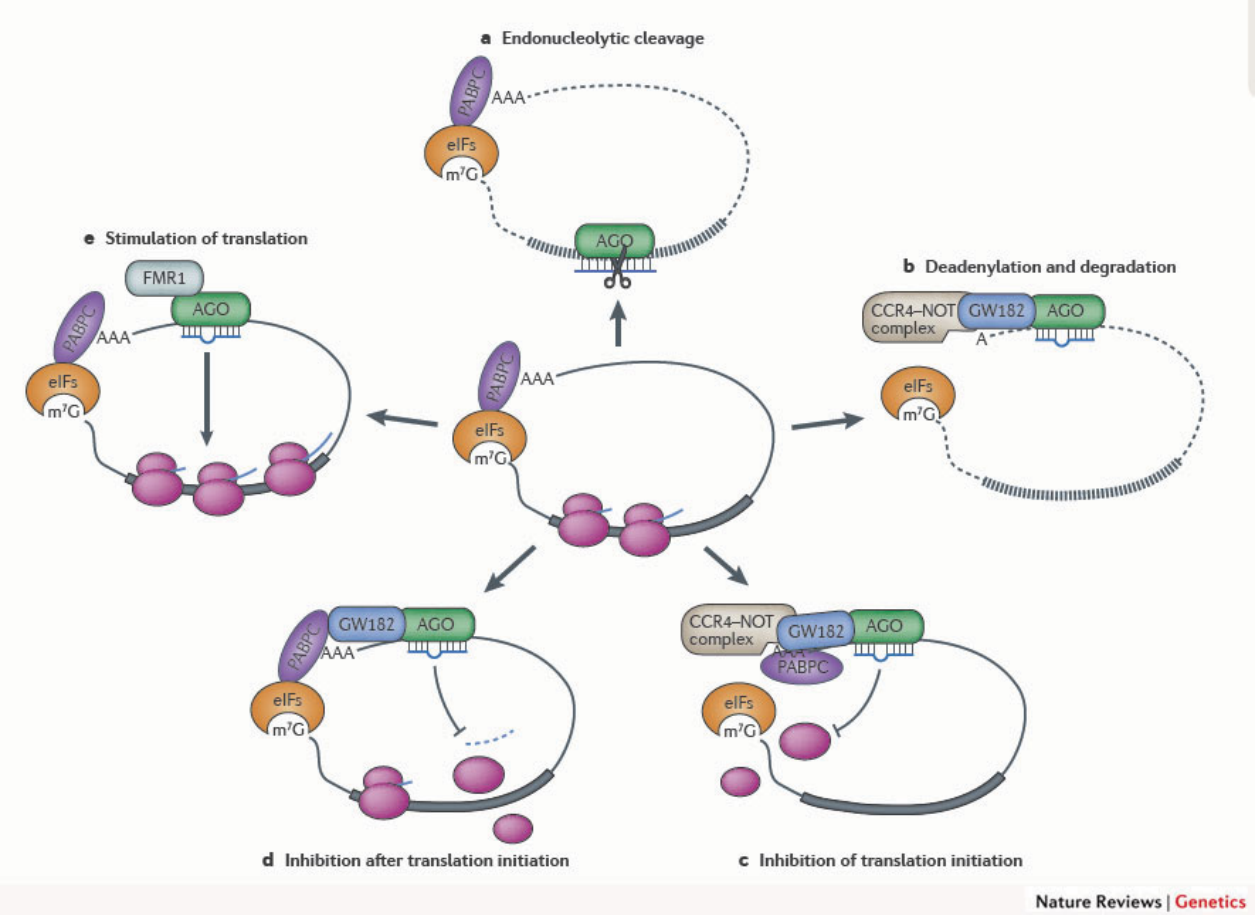
There have also been studies that indicate that STAT3 can transcriptionally regulate miRNA expression. One study demonstrated the transcriptional co-regulation of miR-21 by STAT3 and NF-κB in response to IFNα/β and that overexpression of miR-21 inhibited IFNα/β-induced apoptosis (Yang et al, 2010). STAT3 also regulated miR-181a, which was found to promote skeletal muscle differentiation and suggested to

regulate muscle adaptation during acute endurance exercise (Naguibneva et al, 2006; Safdar et al, 2016). There are also miRNA that target STAT3 and suggests that a regulatory network involving STAT3 and miRNAs may contribute to pathological conditions such as cancer (Haghikia et al, 2012). The effect of a miRNA may also indirectly affect STAT3-mediated gene expression by targeting other components of the STAT3 pathway (Haghikia et al, 2012). This highlights the complexity of STAT3-miRNA networks involved in inflammation and disease.

### 1.6.3: Translation

Translation is the final step in producing proteins. The translation and mRNA turnover are closely linked processes that are regulated by many of the same *trans*-acting factors such as RBPs and miRNA (Wilusz et al, 2001; Wu & Brewer, 2012). *Trans*-acting factors that regulate the translation of specific mRNA can also bind *cis* elements found in the 5' or 3' UTR. In fact, factors such as the poly(A)-binding protein (PABP), which binds the poly(A) tail at the 3' end of the mRNA, interacts with eIF4G at the 5' end to promote mRNA circularization and stabilization, which can enhance the overall initiation rate (Fabian et al, 2010b; Song et al, 2016). The suppression of protein synthesis at the level of translation initiation by regulation of eIF2$\alpha$, eIF4E-BP1, and ribosomal protein S6, is a main contributing factor to muscle wasting previously described in section 1.5.1.

**Figure 1.9: Mechanisms of target regulation by miRNAs.** MicroRNAs (miRNAs) regulate gene expression through multiple pathways. A complex of eukaryotic initiation factors (eIFs) binds the 5′ cap and the cytoplasmic poly(A)-binding protein (PABPC), connecting the 5′ and 3′ ends of mRNAs and stimulating their translation by the ribosome (shown in pink). **(a)** Perfect pairing between an miRNA and its target site induces endonucleolytic cleavage by Argonaute (AGO), leading to rapid degradation of the mRNA. **(b)** Partial pairing of the miRNA complex to target 3′ untranslated region (UTR) sites can result in deadenylation of the mRNA through recruitment of the CCR4–NOT complex by the miRNA-induced silencing complex (miRISC)-associated GW182 proteins. Loss of the poly(A) tail causes dissociation of PABPC and leads to degradation of the mRNA. **(c)** The miRISC can also induce translational repression by blocking initiation via recruitment of CCR4–NOT by GW182. **(d)** Translational repression can also be induced by the miRISC by inhibiting a step after initiation, such as promoting ribosome drop-off or stimulating proteolysis of the nascent peptide. **(e)** miRNAs have also been shown to upregulate target expression under certain conditions through a mechanism that involves Argonaute and fragile X mental retardation protein 1 (FMR1). *Reprinted from Pasquinelli, "MicroRNAs and their targets: recognition, regulation, and an emerging reciprocal relationship", Nature Reviews Genetics, 13: 271-282, copyright (2012), with permission from Elsevier.*


a Endonucleolytic cleavage
PABPC
AAA
eIFs
m7G
AGO
e Stimulation of translation
FMR1
b Deadenylation and degradation
CCR4–NOT complex
GW182
A
d Inhibition after translation initiation
c Inhibition of translation initiation
Nature Reviews | Genetics

During translation initiation, ribosomal scanning along the 5' UTR occurs until the initiator AUG codon is reached (Bugaut & Balasubramanian, 2012). However, secondary structures within the 5' UTR can influence translation. One of these structures is the G-quadruplex, formed in G-rich nucleic acid sequences (Bugaut & Balasubramanian, 2012). An RNA G-quadruplex has been reported in numerous 5'UTRs including the human proto-oncogene *NRAS* where a G-quadruplex was found to inhibit protein expression (Bugaut & Balasubramanian, 2012; Kumari et al, 2007). Another structure that acts as an alternative mechanisms for translation initiation is the internal ribosomal entry sites (IRES) (Bugaut & Balasubramanian, 2012; Song et al, 2016). IRES recruit ribosomes independently of the 5' cap and several initiation factors (Bugaut & Balasubramanian, 2012; Song et al, 2016). Importantly, IRES-dependent translation initiation is important under conditions such as cell stress, growth, or differentiation (Bugaut & Balasubramanian, 2012). The 5' UTR of utrophin A was shown to have an IRES responsible for enhancing translation under GC treatment and suggested as a unique target for Duchenne Muscular Dystrophy (DMD) therapy (Miura et al, 2008; Miura et al, 2010). Reductions to translation is a key hallmark of muscle wasting, though there are limited studies that indicate how other modulators of translation such as secondary structures may be influencing translation in the muscle.

*1.6.3.1: The role of HuR in regulating translation*

HuR has also been shown to regulate the translation of target mRNA's. The 3'UTR of p53 mRNA is a target of HuR in RKO cells exposed to UV light and results in enhanced translation of p53 mRNA (Mazan-Mamczarz et al, 2003). In C2C12 muscle cells, the translation of the pro-myogenic HMGB1 depends on the recruitment of HuR to prevent miR-1192-mediated translation repression (Dormoy-Raclet et al, 2013). While there is evidence that supports the notion that HuR is a translational enhancer, there are also examples of the opposite. The repression of the proto-oncogene c-Myc depends on HuR to recruit the miRNA let-7 (Kim et al, 2009b). Furthermore, HuR has been shown to suppress mRNA translation by binding to the 5'UTR, possibly by disrupting IRES (Kullmann et al, 2002). These studies clearly show that HuR can influence translation and can collaborate with miRNA.

*1.6.3.2: The role of miRNA in regulating translation*

There are several different mechanisms for miRNA-mediated translational repression that have been suggested. Studies have demonstrated that blocking initiation and ribosome recruitment, or promoting ribosome dropoff, or stimulating nascent peptide proteolysis as possibilities (Fig 1.9c, d) (Mathonnet et al, 2007; Pasquinelli, 2012; Ricci et al, 2013). In addition, the stimulation of translation has also

been proposed (Orom et al, 2008) (Fig 1.9e). The precise mechanism of regulation by miRNA is complex.

Studies indicate miRNA-mediated repression of key target mRNAs occurs during muscle atrophy. One study demonstrated the translational repression of MuRF1 and atrogin-1 mRNAs by miR-23a (Wada et al, 2011). Several studies have reported a decrease in miR-23a levels during various models of muscle wasting, suggesting a role for this miRNA in regulating muscle wasting (Hudson et al, 2014; Lin et al, 2009; Wada et al, 2011). Other experimentally validated targets of miR-23 include three adult fast myosin heavy chain genes and PGC-1$\alpha$ (Wang, 2013). The impact of miRNA during myogenesis is well-studied (Horak et al, 2016; Wang, 2013), but their role in promoting atrophy is less clear.

The regulation of gene expression by miRNA and RBPs both occur through their ability to recognize sites of control in the 3'UTR of mRNA. Studies indicate that various RBPs and miRNA can collaborate or compete with each other in the regulation of a target mRNA (Loffreda et al, 2015). Examples of RBPs that have been demonstrated to do so include PUM1, PUM2, FMRP, and HuR. In chapter 3, I have uncovered a role for HuR and miR-330 in the regulation of STAT3 mRNA and its implication in muscle wasting.

## 1.7: Human antigen R (HuR)

HuR (also called HuA) is a member of the embryonic lethal abnormal vision (ELAV)-like family of proteins, which also includes HuB, HuC, and HuD (Hinman & Lou, 2008). These proteins were first identified as the antigens against antibodies found in the nuclei of neurons of cancer patients with paraneoplastic encephalomyelitis (Graus et al, 1987; Graus et al, 2001; Hinman & Lou, 2008). HuC and HuD are expressed specifically in neurons, and HuB is expressed in neurons and gonads (Hinman & Lou, 2008). HuR is ubiquitously expressed and was first characterized in 1996 as a non-neuronal family member (Ma et al, 1996).

### 1.7.1: Regulation of gene expression by HuR

HuR is known to play an important role in various biological functions, including muscle differentiation, apoptosis, senescence, stress response, and cancer, due to its ability to bind to mRNAs that influence those processes (Abdelmohsen & Gorospe, 2010; Grammatikakis et al, 2017). Some of these mRNAs include genes involved in cell cycle, such as p21 and p53, and growth factors, such as c-myc and EGF (Abdelmohsen & Gorospe, 2010). HuR is best known for stabilizing mRNAs that contain AREs but can also influence the localization and translation of its target mRNA (Figure 1.10). The physiological importance of HuR has been shown using the $Apc^{min/+}$ mouse model of intestinal tumorigenesis where mice lacking HuR in the intestines displayed reduced

tumor burden and decreased expression of anti-apoptotic HuR mRNA targets (Giammanco et al, 2014). HuR was also found to play an important role in controlling T cell maturation (Papadaki et al, 2009). These studies demonstrate the importance of HuR in the regulation of its target mRNAs.

### 1.7.2: HuR structure

HuR is able to bind to RNA through its three RNA recognition motifs (RRMs) (Okano & Darnell, 1997). The N-terminal RRM1 and central domain RRM2 are likely to bind to class I and II ARE's based on studies of HuD and HuC and are considered essential for its function (Wang & Tanaka Hall, 2001). Structural studies on the C-terminal RRM3 have found that RRM3 is involved in HuR oligomerization and binding of U-rich elements rather than AREs (Scheiba et al, 2014; Toba & White, 2008). HuR also has a hinge region between RRM2 and RRM3 designated as HNS or "HuR Nucleocytoplasmic Shuttling" domain (Fan & Steitz, 1998).

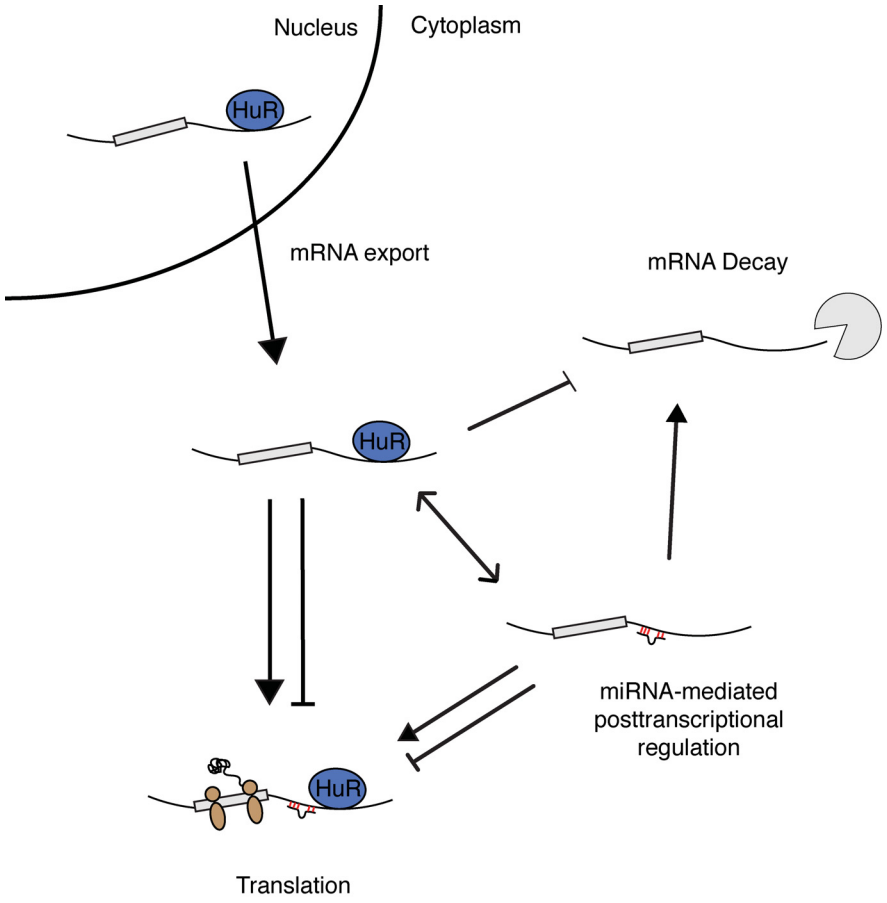



**Figure 1.10: Mechanisms of HuR-mediated posttranscriptional regulation.** HuR has been shown to post-transcriptionally regulate gene expression at different levels. As a shuttling protein, HuR can bind to its target mRNA in the nucleus and export them to the cytoplasm. HuR can stabilize mRNA by protecting them decay machinery. HuR can either inhibit or enhance the level of translation of its mRNA target. HuR is also known to collaborate and compete with miRNA to regulate an mRNA by inhibiting or enhancing translation or promoting mRNA decay.

### 1.7.3: Regulation of HuR function

The function of HuR is regulated primarily at the level of localization by numerous posttranslational modifications, including phosphorylation, methylation, ubiquitination, and neddylation, and by association with protein partners, including pp32, acidic protein rich in leucine (APRIL), transportin-2, and SET$\alpha$ and $\beta$ (Brennan et al, 2000; Grammatikakis et al, 2017). By increasing or decreasing cytoplasmic localization, HuR can influence access of its target mRNA to translational machinery to produce protein.

At least twenty-one residues of HuR have been shown to be modified, resulting in various effects on HuR function, and has been recently reviewed (Grammatikakis et al, 2017). An example of a key modulator of HuR is the cell cycle checkpoint kinase Chk2, which can phosphorylate HuR at residues serine 88, 100, and threonine 118 resulting in enhancement of association with occludin mRNA (Yu et al, 2011). Phosphorylation can also affect the localization of HuR. The G2-phase kinase Cdk1 phosphorylates HuR on residue Ser 202 and increases its association with 14-3-3, which leads to the nuclear localization of pS-HuR (Kim et al, 2008). HuR was also reported to be PARylated by PARP1 at residue D226, resulting in enhanced nucleocytoplasmic shuttling and mRNA binding, promoting mRNA stability (Ke et al, 2017). Interestingly, D226 is also the cleavage site for HuR, which plays an important role in both apoptosis and muscle differentiation (Beauchamp et al, 2010; Mazroui et al, 2008; von Roretz & Gallouzi, 2010). It is clear the posttranslational modifications of HuR have an impact on its function.

## 1.8: Thesis Summary

Muscle wasting is a debilitating disease that results in reduced quality of life and increased morbidity and mortality (Cohen et al, 2015). Pro-inflammatory cytokines are well accepted as key factors in triggering muscle wasting observed in patients with disease such as cancer. Despite the knowledge of the impact of skeletal muscle loss on quality of life and survival, treatment options are still limited and our understanding of the molecular mechanisms that leads to the rapid wasting of skeletal muscle remains incomplete. In this thesis I have aimed to identify novel mechanisms involved in promoting cytokine-induced muscle wasting. In chapter 2, I identify a mechanism through which the signaling molecule STAT3 is activated on its key tyrosine residue by IFN$\gamma$ and TNF$\alpha$ treatment independently of IL-6 and how it relies on NF-$\kappa$B for translocation to the nucleus. This study identifies STAT3 as a down-stream factor involved in promoting muscle wasting by multiple cytokines and may explain why experimental therapies that target one cytokine are not very effective. Following this, I investigated how posttranscriptional regulatory mechanisms are involved in regulating STAT3 under cachectic conditions (chapter 3). I found that STAT3 mRNA is regulated by HuR at the level of translation during cytokine-induced muscle wasting. Furthermore, I also identify that HuR negates the action of miR-330 to maintain elevated STAT3 expression under these condition. These studies advance our knowledge on the role of STAT3 during IFN$\gamma$/TNF$\alpha$-induced muscle wasting and suggest a key posttranscriptional role for HuR in promoting STAT3 expression under these conditions.

## CHAPTER 2

## STAT3 Promotes IFNγ/TNFα-induced muscle wasting in an NF-κB-dependent and IL-6-independent manner

## 2.1: Rationale

The activation of STAT3 has been demonstrated to be a key step in promoting IL-6 dependent muscle wasting. However, pharmacologic inhibition of IL-6 has proven ineffective at preventing cancer-induced muscle wasting in clinical trials. Cachexia-associated muscle wasting is associated with many other factors including the pro-inflammatory cytokines TNF$\alpha$ and IFN$\gamma$, each also shown to activate STAT3. The ability of many different pro-cachectic factors that are present during cachexia to induce STAT3 phosphorylation may explain why targeting only IL-6 was unable to prevent muscle loss. These studies raise the possibility that STAT3 may be a common downstream effector of muscle wasting induced by numerous other cytokines such as IFN$\gamma$/TNF$\alpha$. Therefore, I investigated whether STAT3 is involved in promoting IFN$\gamma$/TNF$\alpha$-induced muscle wasting.

# STAT3 Promotes IFNγ/TNFα-induced muscle wasting in an NF-κB-dependent and IL-6-independent manner

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This research was originally published as: STAT3 Promotes IFNγ/TNFα-induced muscle wasting in an NF-κB-dependent and IL-6-independent manner. *EMBO Molecular Medicine*. 2017, 9(5): 622-637. 

## 2.2: Abstract

Cachexia is a debilitating syndrome characterized by involuntary muscle wasting that is triggered at the late stage of many cancers. While the multifactorial nature of this syndrome and the implication of cytokines such as IL-6, IFNγ, and TNFα is well established, we still do not know how various effector pathways collaborate together to trigger muscle atrophy. Here, we show that IFNγ/TNFα promote the phosphorylation of STAT3 on Y705 residue in the cytoplasm of muscle fibers by activating JAK kinases. Unexpectedly, this effect occurs both *in vitro* and *in vivo* independently of IL-6, which is considered as one of the main triggers of STAT3-mediated muscle wasting. pY-STAT3 forms a complex with NF-κB that is rapidly imported to the nucleus where it is recruited to the promoter of the *iNos* gene to activate the iNOS/NO pathway, a well-known downstream effector of IFNγ/TNFα-induced muscle loss. Together these findings show that STAT3 and NF-κB respond to the same upstream signal and cooperate to promote the expression of pro-cachectic genes, the identification of which could provide effective targets to combat this deadly syndrome.

## 2.3: Introduction

Cancer-related cachexia is a debilitating syndrome characterized by the progressive loss of body weight that is triggered in part by an involuntary loss of skeletal muscle mass, referred to as muscle wasting (Argiles et al, 2016; Dodson et al, 2011; Ma et al, 2012). Cachexia is associated with anorexia, fatigue, skeletal muscle weakness, and an overall reduced quality of life (Argiles et al, 2014a; Argiles et al, 2014b; Tisdale, 2009). Unlike muscle atrophy caused by starvation or physical inactivity, muscle wasting in cachectic patients cannot be reversed by nutritional supplementation (Argiles et al, 2013; Fearon, 2011). Furthermore, the development of cachexia is a strong predictor of poor treatment outcome and mortality for individuals afflicted with cancer, and is estimated to be a direct cause of 20% of patient death (Muscaritoli et al, 2015). Cancer-cachexia is largely considered to be an end-of-life condition. Despite this, there is no standard treatment option available to prevent or treat cachexia. This highlights the importance of delineating the pro-cachectic mechanism of action in order to identify targets to combat this deadly syndrome.

The primary catabolic mediators of cachexia include proinflammatory cytokines such as tumor necrosis factor alpha (TNF$\alpha$), interferon gamma (IFN$\gamma$) and interleukin-6 (IL-6) (Argiles et al, 2013; Cohen et al, 2015; Hall et al, 2011). Numerous studies have been dedicated to develop pharmacological inhibitors to interfere with their pro-cachectic effects both in animals and humans. However, the targeted inhibition of these cytokines, specifically in human, was not efficacious. For example, the use of anti-TNF$\alpha$

inhibitors not only had limited effect on the progression of this condition but also has been associated with unwanted side effects (Jatoi et al, 2010a; Wiedenmann et al, 2008). Additionally, recent clinical trials using a monoclonal anti-IL-6 antibody on patients with lung cancer-induced muscle wasting showed a reversal of anorexia, fatigue, and anemia, but did not prevent the loss of lean body mass (Bayliss et al, 2011). While no anti-IFNγ therapy has been tested in humans so far, some studies using antibodies against this cytokine have shown some successes in interfering with cancer-induced muscle loss in mice (Langstein et al, 1991; Matthys et al, 1991). Inhibition of cytokines may also impinge on their primary roles in the immune response. Indeed, it has been reported that the prolonged suppression of cytokines such as IL-6 or TNFα can result in increased risk of infection and delayed wound healing (Jones et al, 2011; McFarland-Mancini et al, 2010). The limited success of these mono-therapeutic approaches underscores the multifactorial nature of cachexia and highlights the possibility that its pathology could result from the combined activation of common downstream effector pathways.

During the last few decades, several downstream signaling pathways and their effectors have been identified and linked to cytokine-induced muscle loss (Cohen et al, 2015). It has been shown that TNFα in collaboration with IFNγ mediates its pro-cachectic effects through the activation of the NF-κB (Nuclear transcription Factor kappa B) pathway, which promotes the expression of several target genes (Argiles et al, 2016; Dahlman et al, 2010; Fearon et al, 2012; Grivennikov & Karin, 2010; Hall et al,

2011; Ma et al, 2012). Others and we have demonstrated that the inducible nitric oxide (NO) synthase (iNOS) enzyme is one of the main effectors of the TNFα/NF-κB-induced muscle wasting (Buck & Chojkier, 1996; Di Marco et al, 2012; Di Marco et al, 2005). The upregulation of the iNOS/NO pathway correlates with a dramatic decrease in general translation and the loss of promyogenic factor such as MyoD and Myogenin (Hall et al, 2011; Ma et al, 2012). More recently, several studies have indicated that IL-6 triggers muscle atrophy by activating the STAT3 (Signal transducer and activator of transcription 3) pathway (Bonetto et al, 2012; Bonetto et al, 2011; Sala & Sacco, 2016; Zimmers et al, 2016). IL-6 activates signal transduction by binding to the IL-6 receptor alpha-chain and the common receptor subunit gp130. The gp130-associated Janus kinases (JAKs) become activated and mediate the phosphorylation of STAT3 protein on its key tyrosine 705 (Y705) residues. This JAK-mediated phosphorylation allows STAT3 to dimerize and bind to DNA to promote the transcription of several pro-cachectic genes (Bonetto et al, 2012; Bonetto et al, 2011; Sala & Sacco, 2016; Zhang et al, 2009b; Zimmers et al, 2016). Interestingly, IL-6 is not the only activator of STAT3 during muscle wasting. Treating IL6 -/- mice with lipopolysaccharides (LPS), a component of Gram-negative bacteria, also leads to phosphorylation of STAT3 (Bonetto et al, 2012). While these observations raise the possibility that STAT3 can trigger muscle wasting independently of IL-6, a STAT3-mediated and IL-6 independent molecular mechanism remains elusive.

Several studies have shown that NF-κB and STAT3 can collaborate together to mediate cell response to various extracellular challenges. For example, unphosphorylated STAT3 was shown to associate with the p65 subunit of NF-κB to promote the transcription of the serum amyloid A (SSA) gene, the expression of which is associated with serious complications of inflammatory diseases, such as rheumatoid arthritis (RA) and juvenile inflammatory arthritis (Gillmore et al, 2001; Hagihara et al, 2005). It has also been shown that TNFα activates STAT3 in various cell systems in an NF-κB-dependent manner (Guo et al, 1998; Lee et al, 2013; Snyder et al, 2014). TNFα stimulates metastatic pathways in breast cancer cells by triggering the formation of the STAT3-NF-κB complex, which in turn, upregulates the transcription of the actin-bundling protein fascin (Snyder et al, 2014). Therefore, it is possible that the failure of the anti-TNFα and -IL-6 therapies to prevent muscle wasting is due to the fact that both cytokines redundantly activate a common downstream effector such as STAT3. In this work, we show that STAT3 is required for the IFNγ/TNFα-induced muscle wasting and that these effects depend on the collaboration between STAT3 and NF-κB pathways. Interestingly, while this STAT3-NF-κB mediated effect occurs independently of IL6, NF-κB is required for the translocation of STAT3 to the nucleus as well as for the activation of the iNOS/NO pathway, one of the main effectors of IFNγ/TNFα-induced muscle wasting.

## 2.4: Results

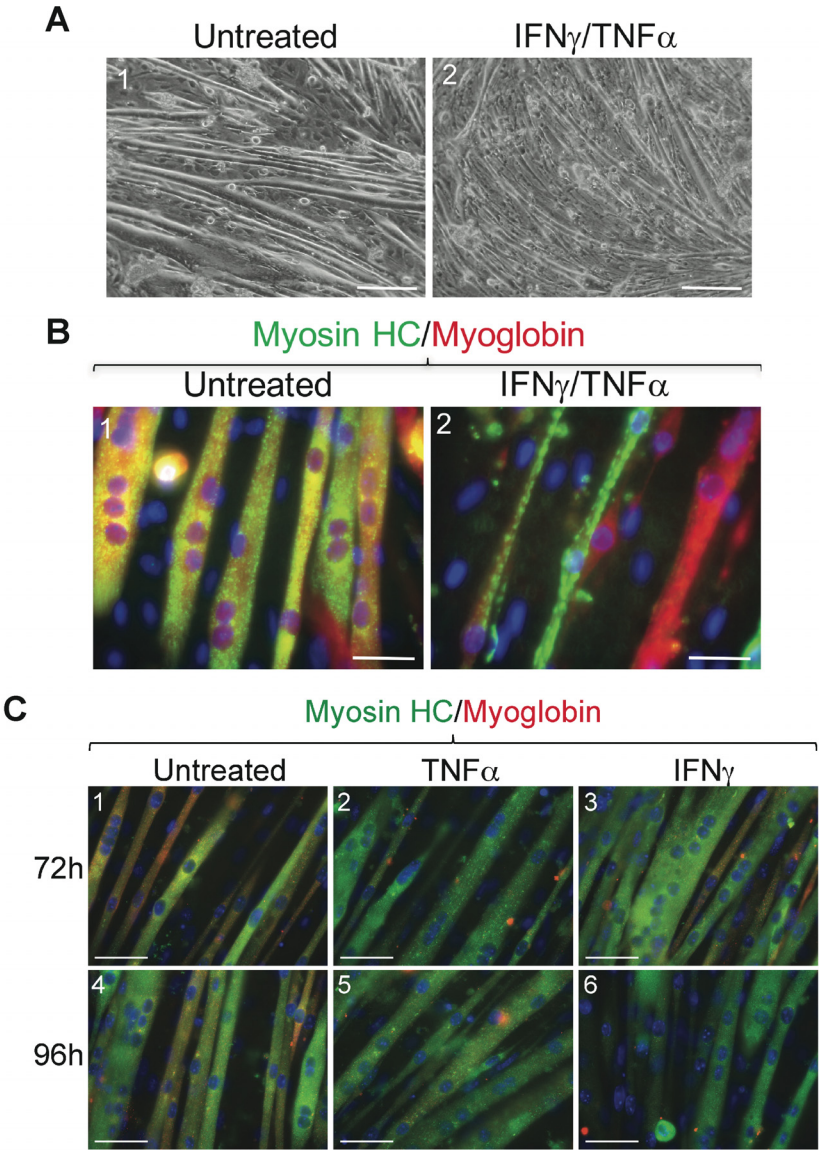
### 2.4.1: TNFα and IFNγ activate STAT3 in muscle fibers in an IL-6 – independent manner

As a first step in assessing the role of the STAT3 pathway in IFNγ and TNFα-induced muscle wasting, we treated fully differentiated muscle fibers with these two cytokines over various period of times and assessed muscle wasting as well as STAT3 activation (Figs. 2.1, 2.2, 2.3, and 2.4). While as expected (Guttridge et al, 2000), IFNγ or TNFα separately did not promote muscle wasting of C2C12 myotubes (Fig. 2.1C), both cytokines together did trigger a dramatic loss of muscle fibers within 72h of treatment (Figs. 2.1A-B) (Di Marco et al, 2012; Di Marco et al, 2005). The observed IFNγ/TNFα-induced muscle wasting was not due to cell death by apoptosis, since these cytokines failed to trigger the cleavage of caspase-3, a well-known marker of caspase-mediated apoptosis (Beauchamp et al, 2010; von Roretz et al, 2013), in C2C12 myotubes even after 72h of treatment (Fig. 2.2).

Next, we assessed the activation of STAT3 in muscle fibers exposed to both cytokines as described above. We followed the phosphorylation status of the tyrosine (Y) 705 and serine (S) 727 residues, two well-characterized phosphorylation sites of STAT3 (Grivennikov & Karin, 2010). While IFNγ and TNFα did not affect the level of pS-STAT3 when compared to untreated muscle fibers, a 30 min treatment with these two cytokines was sufficient to significantly enhance the levels of pY-STAT3 in C2C12 myotubes (Fig. 2.3). Similar results were obtained when we assessed pY-STAT3 levels

in primary muscle fibers treated with IFNγ/TNFα (Fig. 2.4). Together these observations raise the possibility that the phosphorylation of STAT3 on its Y705 residue is an integral part of the downstream signaling pathway used by IFNγ/TNFα to trigger muscle wasting.

**Figure 2.1: IFNγ and TNFα treatment induced muscle wasting together but not separately.** C2C12 cells were grown to confluency than induced for differentiation for four days to form fully differentiated myotubes. These myotubes were exposed or not to IFNγ/TNFα for 72h or 96h. ***(A)*** Phase contrast images of cultured C2C12 myotubes treated with or without IFNγ/TNFα for 72h. Scale bar = 200μm. Images shown are representative of n=4 independent experiments. ***(B)*** Immunofluorescence (IF) images of C2C12 myotubes either untreated or treated with IFNγ/TNFα for 72 were stained with anti-myosin heavy-chain (MyHC; green) and anti-myoglobin (red) antibodies. Scale bar = 50μm. Images shown are representative of n=4 independent experiments. ***(C)*** IF images of C2C12 myotubes treated for 72h (panels 1-to-3) or 96h (panels 4-to-6) with either TNFα (panels 2 and 5) or IFNγ (panels 3 and 6). Treated and untreated (panels 1 and 4) myotubes were stained with Myosin heavy chain (green) and myoglobin (red) as marker for differentiated muscle cells. Images shown are representatives of n=2 independent experiments.


A
Untreated
IFNγ/TNFα
1
2
B
Myosin HC/Myoglobin
Untreated
IFNγ/TNFα
1
2
C
Myosin HC/Myoglobin
Untreated
TNFα
IFNγ
72h
96h
1
2
3
4
5
6

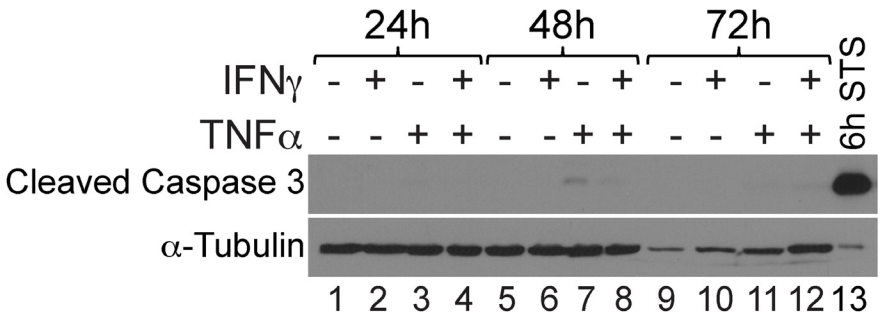



**Figure 2.2: The treatment of C2C12 myotubes with both TNFα and IFNγ for 72h does not trigger caspase 3 cleavage.** Total cell extracts were prepared from C2C12 myotubes treated with IFNγ alone, TNFα alone, or both IFNγ and TNFα for the indicated amount of time. Extracts from C2C12 treated with staurosporine for 6 hrs was also prepared and used as a positive control. Western blotting experiment was performed using these extracts with antibody specific for caspase-3 cleavage product and anti-α-tubulin antibody. The blot shown is a representative of two independent experiments. *Originally published as Appendix figure S1.*

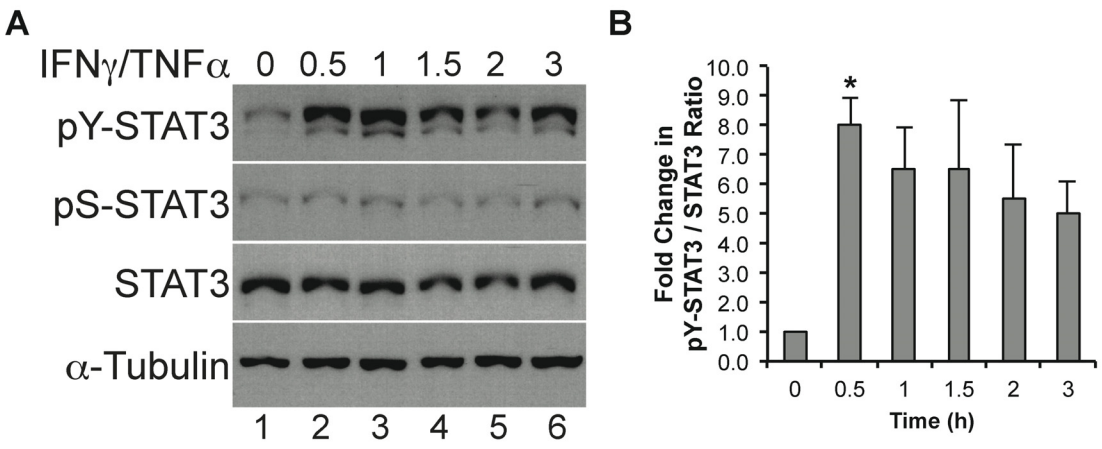



**Figure 2.3: STAT3 is phosphorylated on residue Tyr-705 during IFNγ/TNFα–induced muscle wasting.** ***(A)*** Total extracts of C2C12 myotubes treated with or without IFNγ/TNFα were used for Western blot analysis with antibodies against pY-STAT3, pS-STAT3, total STAT3, and α-tubulin. The blot shown is a representative of n=4 independent experiments. (***B)*** Densitometric quantification of pY-STAT3 signal relative to total STAT3 signal in Fig. 2.3A. Data represented as mean ± SEM (n = 4) with p-value = 0.0135 (*) by one-way ANOVA with Dunnett's post hoc test.

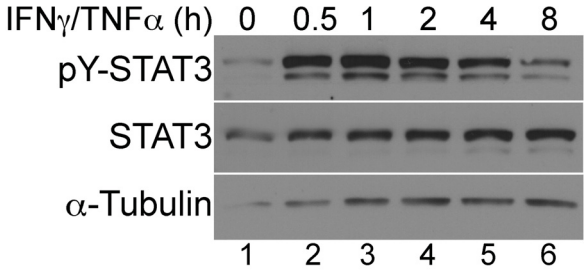



**Figure 2.4: IFNγ/TNFα rapidly triggers the phosphorylation of STAT3 on its Y705 residue (pY-STAT3) in differentiated primary muscle cells.** Total cell extracts from differentiated primary muscle cells stimulated with or without IFNγ/TNFα for 0h to 8h were used for Western blot analysis with antibodies against pY-STAT3, total STAT3, and α-tubulin. The blot shown is a representative of three independent experiments. *Originally published as Appendix figure S2.*

Work from several groups has established pY-STAT3 as a one of the key mediators in the IL-6-induced muscle atrophy (Bonetto et al, 2012; Bonetto et al, 2011; Zimmers et al, 2016). Since IFNγ/TNFα have been demonstrated to induce IL-6 expression and secretion in various cell lines (Alvarez et al, 2002; Sanceau et al, 1991), we wanted to verify whether their ability to activate STAT3 in muscle fibers depends on IL-6 expression and secretion. As a first step, we analyzed the levels of IL-6 secreted by muscle fibers treated with IFNγ/TNFα as described above. Using ELISA assay, we detected a dramatic increase in IL-6 protein levels 24h and 48h post-treatment with amounts raging from ~2ng/ml to ~14ng/ml (Fig. 2.5A). Next, we tested whether these levels of IL-6 are sufficient to induce STAT3 phosphorylation in muscle fibers. These fibers were treated with various concentration of murine recombinant IL-6 (rIL-6, ranging from 1 to 20ng/mL) that reflected the approximate concentrations detected by ELISA during the IFNγ/TNFα treatment. Western blot analysis using the anti-pY-STAT3 antibody indicated no change in the levels of pY-STAT3 with any of these concentrations of recombinant IL-6 (Figs. 2.5B-C). However, pY-STAT3 was detected only when C2C12 muscle fibers or macrophages (used as positive control) were treated with 100ng/mL of IL-6 (Fig. 2.6). Therefore, the failure of low doses of rIL-6 (20ng/ml or lower) to phosphorylate STAT3 in myotubes might be due to instability or inactivity of our rIL-6.

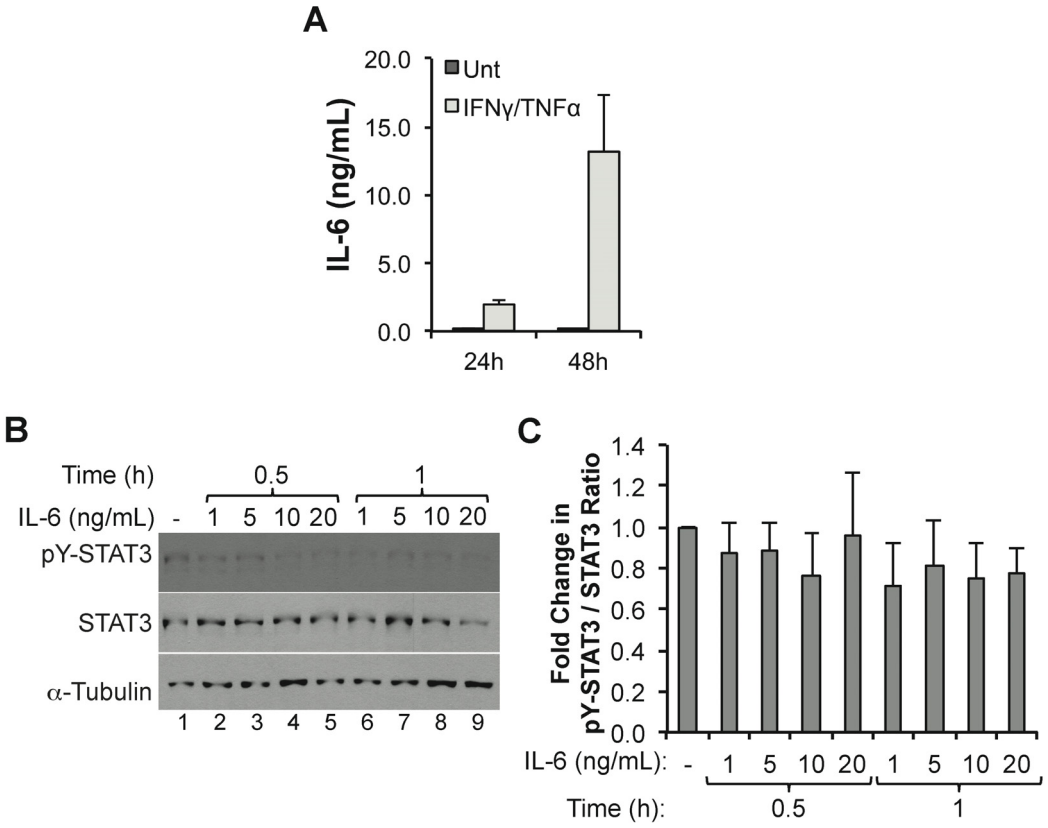



***Figure 2.5:*** **Recombinant IL-6 does not induce STAT3 phosphorylation in C2C12 myotubes at low doses.** ***(A)*** Supernatant was collected from C2C12 myotubes treated with or without IFNγ/TNFα and analysed by ELISA to determine the concentration of IL-6. Data represented as mean ± SEM (n=2). ***(B)*** Total cell extracts of C2C12 myotubes treated with or without recombinant murine IL-6 (rIL-6) were used for western blot analysis with antibodies against pY-STAT3, total STAT3, and α-tubulin. The blot shown is a representative of n=3. ***(C)*** Densitometric quantification of pY-STAT3 signal relative to total STAT3 signal from western blots in Fig. 2B. Data represented as mean ± SEM (n=3) with p-value = N.S. by two-way ANOVA with a Tukey post hoc test.

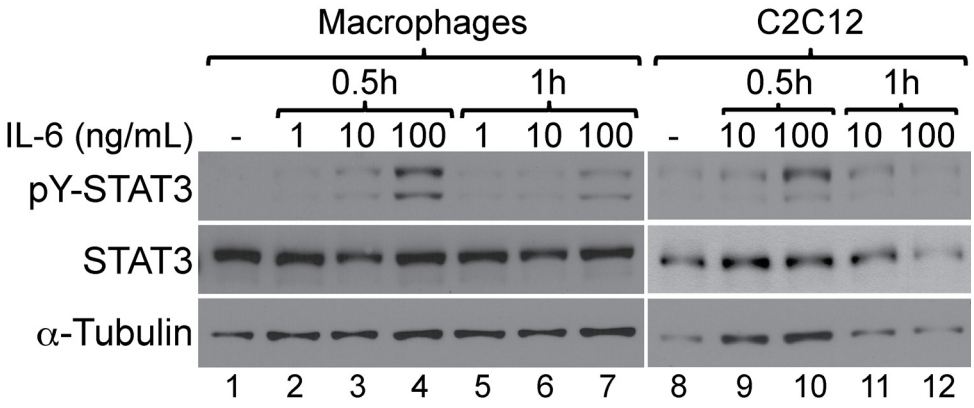


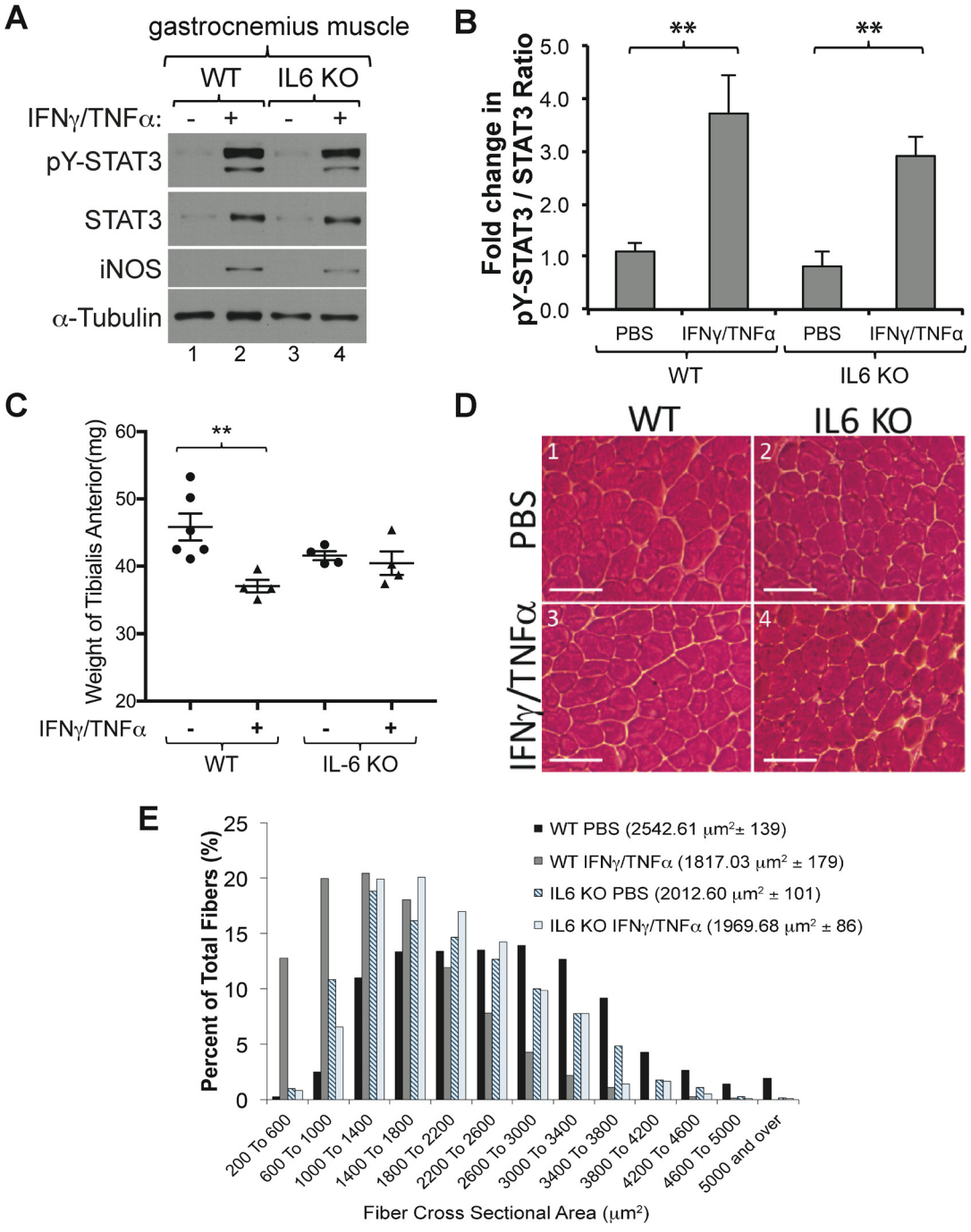

**Figure 2.6: IL-6-induced phosphorylation of STAT3 in both macrophages and C2C12 cells occurs in a dose-dependent manner.** Total cell extract from macrophages or C2C12 myotubes were treated with 10 or 100 ng/mL of recombinant mouse IL-6 for the indicated length of time were used for Western blot analysis with antibodies against pY-STAT3, total STAT3, and α-tubulin. The 100 ng/mL dose of rIL-6 induced pY-STAT3 in both macrophages and C2C12 myotubes while the 10 ng/mL dose was only able to induce pY-STAT3 in macrophages. The blot shown is a representative of two independent experiments. *Originally published as Appendix figure S2.*

To further explore the effect of IL-6 on STAT3 phosphorylation, we assessed pY-STAT3 levels in the muscle of IL-6 knockout (KO) and wild-type mice, which were intramuscularly injected with IFNγ/TNFα or saline for 5 days as described (Di Marco et al, 2012). Interestingly, we observed a significant induction of pY-STAT3 in the gastrocnemius muscle of both wild-type and IL-6 KO animals (Fig. 2.7A, B). However, unlike in wild-type mice, IFNγ/TNFα failed to trigger the wasting of skeletal muscle of IL-6 KO mice as evidenced by the weight and cross-section analyses of the tibialis anterior of these animals (Figs. 2.7C-E). Therefore, our data demonstrate that in muscle fibers IFNγ/TNFα activate STAT3 by triggering the phosphorylation of its Y705 residue in an IL-6-independent manner.

### 2.4.2: IFNγ/TNFα use the JAK signaling pathway to phosphorylate STAT3 and induce muscle wasting

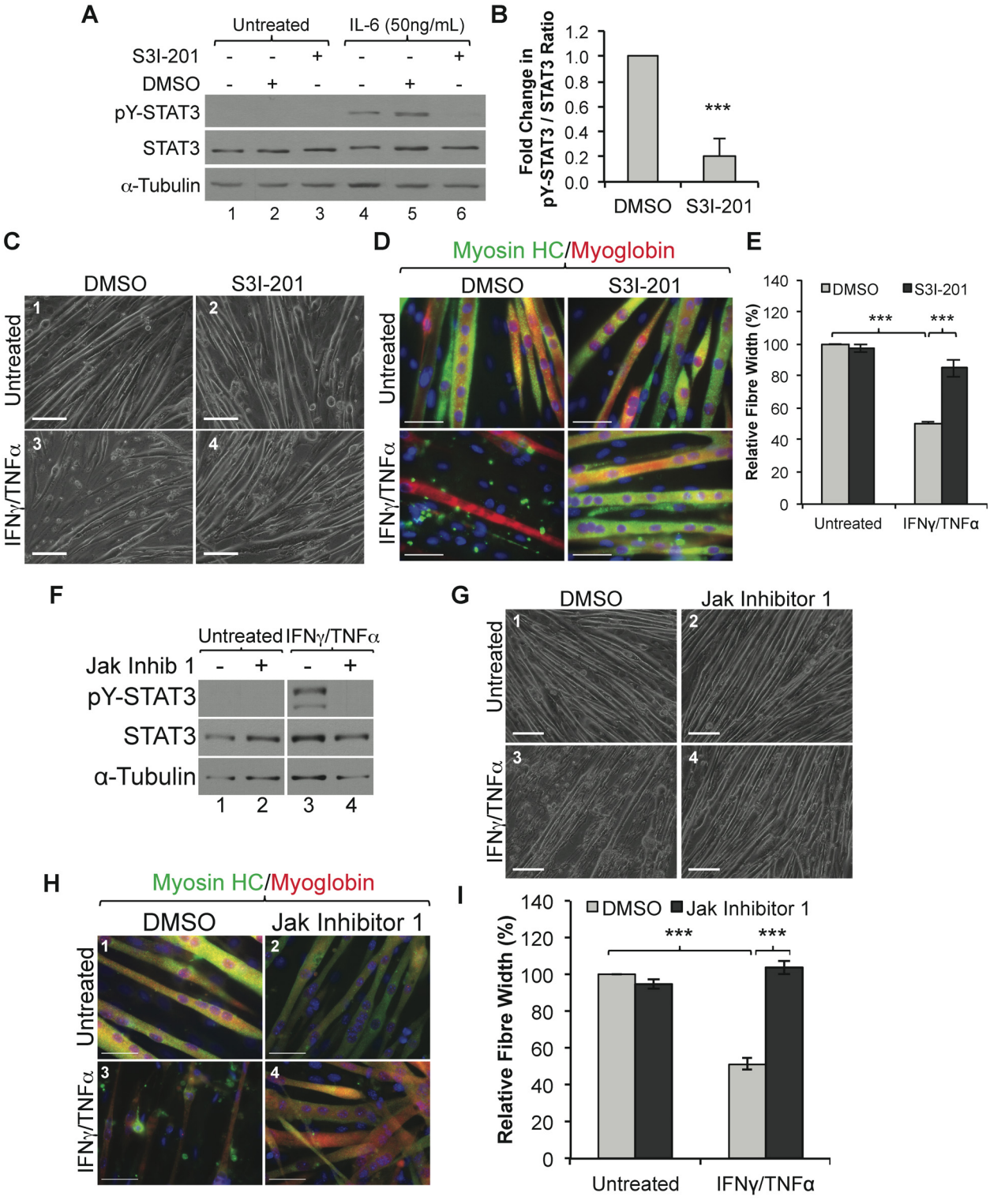
Next, we assessed the possibility that STAT3 activation could play an important part in IFNγ/TNFα-induced muscle wasting. Since it is well-established that knocking down STAT3 expression prevents myogenesis *in vitro* (Sun et al, 2007; Wang et al, 2008), to assess this possibility, we used the STAT3 inhibitor S3I-201 on the C2C12 myotubes. As has been shown in other cell lines (Pang et al, 2010), we observed that this inhibitor was effective in interfering with IL-6-mediated phosphorylation of STAT3 on its Y705 residue in muscle cells (Figs. 2.8A-B).

**Figure 2.7: IFNγ/TNFα induces STAT3 phosphorylation in an IL-6-independent manner.** ***(A)*** Wild-type (WT) and IL-6 KO mice were intramuscularly injected with IFNγ/TNFα for five consecutive days and sacrificed on the sixth day. Gastrocnemius muscle was homogenized and used for Western blot analysis with antibodies against pY-STAT3, total STAT3, iNOS, and α-tubulin. The blot shown is a representative of n=3 mice. ***(B)*** Densitometric quantification of pY-STAT3 signal relative to total STAT3 signal from western blots in Fig. 2.7A. Data represented as mean ± SEM (n=3) with p = 0.0033 (**) and 0.0091 (**) by two-way ANOVA with a Tukey post hoc test. ***(C)*** The tibialis anterior (TA) muscle weight significantly decreased in WT animals but not in IL-6 KO animals. Data represented as mean ± SEM for n = 6 (WT PBS), 4 (WT IFNγ/TNFα), 4 (IL-6 KO PBS), and 4 (IL-6 KO IFNγ/TNFα) with p-value = 0.0081 (**) by two-way ANOVA with a Tukey post-hoc test. ***(D)*** Image of a representative section of the TA muscle from wild type and IL-6 KO mice stained with hematoxylin and eosin. Scale bar = 100 μm. ***(E)*** The cross-sectional areas (CSA) of TA muscle from Fig. 2.7D are represented as a frequency histogram from *n*=2 mice. Nine hundred fibers were analysed for each animal. The mean CSA ± SD is indicated in the legend of the histogram.


A
gastrocnemius muscle
WT
IL6 KO
IFNγ/TNFα: - + - +
pY-STAT3
STAT3
iNOS
α-Tubulin
1 2 3 4
B
Fold change in pY-STAT3 / STAT3 Ratio
5.0
4.0
3.0
2.0
1.0
0.0
**
**
PBS
IFNγ/TNFα
PBS
IFNγ/TNFα
WT
IL6 KO
C
Weight of Tibialis Anterior(mg)
60
50
40
30
20
**
IFNγ/TNFα - + - +
WT
IL-6 KO
D
WT
IL6 KO
PBS
IFNγ/TNFα
1
2
3
4
E
Percent of Total Fibers (%)
25
20
15
10
5
0
WT PBS (2542.61 μm² ± 139)
WT IFNγ/TNFα (1817.03 μm² ± 179)
IL6 KO PBS (2012.60 μm² ± 101)
IL6 KO IFNγ/TNFα (1969.68 μm² ± 86)
200 To 600
600 To 1000
1000 To 1400
1400 To 1800
1800 To 2200
2200 To 2600
2600 To 3000
3000 To 3400
3400 To 3800
3800 To 4200
4200 To 4600
4600 To 5000
5000 and over
Fiber Cross Sectional Area (μm²)

Phase contrast images as well as immunofluorescence experiments on muscle fibers exposed or not to IFNγ/TNFα for 72hrs showed that S3I-201 significantly reduced the muscle fiber loss that is normally observed with these two cytokines (Figs. 2.8C-E). To identify the signaling pathway behind the IFNγ/TNFα mediated activation of STAT3, we assessed the involvement of the Janus kinases (JAK's), an upstream non-receptor tyrosine kinases that, in response to IL-6 and other stimuli, phosphorylate STAT3 on its Y705 residue (Grivennikov & Karin, 2010). We observed that the Jak kinase inhibitor 1 (Pedranzini et al, 2006) was sufficient to prevent both STAT3 phosphorylation (Fig. 2.8F) and the IFNγ/TNFα-induced muscle atrophy (Figs. 2.8G-H). The widths of the muscle fibers simultaneously treated with a Jak inhibitor and IFNγ/TNFα were also significantly higher than the IFNγ/TNFα-treated DMSO control fibers (Fig. 2.8I). These results indicate that IFNγ/TNFα trigger muscle wasting by activating the JAK signaling pathway, which in turn phosphorylates STAT3 on its Y705 residue.

**Figure 2.8**: ***Inhibition of the Jak/STAT3 pathway prevents IFNγ/TNFα-induced muscle wasting. (A)*** Total cell extracts from proliferating C2C12 treated with STAT3 inhibitor S3I-201 then with recombinant murine IL-6 for 30 min were used for western blot analysis with antibodies against pY-STAT3, total STAT3, and α-tubulin. The blot shown is a representative of n=3 experiments. ***(B)*** Densitometric quantification of pY-STAT3 signal relative to total STAT3 signal of western blots in Fig 2.8A. Data represented as mean ± SEM (n = 3) with a p-value = 0.00439 (**) by two-tailed, unpaired Student's t-test. ***(C, D)*** Phase contrast (C) and IF images (D) of C2C12 myotubes treated with or without IFNγ/TNFα and with STAT3 inhibitor S3I-201 or DMSO as a control. IF of C2C12 myotubes were stained with antibodies against endogenous myosin heavy chain (green), myoglobin (red), and counter stained with DAPI for nuclei (blue). Scale bar is 200μm and 50 μm for images in C and D, respectively. Images shown are representatives of n=3 experiments. ***(E)*** Quantification of average myotube width from Fig 2.8D. The average myotube widths are presented as percentage change ± SEM (n=3) relative to the untreated, with p-values = $5.6 \times 10^{-5}$ (***) and $7.56 \times 10^{-4}$ (***) by two-way ANOVA with Tukey post-test. ***(F)*** Total extracts from C2C12 myotubes pre-treated with a Jak inhibitor then with IFNγ/TNFα for 30min was used for western blotting analysis with antibodies against pY-STAT3, total STAT3, and α-tubulin. The blot shown is a representative of n=3 experiments. ***(G, H)*** Phase contrast images (G) and IF (H) of C2C12 myotubes treated with or without a Jak inhibitor or DMSO as a control and with or without IFNγ/TNFα for 72h. *(H)* IF images of cultured C2C12 myotubes stained for myosin heavy chain (green), myoglobin (red), and counter stained with DAPI for nuclei (blue). Scale bar is 200μm and 50 μm for images in G and H, respectively. Images shown are representative of n=3 experiments. ***(I)*** Quantification of C2C12 myotube widths from Fig. 2.8H were determined as describe in Fig 3D. Data represented as average ± SEM (n=3), with ***p value = $1.8 \times 10^{-5}$ and $2.3 \times 10^{-5}$ by two-way ANOVA with Tukey post-test.


A
Untreated
IL-6 (50ng/mL)
S3I-201
DMSO
pY-STAT3
STAT3
α-Tubulin
1 2 3 4 5 6
B
Fold Change in pY-STAT3 / STAT3 Ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
DMSO
S3I-201
C
DMSO
S3I-201
Untreated
IFNγ/TNFα
1
2
3
4
D
Myosin HC/Myoglobin
DMSO
S3I-201
Untreated
IFNγ/TNFα
E
DMSO
S3I-201
Relative Fibre Width (%)
140
120
100
80
60
40
20
0
***
***
Untreated
IFNγ/TNFα
F
Untreated
IFNγ/TNFα
Jak Inhib 1
- + - +
pY-STAT3
STAT3
α-Tubulin
1 2 3 4
G
DMSO
Jak Inhibitor 1
Untreated
IFNγ/TNFα
1
2
3
4
H
Myosin HC/Myoglobin
DMSO
Jak Inhibitor 1
Untreated
IFNγ/TNFα
1
2
3
4
I
DMSO
Jak Inhibitor 1
Relative Fibre Width (%)
140
120
100
80
60
40
20
0
***
***
Untreated
IFNγ/TNFα

### 2.4.3: pY-STAT3 promotes iNOS expression during IFNγ/TNFα-mediated muscle wasting

Previously, iNOS/NO pathway was shown as an important mediator of IFNγ/TNFα-induced muscle wasting (Di Marco et al, 2012; Di Marco et al, 2005). In addition, work from various groups has suggested that STAT3 modulates iNOS expression in non-muscle cells (Park et al, 2010; Yu et al, 2002b; Ziesche et al, 2007). Therefore, we assessed the involvement of STAT3 in IFNγ/TNFα-mediated iNOS expression in C2C12 muscle fibers. First, as expected (Di Marco et al, 2005), when used separately, TNFα but not IFNγ was able to slightly induce iNOS expression in C2C12 myotubes when used for 48h or more. However, when both cytokines were used together for the same period of time, they synergistically induced a high expression level of iNOS in these muscle fibers (Fig. 2.9).

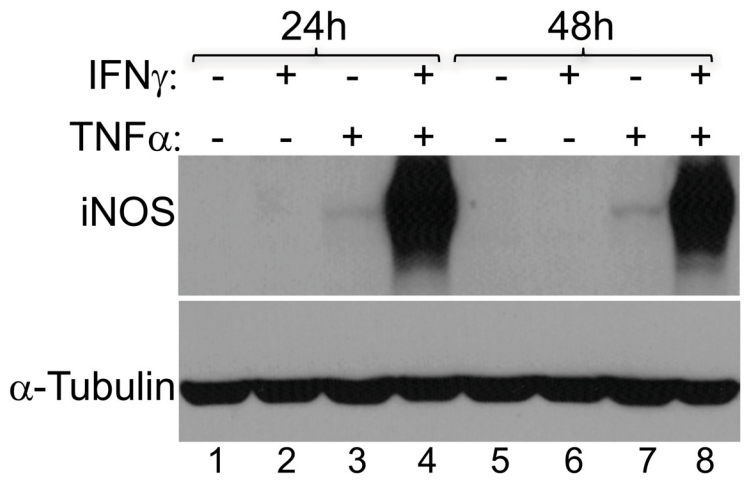



**Figure 2.9**: ***IFNγ and TNFα synergistically induce the expression of iNOS in C2C12 muscle fibers.*** Total cell extracts were prepared from C2C12 myotubes treated with IFNγ/TNFα for the indicated amount of time. These extracts were used for Western blot analysis with antibodies against iNOS and α-tubulin. The blot shown is a representative of two independent experiments. *Originally published as Appendix figure S4.*

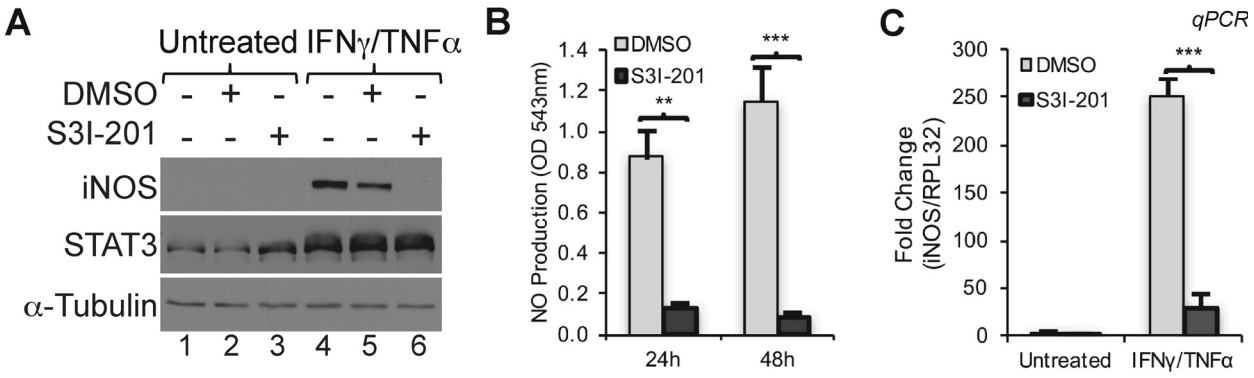



**Figure 2.10**: ***STAT3 promotes the expression of iNOS in IFNγ/TNFα-treated myotubes***. ***(A)*** C2C12 myotubes treated with or without IFNγ/TNFα and with STAT3 inhibitor S3I-201 or DMSO as a control. Total cell extracts from these muscle fibers were used for western blot analysis with antibodies against iNOS, STAT3, and α-tubulin as a loading control. The blot shown is representative of n=3 experiments. ***(B)*** NO levels were measured in supernatant from the myotubes described in Fig. 4A. Data represented as mean ± SEM (n = 3) with p-values = 0.0013 (**) and 0.0006 (***) by two-tailed, unpaired Student's t-test. ***(C)*** Total mRNA from C2C12 myotubes treated with IFNγ/TNFα and with the STAT3 inhibitor S3I-201 or DMSO as a control was used for RT-qPCR analysis of iNOS (*Nos2*) and *Rpl32* mRNA. Data represented as mean ± SEM (n=3) with p value = 0.0007 (***) by two-tailed, unpaired Student's t-test.

Next, we observed that S3I-201 completely inhibited iNOS expression and NO production in muscle fibers treated with IFNγ/TNFα (Figs. 2.10 and 2.11). Moreover, an shRNA that reduced STAT3 expression by ~40% also decreased iNOS protein levels by >35% in muscle fibers treated with IFNγ/TNFα (Fig 2.12A). To further explore the importance of STAT3 phosphorylation, we assessed the effect of two STAT3 mutants

on iNOS expression. We observed that, while expressing a constitutively-active isoform of STAT3 (STAT3-C) (Bromberg & Darnell, 1999) in C2C12 cells failed, on its own, to promote iNOS expression (Fig. 2.12B, lanes 5-6), this isoform dramatically enhanced the level of IFNγ/TNFα-induced iNOS protein. However, the expression of a Tyr 705 to Phe (Y705F)-STAT3 mutant (Wen & Darnell, 1997) dramatically decreased iNOS expression under these conditions (Fig. 2.12B). Additionally, using iNOS KO mice, we observed that although these animals are protected against IFNγ/TNFα-induced loss of muscle mass (Figs. 2.12C-F), a high level of pY-STAT3 was detected in their gastrocnemius muscle (Fig. 2.12C). These data show that, while activating STAT3 alone is not sufficient to trigger iNOS expression, STAT3 collaborates with other signaling pathways to promote IFNγ/TNFα-induced iNOS and muscle atrophy. Moreover, these observations also indicate that iNOS is a key downstream effector of the IFNγ/TNFα-induced muscle wasting *in vivo*.

It is known that in addition to the NF-κB binding sites, the murine iNOS promoter has binding sites for STATs, IRFs and AP-1 (Aktan, 2004). The binding of STAT3 to the murine iNOS promoter has been demonstrated to have, depending on the cell type and the stimuli, both a positive and a negative effect on the transcription of the *iNos* gene (de la Iglesia et al, 2008; Puram et al, 2012; Yu et al, 2009). We, therefore, performed ChIP coupled to qPCR experiments to determine if, similar to what has been shown before in other cell systems (Yu et al, 2002a), the effects of STAT3 on iNOS expression during IFNγ/TNFα induced muscle wasting occurs through a direct binding of STAT3 to

the iNOS promoter (Fig. 2.13A). We observed a ~2.5-fold increase in the recruitment of STAT3 and p65 subunit of NF-κB to the iNOS promoter region in IFNγ/TNFα–treated muscle fibers (Figs. 2.13B-E). The recruitment of STAT3 and p65 to the iNOS promoter were similar to those of RNA polymerase II under these conditions (Fig. 2.13F). These results indicate that in response to IFNγ/TNFα pY-STAT3 and p65 binds to the iNOS promoter to induce its transcription in muscle fibers undergoing wasting.

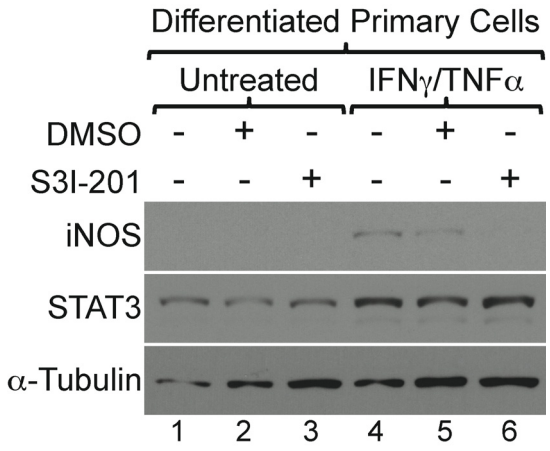



**Figure 2.11:** ***Inhibition of STAT3 activity prevents IFNγ/TNFα-induced iNOS expression in differentiated primary muscle cells.*** Total cell extracts were prepared from differentiated primary muscle cells treated with IFNγ/TNFα in the presence or absence of the STAT3 inhibitor S3I-201 > 3 days. Western blot analysis indicated that inhibiting STAT3 in primary cells blocks IFNγ/TNFα-induced iNOS expression. The blot shown is a representative of three independent experiments. *Originally published as Appendix figure S5.*

**Figure 2.12: STAT3 promotes the expression of iNOS during IFNγ/TNFα-induced muscle wasting.** ***(A)*** C2C12 cells were transfected with a plasmid expressing shRNA against STAT3 or a scramble control. (Left) Total cell extract was used for western blot analysis with antibodies against pY-STAT3, total STAT3, iNOS, and α-tubulin. Densitometric quantification of STAT3 (middle panel) and iNOS (right panel) signals relative to α-tubulin of western blots from left panel. Data represented as mean ± SD (n=2) with p value = 0.0123 (*) by two-tailed, unpaired Student's t-test. ***(B)*** C2C12 were transfected with an empty vector or a STAT3 constitutively-active (STAT3-C) mutant or a Tyr705 to Phe (Y705F) mutant-expressing plasmid and followed by treatment with TNFα alone, IFNγ alone, or both for 24h. Total cell extracts were used for western blot analysis using antibodies against pY-STAT3, total STAT3, iNOS, and α-tubulin. The blot shown is a representative of n=2 experiments. ***(C - F)*** Wild-type (WT) and iNOS KO mice were intramuscularly injected with IFNγ/TNFα for five consecutive days and sacrificed on the sixth day. *(C)* Gastrocnemius muscle was homogenized and used for Western blot analysis with antibodies against pY-STAT3, total STAT3, iNOS, and α-tubulin. The blot shown is a representative of n= 3 (WT PBS), 2 (WT IFNγ/TNFα), 3 (iNOS KO PBS), and 2 (iNOS KO IFNγ/TNFα). *(D)* The gastrocnemius muscle weight significantly decreased in WT animals but not in iNOS KO animals treated with IFNγ/TNFα. Data represented as mean ± SEM (n = 7 (WT, PBS), 4 (WT, IT), 6 (iNOS KO, PBS), 5 (iNOS KO, IT)) with p value = 0.0210 (*) by two-tailed, unpaired Student's t-test. *(E)* Image of representative sections of the gastrocnemius muscle from wild type and iNOS KO mice stained with hematoxylin and eosin. Scale bar = 100 μm. The image shown is a representative of gastrocnemius muscles from each group (n=2). *(F)* The CSA of gastrocnemius muscles from Fig 4G is represented as a frequency histogram. One thousand fibers were analysed for each group (n=2). The mean CSA ± SD is indicated in the legend of the histogram.

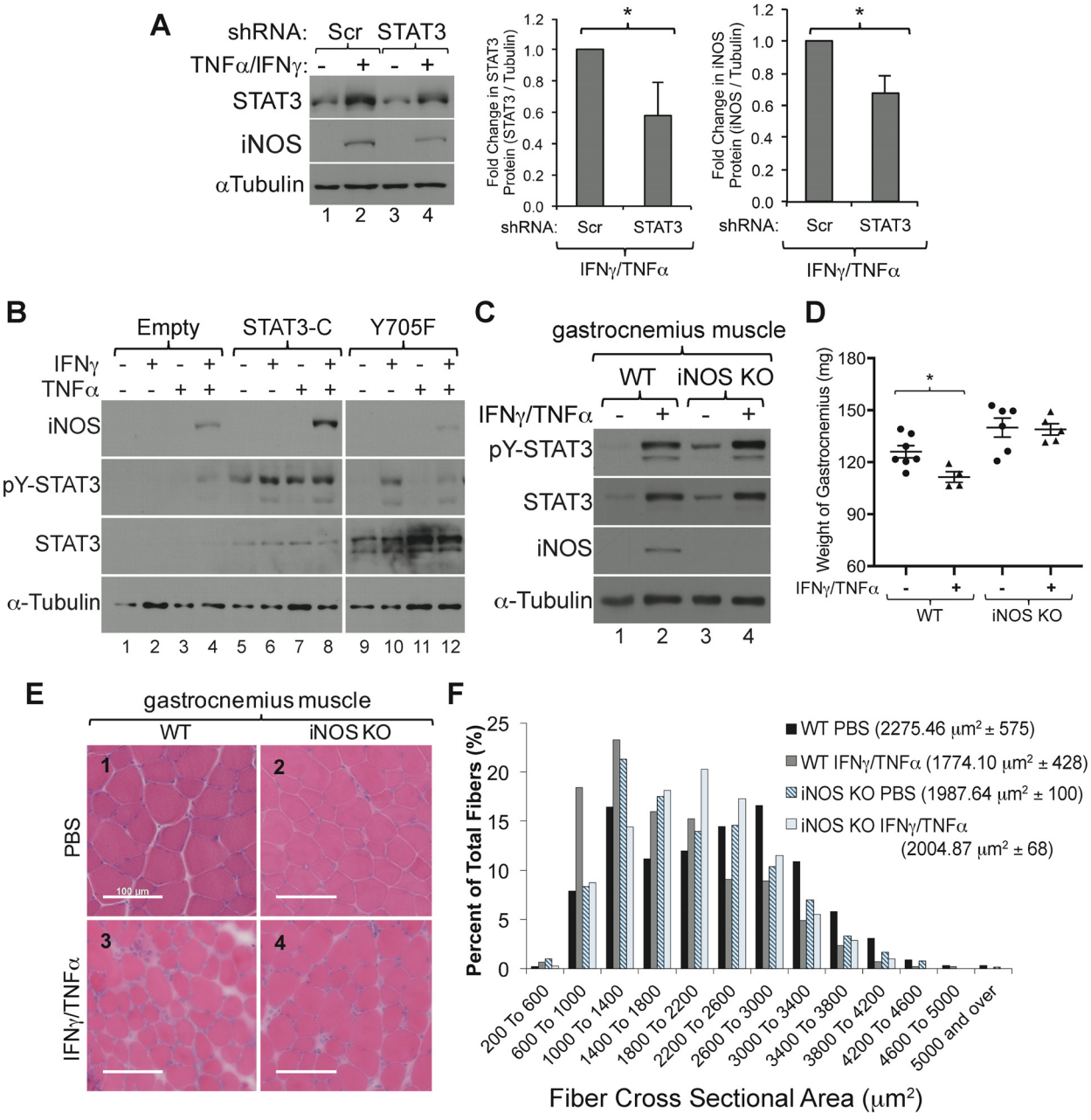
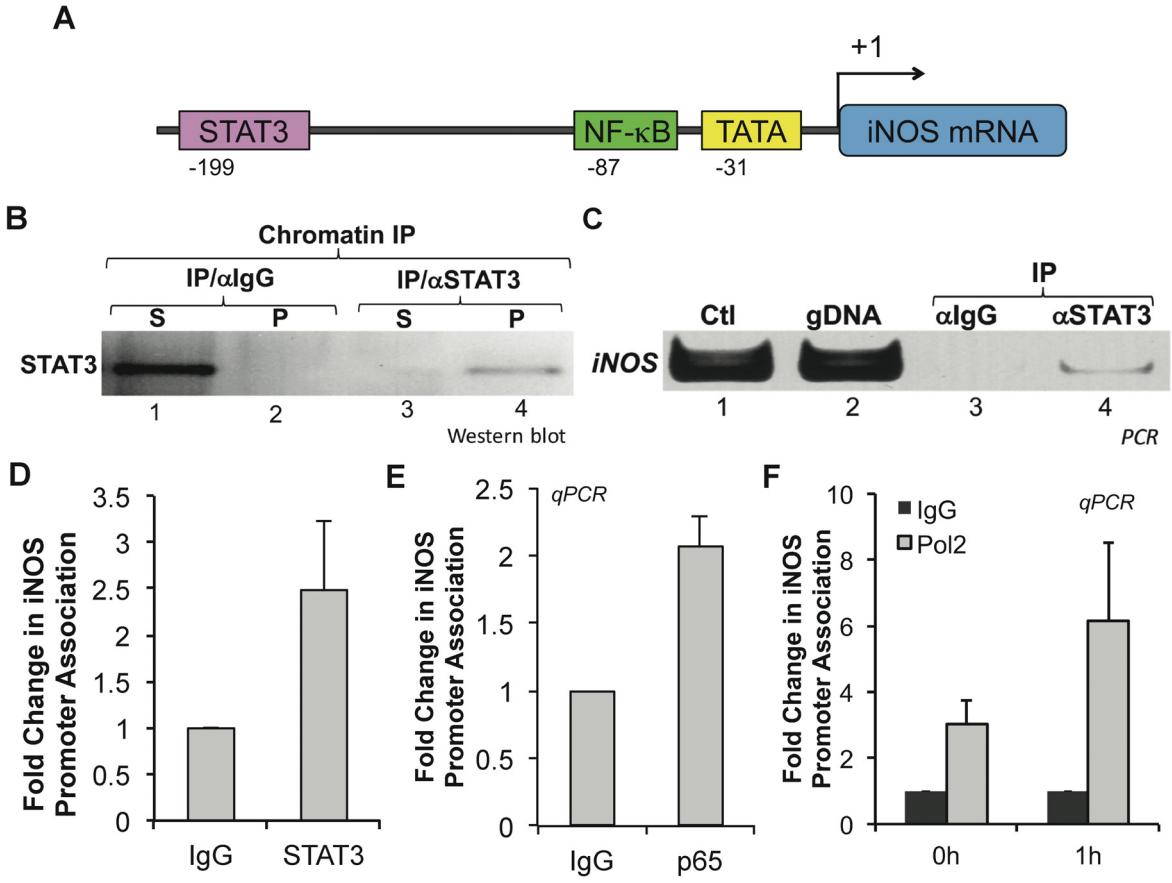

A
shRNA: Scr STAT3
TNFα/IFNγ: - + - +
STAT3
iNOS
αTubulin
1 2 3 4
Fold Change in STAT3 Protein (STAT3 / Tubulin)
Fold Change in iNOS Protein (iNOS / Tubulin)
shRNA: Scr STAT3
IFNγ/TNFα
B
Empty STAT3-C Y705F
IFNγ
TNFα
iNOS
pY-STAT3
STAT3
α-Tubulin
1 2 3 4 5 6 7 8 9 10 11 12
C
gastrocnemius muscle
WT iNOS KO
IFNγ/TNFα
pY-STAT3
STAT3
iNOS
α-Tubulin
1 2 3 4
D
Weight of Gastrocnemius (mg)
IFNγ/TNFα
WT
iNOS KO
E
gastrocnemius muscle
WT
iNOS KO
PBS
IFNγ/TNFα
100 μm
F
Percent of Total Fibers (%)
WT PBS (2275.46 μm² ± 575)
WT IFNγ/TNFα (1774.10 μm² ± 428)
iNOS KO PBS (1987.64 μm² ± 100)
iNOS KO IFNγ/TNFα
(2004.87 μm² ± 68)
200 To 600
600 To 1000
1000 To 1400
1400 To 1800
1800 To 2200
2200 To 2600
2600 To 3000
3000 To 3400
3400 To 3800
3800 To 4200
4200 To 4600
4600 To 5000
5000 and over
Fiber Cross Sectional Area (μm²)

***Figure 2.13*: *STAT3 and NF-κB bind to the iNOS promoter* during cytokine-induced muscle wasting.** ***(A)*** Diagram of the iNOS promoter depicting known binding sites for STAT3 and NF-κB. ***(B)*** Chromatin prepared from C2C12 myotubes treated with or without IFNγ/TNFα was used for chromatin immunoprecipitation (ChIP). Supernatants (S) and pellets (P) of the ChIP were used for western blot with antibodies against STAT3α or p65 or IgG control. ***(C)*** DNA from ChIP assay with anti-STAT3 (STAT3α) and IgG antibodies was analyzed by PCR using primers for the iNOS promoter. Ctl: Plasmid containing iNOS promoter. gDNA: genomic DNA. ***(D)*** Same as C except that DNA was analyzed by qPCR. Data represented as mean ± SD of n=2 experiments. ***(E)*** DNA from ChIP assay with anti-p65 (p65α) and IgG was analyzed by PCR using primers for the iNOS promoter indicated the iNOS promoter is associated with STAT3α. Data represented as mean ± SD (n=2). ***(F)*** RNA polymerase II is recruited to the iNOS promoter in response to IFNγ/TNFα. Data represented as mean ± SD (n=2).


A
+1
STAT3
NF-κB
TATA
iNOS mRNA
-199
-87
-31
B
Chromatin IP
IP/αIgG
IP/αSTAT3
S
P
S
P
STAT3
1
2
3
4
Western blot
C
IP
Ctl
gDNA
αIgG
αSTAT3
iNOS
1
2
3
4
PCR
D
Fold Change in iNOS Promoter Association
3.5
3
2.5
2
1.5
1
0.5
0
IgG
STAT3
E
qPCR
Fold Change in iNOS Promoter Association
2.5
2
1.5
1
0.5
0
IgG
p65
F
qPCR
IgG
Pol2
Fold Change in iNOS Promoter Association
10
8
6
4
2
0
0h
1h

### 2.4.4: IFNγ/TNFα induces the translocation of pY-STAT3 to the nucleus in an NF-κB-dependent manner

The ability of STAT3 to activate transcription relies on its localization from the cytoplasm to the nucleus upon stimulation. Therefore, we assessed the localization of pY-STAT3 in muscle fibers treated with or without IFNγ/TNFα for the indicated periods of time. We observed, by immunofluorescence (IF), a substantial increase in the localization of pY-STAT3 to the nucleus as early as 0.5h posttreatment with IFNγ/TNFα (Fig. 2.14A). Nuclear and cytoplasmic fractionation experiments followed by western blot analysis, showed that the significant increase in the level of pY-STAT3 in the nucleus induced by IFNγ/TNFα persists for the first few hours of the treatment (Figs. 2.14B-C). The increase of pY-STAT3 levels in the nucleus is not due to contamination between the fractions, since as expected hnRNP A1 is only detected in the nucleus, α-tubulin is only detected in the cytoplasm, while β-actin, as previously described (Bettinger et al, 2004), was detected in both fractions (Fig. 2.14B).

***Figure 2.14*: IFNγ/TNFα treatment induces a rapid translocation of pY-STAT3 to the nucleus. *(A)*** Confocal microscopy images of C2C12 myotubes treated with or without IFNγ/TNFα for 30 min and stained for pY-STAT3 (red), myosin heavy chain (MyHC; green) and counterstained with DAPI (blue). Scale bar = 20μm. Images shown are representative of n=3 experiments. ***(B)*** Nuclear and cytoplasmic fractions prepared from C2C12 myotubes treated with IFNγ/TNFα for the indicated amount of time were used for Western blot analysis with antibodies against pY-STAT3, total STAT3, p65, hnRNP A1 (nuclear marker), α-tubulin (cytoplasmic marker), and β-actin (loading control). Images shown are representative of n=3 experiments. ***(C)*** Densitometric quantification of nuclear pY-STAT3 to β-actin in Fig. 14B. Data represented as mean ± SEM (n=3) with p value = 0.0233 (*) by one-way ANOVA with Dunnett's post hoc test.

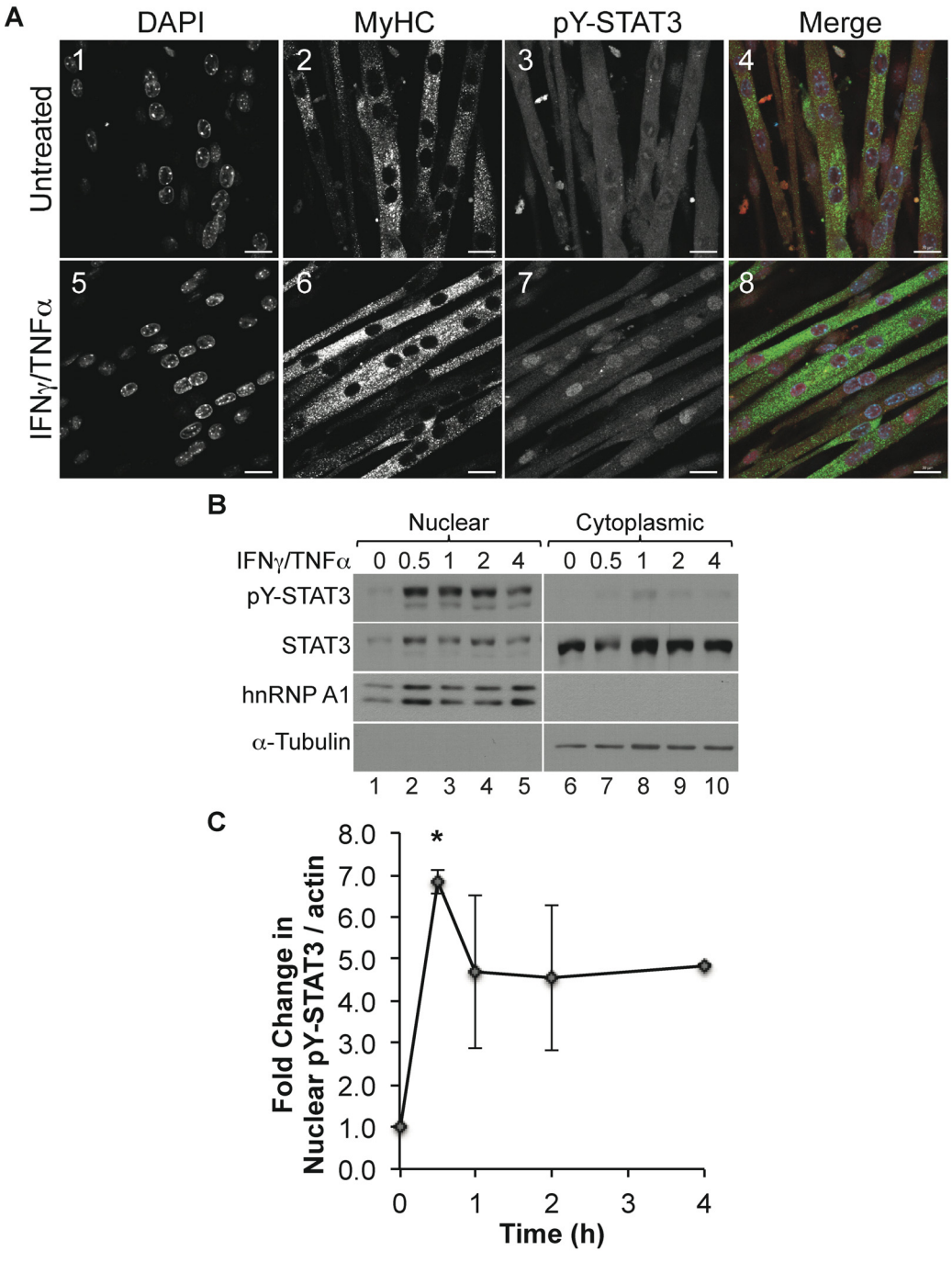
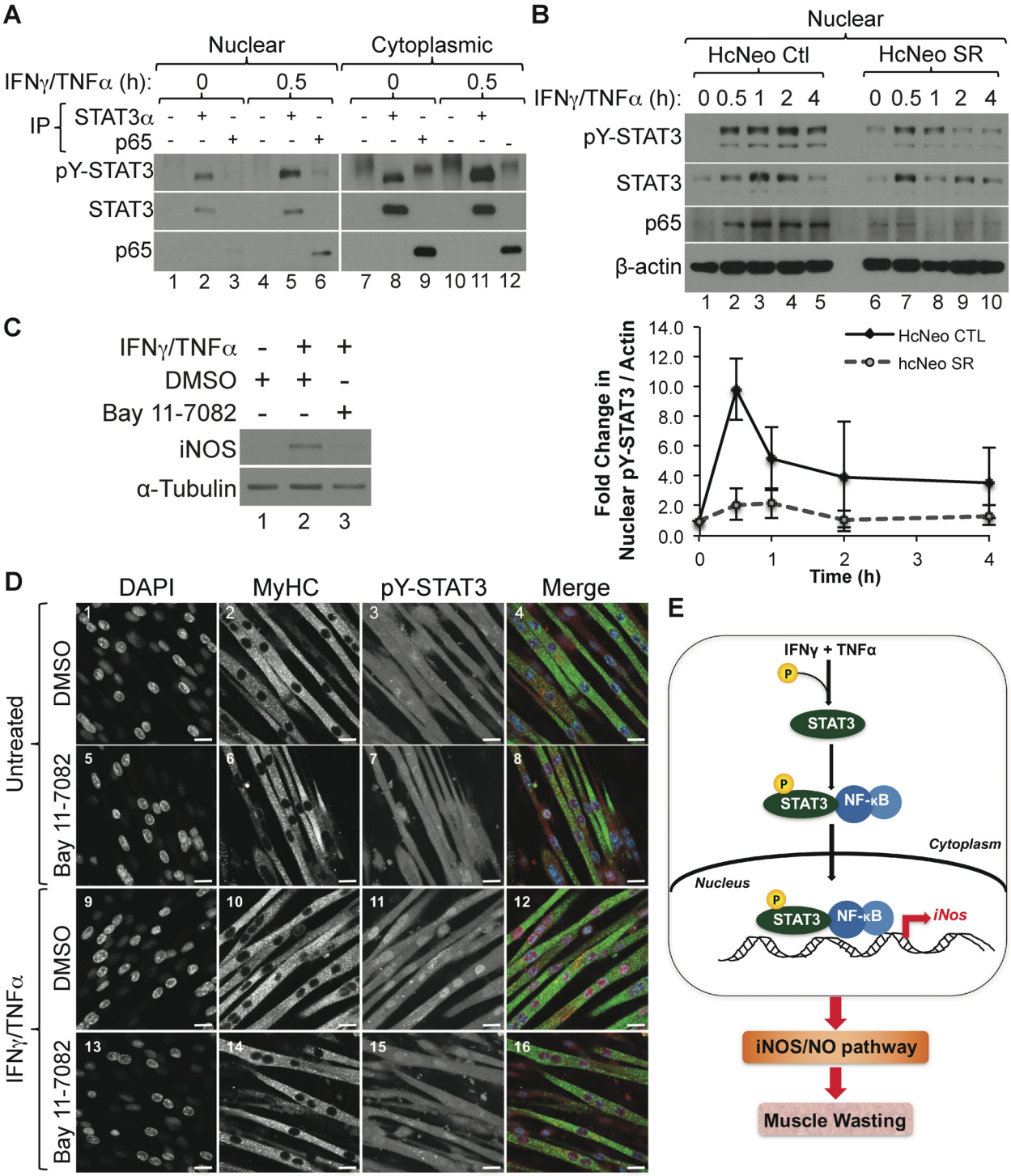

A
DAPI
MyHC
pY-STAT3
Merge
Untreated
IFNγ/TNFα
1
2
3
4
5
6
7
8
B
Nuclear
Cytoplasmic
IFNγ/TNFα 0 0.5 1 2 4 0 0.5 1 2 4
pY-STAT3
STAT3
hnRNP A1
α-Tubulin
1 2 3 4 5 6 7 8 9 10
C
Fold Change in Nuclear pY-STAT3 / actin
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
0 1 2 3 4
Time (h)

***Figure 2.15:*** **Active NF-κB pathway is required for the rapid translocation of pY-STAT3 to the nucleus during IFNγ/TNFα-induced muscle wasting.** (A) Nuclear and cytoplasmic fractions prepared from C2C12 myotubes treated with IFNγ/TNFα followed by immunoprecipitation (IP) with either total STAT3, p65, or an IgG control antibody. Western blot analysis was performed using antibodies against total STAT3, pY-STAT3 and p65. The blot shown is a representative of n=3. (B) (Upper panel) Nuclear and cytoplasmic fractions were prepared from hcNeo control (Ctl) and hcNeo Super Repressor (SR) cells treated with IFNγ/TNFα and used for western blot analysis with antibodies against pY-STAT3, total STAT3, p65 and β-actin. The blot shown is a representatives of n=2. (Lower panel) Densitometric quantification of nuclear pY-STAT3 relative to β-actin in Fig 2.15B. Data are represented as mean ± SD (n=2). (C) Total cell extract from C2C12 myotubes pre-treated with Bay-11-7082 or DMSO as a control then treated with IFNγ/TNFα for 24h were used for western blot analysis with antibodies against iNOS and α-tubulin. The blot shown is a representative of n=2. (D) Confocal microscopy images of C2C12 myotubes pre-treated with Bay 11-7082 or DMSO as a control, then treated with or without IFNγ/TNFα and stained for pY-STAT3 (red), MyHC (green) and counterstained with DAPI (blue). Scale bar = 20μm. Images are representative of n=1 experiment. (E) Model depicting the how STAT3 promotes cytokine-induced muscle wasting. The cytokines IFNγ and TNFα act synergistically by activating STAT3 via phosphorylation on its Y705 residue. Following the degradation of IκBα (not shown here), pY-STAT3 translocates to the nucleus as a complex with NF-κB and upregulates the expression iNOS, leading to the activation of the iNOS/NO pathway, which in turn promotes muscle wasting.


A
Nuclear
Cytoplasmic
IFNγ/TNFα (h):
0
0.5
IP
STAT3α
p65
pY-STAT3
STAT3
p65
1 2 3 4 5 6 7 8 9 10 11 12
B
Nuclear
HcNeo Ctl
HcNeo SR
IFNγ/TNFα (h): 0 0.5 1 2 4 0 0.5 1 2 4
pY-STAT3
STAT3
p65
β-actin
1 2 3 4 5 6 7 8 9 10
Fold Change in Nuclear pY-STAT3 / Actin
HcNeo CTL
hcNeo SR
Time (h)
C
IFNγ/TNFα - + +
DMSO + + -
Bay 11-7082 - - +
iNOS
α-Tubulin
1 2 3
D
DAPI
MyHC
pY-STAT3
Merge
Untreated
DMSO
Bay 11-7082
IFNγ/TNFα
DMSO
Bay 11-7082
E
IFNγ + TNFα
P
STAT3
NF-κB
Cytoplasm
Nucleus
iNos
iNOS/NO pathway
Muscle Wasting

The ability of STAT3 and NF-κB to cooperatively regulate the transcription of common genes is known to occur through direct interaction with each other (Grivennikov & Karin, 2010). Using immunoprecipitation of STAT3 or the p65 subunit of NF-κB, we detected an interaction between STAT3 and NF-κB in both the nuclear and cytoplasmic fractions of cytokine treated or untreated muscle fibers (Fig. 2.15A). This observation raises the possibility that the formation of the STAT3-NF-κB complex occurs in the cytoplasm before translocation to the nucleus. It is well established that the retention of NF-κB in the cytoplasm occurs through its interaction with IκBα (Grivennikov & Karin, 2010). To assess whether the IFNγ/TNFα–induced nuclear translocation of STAT3 depends on the activation of the NF-κB pathway, we used muscle fibers stably over-expressing mutant NF-κB inhibitor protein IκBα (super repressor cells; SR) which cannot be phosphorylated or subsequently degraded. As a result, NF-κB cannot translocate to the nucleus and remains inactive in the cytoplasm (Di Marco et al, 2005; Guttridge et al, 2000). We observed that unlike in control fibers, IFNγ/TNFα treatment of SR muscle fibers resulted in a decrease in the levels of both pY-STAT3 and p65 subunit of NF-κB in the nucleus (Fig. 2.15B). Additionally, western blot analysis and immunofluorescence experiments showed that the inhibition of the NF-κB pathway with Bay 11-7082 prevented the IFNγ/TNFα induced nuclear translocation of pY-STAT3 as well as iNOS expression in muscle fibers undergoing wasting (Figs. 2.15C-D). Taken together, these data demonstrate that NF-κB is required for the

nuclear translocation of pY-STAT3 and for STAT3-mediated activation iNOS expression during IFNγ/TNFα -induced muscle wasting.

## 2.5: Discussion

The failure of anti-TNFα or anti-IL-6 therapies to prevent muscle wasting in humans is a clear indication that the onset of this deadly syndrome is resistant to mono-therapeutic intervention (Bayliss et al, 2011; Jatoi et al, 2010a; Wiedenmann et al, 2008). However, it is not known if this failure is due to activation of unique molecular pathways sufficient to induce atrophy, or if pro-cachectic signals collaborate to activate common downstream effector targets. In this study, we show that STAT3 collaborates with the NF-κB pathway to promote IFNγ/TNFα-induced muscle wasting. While the implication of STAT3 in muscle atrophy has been mainly associated with IL-6 function (Bonetto et al, 2012; Bonetto et al, 2011; Zimmers et al, 2016), our data demonstrate that to trigger muscle fiber loss, IFNγ/TNFα rapidly phosphorylates STAT3 on its Y705 residue. IFNγ/TNFα mediate this effect by activating the JAK kinases independently of IL-6. One of the main effectors of IFNγ/TNFα-induced muscle loss is the iNOS/NO pathway (Di Marco et al, 2012; Di Marco et al, 2005; Hall et al, 2011). Here, not only do we confirm the importance of the iNOS/NO pathway in this process *in vivo*, but we also show that pY705-STAT3 is required for IFNγ/TNFα-mediated expression of iNOS. In response to IFNγ/TNFα, activated STAT3 associates with NF-κB in the cytoplasm and

then translocates to the nucleus, where it is rapidly recruited to the *iNos* promoter. Taken together, this work demonstrates that different pro-cachectic inducers (i.e. IL-6 and IFNγ/TNFα) activate STAT3 signaling independently, and that this signaling can collaborate with the NF-κB pathway to induce cachexia through activation of target genes, like iNOS (Fig. 15E). This suggests that, although there is a diversity of inflammatory factors that can induce cachexia, there may be an integrated network of downstream effectors, such as STAT3-NFκB.

The importance of IL-6 and the mechanisms through which it promotes muscle wasting have been well-established (Bonetto et al, 2012; Bonetto et al, 2011). However, evidence from clinical trials has clearly indicated that blocking IL-6 cannot completely stop or reverse muscle wasting (Bayliss et al, 2011). This may be due to other cytokines that are known to activate similar pathways as IL-6. Indeed, in this study, we found that treatment of muscle fibres with IFNγ/TNFα activates pY705-STAT3 resulting in muscle atrophy. Interestingly, we demonstrated, for the first time, that the activation of pY-STAT3 by IFNγ/TNFα during muscle wasting could occur independently of IL-6. Studies have also demonstrated that other pro-cachectic factors, such as transforming growth factor beta 1 (TGF-β1) can induce muscle wasting by activating STAT3 (Guadagnin et al, 2015). Thus, it is likely that monotherapies targeting any particular factor, such as anti-IL-6 antibodies, would be unable to ablate STAT3 activation in cases where multiple cytokines are driving its activation. However, treatments that directly targeted STAT3 activation may be able to impair the transduction of pro-cachectic signaling from

multiple cytokines simultaneously, potentially providing a much more robust and effective therapy.

While our work and others establishes STAT3 as a key mediator of muscle wasting, the mechanisms by which STAT3 induces atrophy remain elusive. Impairing STAT3 function has been shown to correlate with reduced protein proteolysis and muscle loss (Bonetto et al, 2012; Silva et al, 2015). However, STAT3 is a transcription factor, known to promote the expression of many target genes, and it is unknown which of these genes contribute to IFNγ/TNFα-induced muscle wasting. Here, we identify iNOS as a key target gene of STAT3-mediated cachexia. We found that STAT3 binds directly to the promoter of *iNos* to induce its expression. We have previously shown that iNOS is a key mediator of cytokine driven muscle wasting. Here, we show that iNOS-knockout mice are resistant to IFNγ/TNFα-induced muscle atrophy, suggesting that iNOS is a key gene required for STAT3-mediated wasting. Further studies are needed to identify the other genes that also contribute to the many facets of STAT3-induced muscle wasting.

Induction of gene expression by transcription factors often requires collaboration with partner proteins. Previous studies have found that during cancer-induced inflammation, STAT3 collaborates with other transcription factors such as NF-κB, also a well-known inducer of the cachectic phenotype (Fan et al, 2013). We and others have previously shown that the expression of iNOS depends on NF-κB (Altamirano et al, 2012; Di Marco et al, 2005). This suggests that collaboration between STAT3 and NF-κB could be an important step in promoting cachexia. Indeed, these two factors are

already known central players in promoting inflammation (Bollrath & Greten, 2009; Grivennikov & Karin, 2010) and demonstrated to interact in the nucleus under inflammatory conditions (Bode et al, 2012; Hagihara et al, 2005; Yu et al, 2002b). Our study shows that the localization of pY-STAT3 to the nucleus of IFNγ/TNFα-treated fibers is likely to dependent on the degradation of IκBα and subsequent release of NF-κB, indicating the importance of cytoplasmic STAT3-NF-κB complex formation in facilitating the translocation of STAT3 and NF-κB to the nucleus. The nuclear localization of the STAT3- NF-κB-IκBα complex was recently shown to be regulated by Rac-1 in starved cancer cells (Kim & Yoon, 2016). Furthermore, it is known that the nuclear retention of NF-κB is prolonged by its interaction with STAT3, which is central to maintaining NF-κB activity in tumors (Lee et al, 2009). These results, together with our observations described above, suggest that cytoplasmic STAT3-NF-κB complex formation and its translocation are key steps in promoting muscle atrophy. Further investigation into the genes targeted by STAT3-NF-κB complex is needed to identify the networks of downstream genes through which these two transcription factors promote muscle wasting.

In our studies, we demonstrate that pY-STAT3 modulates IFNγ/TNFα induced muscle wasting in an IL-6 and iNOS dependent manner. Indeed, we showed that both IL-6 and iNOS knockout mice, despite the phosphorylation of STAT3, are resistant to muscle wasting (Figs. 5, 6, 7, 10, 11, 12). This would suggest that both IL-6 and iNOS collaborate downstream of STAT3 activation to modulate IFNγ/TNFα -induced muscle

atrophy. Indeed, we and others have shown that both iNOS and IL-6 are targets of STAT3 transcriptional activity (Figs. 12, Lee et al, 2013). Therefore, our results support the idea that targeting factors such as STAT3 may be a therapeutic avenue to prevent muscle wasting due to its ability to activate multiple downstream procachectic factors concurrently. Furthermore, our data suggests that the STAT3-NF-κB complex and their downstream target genes could help design novel therapeutic strategies to combat this deadly syndrome. Indeed, the fact that iNOS KO mice are protected from cytokine-induced muscle wasting is a clear indication that the iNOS/NO pathway should be considered as a potential target for such a strategy. Ultimately, the targeting of these integrated downstream effectors of the cachectic phenotype may prove more effective than mono-therapies against pro-cachectic inducers for the treatment of cachexia.

## 2.6: Materials and Methods

### 2.6.1: Cell culture and plasmids

C2C12 cells were obtained from American Type Culture Collection (VA, USA) and grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing high glucose, L-glutamine, sodium pyruvate, and supplemented with 20% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were routinely tested for mycoplasma. Cells were plated on tissue culture plates (Corning) coated with 0.1% gelatin (Sigma-Aldrich) for differentiation. To induce myotube formation, cells were

grown to 100% confluency and switched to DMEM containing 2% horse serum (Gibco) and 1% penicillin/streptomycin for up to four days. Phase contrast pictures were taken with a 10X objective lens on an inverted Zeiss Axiovert 25 and a Sony Cyber-shot DSC-S75 Digital still camera.

A mixture of IFN$\gamma$ (100 U/mL) (R&D Systems) and TNF$\alpha$ (20 ng/mL) (R&D Systems or BioBasic, Inc.) was used for the treatment of cells for various periods of time. S3I-201 and Jak Inhibitor 1 (EMD Millipore) were used to pre-treat cells as indicated. Short-hairpin RNA (shRNA) that specifically targets STAT3 or a scramble hairpin were purchased from Sigma. The pMXs-STAT3-C was a gift from Shinya Yamanaka (Addgene plasmid #13373) (Davis et al, 2006). STAT3 Y705F Flag pRC/CMV was a gift from Jim Darnell (Addgene plasmid #8709) (Wen & Darnell, 1997). C2C12 transfected with plasmid using jetPRIME$^{TM}$ DNA and siRNA transfection reagent (Polyplus Transfection) according to manufacturer's protocol.

### 2.6.2: Study Approval

The experiments using animal studies were approved by the McGill University Faculty of Medicine Animal Care Committee and comply with guidelines set by the Canadian Council of Animal Care.

### 2.6.3: Mice

C56BL/6J wild-type or Interleukin-6 knock-out (IL-6 KO; B6.129S2-Il6$^{tm1Kopf}$/J, #002650) or inducible nitric oxide synthase KO mice (iNOS KO; B6.129P2-Nos2$^{tm1Lau}$/J,

#002609, Jackson Laboratories) were housed in a room with a 12h light – 12h dark cycle. All mice were housed in a sterile cage with corn-cob bedding and had free access to water and rodent chow (#2920, Envigo). Testing for rodent-related pathogens were routinely performed by McGill University's Comparative Medicine and Animal Resources Center.

Male mice (21 – 25g) aged 7 – 10 weeks were size-matched and randomly assigned to treatment with either saline (Sigma) or with 7500 units of IFN$\gamma$ (R&D Systems) and 3 µg of TNF$\alpha$ (R&D Systems). Mice were injected with a 30G needle every day for five days as described (Di Marco et al, 2012). On the sixth day, mice were sacrificed by $CO_2$ inhalation and the gastrocnemius muscle was dissected and frozen on dry ice when used for western blot analysis. Muscle used for cross-sectional area (CSA) analysis was frozen in isopentane (Sigma) chilled in liquid nitrogen. Each muscle was sectioned at 8 or 10 µm thickness and stained with hematoxylin and eosin. Wide-field images were taken with a 20X objective lens on an inverted Zeiss Axioskop microscope with an Axiocam MRc colour camera in the McGill University Life Sciences Complex Advanced BioImaging Facility. The CSA of muscle was analyzed blindly. Each animal was considered one experimental unit.

**2.6.4: Western blot analysis**

Whole cell lysates were prepared by lysis in buffer containing 50 mm HEPES (pH 7.0), 150 mm NaCl, 10% glycerol, 1% Triton X-100, 10 mm sodium pyrophosphate, 100 mm NaF, 1 mm EGTA, 1.5 mm $MgCl_2$, 0.1 mM sodium orthovanadate, and complete

EDTA-free protease inhibitors (Roche Applied Science). Primary antibodies used were phospho-Tyr$^{705}$-STAT3 (1:2000; #9145, Cell Signaling), total STAT3 (1:1500; #9132 or 9139, Cell Signaling), phospho-Ser$^{727}$-STAT3 (1:1000; #9134, Cell Signaling), STAT3α (1:1000; C-20, Santa Cruz), α-tubulin (1:2000; clone 6G7 deposited by Halfter WM to Developmental Studies Hybridoma Bank), iNOS (1:3000; Clone 6, BD Transduction Laboratories), hnRNP A1 (1:1000; ab5832, Abcam), p65 (1:1000; 06-418, EMD Millipore) and 3A2 (anti-HuR, 1:10 000; (Gallouzi et al, 2000)).

### 2.6.5: Immunofluorescence

Cells were plated in 6-well plates coated with 0.1% gelatin and induced for differentiation. After treatment, cells were fixed in 3% para-formaldehyde for 30min, permeabilized with 0.1% Triton-X 100 in PBS, and then incubated with myoglobin (1:250; ab77232, Abcam) and myosin heavy chain (1:1000; clone MF-20 deposited by Fischman DA to Developmental Studies Hybridoma Bank). Images were taken with a 40X objective lens on an inverted Zeiss Observer.Z1 microscope with an Axiocam MRm camera. The fiber widths were measured using the Axiovision software (release 4.8.2 SP2). Only fibers with at least three nuclei were measured in three places to obtain an average width for each fiber. At least three fields per condition for each experiment were measured. Measurements were analyzed in Microsoft Excel. For localization studies, cells were plated into an 8-well µ-slide (ibidi) coated with Matrigel (Corning) diluted to 1 mg/mL. Differentiated C2C12 were treated at various times and stained according to manufacturer's protocol. Images were taken with a 63X objective lens on an inverted

Zeiss confocal laser scanning microscope 800 in the McGill University Life Sciences Complex Advanced BioImaging Facility.

### 2.6.6: ELISA

The supernatant of IFNγ/TNFα-treated C2C12 myotubes were assayed for IL-6 production using the Mouse IL-6 ELISA Ready-SET-Go!® Kit (eBioscience, Inc.) according to the manufacturer's protocol.

### 2.6.7: Quantitative PCR (qPCR)

One microgram of total RNA was reverse transcribed with the M-MuLV Reverse Transcriptase (New England Biolabs) according to the manufacturer's protocol. Each sample was diluted 1/20 and used to detect the mRNA levels of iNOS and RPL32. Expression of iNOS mRNA was normalized to RPL32 as a reference. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCt is the difference in Ct values between the target and reference genes. Primers for detection of mRNA are as follows: iNOS Forward: 5'-GTG CGC ATG GCT CGG GAT GT-3', iNOS Reverse: 5'-GGC TGT CAG AGC CTC GTG GC-3', RPL32 Forward: 5'-TTC TTC CTC GGC GCT GCC TAC GA-3', and RPL32 Reverse: 5'-AAC CTT CTC CGC ACC CTG TTG TCA-3'.

### 2.6.8: Subcellular fractionation

Pellets of C2C12 myotubes were immediately resuspended in EBKL buffer and incubated on ice for 15 min without agitation. The cellular membrane was lysed using a glass dounce with the tight pestle. The lysate was centrifuged and the supernatant

(cytoplasmic fraction) was removed. The pellet (nuclear fraction) was washed three times then lysed in nuclear lysis buffer. The cytoplasmic fraction was centrifuged again at 10000g to remove nuclear contamination.

### 2.6.9: Co-Immunoprecipitation

Fresh pellets of C2C12 myotubes were collected and immediately fractionated. 50uL of protein A Sepharose beads pre-incubated with primary antibody for four hours and washed three times before incubation with lysates overnight at 4°C. The following day, each IP was washed with once with low salt buffer and twice with medium salt buffer before adding equal volume of 2x Laemmli dye. Each sample was then vortexed and boiled. Samples were analyzed by western blot analysis.

### 2.6.10: Chromatin Immunoprecipitation

One 10 cm dish of C2C12 myotubes was fixed in 1% formaldehyde and washed with PBS. Fixed cells were scraped in PBS and cell pellets were frozen at -80°C. The Magna ChIP™ A/G One-Day Chromatin Immunoprecipitation Kit (Millipore) was used to perform ChIP according to manufacterer's protocol. Briefly, cell pellets were resuspended in 1 mL of cell lysis buffer and sonicated with a Branson Sonifier 450 attached to a cup horn for 30sec on / 30sec off for 8min total. 5% of each chromatin preparation was incubated with 2 µg of primary antibody against STAT3 or p65 or 1 µg of primary antibody against RNA polymerase II and 20 µL of protein A/G magnetic beads overnight. Then, beads were washed once with low salt buffer, high salt buffer,

LiCl buffer, and TE buffer. Protein-DNA complexes were eluted and reverse cross-linked with elution buffer containing proteinase K by rotating at 62°C for 2h. DNA was isolated from the supernatant using the spin column provided and eluted in 50 µL of distilled $H_2O$. Using qPCR, 2 µL of ChIP DNA was amplified using the SsoFast EvaGreen Supermix (Bio-Rad). Raw Ct values were analysed using the fold enrichment method relative to IgG. The *iNOS/Nos2* promoter was detected using specific primer pair for the region containing putative STAT3 binding site (forward: 5'-CCAGAACAAAATCCCTCAGC-3', reverse: 5'-CTCATGCAAGGCCATCTCTT-3') or the TATA box (forward: 5'-GAGCTAACTTGCACACCCAAC-3', reverse: 5'-GCAGCAGCCATCAGGTATTT-3'). Primary antibodies used for ChIP were against STAT3α (C-20X; Santa Cruz), p65 (ab7970, abcam), normal rabbit IgG (12-370, Millipore), RNA polymerase II (clone CTD4H8, #05-623, Millipore), and normal mouse IgG (12-371, Millipore).

### 2.6.11: Detection of nitric oxide release

The detection of NO in the media was performed using Griess reagent as previously described. The O.D. was measured on a spectrophotometer at 543nm (Di Marco et al, 2005).

### 2.6.12: Primary myoblast cell culture

The protocol used for skeletal muscle satellite cell isolation was previously described (Demoule et al, 2005). Briefly, the tibialis anterior muscle was dissected out

and digested in 0.2% collagenase for 1h 45m. The digested tissue was gently pipetted to liberate single fibers were plated onto Matrigel-coated flasks. After four days of incubation at 37°C and 5% $CO_2$ in plating medium (10% horse serum, 0.5% chick embryo extract in DMEM), the cells began sub-culturing every two days. Proliferating myoblasts were maintained in proliferation medium (20% FBS, 10% HS, 1% CEE in DMEM). To differentiate, the cells were switched to differentiation media (2% FBS, 10% HS, 0.5% CEE in DMEM) for four days then to DMEM with 2% HS for one day before treatment.

### 2.6.13: Statistics

Data are represented as mean ± SEM for n = 3 experiments unless otherwise stated. Quantification of band intensities was performed using ImageJ software (National Institute of Health). Statistical significance was evaluated using analysis of variance (ANOVA) or two-tailed, unpaired Student's *t*-test depending on the number of groups or factors to be analyzed. Data was considered statistically different with the following: * if $p < 0.05$, ** if $p < 0.01$, and *** if $p < 0.001$.

### 2.6.14: Conflict of interest Statement

The authors have declared that no conflict of interest exists.

## 2.7: Acknowledgments

We are grateful to Erzsebet Nagy Kovacs for advice and assistance with the animal studies. We thank Mr. Xian J. Lian for his technical help with immunoprecipitation experiments. This work is funded by a Prostate Cancer Canada Discovery Grant: D2014-14, a CIHR operating grant (MOP-142399), and a Qatar National Research Fund (QNRF) (NPRP8-457-3-101) to I.E.G. J.F.M. was supported by the CIHR/FRSQ training grant in cancer research FRN53888 of the McGill Integrated Cancer Research Training Program. D.T.H. was funded by a scholarship received from the Canadian Institute of Health Research (CIHR) funded Chemical Biology Program at McGill University.

## CHAPTER 3

## The interplay between HuR and miR-330 modulates STAT3 translation during TNFα/IFNγ-induced muscle wasting

### 3.1: Rationale

In chapter 2, we identified STAT3 as a critical regulator of muscle wasting induced by TNF$\alpha$ and IFN$\gamma$. In addition to the importance of STAT3 phosphorylation in promoting disease such as cancer and muscle wasting, aberrant STAT3 signaling caused by an increase in the expression level of STAT3 protein can also contribute to disease and may be important in promoting muscle wasting. Our lab previously identified the RNA binding protein HuR as a key factor in promoting muscle wasting through its ability to stabilize the inducible nitric oxide synthase (iNOS) mRNA (Di Marco et al, 2005). This discovery suggested that HuR could be involved in promoting muscle loss by regulating a group of mRNA that collectively promote the loss of muscle. To address this, we used RNA immunoprecipitation coupled to RT-qPCR to identify novel mRNA targets for HuR during cytokine treatment, which included the STAT3 mRNA. In this study, we aimed to characterize how STAT3 mRNA is post-transcriptionally regulated by HuR under these conditions.

**The interplay between HuR and miR-330 modulates STAT3 translation during TNFα/IFNγ-induced muscle wasting**

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This research is *in preparation*.

## 3.2: Abstract

Pro-inflammatory diseases, such as cancer, AIDS, and COPD, are often associated with progressive muscle wasting, a syndrome also known as cachexia. It is known that pro-inflammatory cytokines, such as TNFα and IFNγ, trigger muscle loss by activating downstream inflammatory pathways. Signal transducer and activator of transcription (STAT) 3 protein is known to be one of the main effectors of TNFα/IFNγ-induced muscle atrophy. Although, STAT3 abundance and activation are both required for its pro-cachectic effect, the mechanisms modulating STAT3 expression in muscle remain elusive. Here, we show that the expression of STAT3 during TNFα and IFNγ mediated muscle atrophy is dependent on the RNA binding protein HuR. We observed that HuR promotes the translation of STAT3 mRNA by directly binding to U-rich elements in the *STAT3 3'UTR*. HuR mediates this effect by inhibiting the suppressive effect of miRNA-330 on STAT3 translation. Together our data suggest that the interplay between HuR and miR-330 represents a novel regulatory mechanism modulating STAT3 expression during TNFα and IFNγ mediated muscle loss.

### 3.3: Introduction

Skeletal muscle is the largest organ in the body, accounting for at least 40% of the total body mass of healthy individuals (Frontera & Ochala, 2015). The maintenance of skeletal muscle mass requires a balance between protein synthesis and protein degradation to ensure continuous renewal of muscle proteins (Robert et al, 2012; Sandri, 2013). Chronic diseases such as cancer, AIDS, and COPD (chronic obstructive pulmonary diseases) can disrupt this balance to favor protein degradation, leading to the onset of cachexia, a syndrome characterized by rapid muscle wasting and deterioration (Tsoli & Robertson, 2013). Patients that develop muscle wasting experience weakness, a lower quality of life, a decreased response to therapy, and reduced survival rates (Fearon et al, 2013). Despite its deleterious effects, there are currently no effective treatment options available, highlighting the need to better understand the molecular mechanisms mediating muscle wasting to identify novel targets for therapy.

It is well-accepted that one of the most common promoter of cachexia-induced muscle wasting is the excessive production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF$\alpha$), interferon gamma (IFN$\gamma$), and interleukin 6 (IL-6), that results from the underlying chronic diseases (Fearon et al, 2013; Hall et al, 2011). One of the main effectors of cytokine-induced muscle wasting is the transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) protein, a member of the STAT family (Ma et al, 2017; Zimmers et al, 2016). It is well-established that the

phosphorylation of STAT3 on Y705 residue induces muscle wasting by promoting the transcription of procachectic genes (Bonetto et al, 2012; Ma et al, 2017; Yu et al, 2014). Recently, we observed that the activation of STAT3 in skeletal muscles exposed to IFN$\gamma$ and TNF$\alpha$ was associated with an increase in STAT3 expression (Ma et al, 2017). Elevated STAT3 protein has been shown to enhance STAT3 activity and also correlates with poor prognosis in cancer patients (Kim et al, 2009a; Wu et al, 2016; Xu & Lu, 2014). Taken together, these observations suggest that the modulation of STAT3 expression could also be an important molecular event in the progression of cachexia-induced muscle loss.

The control of gene expression can occur at numerous levels, including transcriptionally and post-transcriptionally. STAT3 expression is known to be regulated at the transcriptional level by numerous factors, including STAT3 itself (Ichiba et al, 1998; Narimatsu et al, 2001; Pakala et al, 2013). Recent studies have indicated that STAT3 is also regulated posttranscriptionally by both RNA-binding proteins (RBPs) and miRNAs. For example, the RBP cytoplasmic polyadenylation element binding protein 1 (CPEB1) mediates the repression of *STAT3* mRNA translation, which in turn promotes insulin signaling in the liver (Alexandrov et al, 2012). Another study showed that in response to IL-6 signal, Arid5a (AT-rich interactive domain-containing protein 5a) stabilizes *STAT3* mRNA to promote the differentiation of naïve $CD4^+$ T cells into pro-inflammatory Th17 cells (Masuda et al, 2016). Additionally, miRNA-17 and -20a have been shown to downregulate *STAT3* message in acute myeloid leukemia cells to

promote their differentiation (He et al, 2013). While these studies clearly show that the posttranscriptional regulation of *STAT3* mRNA represents a key regulatory step, the implication of this mode of regulation in its pro-cachectic function has never been tested.

Various posttranscriptional events are mediated, in part, by the presence of cis-elements (including A/U and/or U-rich elements) in the 3'untranslated regions (3'UTRs) of mRNAs that associate with RNA-binding proteins (RBPs), such as HuR, a well-established posttranscriptional regulator (Abdelmohsen et al, 2008). HuR is best known for its role in stabilizing and regulating the translation of mRNAs that influence various cellular processes, including cell cycle, apoptosis, differentiation, stress response, tumorigenesis, and inflammation (Abdelmohsen et al, 2008; Artman et al, 2014; Cammas et al, 2014; Dormoy-Raclet et al, 2013). Our work has previously shown that HuR affects the fate of muscle fibers by modulating the stability and translation of mRNAs that either promote or hinder muscle differentiation and integrity (Beauchamp et al, 2010; Cammas et al, 2014; Di Marco et al, 2005; Dormoy-Raclet et al, 2013; van der Giessen et al, 2003; von Roretz et al, 2011a). We have also observed that HuR plays a key role in TNF$\alpha$/IFN$\gamma$-induced muscle wasting by increasing the half-life of the mRNA encoding for inducible nitric oxide synthase (iNOS) mRNA (Di Marco et al, 2005). Several studies have demonstrated that HuR differentially regulates mRNA targets by interacting cooperatively or antagonistically with *trans-acting* factors such as other RBPs or small non-coding RNAs such as miRNAs. For example, we have showed that HuR mediates the translation of the alarmin HMGB1 by negating the effects of miR-

1192 to promote myogenesis (Dormoy-Raclet et al, 2013). However, the exact mRNA network of HuR during muscle wasting remains elusive. In this study we addressed this issue and identified STAT3 mRNA as a novel HuR target during cytokine-induced muscle loss. We showed that while HuR does not affect the half-life of STAT3 mRNA, HuR binds specific elements in the 3'UTR to prevent the miR-330-mediated translation inhibition of STAT3. We also discuss the HuR-mediated regulation of STAT3 expression and its implication for the onset of muscle wasting.

## 3.4: Results

### 3.4.1: The *STAT3* mRNA is a novel HuR mRNA target in myotubes undergoing wasting

To assess the association between HuR and the *STAT3* mRNA during muscle wasting, we performed RIP experiments (immunoprecipitation using the anti-HuR antibody or IgG as negative control) coupled to RT-qPCR analysis (Tenenbaum et al, 2000; Tenenbaum et al, 2002) using total extracts from C2C12 myotubes treated with or without IFNγ/TNFα for 24 hours. This type of treatment was chosen because exposing myotubes to IFNγ/TNFα for 24h has been shown to be sufficient to induce the expression of the HuR mRNA target iNOS, a well-known promoter of IFNγ/TNFα-induced muscle wasting (Bakkar et al, 2008; Di Marco et al, 2012; Di Marco et al, 2005; Guttridge et al, 2000). This *in vitro* cell model of muscle wasting is routinely used to

mimic the effects of cytokines, such as IFNγ and TNFα, on the integrity of muscle fibers (Bakkar et al, 2008; Di Marco et al, 2012; Di Marco et al, 2005; Guttridge et al, 2000; Ma et al, 2017), as seen during cachectic conditions (Fig. 3.1A). We observed that HuR associates with *STAT3* mRNA in both untreated and IFNγ/TNFα-treated C2C12 myotubes (Figs. 3.1B-C) and cells (Fig. 3.2). We observed that immunoprecipitated HuR (Fig. 3.1B) is associated with *STAT3* mRNA in both untreated as well as treated myotubes (Fig. 3.1C). The association of the *STAT3* mRNA to HuR is however ~2-fold higher in myotubes treated with IFNγ/TNFα than their untreated control counterparts (Fig. 3.1C). While these experiments clearly suggest that the *STAT3* message is a novel HuR target during muscle wasting, they do not confirm whether HuR associates to this mRNA in a direct or indirect manner.

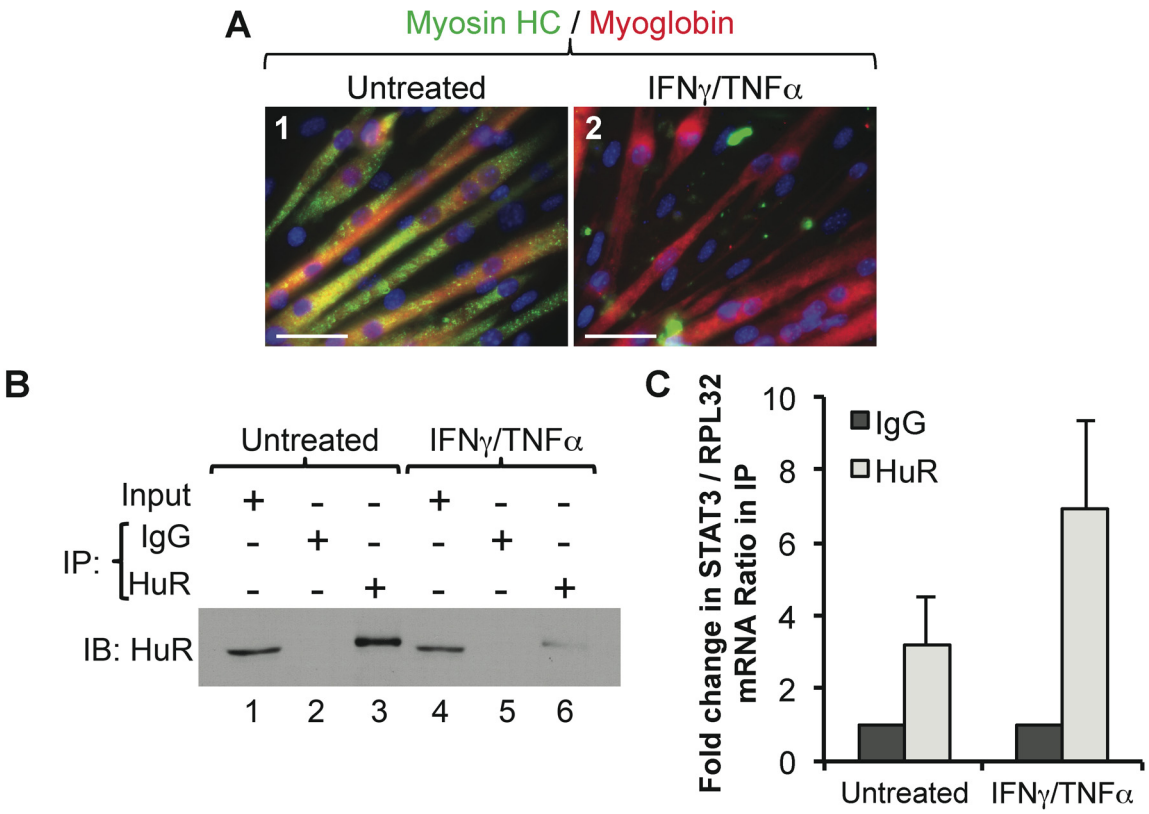



***Figure 3.1:*** **HuR associates with STAT3 mRNA in C2C12 myotubes during muscle wasting. (A)** Immunofluorescence (IF) images of C2C12 myotubes treated with or without IFNγ/TNFα for 72h that were stained with antibodies detecting myosin heavy-chain (MyHC) and myoglobin as well as DAPI. Images are representative of n = 3 experiments. Scale bar = 50 μm. **(B-C)** Lysates obtained from C2C12 myotubes treated with or without IFNγ/TNFα for 24h were used for immunoprecipitation experiment using antibodies against HuR or IgG as a negative control. Western blot experiments demonstrating immunoprecipitated HuR (B) and analysis by RT-qPCR of *STAT3* mRNA associated to HuR (C) are shown. Levels were standardized to and *RPL32* mRNA levels. Data represented as mean ± SEM (*n* = 3).

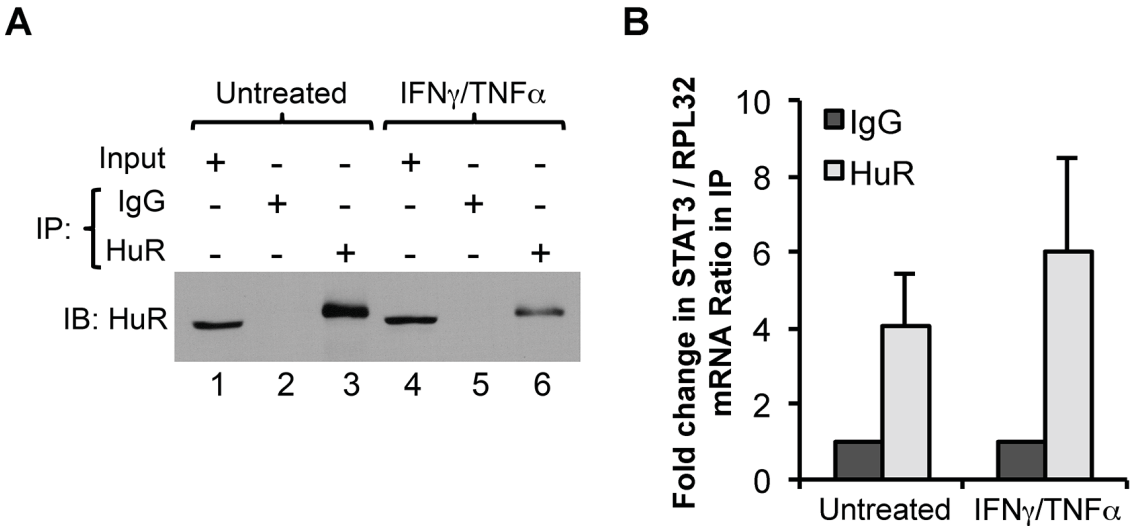
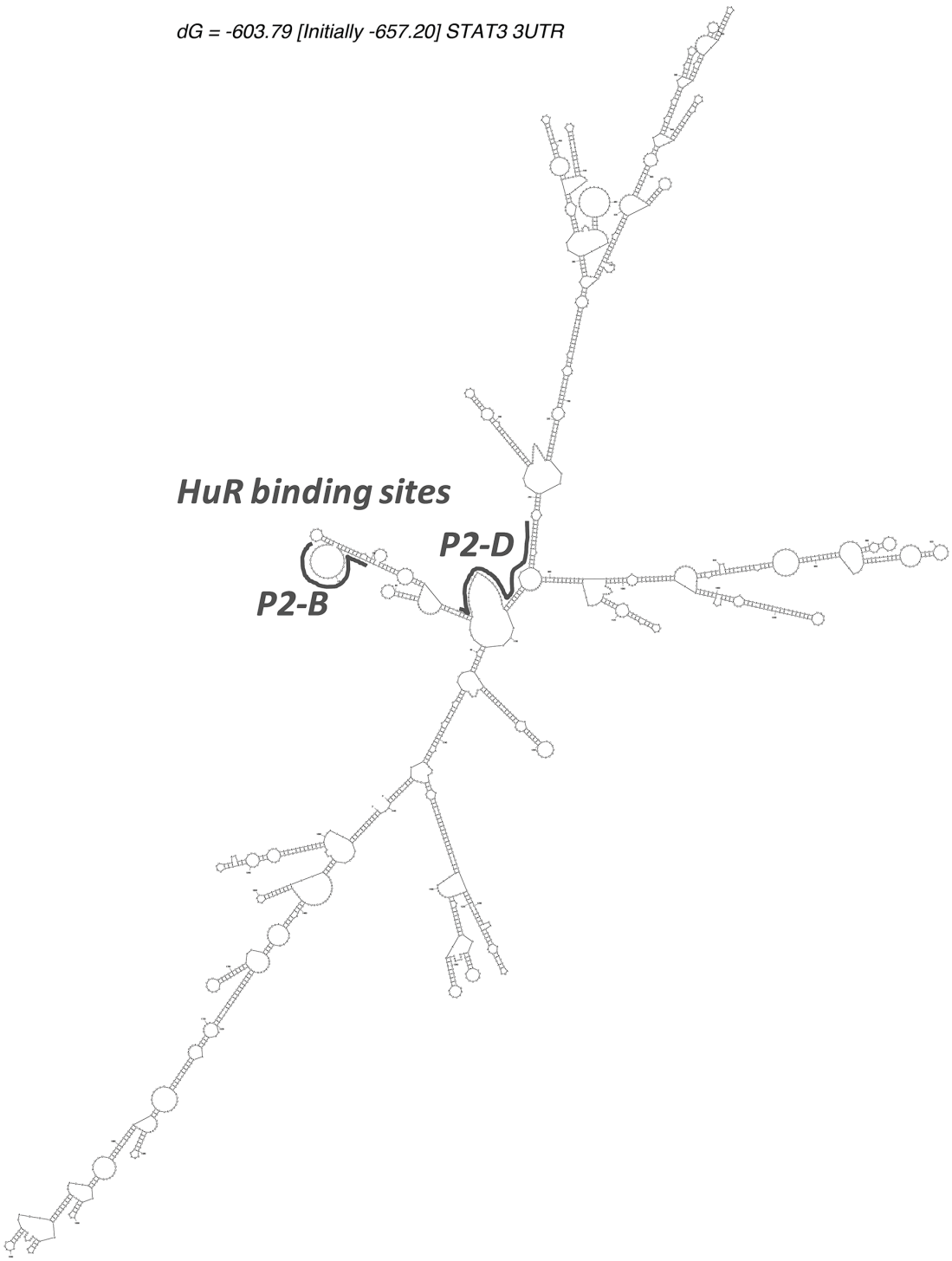


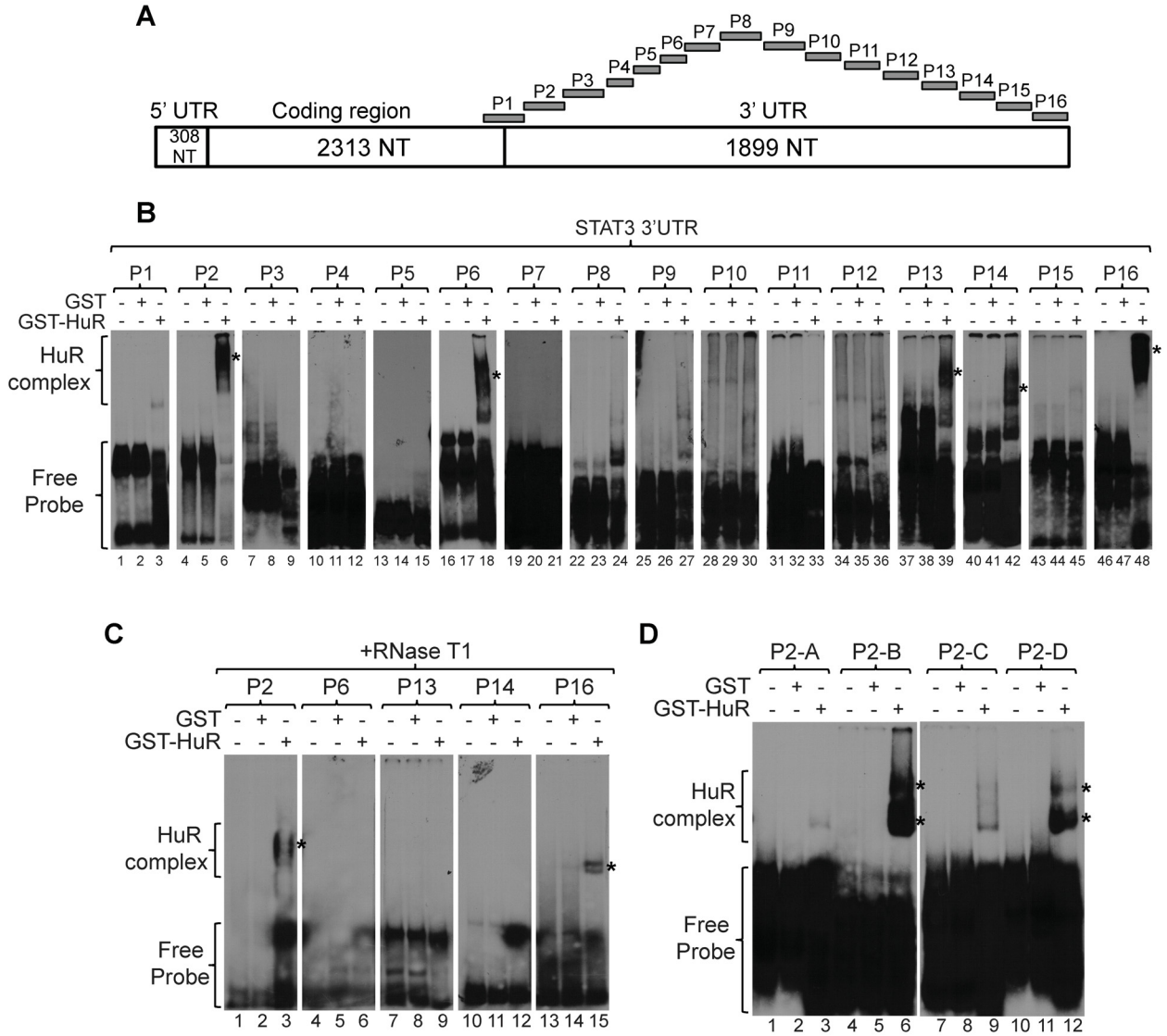

***Figure 3.2:*** **HuR associates with the STAT3 mRNA in muscle cells treated with or without IFNγ/TNFα. (A, B)** Immunoprecipitation experiments were performed using antibodies against HuR or IgG as a negative control with lysates obtained from C2C12 muscle cells treated with or without IFNγ/TNFα for 24h were used for. Western blot experiments demonstrating immunoprecipitated HuR (A) and analysis by RT-qPCR of *STAT3* mRNA associated to HuR (B) are shown. Levels were standardized to and *RPL32* mRNA levels. Data represented as mean ± SEM (*n* = 3). *Originally prepared as Supp. Fig S1.*

***Figure 3.3:*** **Secondary structure of the *STAT3* mRNA 3'UTR as predicted by mfold software.** The location of the HuR binding sites in the structure are indicated by the dark gray lines (Zuker, 2003). *Originally prepared as Supp. Fig. S2.*


dG = -603.79 [Initially -657.20] STAT3 3UTR
HuR binding sites
P2-D
P2-B

It is well-established that HuR modulates the expression of its mRNA targets by binding U/AU-rich elements in their 3'UTRs (von Roretz et al, 2011a). Sequence analysis of the *STAT3* 3'UTR revealed both U- and AU-rich elements to which HuR could potentially bind (Fig. 3.3 and Fig. 3.4A). To determine whether HuR directly binds to the *STAT3* mRNA through any of these elements, we performed RNA electrophoretic mobility shift assays (REMSA) (Gutierrez et al, 2007; Kandouz et al, 2013) using purified recombinant GST-HuR and sixteen radiolabelled probes that spanned the entire STAT3 3' UTR sequence (Fig. 3.4A and Table 3.2). We found that HuR interacts with only five RNA probes (P2, P6, P13, P14 and P16) (Fig. 3.4B). Next, the strength of HuR binding to these probes was tested by introducing RNase T1 into the REMSA. In this assay, HuR-mRNA complexes are formed prior to digestion with RNase T1, which cleaves specifically after G residues. It is well-accepted that resistance to RNAse T1 treatment will only occur as result of strong and direct binding of HuR to RNA probe (Di Marco et al, 2001). We observed that only the P2 probe resisted RNAse T1 treatment and maintained a strong binding to HuR (Fig. 3.4C). To further delineate the HuR binding site within P2, we divided this probe into four smaller fragments, P2-A, -B, -C, and -D. We observed a complex formation with P2-B and to a lesser extent to both P2-C and -D (Fig. 3.4D). Together these experiments identify *STAT3* mRNA as a novel, *bona fide* HuR target in both untreated and cytokine-treated muscle fibers.

***Figure 3.4:*** **HuR directly binds to a U-rich element in the 3'UTR of *STAT3* mRNA.** (**A**) Schematic representation of the *STAT3* mRNA sequence. The location of the cRNA probes covering the 3'UTR of *STAT3* mRNA used for RNA electrophoretic mobility shift (REMSA) assays are also indicated (P1 to P16). (**B**) Gel shift assay was performed using recombinant GST-HuR protein or GST as a control incubated with radiolabelled RNA probes as indicated in (A). Representative gels of each probe from n = 3 experiments. HuR/cRNA complex are indicated with an asterisk (*). **(C)** Representative gels of REMSA assays performed with radiolabelled cRNA probes (P2, P6, P13, P14, and P16) incubated with purified GST or GST-HuR prior to treatment with RNaseT1 to assess strength of complex formation seen in (B). (**D**) REMSA with radiolabelled probes spanning P2 region (P2-A, P2-B, P2-C, and P2-D) to further delineate HuR binding site. Lower panel indicates the nucleotide sequence of each probe used for gel shift in panel D.




A: CGAACCCCATATCGTCTGAAACTCCTAACTTTGT

B: GGTTCCAGATTTTTTTTTTTAATTTCCTACTTCTG

C: CTATCTTTGGGCAATCTGGGCACTTTTTAAAATAGAGAAATGAGTGAGT

D: GTGGGTGATAAACTGTTATGTAAAGAGGAGAGCACCTCTGAGTCTGGGG

### 3.4.2: HuR promotes the translation of *STAT3* mRNA

Previous studies have shown that STAT3 protein levels increase during muscle wasting and that this increase is associated with STAT3 activation (Ma et al, 2017; Zhang et al, 2013). We confirmed these effects and observed an increase in STAT3 protein levels over time when treated with IFNγ/TNFα (Figs. 3.5 and 3.6). The fact that HuR directly associates with *STAT3* mRNA suggests its involvement in regulating STAT3 expression during IFNγ and TNFα–induced muscle wasting. To investigate this possibility, we used C2C12 muscle cells treated or not with these cytokines in which we depleted HuR expression using a specific siRNA (Gherzi et al, 2010; van der Giessen et al, 2003). We observed, by performing RT-qPCR and western blot experiments respectively, that knocking down HuR expression significantly reduced *STAT3* mRNA (Fig. 3.7A) and protein (Figs. 3.7B, C) levels in untreated as well as IFNγ/TNFα treated conditions. This suggests that HuR is required for STAT3 expression during cytokine-induced muscle wasting.

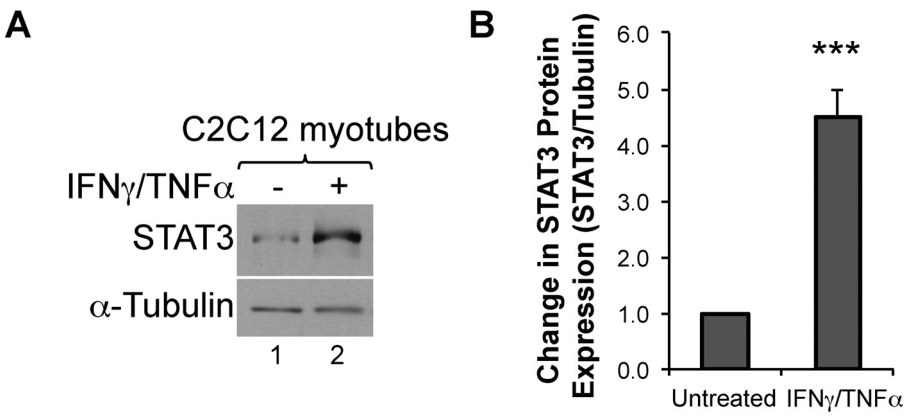



***Figure 3.5:*** **The expression of STAT3 protein increases in myotubes treated with or without IFNγ/TNFα. (A)** Lysates obtained from C2C12 myotubes treated with or without IFNγ/TNFα for 24h were used for western blot analysis with antibodies against STAT3 and α-tubulin. **(B)** Densitometric quantification of STAT3 signal relative to α-tubulin signal in Fig. 3.5A. Data represented as mean ± SEM (*n* = 3) with p = 0.0020 (***) by unpaired *t*-test.

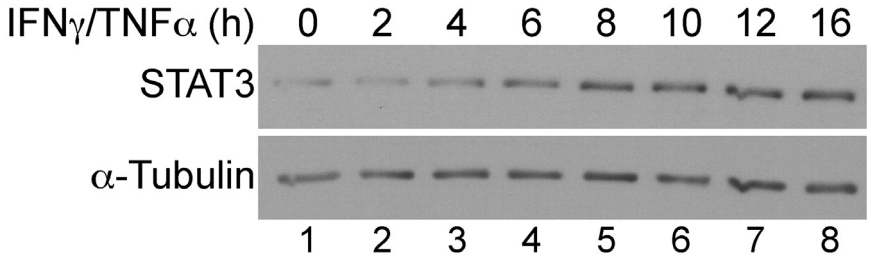



***Figure 3.6:*** **The expression of STAT3 protein increases over time in myotubes treated with or without IFNγ/TNFα.** Total lysates obtained from myotubes treated for the indicated period of time with IFNγ/TNFα were used in western blot experiments using antibodies against STAT3 and α-tubulin (assessed as a loading control). *Originally prepared as Supp. Fig. S3.*

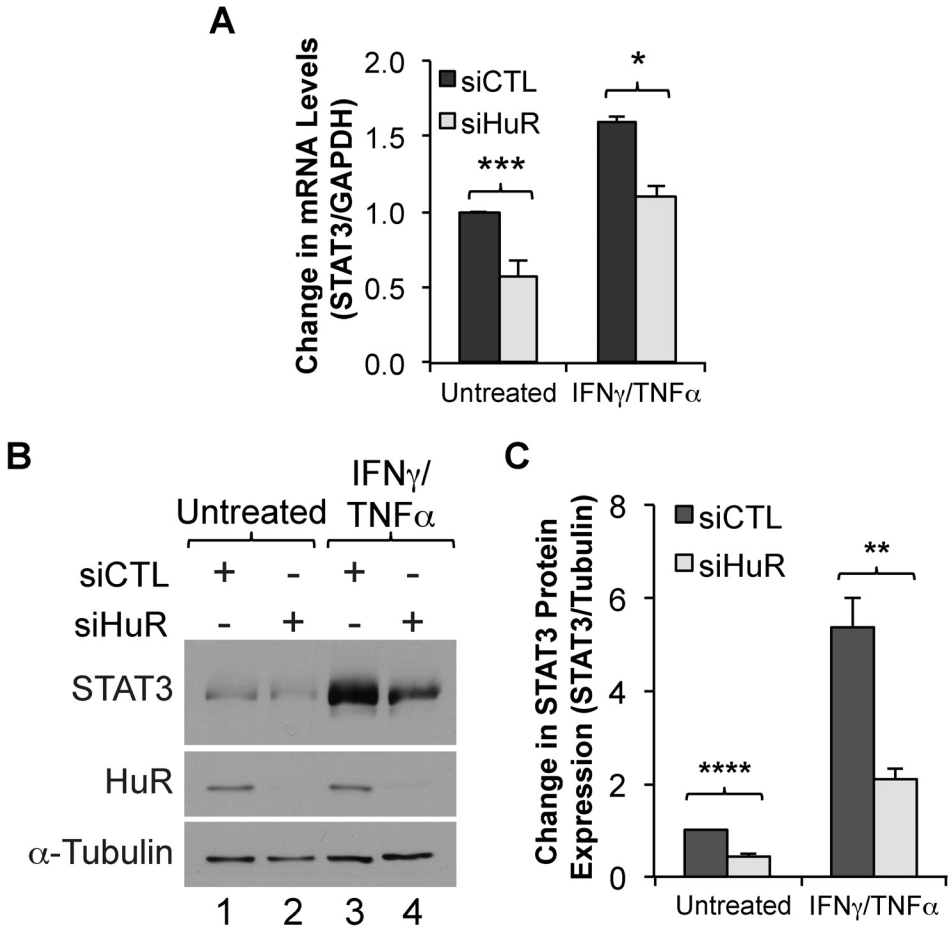
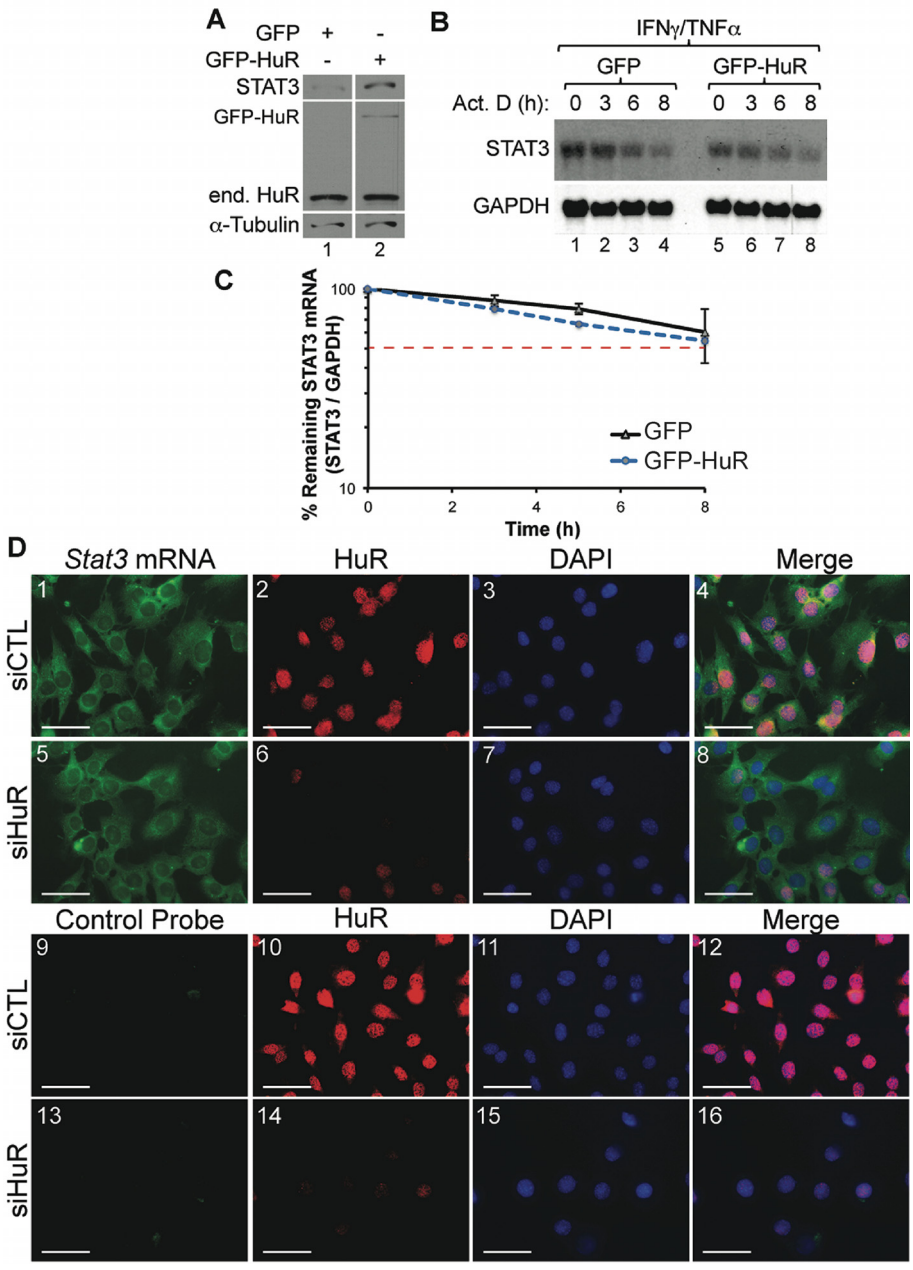



***Figure 3.7:*** **HuR regulates the expression of STAT3 during cytokine-induced muscle wasting. (A)** Total RNA extracted from C2C12 cells depleted or not of HuR treated with or without IFNγ/TNFα for 24h were analyzed by RT-qPCR using primers specific for STAT3 and GAPDH cDNAs. Data are represented as mean ± SEM (*n* = 3) with $p < 0.0001$ (****) and $p = 0.0184$ (*) by unpaired *t*-test. **(B)** Total cell lysate from C2C12 cells treated as described in (A) were used for Western blot analysis with antibodies against STAT3, HuR, and α-tubulin. The blot shown is representative of n = 3 experiments. **(C)** Densitometric quantification of STAT3 signal relative to α-tubulin signal in Fig. 3.7B (n = 3). Data are represented as mean ± SEM (*n* = *3*) with $p < 0.001$ (***) by unpaired *t*-test.

HuR is known to influence gene expression by modulating the stability, export, and/or the translation of its target mRNAs (Srikantan & Gorospe, 2012). Since we observed that silencing HuR decreased the steady-state levels of the *STAT3* mRNA, we first determined whether HuR regulates its stability in IFNγ/TNFα treated muscle cells. We performed Actinomycin D pulse-chase experiments (Drury et al, 2010) using muscle cells overexpressing exogenous HuR and treated or not with IFNγ/TNFα. Our data indicated that although the overexpression of a recombinant GFP-HuR increases STAT3 protein levels (as compared to the GFP control plasmid) (Fig. 3.8A), it had no effect on the stability of the *STAT3* mRNA (Figs. 3.8B, C). We next investigated if the decrease in STAT3 expression was the result of a change in HuR-mediated mRNA export. *In situ* hybridization experiments indicated that the cellular movement of *STAT3* message is not affected by the absence or presence of endogenous HuR (Fig. 3.8D). Our results therefore suggest that HuR does not affect the stability nor the localization of *STAT3* mRNA.

***Figure 3.8:*** **HuR does not affect the stability or localization of *STAT3* mRNA.** C2C12 myoblasts were transfected with GFP-HuR or GFP (as a control) for 24h then treated with IFNγ/TNFα for an additional 24h. **(A)** Western blot analysis with antibodies against STAT3, HuR, and α-tubulin. **(B)** The stability of the STAT3 mRNA in cells treated as described in (A) was determined by performing Actinomycin D pulse chase experiments. Cells were incubated with Actinomycin D for the indicated period of time. Total RNA was extracted and used for Northern blot analysis with radiolabelled RNA probes against *STAT3* and *GAPDH* mRNA. **(C)** Densitometric quantification of *STAT3* signal relative to *GAPDH* signal in Fig. 3.8B. Levels are shown as the percentage of mRNA remaining when compared to levels at 0 hour time point (shown as 100%). The dashed line indicates time point where 50% of mRNA is remaining. Data are represented as mean ± SD (*n* = 2) experiments. **(D)** C2C12 cells depleted or not of HuR were fixed and then stained with the anti-sense or sense probe for *STAT3* mRNA, HuR protein, and counterstained with DAPI (nuclear marker). Images are representative of *n* = 2 experiments. Scale bar = 50 μm.


A
GFP + -
GFP-HuR - +
STAT3
GFP-HuR
end. HuR
α-Tubulin
1 2
B
IFNγ/TNFα
GFP
GFP-HuR
Act. D (h): 0 3 6 8 0 3 6 8
STAT3
GAPDH
1 2 3 4 5 6 7 8
C
% Remaining STAT3 mRNA (STAT3 / GAPDH)
100
10
0 2 4 6 8
Time (h)
GFP
GFP-HuR
D
Stat3 mRNA
HuR
DAPI
Merge
siCTL
siHuR
Control Probe
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

We then assessed whether HuR affects the translation of the STAT3 mRNA in muscle cells under wasting conditions. Using sucrose fractionation experiment we followed the distribution of *STAT3* mRNA in polysome (P) and non-polysome (NP) fractions in muscle cells depleted or not of HuR and treated with or without IFNγ/TNFα (Figs. 3.9A, B). Neither the knockdown of HuR nor the treatment of muscle cells with cytokines affected general translation as determined by the profile of the polysome (P) and non-polysome (NP) fractions (Fig. 3.9A). Consistent with an increase in STAT3 protein levels as shown in Fig. 3.5A however, the levels of *STAT3* mRNA on polysomes dramatically increases in muscle cells treated with IFNγ/TNFα (Fig. 3.9B). The depletion of HuR in both untreated and IFNγ/TNFα-treated cells resulted in an overall shift of the *STAT3* mRNA from the P to the NP fractions when compared to control siRNA treated cells (Fig. 3.9B). Together, these data strongly support the idea that HuR is required for the translation of the *STAT3* mRNA in muscle cells exposed to wasting conditions.

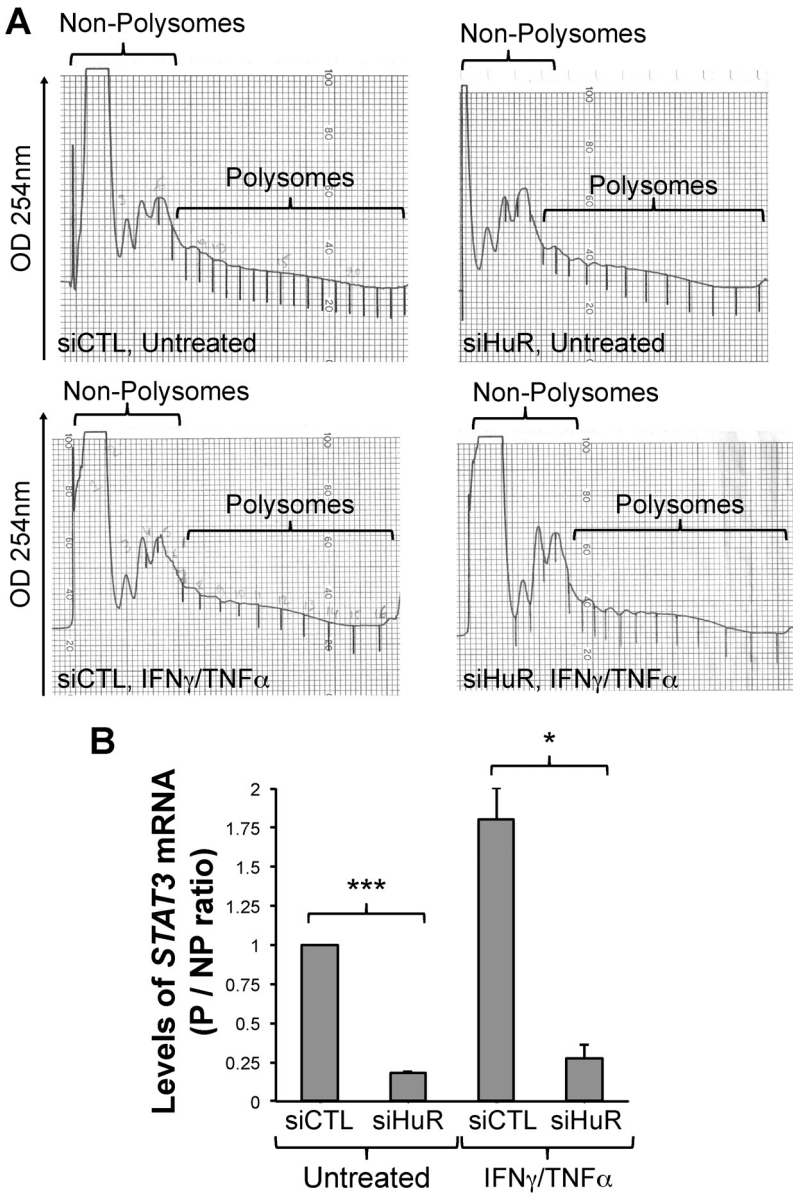



***Figure 3.9:*** **HuR promotes the translation of *STAT3* mRNA. (A)** Polysome profile of extracts of C2C12 cells either depleted of HuR or not and treated with or without IFNγ/TNFα for 24h. Profiles are representative of n = 2 experiments. **(B)** RNA was extracted from each fraction and RT-qPCR analysis was performed using primers for *STAT3* and *5.8S* mRNA. The levels of STAT3 relative to 5.8S were graphed as the Polysomal (P) to Non-polysomal (NP) ratio. Data are represented as mean ± SD (n = 2) with ***p-value = 0.001 and *p-value = 0.020 by two-tailed, unpaired Student's t-test.

### 3.4.3: HuR prevents miR-330-mediated translation inhibition of the STAT3 mRNA

Previous studies have demonstrated that miRNAs can compete or collaborate with HuR to modulate the translation of their target mRNAs (Meisner & Filipowicz, 2011; Srikantan et al, 2012). Therefore, we investigated whether there were any miRNAs in C2C12 myotubes that could impact the HuR-mediated translational regulation of *STAT3* mRNA. As a first step, we identified miRNA(s) that associate with HuR in C2C12 myotubes by performing a RIP experiment coupled to miRNA sequencing analysis using antibodies against HuR or IgG as a negative control. These experiments identified 15 miRNAs that associate with HuR in C2C12 muscle cells (Table 3.1). Interestingly, only one of these miRNAs, miR-330, is predicted by three online prediction programs (TargetScan, miRBase, and microrna.org) to target the *STAT3* mRNA 3'UTR (Fig. 3.10A). Interestingly, scanning the *STAT3-3'UTR* indicated that the seed element of miR-330 is found in close proximity to the HuR binding site. By repeating the RIP experiment followed by RT-qPCR using primers specific for miR-330 and snRNP U6 (negative control), we validated the association of HuR with miR-330 in both untreated as well as IFN$\gamma$/TNF$\alpha$-treated C2C12 myotubes (Figs. 3.10B, C) and muscle cells (Fig. 3.11). These data show that HuR associates with miR-330 in myotubes, raising the possibility that the translation of *STAT3* mRNA involves an interplay between these two trans-acting factors. To assess this possibility, we first determined the functional impact of miR-330 on STAT3 expression. We overexpressed a mimic of miR-330 in C2C12 cells and observed a significant decrease (~45%) in the level of STAT3 protein (Figs. 3.12A, B). This result suggested that miR-330 is involved in inhibiting STAT3

expression. These data coupled with the fact that the depletion of HuR decreased STAT3 protein level (Fig. 3.7B, C), suggest that HuR promotes STAT3 expression by interfering with the effects of miR-330. If this is true, silencing miR-330 should rescue the expression of STAT3 in muscle cells depleted of endogenous HuR. We showed that this is indeed the case and observed that an anti-miR-330 (antagomir) increased by at least two-fold the STAT3 protein level in HuR knock down cells when compared to cells treated with siHuR alone (Figs. 3.12C, D). These results therefore indicate that HuR promotes STAT3 expression in muscle cells by negating the effects of miR-330.

**Table 3.1: Complete list of miRNA that immunoprecipitate with HuR in C2C12 myotubes identified by RIP coupled to sequencing with a two-fold or higher signal.** *Originally prepared as Supp. Fig. S4.*

| miRNA that associate with HuR | Ratio of HuR Signal / IgG Signal |
|---|---|
| mmu-miR-3470b | 19.0 |
| mmu-miR-5114 | 14.0 |
| mmu-miR-3066 | 6.0 |
| mmu-miR-1231 | 3.0 |
| mmu-miR-3473b | 2.66 |
| mmu-miR-330 | 2.57 |
| mmu-miR-1948 | 2.40 |
| mmu-miR-92a-2 | 2.40 |
| mmu-miR-5126 | 2.38 |
| mmu-miR-503 | 2.32 |
| mmu-miR-155 | 2.27 |
| mmu-miR-1198 | 2.15 |
| mmu-miR-450b | 2.14 |
| mmu-miR-92a-1 | 2.03 |
| mmu-miR-700 | 2.00 |

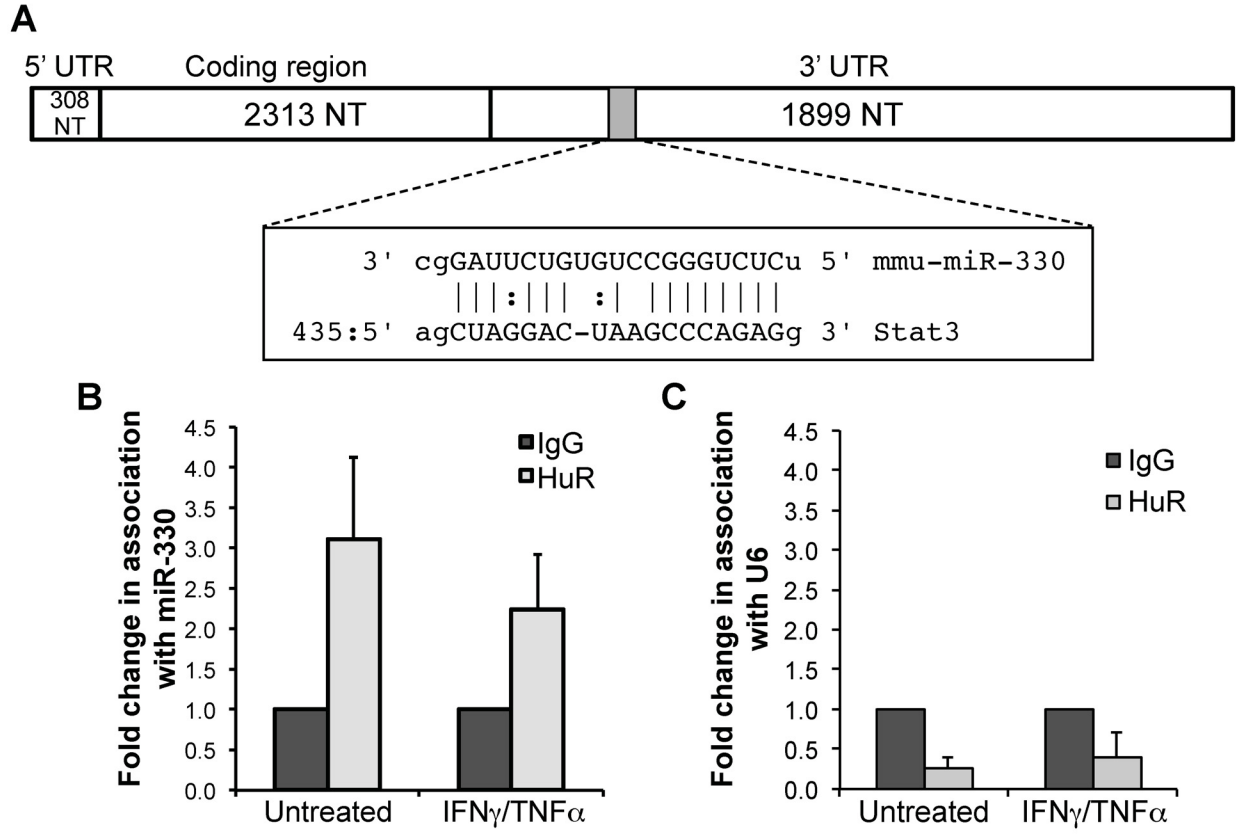



***Figure 3.10:*** **HuR associates with miR-330 in C2C12 myotubes treated with or without IFNγ/TNFα. (A)** Schematic demonstrating the alignment between the sequence of mmu-miR-330-5p with the predicted seed element in the 3' UTR of mouse *STAT3* mRNA. **(B, C)** Lysates obtained from C2C12 myotubes treated with or without IFNγ/TNFα for 24h were used for immunoprecipitation experiment using antibodies against HuR or IgG as a negative control. Analysis of miRNA isolated from the immunoprecipitate was performed by RT-qPCR using primers specific for miR-330 (B) or for U6 (C) as a negative control. Data are represented as mean ± SEM (*n* = 3).

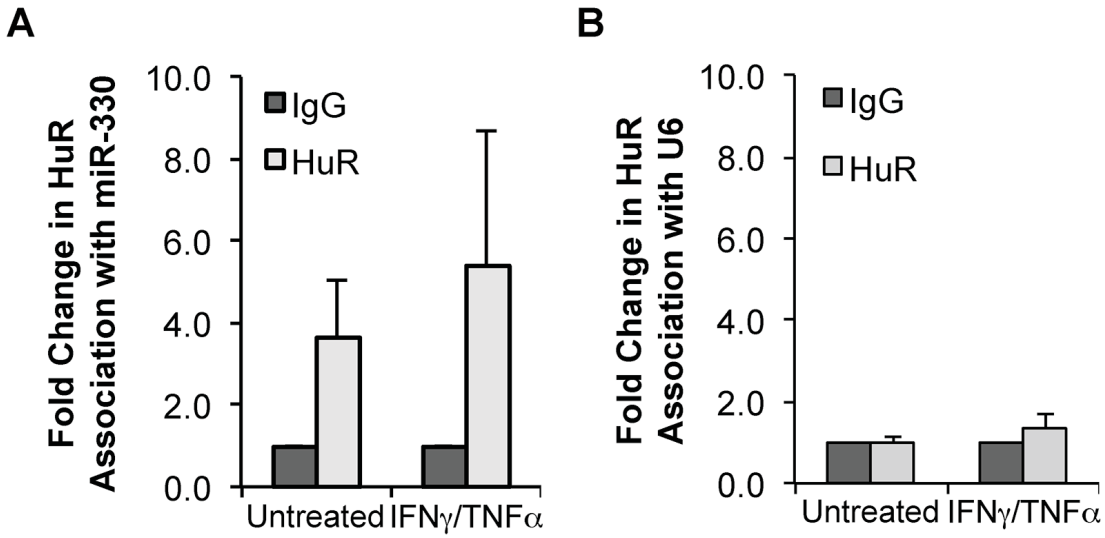



***Figure 3.11:*** **HuR associates with miR-330 in muscle cells treated with or without IFNγ/TNFα. (A, B)** Lysates from C2C12 muscle cells treated with or without IFNγ/TNFα for 24h were used for immunoprecipitation experiment using antibodies against HuR or IgG as a negative control. Analysis of miRNA isolated from the immunoprecipitate was performed by RT-qPCR using primers specific for miR-330 (A) or for U6 (B) as a negative control. Data are represented as mean ± SEM (*n* = 3). *Originally prepared as Supp. Fig. S5.*

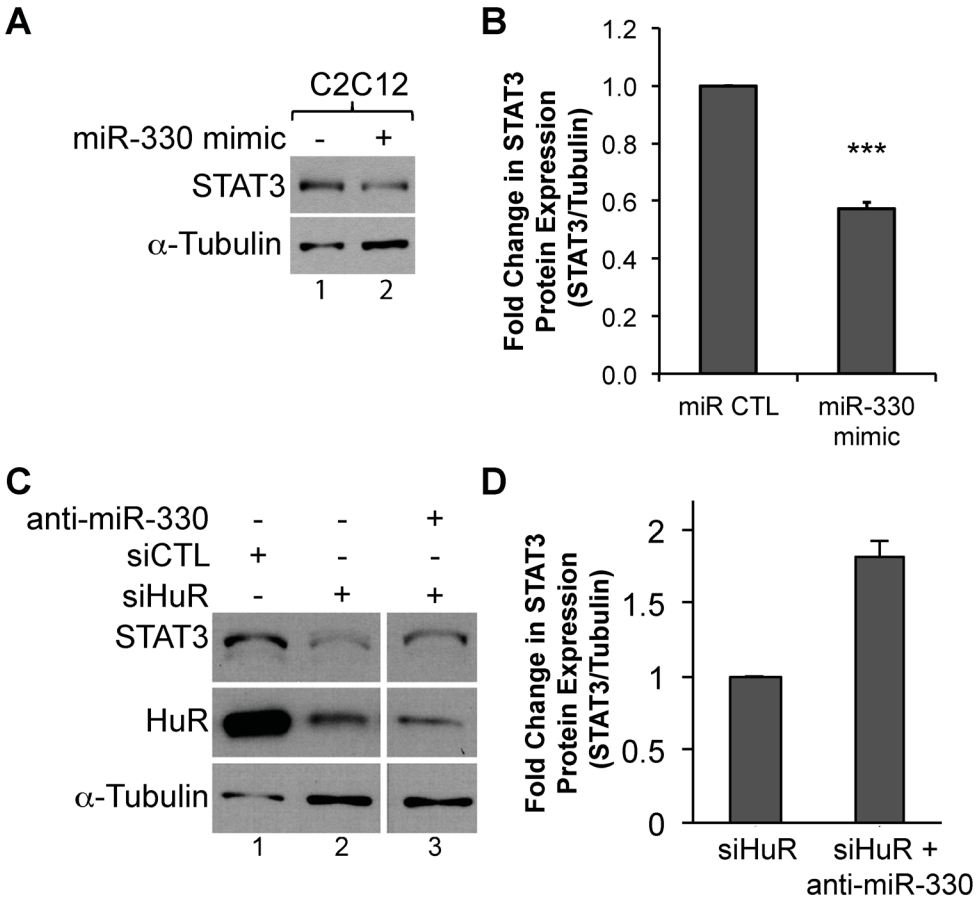



***Figure 3.12:*** **HuR negates the effect of miR-330 on the expression of STAT3. (A)** Total cell lysate from C2C12 cells transfected with a miR-330 mimic or a control miRNA were used for Western blot analysis with antibodies against STAT3 and α-tubulin. **(B)** Densitometric quantification of STAT3 signal relative to α-tubulin signal in Fig. 3.12A. Data are represented as mean ± SEM (n = 3) with $p < 0.0001$ (***). **(C)** C2C12 cells were transfected with siRNA targeting HuR or a non-specific control siRNA. The cells were then transfected with an antagomiR against miR-330. Total cell lysate was used for Western blot analysis using antibodies against STAT3, HuR, and α-tubulin. **(D)** Densitometric quantification of STAT3 signal were normalized to α-tubulin (C), and the STAT3 protein level in anti-miR-330 + siHuR treated cells is shown relative to siHuR. Data are represented as mean ± SD (n = 2).

### 3.5: Discussion

The correlation between the expression level of STAT3 and its function has been observed by several groups in various systems. We recently demonstrated that IFNγ and TNFα trigger the wasting of muscle fibers by promoting the phosphorylation of STAT3 on its tyrosine 705 residue (Ma et al, 2017). While the role of phosphorylated-STAT3 in cancer-induced muscle wasting is well-documented, the regulatory mechanisms modulating STAT3 expression under these conditions are still unknown. In this study, we address this gap and demonstrate the implication of posttranscriptional regulatory mechanisms in ensuring the high expression level of STAT3 protein in muscles undergoing atrophy. We show that, in response to IFNγ and TNFα, the well-known posttranscriptional regulator HuR associates with *STAT3* mRNA to promote its translation. To achieve this, HuR interferes with miR-330-mediated inhibition of STAT3 translation by binding to U-rich elements in the *STAT3*-3'UTR. Together our data support a model whereby maintaining a high expression level of STAT3 protein in an HuR-dependent manner represents one of the key steps required for IFNγ/TNFα-induced muscle loss.

In this study, we suggest that HuR-dependent STAT3 translation represents one of the ways by which cytokines such as IFNγ/TNFα ensure that enough STAT3 is available in muscle cells to promote wasting. While HuR directly promotes STAT3 translation by preventing miR-330 inhibitory effect (Figs. 3.9, 3.11, and 3.12), our data also indicate that HuR indirectly regulates the transcription of *Stat3* gene. We observed

the knockdown of HuR reduces the steady-state level of *STAT3* mRNA, the overexpression of HuR does not affect the stability of this message (Figs 3.7A and 3.8B-C). This indirect effect could simply be explained by an HuR-dependent feedback mechanism whereby, as previously shown, the newly translated STAT3 auto-promotes the transcription of its own genes (Narimatsu et al, 2001; Zimmers et al, 2016). Alternatively, this indirect effect on the *STAT3* mRNA expression could involve other HuR targets such as, the alarmin HMGB1, a known promoter of *Stat3* transcription (Dormoy-Raclet et al, 2013; Xu et al, 2015). Therefore, a better understanding of the direct and indirect role of HuR in modulating the expression of STAT3 during muscle wasting will provide more insight into the mechanisms involved in modulating the pro-cachectic function of STAT3.

Our data suggest that HuR mediates its effect on STAT3 expression by directly binding to U-rich elements (P2-B and to a lesser extent P16). One of the consequences of this binding is the inhibition of the miR-330 effect on STAT3 translation. There are numerous examples of cross-talk between HuR and miRNA in the regulation of HuR mRNA targets. For example, HuR and miR-16 were found to regulate *cyclin E1* mRNA while simultaneously binding to the message in breast cancer cells (Guo et al, 2015). In contrast, HuR has been found to compete with miR-195 for binding to the *stim1* mRNA (Zhuang et al, 2013). Our laboratory has previously shown that HuR negates the affect of miR-1192 on HMGB1 expression despite the fact that they both simultaneously associate to the HMGB1 mRNA in muscle cells (Dormoy-Raclet et al, 2013). In this study, we demonstrate that the overexpression of miR-330 decreased STAT3 protein

levels while the use of antagomirs targeting this miRNA rescued the decreased expression of STAT3 in cells depleted of HuR (Fig. 3.12). These results clearly demonstrate that the interplay between HuR and miR-330 impacts STAT3 expression. However, we also show that depleting miR-330 activity did not fully restore STAT3 protein levels in HuR-knockdown muscle cells (Fig. 3.12C-D). This observation raises the possibility that other *trans*-acting factors are involved in regulating STAT3 expression. Indeed, it has been shown that the HuR-binding sites P2-B and P16 that we describe in this study associate with other RBPs, such as Arid5a and CPEB1. Arid5a stabilizes *STAT3* mRNA by competing with the endoribonuclease Regnase-1 for binding to a stem-loop structure (1738-1765) that is found within the P16 element (Masuda et al, 2016). On the other hand, CPEB1 prevents the synthesis of STAT3 protein by binding to two putative U-rich-elements, one of which is located within the HuR-binding site P2-B (Alexandrov et al, 2012). Therefore, the possibility exists that competition or collaboration with these or other factors contribute to the HuR-mediated modulation of STAT3 expression in response to various stimuli. Together, our data indicate that uncovering these mechanisms may lead to the identification of novel therapeutic options that can be exploited to interfere with STAT3-induced muscle wasting.

### 3.6: Materials and methods

#### 3.6.1: Cell culture and transfection

C2C12 cells were obtained from American Type Culture Collection (VA, USA) and grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing high glucose, L-glutamine, sodium pyruvate, and supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin. Transfections with siRNA specific for HuR or as well as plasmids expressing GFP or GFP-HuR were performed using jetPrime reagent (PolyPlus Transfection) according to manufacturer's protocol. The siRNA against HuR (5'- CAUCAACACCGAGAUCAAAdTdT -3') were custom synthesized (van der Giessen et al, 2003). The mmu-miR-330-5p antagomir or mimic (Invitrogen-Ambion) were transfected at a final concentration of 200 nM using jetPrime reagent (PolyPlus Transfection) according to manufacturer's protocol.

#### 3.6.2: miRNA Prediction

The miRNA predicted to associate with the STAT3 3'UTR was determined on the TargetScan (www.targetscan.org), miRanda (www.miRbase.org) and microrna.org websites.

#### 3.6.3: Western blot analysis

Whole cell lysates were prepared by lysis in buffer containing 50 mm HEPES (pH 7.0), 150 mm NaCl, 10% glycerol, 1% Triton X-100, 10 mm sodium pyrophosphate, 100 mm NaF, 1 mm EGTA, 1.5 mm $MgCl_2$, 0.1 mM sodium ortho-vanadate, and complete

EDTA-free protease inhibitors (Roche Applied Science). Primary antibodies used were total STAT3 (#9132 or 9139; Cell Signaling), α-tubulin (Developmental Studies Hybridoma Bank), and 3A2 (anti-HuR) (Cammas et al, 2014).

### 3.6.4: Quantitative PCR (qPCR)

One microgram of total RNA was reverse transcribed with the M-MuLV Reverse Transcriptase (New England BioLabs) according to the manufacturer's protocol. Each sample was diluted 1/20 and used to detect the mRNA levels of *STAT3* and *RPL32* or *GAPDH* as a loading control. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCt is the difference in Ct values between the target and reference genes. Primers used for qPCR are as follows. STAT3 (F: 5' TAT CTT GGC CCT TTG GAA TG – 3', R: 5' - GCT GCT TGG TGT ATG GCT CT – 3'), GAPDH (F: 5' - AAG GTC ATC CCA GAG CTG AA - 3', R: 5' - AGG AGA CAA CCT GGT CCT CA – 3'), RPL32 (F: 5' - TTC TTC CTC GGC GCT GCC TAC GA – 3', R: 5' - AAC CTT CTC CGC ACC CTG TGG TCA – 3').

### 3.6.5: RNA-Immunoprecipitation (RIP)

Total cell extract (TCE) from C2C12 cells either untreated or treated with IFNγ/TNFα were prepared as previously described (Cammas et al, 2014). Immunoprecipitation was performed with an antibody against HuR (3A2) or IgG (Jackson ImmunoResearch Laboratories) as previously described. RT-qPCR experiments were then performed on mRNA or miRNA that was purified from the

immunoprecipitate. Analysis of miRNA was performed using the Universal cDNA synthesis kit (Exiqon) followed by qPCR analysis for miRNA using the SyBr Green Master Mix kit (Exiqon) as we have previously described (Dormoy-Raclet et al, 2013).

### 3.6.6: RNA-IP coupled to RNA-Sequencing (RIP-Seq)

IP was performed as described above, with alterations to the isolation of RNA method. miRNA was isolated using a miRNasy mini kit (Qiagen). Sequencing and analysis were performed at Institute for Research in Immunology and Cancer (IRIC), Université de Montréal (Montreal, QC, Canada).

### 3.6.7: Actinomycin D Pulse-Chase and Northern blot analysis

Actinomycin D pulse-chase experiments were performed as previously described (Di Marco et al, 2012). Briefly, C2C12 muscle cells were transfected with GFP only or GFP-HuR using jetPrime reagent (PolyPlus Transfection) according to manufacturer's protocol. After 24h, cells were treated with IFN$\gamma$/TNF$\alpha$ for 24h before treatment with 5 $\mu g\ mL^{-1}$ of ActD, a transcriptional inhibitor, for the indicated periods of time. Total RNA was then isolated using TRIzol reagent (Invitrogen). Northern blot analysis was used to determine the *STAT3* and *GAPDH* mRNA levels. Briefly, samples were separated on a 1.2% formaldehyde-agarose gel. RNA was transferred to a nylon membrane by capillary blot then cross-linked before hybridization with radiolabeled cDNA probes to detect *STAT3* or *GAPDH* mRNA as previously described (Di Marco et al, 2005).

### 3.6.8: Polysome fractionation

Forty million C2C12 cells either untreated or treated with IFNγ/TNFα and either depleted of HuR or not were prepared for each sample. Cells were treated with the protein synthesis inhibitor cycloheximide (CHX; 100 mg $mL^{-1}$) for 15 min at 37°C immediately before harvesting. The cytoplasmic extracts of C2C12 cells were obtained by lysing pellets with a Dounce homogenizer and tight pestle. Isolated cytoplasmic fractions were centrifuged at 130 000g for 2h on a sucrose gradient (15-50%) and fractionated using a gradient fractionation system (Brandel) and absorbance was monitored at 254nm. RNA was extracted using phenol/chloroform from each fraction and the non-polysome (NP) fractions and polysome (P) fractions were combined. The levels of *STAT3* and *5.8S* mRNA were analysed using RT-qPCR. The *STAT3* mRNA levels were normalized to *5.8S* mRNA levels then the polysome / non-polysome (P/NP) ratio was determined and further normalized to the control siRNA, untreated condition.

### 3.6.9: Immunofluorescence

Cells were fixed in 3% para-formaldehyde for 20min, permeabilized with 0.1% Triton-X100 in PBS for 20 minutes then incubated with myoglobin (1:250; ab77232, Abcam) and myosin heavy chain (1:1000; clone MF-20, Developmental Studies Hybridoma Bank) (Ma et al, 2017). Images were taken with a 40X magnification lens on an inverted Axiovert 200M microscope with an Axiocam MRm camera (Zeiss).

### 3.6.10: Fluorescent *in-situ* hybridization

*in-situ* hybridization were performed as previously described (Dormoy-Raclet et al, 2013). Briefly, the sense and anti-sense probes for *STAT3* mRNA were generated from PCR fragments with the following sequence and fused to the indicated RNA polymerase promoter: anti-sense fused to T7 promoter (5' – TAA TAC GAC TCA CTA TAG GGT GCA CCA GCT GTA CAG CGA AC – 3'), sense fused to T3 promoter (5' AAT TAA CCC TCA CTA AAG GGG ATC CTG CAC TCG CTT CCG G – 3'). Probes were DIG-labeled according to manufacturer's instructions (Roche). C2C12 cells depleted or not of HuR were fixed and permeabilized as described above. Cells were incubated with DIG-labeled anti-sense or control sense probe to detect *STAT3* mRNA as well as the 3A2 (anti-HuR) antibody (1:1000). Cells were blocked with 1% goat serum for 10 min and incubated with anti-DIG and goat anti-mouse secondary antibody.

### 3.6.11: RNA Electrophoretic Mobility Shift Assay (REMSA)

cDNA templates for the 16 cRNA probes spanning the STAT3 3'UTR were generated as previously described (Dormoy-Raclet et al, 2013). Briefly, cDNA for 14 of the 16 cDNAs spanning the STAT3 3'UTR, with the exception of P4 and P5, were amplified by PCR using the complete STAT3 3'UTR as a template and individual primer sets specific for each region (Table 3.2). PCR products for these regions were purified with an EZ-10 PCR product spin column purification kit (BioBasic). The P4 and P5 cDNA templates were obtained by annealing sense and anti-sense oligonucleotides spanning these regions. The radiolabelled probes were generated from these DNA

templates by *in vitro* transcription reaction using radiolabelled $\alpha$-$^{32}$P-UTP and T7 RNA polymerase (Promega). Recombinant GST or GST-HuR were incubated with 100 000 cpm of radiolabelled probe per reaction for 15 minutes and then with 100 $\mu$g of heparin for 15 min. After, samples were run for 2.5hrs at 180V on a native 5% acrylamide gel that had been pre-run for 1h at 80V. After fixation for 15 min, the gels were dried at 80°C for 1h and exposed at -80°C. For REMSAs performed with RNAse T1, recombinant GST or GST- HuR was first incubated with the radiolabelled mRNA for 15 min to allow complex formation, then with heparin, followed by digestion with 300U RNAse T1 (Thermo Fisher) for 10 min at room temperature.

### 3.7: Acknowledgements

We would like to thank D. Hall for helpful discussions and comments on the manuscript. This work is funded by a CIHR operating grant (MOP-142399) and a Qatar National Research Fund (QNRF) (NPRP8-457-3-101) to I.E.G. J.F.M. was supported by the CIHR/FRSQ training grant in cancer research FRN53888 of the McGill Integrated Cancer Research Training Program.

**Table 3.2:** List of primers used to generate STAT3-3'UTR probes for REMSA. Bolded sequences represent the T7 promoter sequence used for in vitro transcription.

| PROBES | PRIMERS | SEQUENCE |
|---|---|---|
| ***P1*** | For | **TAATACGACTCACTATAGGG**TTGAGTCGCTCACGTTTGAC |
| | Rev | TTCAGACGATATGGGGTTCG |
| ***P2*** | For | **TAATACGACTCACTATAGGG**CGAACCCCATATCGTCTGAA |
| | Rev | CCCCAGACTCAGAGGTGCT |
| ***P3*** | For | **TAATACGACTCACTATAGGG**ATGGGGCTGAGAGCAGAAGG |
| | Rev | CCTGGGGTATCAGCTCACAG |
| ***P6*** | For | **TAATACGACTCACTATAGGG**TTAAAGGGCAAAACACACTGA |
| | Rev | ATATAGATGATCAGCCTGAAGACA |
| ***P7*** | For | **TAATACGACTCACTATAGGG**AAACCCTGGAATGGTTGCAG |
| | Rev | CGACAAGCATACTGGGTAAA |
| ***P8*** | For | **TAATACGACTCACTATAGGG**CTGGGTTTTGTTTACCCAGT |
| | Rev | TGACCCTAGGCCCAGTAAG |
| ***P9*** | For | **TAATACGACTCACTATAGGG**CCTGTGGGTGCCTTACTGG |
| | Rev | CAATCCCTCTCGACACAAGG |
| ***P10*** | For | **TAATACGACTCACTATAGGG**CCTTGTGTCGAGAGGGATTG |
| | Rev | TGAGGGCAGACCTGGAGTAT |
| ***P11*** | For | **TAATACGACTCACTATAGGG**ATACTCCAGGTCTGCCCTCA |
| | Rev | AGCAAGCAAGCGACATGG |
| ***P12*** | For | **TAATACGACTCACTATAGGG**CCTCCATGTCGCTTGCTT |
| | Rev | TGGAAGGAAACAATTCCACAG |
| ***P13*** | For | **TAATACGACTCACTATAGGG**AGCTGATCAGTGTCTGTGGA |
| | Rev | AGAGCAGTAACTGTCTCGAAG |
| ***P14*** | For | **TAATACGACTCACTATAGGG**CCACCCCATCAATGTTCTTT |
| | Rev | GTACAACACCAGCCCCAGAG |
| ***P15*** | For | **TAATACGACTCACTATAGGG**GTTGTACCTCAGGGGCTCT |
| | Rev | GCACTGAAAGGCTAAGCAGT |
| ***P16*** | For | **TAATACGACTCACTATAGGG**AGTGGCTTGTGTTCTGGC |
| | Rev | GACAGTTCCTATATAATCTTT |
| *Oligonucleotide fragments (sense and antisense) of P4 and P5 probes were chemically synthesized and annealed as described in Materials and methods sections.* | | |
| ***P4*** | **TAATACGACTCACTATAGGG**CTGGGAACTCCTGGCTCTGCACT<br>TTCAACCTTGCTAATAT | |
| ***P5*** | **TAATACGACTCACTATAGGG**CCACATAGAAGCTAGGACTAAGC<br>CCAGAGGTTCCTCTTTAAATTAAAAAAAAAAAAAATAAGAATTAAA | |

# CHAPTER 4

# General Discussion and Future Directions

## 4.1: Overview

Cachexia is largely considered an end-of-life syndrome where afflicted individuals were traditionally treated with palliative care. Clinical trials assessing therapies that block pro-inflammatory cytokines individually have proven ineffective at preventing or improving cachexia and the associated muscle wasting that occurs, highlighting a need for a better understanding of how pro-inflammatory cytokines are triggering muscle loss. My work in chapter 2 has uncovered that STAT3 is a key mediator of muscle wasting induced by IFNγ and TNFα and is phosphorylated independently of IL-6. Additionally, I show that in this model of muscle wasting, STAT3 localization to the nucleus is NF-κB dependent and the expression of iNOS, a pro-cachectic gene, is synergistically activated by STAT3 and NF-κB. Together, this work demonstrates how multiple cytokines known to promote muscle wasting other than IL-6 contribute to the activation of a common downstream factor such as STAT3 and further indicates that STAT3 is likely a better therapeutic target rather than any of its upstream activators. In chapter 3, I investigate how the posttranscriptional regulation of STAT3 by the RNA-binding protein HuR contributes to its expression during IFNγ/TNFα-induced muscle wasting. In this work, I uncover that HuR promotes the translation of STAT3 by blocking the inhibitory effect of miR-330, implicating a novel molecular mechanism that promotes muscle wasting.

## 4.2: An overall role for STAT3 and its regulation during muscle wasting

In this thesis, we show that during IFNγ/TNFα-induced muscle wasting, the activation of STAT3 plays a crucial role in promoting muscle wasting, inducing the expression of genes such as iNOS. The expression of STAT3 is also induced by IFNγ/TNFα and posttranscriptionally regulated by HuR and miR-330. The work presented here suggests that the induction of IFNγ/TNFα-induced muscle wasting may occur in two distinct phases of STAT3-dependent signaling. The early phase occurs immediately in response to IFNγ/TNFα stimulation through pY-STAT3-NF-κB-dependent transcription while the late phase of STAT3 signaling would occur as a result of the accumulation levels of STAT3 that is dependent on HuR and producing a positive feedback loop, as suggested by a previous study (Yang et al, 2007). Although this body of work provides significant insight into how STAT3 is involved in promoting muscle wasting and a mechanism involved in its regulation, it remains unclear how STAT3-dependent mechanisms contributes to muscle wasting and warrant further investigation.

## 4.3: The role of STAT3 in mediating muscle wasting

### 4.3.1: STAT3 and NF-κB - dependent gene expression

The main function of STAT3 and NF-κB is to drive gene expression by binding to short DNA sequence motifs (Hutchins et al, 2013a). Numerous studies have demonstrated that STAT3 activates pathways that lead to muscle loss (Bonetto et al,

2011; Silva et al, 2015; Zhang et al, 2013). The expression of genes is dependent on whether their promoters contain binding sites for either STAT3 or NF-κB or both, resulting in control of these genes by either one or both STAT3 and NF-κB (Grivennikov & Karin, 2010). This also results in groups of genes controlled by either STAT3 or NF-κB or both. My work shown in chapter 2 indicates that STAT3 and NF-κB are important promoters of muscle wasting, in part, by activating the pro-cachectic gene iNOS. However, the network of genes that are regulated by STAT3 and NF-κB during muscle wasting remains elusive.

In order to identify novel candidate genes that are regulated during IFNγ/TNFα-induced muscle wasting specifically by STAT3 and NF-κB, we performed chromatin immunoprecipitation coupled to massively parallel sequencing (ChIP-Seq). Preliminary analysis (*performed at IRIC*) indicates that both STAT3 and p65 are associated with known and novels candidate genes after 1h IFNγ/TNFα treatment. A partial list of genes with a STAT3 binding site is provided in Table 4.1. A technique used to highlight biological processes within a gene set is gene ontology (GO) analysis (Harris et al, 2004). For STAT3-associated genes, GO analysis shows a bias towards the regulation of various metabolic processes (Table 4.2). Interestingly, STAT3 has been previously implicated in the regulation of cellular metabolism (Demaria et al, 2014) and raises the possibility of the role of STAT3 in regulating cellular metabolism during muscle wasting.

**Table 4.1: Selected genes with STAT3 binding sites identified by ChIP-Seq.**

| ID | Gene name |
|---|---|
| Ptpn1 | Protein tyrosine phosphatase, non-receptor type 1 |
| Ppp2cb | Protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform |
| Ccl28 | Chemokine (C-C motif) ligand 28 |
| Akt1 | Thymoma viral proto-oncogene 1 |
| Irf2bp2 | Interferon regulatory factor 2 binding protein 2 |
| Ifngr2 | Interferon gamma receptor 2 |
| Nfkbiz | Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, zeta |
| Col15a1 | Collagen, type XV, alpha 1 |
| Pax3 | Paired box 3 |
| Fbxo45 | F-box protein 45 |
| Trim27 | Tripartite motif-containing 27 |
| Trim67 | Tripartite motif-containing 67 |
| Bcl6 | B cell leukemia/lymphoma 6 |
| Zfp217 | Zinc finger protein 217 |
| Tiparp | TCDD-inducible poly(ADP-ribose) polymerase |
| Arl8a | ADP-ribosylation factor-like 8A |
| Rac1 | RAS-related C3 botulinum substrate 1 |
| Myo6 | Myosin VI |

**Table 4.2: Gene ontology analysis of genes associated with STAT3 sites using HOMER.**

| TermID | Term | logP |
|---|---|---|
| GO:0031323 | regulation of cellular metabolic process | -2.1716E+02 |
| GO:0080090 | regulation of primary metabolic process | -1.8920E+02 |
| GO:0019222 | regulation of metabolic process | -1.8859E+02 |
| GO:0019219 | regulation of nucleobase-containing compound metabolic process | -1.7567E+02 |
| GO:0051171 | regulation of nitrogen compound metabolic process | -1.7454E+02 |

A particular gene of interest is hypoxia responsive factor 1 alpha (Hif1$\alpha$), known to promote a glycolysis-like state and downregulate mitochondrial activity (Demaria et al, 2014; Demaria et al, 2010). Reduced mitochondrial oxidative capacity and muscle dysfunction are associated with various types of muscle wasting disorders mediated by systemic inflammation (Basic et al, 2014; Rocheteau et al, 2015; Sato et al, 2013). It seems possible that the reduced mitochondrial oxidative capacity that contributes to increased muscle fatigue and long-term patient disability results from STAT3-mediated Hif1$\alpha$ expression. It would be interesting to validate the association of STAT3 to the Hif1$\alpha$ promoter and explore whether Hif1$\alpha$ contributes to STAT3-mediated muscle wasting.

In addition, there may be genes that STAT3 and p65 are regulating together. A comparison of genes found in the STAT3 and p65 lists indicates that there are 554

genes that are the same (Fig. 4.1), suggesting that cooperativity between STAT3 and p65 is occurring (partial list in Table 4.3). Using GO analysis of the overlapping genes indicated a bias for the nervous system development and regulation of transcription from RNA Pol II promoter (Huang da et al, 2009a; Huang da et al, 2009b). Notably, neuropathy is a known complication of critical illness that can worsen and prolong muscle dysfunction (Latronico & Bolton, 2011). A factor found in the list of overlapping genes is the glial cell line-derived neurotrophic factor (Gdnf), produced by the skeletal muscle and involved in regulating adult neuromuscular junction (Zwick et al, 2001). It would be interesting to validate *Gdnf* as a STAT3 and NF-κB target and explore the role of Gdnf during muscle wasting. Further investigation is needed to assess the possibility that STAT3-NF-κB-regulated genes contribute to muscle wasting.

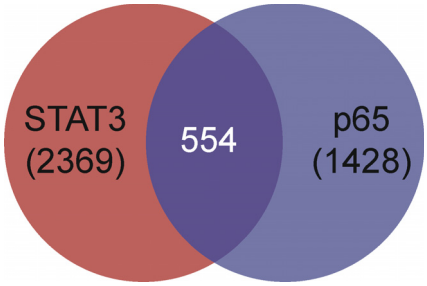



**Figure 4.1: The number of overlapping genes between STAT3 and p65 from IFNγ/TNFα-treated C2C12 myotubes.**

**Table 4.3: Select genes that contain both STAT3 and p65 binding sites**

| ID | Gene name |
|---|---|
| Ccl28 | Chemokine (C-C motif) ligand 28 |
| Filip1l | Filamin A interacting protein 1-like |
| Trim8 | Tripartite motif-containing 8 |
| Nfatc1/2 | Nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1/2 |
| Relb | avian reticuloendotheliosis viral (v-rel) oncogene related B |
| Jund | Jun D proto-oncogene |
| Pink1 | PTEN induced putative kinase 1 |
| Pex2 | Peroxisomal biogenesis factor 2 |
| Fbxo4 | F-box protein 4 |
| Eif5 | Eukaryotic translation initiation factor 5 |
| Eif4a3 | Eukaryotic translation initiation factor 4A3 |
| Cxxc5 | CXXC finger 5 |
| Celf5 | CUGBP, Elav-like family member 5 |
| Camk2n1 | Calcium/calmodulin-dependent protein kinase II inhibitor 1 |
| Bnip2 | BCL2/adenovirus E1B interacting protein 2 |
| Aggf1 | Angiogenic factor with G patch and FHA domains 1 |
| Nfkb1 | Nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105 |
| Pax7 | Paired box 7 |

### 4.3.2: Non-transcriptional mechanisms involved in STAT3-mediated muscle wasting

We show that IFNγ/TNFα induces the translocation of pY-STAT3 to the nucleus (Fig 2.14B). However, this data also indicates that total STAT3 levels remain largely cytoplasmic. How STAT3 localized to other subcellular compartments is involved in muscle wasting is unknown, though, several possibilities for STAT3 have been described. One possibility is non-phosphorylated cytoplasmic STAT3, which was shown to repress autophagy by directly binding and inhibiting PKR in cancer cells (Niso-Santano et al, 2013; Shen et al, 2012). In contrast, a study linked the activation of the Fyn-STAT3-Vps34 pathway to increase autophagic flux and muscle wasting in fast-twitch myofibers during starvation (Yamada et al, 2012). Another possibility is mitochondrial STAT3, which was shown to enhance coupled Complex I and II activity and reduce reactive oxygen species production (Boengler et al, 2010; Gough et al, 2009; Wegrzyn et al, 2009). These numerous possibilities suggest that STAT3 may be involved in non-transcriptional pathways that lead to muscle wasting and merit further investigation.

### 4.3.3: Other mechanisms of IFNγ/TNFα-induced muscle wasting

My work has demonstrated that IFNγ/TNFα synergistically induce the expression of iNOS and that the knockout of iNOS prevented muscle wasting induced by IFNγ/TNFα. Although the inhibition of iNOS/NO has been linked to the prevention of muscle wasting in various models (Bae et al, 2012; Di Marco et al, 2005), our

understanding of the molecular mechanisms of how this occurs is poorly understood. One mechanism in which the iNOS/NO pathway could contribute to the progression of muscle wasting is through the ability of NO to react with oxygen radicals to form peroxynitrite, a toxic molecule that can nitrate other molecules and cause DNA damage (Litvinova et al, 2015). Importantly, peroxynitrite is known to promote mitochondrial damage and irreversibly inhibit the mitochondrial respiratory chain, particularly complexes I and IV (Brown & Borutaite, 2004; Tengan et al, 2012). It is possible that iNOS/NO may be contributing to muscle wasting by damaging mitochondria, causing mitochondrial dysfunction and decreased mitochondrial oxidative capacity, which is recognized as a major contributing factor to muscle wasting (Brown & Borutaite, 2004; Fontes-Oliveira et al, 2013; Julienne et al, 2012). Further experiments to investigate this possibility would clarify this.

Another possibility by which the iNOS/NO pathway may contribute to muscle wasting is through the S-nitrosylation of proteins, a posttranslational modification known to cause protein inactivation (Benischke et al, 2014). Studies have linked S-nitrosylation of contractile proteins to reduced force production and movement in skeletal muscle (de Winter & Ottenheijm, 2017; Dutka et al, 2016; Figueiredo-Freitas et al, 2015). Indeed, muscle weakness is a common feature in cachectic patients and further studies are needed to clarify whether S-nitrosylation of proteins leads to muscle dysfunction during cachexia.

In addition, IL-6 is a crucial cytokine that promotes cachexia and muscle loss. We demonstrate that IL-6 KO mice are resistant to IFNγ/TNFα-mediated muscle wasting, indicating that there are IL-6-dependent pathways that are required for atrophy to occur. Previous studies demonstrate that increased levels of IL-6 are associated with reduced muscle oxidative capacity and correlated with an overall reduction in oxidative capacity in cachectic skeletal muscle (White et al, 2010). Furthermore, IL-6 was found to regulate mitochondrial remodeling/dysfunction observed in cachectic skeletal muscle (White et al, 2012). It is interesting that IL-6 also has known effects on mitochondrial health and suggests a possible collaboration between IL-6 and iNOS, whose expression is not affected in IL-6 KO mice (Fig 2.12 and Ma et al, 2017), that leads to mitochondrial dysfunction during muscle wasting. Investigation into the collaboration between IL-6 and iNOS/NO on mitochondrial function may clarify this further.

## 4.4: Posttranscriptional regulation of STAT3 expression

In chapter 3, we show that HuR associates with STAT3 mRNA in muscle cells and promotes the translation of STAT3 mRNA, suggesting that this HuR-dependent STAT3 translation is an important step by which IFNγ/TNFα induces muscle wasting. Although we identify that STAT3 expression depends on HuR, we do not fully define the HuR binding motif within the P2 region of the STAT3 3'UTR. To further investigate the mechanism through which HuR regulates STAT3, further experiments should be carried out to verify the functionality *in vivo* by performing a luciferase reporter assay using a

construct containing the full STAT3 3'UTR with a mutation in the P2-B putative HuR binding site and comparing to the activity of the wild-type 3'UTR.

In addition to P2, four additional direct binding sites within the STAT3 3'UTR were identified, but their impact on STAT3 expression remains unknown. Other mRNAs, such as P53 and COX-2, are also known to be regulated by HuR via multiple binding sites (Abdelmohsen et al, 2014; Mazan-Mamczarz et al, 2003; Sengupta et al, 2003; Wang et al, 2000). It is possible that multiple HuR binding sites may provide a cell with numerous points of regulation to fine-tune the expression of its target mRNA. Determining the functionality of the other binding sites specifically in muscle cells should also be verified *in vivo* using reporter constructs with individual mutations would provide better insight into whether the other sites also contribute to the regulation of STAT3 expression.

STAT3 is known to be regulated by miRNA during disease such as experimental ulcerative colitis and cancer (He et al, 2013; Koukos et al, 2013). In this study, we identified miR-330-5p, a conserved small non-coding RNA that associates with HuR in muscle cells and targets the STAT3 3'UTR, down-regulating the expression of STAT3 (Fig 3.7, 3.8). However, the direct binding of miR-330 to its predicted binding site in the STAT3 3'UTR should be confirmed by luciferase reporter assay using a luciferase reporter construct containing the STAT3 3'UTR, with or without a mutation in the miR-330 binding site.

Furthermore, previous studies indicate that miR-330 plays a role in disease such as cancer (Su et al, 2016; Trehoux et al, 2015). However, it is not known whether miR-330 expression is dysregulated during IFNγ/TNFα-induced muscle wasting. Also unknown is whether miR-330 can target other mRNA, in additional to STAT3, which may be involved in promoting muscle loss. Further investigation into miR-330 and its target mRNA may clarify whether miR-330 is capable of modulating muscle wasting.

In chapter 3, we implicate the biological significance of HuR-miR-330-mediated regulation of STAT3 expression during IFNγ/TNFα-induced muscle wasting. However, these studies were all conducted in cultured myotubes. Therefore, we wish to further study the dependency of the increase in STAT3 expression on HuR to the loss of muscle under cachectic conditions *in vivo*. We plan to use muscle-specific HuR KO mice, which we recently generated in the lab, and inject them with IFNγ/TNFα or use the LLC model. Analysis of the weight of skeletal muscle and biochemical analysis of STAT3 levels would reveal whether induction of STAT3 expression is abrogated under cachectic conditions in the absence of HuR. Importantly, this would also provide *in vivo* evidence of whether HuR is involved in promoting muscle wasting.

## 4.5: Concluding Remarks

In this body of work, I have highlighted novel mechanisms involved in STAT3-mediated-muscle wasting. In chapter 2, we identify that IFNγ/TNFα triggers muscle

wasting through the activation of STAT3 independently of IL-6, which suggests that other non-IL-6 dependent models of muscle wasting may still depend on STAT3 to promote muscle loss. This work could be further investigated by also looking into the novel genes suggested by our ChIP-Seq data, which will likely lead to the identification of crucial genes that lead to muscle loss. Our work also provides insight into the collaboration of STAT3 with its protein partner NF-κB and its influence on STAT3 localization to the nucleus. In addition, with known non-transcriptional functions in other subcellular compartments, it raises the possibility that STAT3 also contributes to muscle wasting in a non-transcriptional manner. How do these different forms of STAT3 work together to regulate muscle wasting? Are there other STAT3 protein partners that influence its pro-cachectic function? Further studies to address these questions could provide valuable insight into the role of STAT3 during muscle wasting.

Also in this work, I have provided evidence that indicates that STAT3 expression is regulated by HuR, likely by inhibiting the action of miR-330. While a specific role for HuR is demonstrated here, there are questions regarding whether HuR itself is regulated during cytokine-induced muscle wasting that have not yet been addressed. Are there kinases such as IKKα that are activated and target HuR under these inflammatory conditions (Adli et al, 2010)? If this occurs, how does the phosphorylation status of HuR modulate its function? Can phosphorylation of HuR change how it interacts with miRNA such as miR-330? Is it possible to inhibit HuR to prevent muscle

wasting *in vivo*? Further investigation is needed to achieve the answers to these questions and to fully understand the role of HuR in muscle wasting.

In conclusion, this work characterizes an IFN$\gamma$/TNF$\alpha$-mediated signaling pathway that involves the activation of the JAK-STAT3-iNOS pathway, and identifies HuR and miR-330 as key regulators of STAT3 expression. Although there are still unanswered questions on the precise mechanism of how HuR and miR-330 regulated STAT3, these mechanisms contribute to our knowledge of an important mechanism that regulates STAT3 under cachectic conditions. As our knowledge of muscle wasting increases, preventing muscle deterioration altogether may be possible through the identification of therapeutic targets, improving the quality of life of those afflicted with disease.

## Original contributions to knowledge

Chapter 2

- Demonstrated that JAK/STAT3 is a key pathway that mediates IFNγ/TNFα-induced muscle wasting. This study is the first to demonstrate how the synergy of these two cytokines activates STAT3 and NF-κB independently of IL-6 during muscle wasting.
- Identified that IFNγ/TNFα trigger the formation of a STAT3 and NF-κB complex. We further show that NF-κB is required for the translocation of STAT3 to the nucleus, where STAT3 mediates the expression of iNOS to promote IFNγ/TNFα-induced muscle wasting. This provides further insight into the molecular mechanism of how STAT3 can promote muscle wasting.

Chapter 3

- Demonstrated that the increase in STAT3 expression induced by IFNγ/TNFα depends the RNA-binding protein HuR. We show that HuR regulates STAT3 expression posttranscriptionally by promoting the translation of STAT3 mRNA. We further show that miR-330 associates with HuR in C2C12 cells and represses STAT3 expression. These data describe a novel method of regulation of STAT3 that occurs under conditions that lead to muscle wasting.

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# Appendix 1

Unrelated publications and manuscripts

The following papers are not directly related to the work described in this thesis.

**Direct activation of AMPK prevents cancer-induced muscle wasting**

Derek T. Hall[1], Takla Griss[2], **Jennifer F. Ma[1]**, Amr Omer[1], Brenda J. Sanchez[1], Rebecca J. Ford[3], Nathalie Bedard[5], Simon S. Wing[5], Sergio di Marco[1], Gregory R. Steinberg[3, 4], Russell G. Jones[2], Imed-Eddine Gallouzi[1].

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This research is in preparation.

# Destabilization of nucleophosmin mRNA by the HuR/KSRP complex is required for muscle fibre formation

Anne Cammas[1,2], Brenda J. Sanchez[1], Xian Jin Lian[1], Virginie Dormoy-Raclet[1], Kate van der Giessen[1], Isabel Lopez de Silanes[3], **Jennifer Ma[1]**, Carol Wilusz[4], John Richardson[5], Myriam Gorospe[6], Stefania Millevoi[2], Matteo Giovarelli[7], Roberto Gherzi[7], Sergio Di Marco[1], and Imed-Eddine Gallouzi[1]

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This research was originally published in ***Nature Communications*** 2014. 5: 5190, DOI: 10.1038/ncomms5190

**Regulation of eukaryotic initiation factor 4AII by MyoD during murine myogenic cell differentiation**

Gabriela Galicia-Vasquez, Sergio Di Marco, Xian Jin Lian, **Jennifer F. Ma**, Imed-Eddine Gallouzi, and Jerry Pelletier

McGill University, Department of Biochemistry, Rosalind and Morris Goodman Cancer Centre, Montreal, Quebec, Canada

This research was originally published in ***PloS One*** 9 (1): e87237, DOI: 10.1371/journal.pone.0087237. eCollection 2014.

**Apoptotic-induced cleavage shifts HuR from being a promoter of survival to an activator of caspase-mediated apoptosis**

Chris von Roretz, Xian Jin Lian, Angelo M. Macri, Nahid Punjani, Eveline Clair, Olivier Drouin, Virginie Dormoy-Raclet, **Jennifer F. Ma**, and Imed-Eddine Gallouzi.

McGill University, Department of Biochemistry, Rosalind and Morris Goodman Cancer Centre, Montreal, Quebec, Canada

This research was originally published in ***Cell death and differentiation*** 2013. 20(1): 154-168.

**The impact of mRNA turnover and translation on age-related muscle loss**

**Jennifer F. Ma**[1], Derek T. Hall[1], and Imed-Eddine Gallouzi[1,2]

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This invited review was originally published in ***Ageing Research Reviews*** *2012.* 11: 432 – 441.

# The translation inhibitor pateamine A prevents cachexia-induced muscle wasting in mice

Sergio Di Marco[1,*], Anne Cammas[1,*], Xian Jin Lian[1], Erzsebet Nagy Kovacs[1], **Jennifer F. Ma[1]**, Derek T. Hall[1], Rachid Mazroui[2], John Richardson[3], Jerry Pelletier[1], and Imed-Eddine Gallouzi[1]

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This research was originally published in ***Nature Communications*** 2012. 3: 1899, DOI: 10.1038/ncomms1899

# Inducible nitric oxide synthase (iNOS) in muscle wasting syndrome, sarcopenia, and cachexia

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This review was originally published in ***Impact ageing.*** 2011. 3 (8): 1-14