Evaluating CCAAT Enhancer Binding Protein Delta and the Glucocorticoid Receptor as Potential Substrates of the USP19 Deubiquitinating Enzyme

Si Han Li
Faculty of Medicine
Department of Biochemistry
McGill University
Montreal, Quebec, Canada
August 2017

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

© Si Han Li 2017
# Table of Contents

Acknowledgements .................................................. 5

Abstract .............................................................. 6

Résumé ................................................................. 8

List of Abbreviations ................................................ 11

Introduction ................................................................

1. Skeletal Muscle ...................................................... 17

2. Muscle Atrophy ...................................................... 19

3. Mechanisms of Muscle Atrophy ............................... 20

4. Signaling Pathways Controlling Muscle Atrophy .......... 22

4.1 IGF-1/PI3K/Akt/mTOR Signaling Pathway ............... 22

4.2 Myostatin ............................................................ 25

4.3 Inflammatory Cytokines and NF-κB ......................... 27

5. Glucocorticoids and Glucocorticoid Receptor ............. 29

5.1 Structure, Secretion and Function of Glucocorticoids ... 29

5.2 Glucocorticoid Treatment and Muscle Wasting .......... 31

5.3 Glucocorticoid Receptor ....................................... 33

6. The Ubiquitin Proteasome System ............................ 36

6.1 Structure and Function of E1, E2 and E3 Enzymes ...... 38

6.2 The Proteasome ................................................... 39

6.3 Deubiquitinating Enzymes .................................... 41

6.4 Function of Deubiquitinating Enzymes .................... 44
6.5 Regulation of Deubiquitinating Enzymes 47

6.6 The Ubiquitin Proteasome System in Muscle Atrophy 49

7. USP19 51

7.1 Structure of USP19 51

7.2 Function of USP19 53

7.2.1 Regulation of Cell Cycle 53

7.2.2 Stabilization of Hypoxia Inducible Factor-1α 54

7.2.3 Stabilization of c-IAP1 and c-IAP2 55

7.2.4 Role in ERAD 56

7.2.5 Chaperone and Co-chaperone Activity 57

7.5 Myogenesis and Muscle Atrophy 58

8. Objectives of This Thesis 60

II. Materials and Methods

1. Plasmids, Cell Culture and Transfection 62

2. Silencing in Rat L6 cells 64

3. Western Blot 65

4. Quantitative Real-Time PCR 66

5. Co-Immunoprecipitation 66

6. CRISPR-CAS9 67

III. Results

1. Evaluation of C/EBPδ as a Potential Substrate of USP19 69

2. USP19 Regulates the Level of GR Independent of Its Catalytic Activity 73
3. Heterozygous Knockout of USP19 Does Not Induce Change in GR Level 77

4. USP19 Interacts with GR and HSP90 Independent of Its Two CS Domains 79

IV Discussion 83

V. Reference 88
Acknowledgements

I would like to thank my supervisor, Dr. Simon Wing, for his support and guidance throughout my Masters study.

I would like to thank our chief research technician in the Wing Laboratory, Nathalie Bedard, for her invaluable assistance in the collection of materials and data, technical support and help with preparation of my thesis.

I would like to thank my colleague in the Wing Laboratory, Erin Coyne, for her insightful advice and suggestions throughout my research.

I am grateful to the members of my Research Advisory Committee, Dr. Jason Young and Dr. Barry Posner, for their advice and encouragement.

I would also like to thank McGill University Department of Biochemistry and The Research Institute of the McGill University Health Centre for their financial support.
Abstract

Muscle atrophy occurs as a result of disuse, fasting, aging, many common diseases such as cancer, COPD and treatments such as glucocorticoids. Activation of the ubiquitin-proteasome system (UPS) and, in particular, the induction of several ubiquitin ligases appears to be a major cellular mechanism facilitating the increase in muscle protein breakdown and atrophy of the muscle fibers. However, the role of deubiquitinating enzymes remains poorly explored. Interestingly, we have identified ubiquitin-specific protease 19 (USP19) as a deubiquitinating enzyme that is upregulated in several conditions of muscle wasting[1]. Silencing of USP19 in cells increases expression of myofibrillar proteins[2], and its knockout in mice reduces the severity of muscle wasting in response to glucocorticoids, denervation and fasting[3]. These data indicate that USP19 plays an essential role in promoting muscle wasting, but the mechanisms underlying this effect is unknown.

The transcription factor CCAAT Enhancer Binding delta (C/EBPδ) is upregulated in mouse muscle by glucocorticoid and can promote muscle atrophy by inducing myostatin[4, 5]. Importantly, this upregulation of C/EBPδ was blunted in USP19 knockout mice and the level of myostatin was also lower in KO mice compare to WT upon fasting. Therefore, we tested whether C/EBPd is a substrate of USP19. Results from co-expression, silencing and co-immunoprecipitation experiments suggested that USP19 does not interact with C/EBPδ and has no significant effect on its stability.

We also observed decreased glucocorticoid signaling and lower levels of glucocorticoid receptor (GR) in USP19-KO mice compare to WT. Furthermore, the mRNA levels of GR were unaffected, suggesting that its rate of degradation is accelerated upon the loss of USP19. Therefore, we tested whether GR is a substrate of USP19. We found that the level of the GR is
decreased in USP19 knockout HEK293T cells compared to wild-type cells. Conversely, the GR level is increased when it was co-expressed with USP19 isoforms. This effect was independent of USP19’s deubiquitinating activity, as a catalytically inactive USP19 mutant induced a similar increase in GR level as wild-type USP19. However, the catalytic domain of USP19 was required because a USP19 mutant missing this domain did not induce any significant change in GR level. Since the GR is a well-known client of HSP90[6] and the structure of USP19 contains two CS domains that are homologous to two co-chaperones of HSP90, SGT1 and p23, we tested whether USP19 regulates GR by interacting with HSP90. Co-immunoprecipitation experiments in HEK293 cells showed USP19 interacting with both HSP90 and GR. Removal of the two CS domains in USP19’s structure did not disrupt its interaction with HSP90 or GR. Therefore, USP19 may mediate its effects on GR by interacting with Hsp90 through its catalytic domain and assists HSP90 in stabilizing unbound GR. In conclusion, our studies argue that USP19 mediates its effects on muscle atrophy through GR.
Résumé

L’atrophie musculaire se produit lorsque les muscles ne sont pas utilisés, lors d’un jeûne, avec le vieillissement, dans le cadre de maladies tels que le cancer, la MPOC ou encore lors de certains traitements tel que l’administration de glucocorticoïdes. L’activation du système ubiquitine-protéasome (UPS) et plus particulièrement la formation de plusieurs ubiquitine ligases semble constituer un des mécanismes cellulaires prépondérant pour augmenter la dégradation des protéines et l’atrophie des fibres musculaires. Toutefois, le rôle d'enzymes qui possède une activité de désubiquitination dans le contexte de l’atrophie musculaire demeure peu connu. Fait intéressant, nous avons identifié une protéase spécifique de l'ubiquitine (USP19) possédant une activité de désubiquitination régulée positivement en réponse à une perte de masse musculaire associée à plusieurs conditions. La désactivation du gène codant USP19 dans les cellules produit une augmentation de l’expression des protéines myofibrillaires et l’inactivation de USP19 chez des souris knockout réduit la sévérité de la perte musculaire en réponse à un traitement de glucocorticoïdes, lors de la dénervation des muscles ou en conséquence d’un jeûne. Ces données suggèrent que USP19 joue un rôle primordial dans la régulation de la perte musculaire mais les mécanismes sous-jacents demeurent inconnus.

Le facteur de transcription CCAAT Enhancer Binding delta (C/EBPδ) est régulé positivement dans le tissu musculaire chez la souris traitée aux glucocorticoïdes et favoriserait l’atrophie musculaire par l’activation de la myostatin. Fait important, cette régulation positive de C/EBPδ est atténuée chez les souris knockout USP19 (KO-USP19) et les quantités de myostatin sont également plus faibles chez les souris knockout comparativement aux souris de souche sauvage suivant un jeûne. En conséquence, nous avons testé l’hypothèse que C/EBPδ est un substrat de USP19. Les résultats d’expériences de co-expression protéinique, de désactivation du
gène et de co-immunoprécipitation suggèrent que USP19 n’interagit pas avec C/EBPδ et n’a aucun effet sur la stabilité de la protéine.

Nous avons également observé une diminution de la signalisation activée par les glucocorticoïdes ainsi qu’une expression réduite du récepteur des glucocorticoïdes (RG) chez les souris KO-USP19 comparativement aux souris de souche sauvage. De plus, les niveaux d’ARNm de RG n’étaient pas affectés, suggérant que la dégradation des récepteurs est accélérée en l’absence de USP19. Nous avons donc testé l’hypothèse que RG est un substrat de USP19. Nous avons constaté que l’expression de RG est réduite dans les cellules HEK293T avec un gène USP19 inactif comparativement à la souche de cellules exprimant le gène. Au contraire, l’expression de RG était augmenté lorsque co-exprimé avec des isoformes de USP19. Cet effet était indépendant de l’activité de désubiquitination de USP19 puisqu’un USP19 mutant inactivant la fonction catalytique de la protéine démontrait une augmentation similaire de l’expression de RG comparativement à celle de la souche sauvage de USP19. Toutefois, le domaine catalytique de USP19 était nécessaire pour provoquer cet effet puisqu’une forme mutante de USP19 n’exprimant pas le domaine catalytique n’affectait pas significativement l’expression de RG. Étant donné l’interaction connue entre RG et HSP90 et que la structure protéinique de USP19 contient deux domaines CS homologues aux deux co-chaperons de HSP90, SGT1 et p23, nous avons testé l’hypothèse que USP19 peut réguler RG par interaction avec HSP90. Les expériences de co-immunoprécipitation dans les cellules HEK293 ont montrées que USP19 interagit autant avec HSP90 et RG. L’ablation des deux domaines CS de la structure protéinique de USP19 n’a pas démontré d’impact sur l’interaction avec HSP90 ou RG. En conséquence, USP19 pourrait produire ses actions sur RG en interaction avec HSP90 via son domaine catalytique et assister HSP90 à stabiliser RG non-lié. En conclusion, nos études...
suggèrent que l’interaction entre USP19 et RG est responsable des effets de USP19 observés sur l’atrophie musculaire.
List of Abbreviations:

4EBP1: Eukaryotic translation initiation factor 4E-binding protein 1

ActRIIA/B: Activin receptor type-2A/B

Akt: Protein kinase B

ALK: Activin receptor-like kinase

ALS: Autophagy lysosome system

AMSH: Associated molecule with the SH3 domain of STAM

ATM: Ataxia telangiectasia-mutated

ATP: Adenosine triphosphate

ATR: Ataxia telangiectasia and rad3-related

BCAA: Branched chain amino acid

BCAT: Branched chain aminotransferase

bHLH: Basic helix-loop-helix

Bnip3: BCL2/adenovirus E1B 19 kDa protein-interacting protein 3

C/EBPδ: CCAAT enhancer binding protein delta

CBG: Corticosteroid binding globulin

Cdk: Cyclin-dependent kinase

CFTR: Cystic fibrosis transmembrane conductance regulator

CHO: Chinese hamster ovary

c-IAP: Cellular inhibitor of apoptosis

Co-IP: Co-immunoprecipitation

COPD: Chronic obstructive pulmonary disease

CRISPR: Clustered regularly interspaced short palindromic repeats
CS: CHORD-containing proteins and SGT1

CTL: Control

DBD: DNA binding domain

DUB: Deubiquitinating enzyme

E1: Ubiquitin activating enzyme

E2: Ubiquitin conjugating enzyme

E3: Ubiquitin ligase

eEF2K: Eukaryotic elongation factor 2 kinase

EGFR: Epidermal growth factor receptor

eIF: Eukaryotic translation initiation factor

ER: Endoplasmic reticulum

ERAD: ER-associated degradation

ERBB3: Erb-b2 receptor tyrosine kinase 3

ESCRT: Endosomal sorting complex required for transport

FAK: Focal adhesion kinase

FKBP: FK506-binding protein

FoxO: Forkhead box protein O

GDF: Growth differentiation factor

GFP: Green fluorescent protein

GR: Glucocorticoid receptor

GR: Glucocorticoid receptor

GRE: Glucocorticoid response element

GSK3β: Glycogen synthase kinase-3 Beta
HECT: Homologues to the E6AP carboxyl terminus
HEK: Human embryonic kidney
HIF: Hypoxia inducible factor
HIV: Human immunodeficiency virus
HOP: Hsp70-Hsp90 organizing protein
HSP: Head shock protein
IGF-1: Insulin-like growth factor 1
IKK: IκB kinase
IL6: Interleukin 6
IRS: Insulin receptor substrate
IκB: Inhibitor of kappa B
JAK: Janus kinase
JAMM: JAB1/MPN/Mov34 metalloenzyme
KLF: Krüppel-like factor
KO: Knockout
KPC1: Kip1 ubiquitination-promoting complex
LBD: Ligand binding domain
LC3: Microtubule-associated protein light chain 3
LUBAC: Linear ubiquitin chain assembly complex
MAFbx: Muscle atrophy F-box
MAPS: Misfolded-associated protein secretion
MARCH6: Membrane Associated Ring-CH-Type Finger 6
MHC: Myosin heavy chain
MJD: Machado-Joseph Disease protein domain
MRF: Myogenic regulatory factor
mTOR: Mammalian target of rapamycin
MuRF: Muscle-Specific ring finger
Myf5: Myogenic factor 5
MYND: Myeloid-Nervy-DEAF1
MyoD: Myogenic differentiation
NBR1: Next to BRCA1 gene 1
NEMO: NF-κB essential modulator
NFAT: Nuclear factor of activated T-cells
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
Nsp: Non-specific control
NTD: N-terminal domain
OTU: Ovarian tumor protease
PDCD4: Programmed cell death protein 4
PI3K: Phosphatidylinositol 3-kinase
qPCR: Quantitative real-time PCR
REDD1: Regulated in development and DNA damage response 1
RING: Really interesting new gene
RIP: Receptor Interacting Protein
Rpn: Regulatory particle non-ATPase protein
Rpt: Regulatory particle ATPase
S6K: P70S6 kinase
SGT1: Suppressor of G2 allele of SKP1
SIAH: Seven in absentia homolog
siRNA: Small interfering RNA
SMAC: Second Mitochondria-derived activator of caspases
STAT: Signal transducer and activator of transcription protein
TA: Tibialis anterior
TAK: TGF-β activated kinase
TCRα: T-cell receptor α
TGF: Tumor growth factor
TNF: Tumor necrosis factor
TRAF: tumor necrosis factor-receptor associated factor
TRAF: TNF-receptor associated factor
TSC: Tuberous sclerosis protein
TWEAK: TNF-like weak inducer of apoptosis
UBL: Ubiquitin like domain
UCH: ubiquitin C-termina hydrolase
UPS: Ubiquitin proteasome system
USP: Ubiquitin specific protease
USP19-CA: catalytically inactive USP19 mutant
USP19-CS: USP19 mutant containing only CS domains
USP19-CYT: cytoplasmic USP19 isoform
USP19-ER: ER-localized USP19 isoform
USP19-ΔN1: USP19 mutant missing the first CS domain
USP19-ΔN2: USP19 mutant missing both CS domains

VHL: Von Hippel-Lindau ligase

WT: Wild-type
I. Introduction

1. Skeletal Muscle

Skeletal muscle is the most abundant muscle type in the body and represents approximately 40% of body weight in non-obese humans[7]. It is the major organ of the body’s locomotor system and functions to convert chemical energy into force and directed movement. Skeletal muscle also serves as the largest protein reservoir in the body and its metabolic activities play critical roles in maintaining energy homeostasis. Furthermore, skeletal muscle has been identified as a secretory organ with the ability to produce and release myokines that can exert endocrine, paracrine or autocrine effects on a variety of targets[7].

The functional unit of skeletal muscle is the sarcomere, which is primarily comprised of thin actin filaments, thick myosin filaments and their associated accessory proteins[8]. Within the sarcomere, thick and thin filaments are arranged in an overlapping antiparallel order and interlinked through the action of cytoskeletal proteins titin and alpha-actinin[8](Figure 1). This highly ordered array of myosin and actin filaments gives skeletal muscles their characteristic striated appearance and serves as their elemental force producing unit. During muscle contraction, the ATPase activity of myosin plays the central role in the conversion of chemical energy into directed movement[9]. Different types of muscle fibers are characterized primarily according to their myosin profiles, more specifically their myosin heavy chain (MHC) isoforms.[10]. Muscle fibers containing only one specific isoform of MHC are known as pure fibers, while those containing two or more MHC isoforms are referred to as hybrid fibers[10]. Among pure fibers, four types exist: a single Type I slow fiber containing MHCIβ and three Type II fast fibers expressing MHCIIa, MHCIIb and MHCIIId respectively[10]. Hybrid fibers co-express specific pairs of the four major MHC isoforms, with one isoform often expressing
predominantly over the other[10]. In addition to myosin compositions, the diversity between muscle fibers also extends to domains such as contractile response and metabolism. In general, Type II muscle fibers have higher myosin ATPase activity[11], faster contractile velocity[12] and lower oxidative capacity[13] compared to Type I. The existence of multiple myosin isoforms and muscle fiber types allows for functional diversity and specialization.

**Figure I: The Structure of Sarcomere.** Within the sarcomere, thin actin filament and thick myosin filament overlap with each other to give skeletal muscle its characteristic striated appearance.

In addition to structure and force generation, sarcomere proteins also play a variety of regulatory roles. For example, titin contains a tyrosine kinase domain that is activated by a dual mechanism consisting of tyrosine phosphorylation and calcium/calmodulin binding to its regulatory tail[14]. Following activation, this kinase domain can regulate muscle protein turnover and gene expression by interacting with the zinc-finger protein nbr1[15]. Another
important regulatory component within the sarcomere is the troponin-tropomyosin complex, which governs skeletal muscle contraction in response to changes in intracellular Ca\(^{2+}\) levels. Upon Ca\(^{2+}\) binding to troponin, tropomyosin shifts its position to expose myosin-binding sites on actin filaments, thus allowing myosin cross-bridge cycling and muscle contraction[16].

2. Muscle Atrophy

A general loss of skeletal muscle mass is a characteristic and debilitating response to fasting, aging, disuse, as well as many diseases such as cancer, sepsis and diabetes[17]. Some common consequences of muscle wasting include weakness, poor mobility, reduced quality of life, fractures and injuries. In severe cases of muscle wasting such as cachexia, the loss of muscle mass can have far reaching impact that extends beyond impaired physical movement, function and strength to poor disease prognosis, treatment complications or even death.

Cachexia is defined as: “a multifactorial syndrome associated with underlying illness and characterized by an ongoing loss of muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support”[18]. Its most prominent clinical feature is weight loss in adults or stunted growth in children, yet cachexia as a wasting disease is distinct from starvation, age-related loss of muscle mass, primary depression, malabsorption and hyperthyroidism[19]. In cancer patients, the severe loss of body weight due to cachexia is an independent variable that predicts mortality[20]. Moreover, cancer cachexia is associated with poor prognosis, increased morbidity, dose limiting drug toxicity and early tumor relapse [21-24]. Similar adverse effects also extend to other diseases that are commonly associated with cachexia. In patients suffering from chronic heart failure, cardiac cachexia is associated with increased mortality and poor prognosis, independently of functional disease severity, age, exercise capacity
and cardiac function[25, 26]. There is also evidence that HIV-related wasting is an important comorbidity in many patients, and despite the effectiveness of highly active antiretroviral therapy in treating HIV, weight loss remains an independent predictor of mortality for this disease[27].

Weight loss in cachexia is largely due to the rapid depletion of skeletal muscle mass, which in turn is the result of abnormalities in muscle protein metabolism. Under normal conditions, muscle protein turnover occurs in a tightly regulated manner and adjusts in response to different stimuli. For example, resistance training increases both protein breakdown and synthesis simultaneously but to a different degree, synthesis is increased by ~100%, whereas breakdown is only increased by ~50%[28]. Nutrient deprivation is an example where the rate of muscle protein turnover is adjusted in response to metabolic needs, in this state, muscle protein breakdown occurs at an increased rate to provide the body with free amino acids, which then can be oxidized to sustain ATP synthesis. Clearly, muscle fiber size and protein content are governed by multiple complex and adaptive systems. These systems adjust the rate of muscle protein synthesis and degradation in response to the contractile and metabolic needs of skeletal muscles under diverse conditions. Therefore, accelerated or exaggerated loss of skeletal muscle mass, which is key in the pathophysiology of cachexia[19], must be the result of negative balance in muscle protein turnover. Indeed, multiple studies on experimental models of cachexia show both increased protein degradation and decreased protein synthesis to be the cause of muscle atrophy. Thus, treatment of cachexia will need to address both catabolic and anabolic systems in skeletal muscle.


As mentioned previously, shrinkage of myofibers and the consequent loss of muscle mass are
the results of an imbalance in protein metabolism. In atrophying muscles, there is an increase in protein degradation and a reduction in protein synthesis, leading to a net loss of proteins, organelles and cytoplasm. The increase in protein degradation is due to the activation of two major proteolytic pathways, the ubiquitin-proteasome system (UPS) and the autophagy-lysosome system (ALS)[29]. This pivotal change starts at the transcriptional level through increased expression of genes encoding key components of the two proteolytic systems[30, 31]. On the other end, the decrease in protein synthesis can be primarily attributed to the downregulation of insulin-like growth factor-1(IGF-1)-Akt-Mammalian Target of Rapamycin (mTOR) signaling pathway[32, 33]. Reduced stimulation of Akt and mTOR kinase activities, due to either a lack of growth factors (e.g. insulin, IGF) or an over-abundance of catabolic stimuli (e.g. glucocorticoids, inflammatory cytokines), leads to decreased phosphorylation of GSK3β, P70S6 kinase (S6K) and PHAS-1/4EBP (4E-BP1), all of which are critical for protein translation[33]. Interestingly, the IGF-1/Akt pathway also regulates activation of the UPS and the ALS at the transcriptional level through Akt-mediated inhibition of the FoxO family of transcription factors[34-36]. Therefore, the upregulation of protein breakdown and downregulation of protein synthesis in wasting muscles cannot be viewed as independent events; instead they are a series of coordinated and interlinked adaptations that ultimately lead to the rapid depletion of skeletal muscle mass.

In recent years, studies have begun to unravel the signaling pathways controlling the activity of the UPS and the ALS, as well as the involvement of these two proteolytic machineries in promoting muscle atrophy. In healthy muscles, protein degradation by the UPS and the ALS serves several important regulatory and homeostatic functions. Their activities in this state are highly selective and precisely regulated to prevent the un-wanted removal of proteins that are essential for muscle function[37]. But in muscle atrophy, this regulation and selectivity are
disrupted, resulting in hyperactivation of the two proteolytic systems and accelerated protein catabolism that seems to affect all muscle cell components[38, 39]. The induction of UPS and ALS during muscle atrophy is coordinately regulated by the FoxO family of transcription factors, however the proteolytic activities of these two pathways serve distinct roles within the overall increase in muscle proteolysis[36]. The UPS is responsible for the breakdown of most muscle proteins, particularly sarcomere components, and the loss of contractile machinery accounts for the reduction in muscle strength[40, 41]. In contrast, ALS-mediated proteolysis facilitates the degradation of mitochondria (and other cell organelles), which explains the decreased endurance capacity of atrophied muscles[29, 37]. Interestingly, while both the UPS and the ALS are recognized to play important roles in mediating muscle wasting, targeting the UPS appears to be the more attractive therapeutic option, as pharmacological inhibition of autophagy and/or lysosomal function leads to dystrophic muscle due to impaired clearance of damaged organelles and aggregated proteins[42-44].


The activation of protein degradation during muscle atrophy is mediated by a set of transcription-dependent changes in the levels of rate limiting enzymes of key proteolytic systems[29]. This process involves a complex network of catabolic signalling pathways that not only modulate each other at different levels, but also communicate with protein synthetic pathways at various points[29]. In this chapter, we will describe some of the signalling pathways that regulate the balance between muscle hypertrophy and atrophy.

4.1 IGF-1/PI3K/Akt/mTOR Signalling Pathway.

One of the most critical regulators of muscle mass is the IGF-1/PI3K/Akt signalling cascade,
which is initiated by the binding of IGF-1 or insulin to their respective cell surface receptors, and it proceeds through the sequential activation of Insulin Receptor Substrate-1 (IRS-1), phosphatidylinositol-3-kinase (PI3K), and Akt. This pathway exerts its effect on muscle protein balance through regulating both protein synthetic and degradation pathways. The binding of insulin or IGF-1 to their respective cell surface receptors and the consequent phosphorylation of IRS-1 trigger the activation of several downstream kinases, one of which is PI3K[45]. Activated PI3K can phosphorylate the D-3 position of the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P$_2$) to generate phosphatidylinositol-3,4,5-triphosphate (PtdIns-3,4,5-P$_3$)[45]. This modification creates a lipid binding site which recruits Akt to the cell membrane and facilitates its phosphorylation by phosphoinositide-dependent kinase 1 (PDK1)[45]. Phosphorylation of Akt stimulates its catalytic activity, leading to the phosphorylation of a wide array of downstream targets that affect muscle protein balance[45].

The IGF-I/PI3K/Akt signalling cascade is upregulated during muscle growth and downregulated in muscle atrophy[46]. More importantly, activation of this pathway, by expressing a constitutively active form of Akt, in tibialis anterior (TA) muscle of adult mice promotes muscle hypertrophy and prevents denervation-induced atrophy in vivo[32]. Conversely, Akt inactivation through overexpressing the inositol 5-phosphatase SHIP-2 blocks compensatory hypertrophy in normal mice TA muscle[32]. Inhibition of this signalling cascade at various points, through blocking PI3K activity with a pharmacological inhibitor, treatment with rapamycin or expression of a dominant kinase-negative Akt, lead to reduced myotube diameter in vitro[33]. Furthermore, insulin and its homolog IGF-1 prevent the stimulation of proteolysis by glucocorticoid in C2C12 muscle cells[47], and muscle specific expression of IGF-1 blocks angiotensin II-induced skeletal muscle wasting that is associated with chronic heart failure[48],
indicating the potential of the Akt pathway to be dominant over catabolic stimuli.

A key hypertrophy mediator downstream of Akt is mTOR, a kinase that also activates in response to a variety of other growth signals, including simple nutrient stimulation and extracellular growth factors[46]. Within the regulation of mTOR, Akt phosphorylates tuberous sclerosis (TSC) protein 2 and disrupts its interaction with TSC1[49]. This releases mTOR from TSC1/TSC2-mediated inhibition and allows it to phosphorylate S6K as well as 4E-BP1[33]. S6K promotes protein synthesis by targeting a number of factors (eIF4B, PDCD4 and eEF2K) that control protein translation[50]. Conversely, phosphorylated 4E-BP1 dissociates from eIF4E, which is then available to interact with eIF4G to initiate cap-dependent translation[51]. Akt can also promote muscle hypertrophy in a mTOR-independent manner by directly phosphorylating and thereby inhibiting glycogen synthase kinase-3β (GSK3β)[52]. Active GSK3β negatively regulates the translation initiation factor eIF2B as well as hypertrophic transcriptional effectors such as GATA-4, c-Myc, β-catenin and NFAT[53]. A third downstream target of Akt in skeletal muscle is the FoxO family of transcription factors: FoxO1, FoxO3 and FoxO4[54]. Akt’s phosphorylation of FoxO proteins takes place in the nucleus, and it targets threonine 24, serine 256 and serine 319 on FoxO1 along with their equivalents on FoxO3 and FoxO4[54]. Phosphorylated FoxO members are bound by 14-3-3 proteins in the nucleus and exported to the cytoplasm, which effectively blocks FoxO-mediated transcription of target genes[54]. In muscle atrophy, increased nucleus translocation and activity of FoxO members are required for the upregulation of atrophy-related ubiquitin ligases MuRF-1 and atrogin-1/MAFbx[55]. Furthermore, activation of FoxO transcription factors also induces protein breakdown by the autophagy-lysosomal system in muscle fibers and increases the transcription of autophagy-related genes LC3 and Bnip3[35]. Collectively, these observations indicate that the IGF-1/Akt
signalling pathway is a critical mediator of muscle growth by simultaneously activating anabolic processes and suppressing catabolic ones.

4.2 Myostatin

Myostatin, also known as growth/differentiation factor-8 (GDF-8), is a part of the transforming growth factor-β (TGF-β) superfamily and functions as an essential negative regulator of skeletal muscle mass[56]. Myostatin is expressed in many different muscles throughout the body, and its genetic disruption in a number of mammalian species, including cattle, sheep and mice, leads to excessive skeletal muscle growth through hypertrophy and hyperplasia[55]. Loss-of-function mutations in the human myostatin gene also induces the same effect, as a newborn child identified as carrying a myostatin null mutation is extraordinarily muscular at birth[57]. Similar to mutations in the gene, pharmacological inhibition of myostatin in adult mice via treatment with an anti-myostatin antibody results in increased skeletal muscle mass and grip strength as well[58]. Conversely, male transgenic mice with muscle-specific overexpression of myostatin displays decreased muscle fiber diameter and myonuclear number[59]. High levels of circulating myostatin in mice, achieved by injection of a myostatin-releasing CHO (Chinese Hamster Ovary) cell line into the thighs, also induces a global decline in skeletal muscle mass that is characterized by decreased fiber size as well as increased expression of cell cycle inhibitor and pro-apoptotic genes[60]. Most importantly, upregulation of myostatin is associated with glucocorticoid-induced muscle wasting as well as that due to a diverse set of diseases including HIV, cancer and heart failure[61-64]. Consistent with in vivo observations, treating C2C12 muscle cells with myostatin inhibits myoblast proliferation and differentiation in vitro[65, 66]. Furthermore, exposure of differentiated C2C12 myotubes to tumor-secreted myostatin induces myotube wasting through decreased protein synthesis and increased protein
degradation[67]. Given the overwhelming evidence showing the importance of myostatin as a powerful negative regulator of muscle mass, considerable effort has gone into studying the molecular mechanisms underlying its effect. To date, downstream targets of myostatin that have been identified include the IGF-1/PI3K/Akt signalling pathway, myogenic regulatory factors, FoxO proteins, the UPS and the ALS[68]. In summary, myostatin is capable of inducing muscle atrophy via inhibiting myogenesis, suppressing protein synthesis and increasing protein breakdown.

Myostatin is initially synthesized in muscle cells in an inactive form and must go through proteolytic processing by the calcium-dependent serine protease furin before its secretion[69]. Similar to other members of the TGF-β superfamily, myostatin interacts with heterodimeric activin receptor complexes that consist of type I and type II receptors[69]. Activated myostatin initiates its intracellular signalling by binding to activin type II receptors ActRIIB and, to a lesser extent, ActRIIA[69]. Upon binding of myostatin, the serine/threonine kinase activity of ActRIIB phosphorylates the GS domain of activin type I receptors ALK4 and ALK5[70]. Phosphorylated ALK4 and ALK5 then phosphorylate mothers against decapentaplegic homolog (Smad) transcription factors Smad1 and Smad2, promoting them to form hetero-oligomers with Smad4 and translocate to the nucleus where the transcription of target genes will be altered[71, 72]. Myostatin-induced activation of Smad signalling represses the level of MyoD, myogenin and Myf5 in muscle cells, leading to inhibition of myogenic differentiation[71]. Furthermore, in myoblasts and muscle satellite cells, myostatin prevents the progression of cell cycle at the G1-to-S phase via upregulating p21, a cyclin-dependent kinase inhibitor, and downregulating cdk2[73, 74]. This, in turn, results in decreased myoblast proliferation as well as impaired satellite cell activation and self-renewal[73, 74]. In addition to its role in myogenesis, myostatin
also attenuates IGF-1-stimulated phosphorylation of Akt, which consequently inhibits protein synthesis through deactivating mTOR and activating GSK3β[75]. Moreover, high levels of dephosphorylated (active) FoxO proteins contribute to the increase in protein degradation following myostatin treatment, since nuclear-localized FoxO proteins promote transcription of atrogenes that encode key components of the UPS and the ALS[35, 55]. Therefore, myostatin can be viewed as a central switch in the cross-talk between myogenesis, protein synthetic pathways and catabolic processes. With its ability to simultaneously modulate several key factors that control skeletal muscle mass, myostatin may prove to be a valuable therapeutic target for treating cachexia and other types of muscle atrophy.

4.3 Inflammatory Cytokines and NF-κB

The third muscle atrophy-inducing signaling pathway involves the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factors[76]. NF-κB is the central regulator of inflammatory responses, and its activity in skeletal muscle is regulated by the binding of proinflammatory cytokines, most notably tumor necrosis factor (TNF-α), to the appropriate cell surface receptors[77]. TNF-α-mediated activation of NF-κB is shown to downregulate the expression of MyoD in undifferentiated myoblasts, and induce protein degradation in differentiated myotubes[78, 79]. Transgenic mice with muscle specific activation of NF-κB displays severe muscle wasting, which can be rescued by transgenic/pharmacological reversal of NF-κB activation[76]. Conversely, muscle specific inhibition of NF-κB leads to increased muscle strength, protection against denervation-induced muscle atrophy and enhanced muscle regeneration following injury[80]. Similarly, mice lacking the p105/p50 subunit of NF-κB is resistant to muscle atrophy induced by hindlimb unloading[81]. TNF-like weak inducer of apoptosis(TWEAK) is found to be another potent mediator of skeletal muscle wasting that acts
through NF-κB, and its knockout in mice leads to reduced muscle loss under denervation condition[82].

Within the cell, the activity of NF-κB is tightly regulated by its interaction with inhibitory IkB proteins[83]. This interaction generally conceals the nuclear localization signal on NF-κB and interferes with its DNA binding capabilities[84]. Therefore, NF-κB normally exists as an inactive and IkB-bound complex in the cytoplasm. The binding of inflammatory cytokines to their cell surface receptors triggers the activation of an IkB kinase (IKK) complex, which is composed of a catalytic kinase subunit (IKKβ or IKKα) and a scaffold protein known as NF-κB essential modulator (NEMO)[83]. Activated IKK complex mediates the rapid phosphorylation of IkB proteins and targets them for proteasome degradation[85]. This results in liberation of NF-κB, thus allowing it to translocate to the nucleus where it can activate gene transcription[85]. In the context of muscle wasting, activation of NF-κB leads to upregulation of MuRF1 ubiquitin ligase and increased activity of the UPS. Furthermore, there is evidence suggesting that NF-κB engages in cross-talk with the IGF-1/Akt pathway, since IKKβ conditional knockout mice are both resistant to muscle atrophy and display hyperphosphorylation of Akt[80]. In addition to elevated levels of classical inflammatory cytokines (e.g. TNF-α), specific conditions of atrophy such as denervation or immobilization also involve the upregulation of fibroblast growth factor inducible 14(Fn14), the cell surface receptor for TWEAK[86]. Increased Fn14 expression leads to TWEAK-induced activation of NF-κB, which then modulates its downstream catabolic effects through transcriptional changes[86].

Alongside the activation of NF-κB activity, several inflammatory cytokines, particularly interleukin-6 (IL-6), also induces muscle wasting via an alternative pathway that involves the activation of Janus kinase/signal transducer and activator of transcription (JAK/STAT)
pathway[5, 87]. The receptor complex for IL-6 is composed of an 80kDa (gp80) ligand binding subunit and a 130kDa (gp130) signal-transducing component[88]. Binding of IL-6 to gp80 triggers homodimerization of gp130 to the receptor-ligand complex, and subsequently activates gp130-associated JAK[88]. JAK phosphorylates STAT-3 on tyrosine 705, leading to its dimerization, nuclear localization, DNA binding and target gene regulation[87]. In muscle atrophy, one proposed target of phosphorylated STAT-3 (p-STAT3) is CCAAT/enhancer binding protein delta (C/EBPδ), a transcription factor whose expression and DNA binding activity are increased in response to excess glucocorticoid[4, 5]. This study uncovered a signaling cascade where p-STAT3 increases the expression of C/EBPδ in the initial step, and then C/EBPδ induces muscle atrophy by upregulating myostatin[5]. Clearly, inflammatory cytokines play pivotal roles in the pathogenesis of muscle atrophy, and they act through multiple pathways that are interconnected with other catabolic and anabolic signals.

5. Glucocorticoids and Glucocorticoid Receptor

5.1 Structure, Secretion and Function of Glucocorticoids

Glucocorticoids, along with mineralocorticoids, belong to the corticosteroid class of hormones. It possesses the steroid core structure of seventeen carbons arranged into four rings: three six-member cyclohexane rings and one five-member cyclopentane ring. Different glucocorticoids are distinguished from each other by the functional groups attached to their core structure. The secretion of glucocorticoids is controlled by the hypothalamic-pituitary-adrenal axis[89]. First, different systemic signals are processed at the periventricular nucleus of the hypothalamus to modulate the secretion of corticotropin-releasing hormone[89]. Next, corticotropin-releasing hormone signals the anterior pituitary to release corticotropin[89].
Finally, corticotropin stimulates the production and secretion of cortisol by the zona fasciculata of the adrenal cortex[89]. Once it is in circulation, the availability of cortisol to target tissues is further regulated by interaction with corticosteroid binding globulins (CBG) in the plasma[90]. Corticosteroids bound to CBGs are unavailable to tissues, and only free (unbound) cortisol is able to enter cells, activate glucocorticoid receptors, and also be metabolized by the liver[90]. The name “glucocorticoid” originated from early observations that these steroid hormones play important roles in regulating glucose metabolism. In the liver, glucocorticoids promote gluconeogenesis and increase glycogen storage[91]. In skeletal muscle and white adipose tissues, glucocorticoids inhibit their glucose uptake and utilization by counteracting insulin activity[91]. Furthermore, glucocorticoids induce proteolysis in skeletal muscle and lipolysis in white adipose tissue to provide precursors for gluconeogenesis[92]. Moreover, glucocorticoids modulate the activities of pancreatic α and β cells to control the secretion of glucagon and insulin respectively[91]. Together, these effects are critical for maintaining blood glucose levels and energy homeostasis during stress (e.g. fasting).

In addition to their effects on metabolism, glucocorticoids can suppress immune response and inflammation by controlling the activity of multiple signalling pathways. First, glucocorticoids can activate the transcription of genes encoding anti-inflammatory or immunosuppressive proteins, such as lipocortin-1, interleukin-10, interleukin-1 receptor antagonist and neutral endopeptidase[93]. Second, glucocorticoids inhibit the expression of genes for enzymes, receptors, adhesion molecules, and cytokines that are essential for immune-activation and inflammation [93]. This inhibitory effect is associated with two molecular mechanisms: inhibition of activator protein 1 (AP-1) and inhibition of NF-κB[94]. Activated glucocorticoid receptors interact with AP-1 and block its interaction with other transcription
factors[94]. Similarly, glucocorticoids inhibit NF-κB either by stimulating the transcription of IκB or through binding of activated glucocorticoid receptor to the p65 subunit of NF-κB[94]. Collectively, glucocorticoid-mediated transactivation and transrepression lead to increased lymphocyte apoptosis, reduced synthesis of lymphokines, decreased expression of major histocompatibility class I antigens, and lower production of proinflammatory humoral factors[95]. Outside of these most well-characterized functions, other physiologic processes affected by glucocorticoids include but are not limited to: (1) lung development in preterm infants, (2) modulation of bone density, and (3) acquisition, storage, as well as retrieval of memory[96-98].

5.2 Glucocorticoid Treatment and Muscle Wasting

Given their pleiotropic effects on a wide array of physiologic systems, glucocorticoids are used to treat many different diseases. Naturally, glucocorticoid replacement therapy is required to treat patients with adrenal insufficiency. More importantly, glucocorticoids are one of the most widely prescribed immunosuppressant drugs in the treatment of graft rejection, severe allergic reactions, autoimmune diseases, and certain side effects of chemotherapy[99]. However, despite their cost-effectiveness and potent immunosuppressive properties, high dose and/or chronic use of glucocorticoids can result in a plethora of adverse reactions. These include: diabetes mellitus, osteoporosis, myopathy, hypertension, higher risk of infection, and undesired effects on the central nervous system[100].

Glucocorticoid treatment is a powerful catabolic stimuli that has been shown to cause muscle wasting both in vitro and in vivo[55]. Furthermore, the loss of muscle mass under a variety of catabolic conditions such as sepsis, cachexia, starvation, and severe insulinopenia, is associated with elevated levels of circulating glucocorticoids[101]. More importantly, adrenalectomy,
treatment with a glucocorticoid receptor antagonist (RU-486), or muscle specific deletion of glucocorticoid receptor reduces the severity of muscle wasting during these conditions[101, 102]. Interestingly, the catabolic effects of glucocorticoids appear to preferentially target type II muscle fibers, and thus fast-twitch glycolytic muscles are more susceptible to glucocorticoid-induced atrophy than slow-twitch oxidative muscles[103]. Even in muscles with mixed fiber types, such as gastrocnemius muscle, type II fibers are still specifically targeted over type I via a mechanism that is unclear as of yet[104].

Similar to many other catabolic stimuli, glucocorticoids induce muscle atrophy by activating protein degradation and suppressing protein synthesis. The inhibitory effect on protein synthesis results primarily from interference with the IGF-1/PI3K/Akt/mTOR signalling pathway. First, glucocorticoids block IGF-1/insulin-stimulated phosphorylation of Akt, and this consequently leads to inhibition of mTOR and activation of GSK3β[34]. Second, activated glucocorticoid receptors enter the nucleus and upregulate the transcription of three genes that can modulate the Akt pathway: p85, REDD1 and KLF15[38, 105]. p85 is the regulatory subunit of PI3K, and in its monomeric form, p85 competes with PI3K for the same binding site on IRS-1[106]. Therefore, excess levels of p85 monomers can block IGF-1/insulin-stimulated activation of PI3K by acting as a competitive inhibitor[106]. REDD1 activates the TSC1/TSC2 complex, and thereby facilitates the hydrolysis of Rheb·GTP to Rheb·GDP[107]. Rheb·GDP then binds to mTOR and inhibits its activity, resulting in decreased phosphorylation of 4E-BP1 and S6K1[107]. On the other hand, KLF15 upregulates the expression of branched-chain aminotransferase (BCAT) to catalyze the breakdown of branched-chain amino acids (BCAA)[108]. The resulting decrease in intracellular BCAA level leads to downregulation of mTOR signalling and reduced protein synthesis[108]. In addition to REDD1 and KLF15, glucocorticoids also stimulate the production
of myostatin, which is another key inhibitor of protein synthesis due to its ability to block IGF-1-mediated phosphorylation of Akt[75].

The activation of protein degradation by glucocorticoids is in part mediated by three distinct mechanisms: (1) glucocorticoids directly stimulate the expression of FoxO1, FoxO3 and KLF15, which can work together to upregulate the expression of atrophy-inducing E3 ligases MuRF1 as well as atrogin-1, (2) ligand-bound glucocorticoid receptors can bind to the glucocorticoid response element (GRE) in the MuRF1 proximal promoter region to directly induce the transcription of MuRF1, and (3) glucocorticoids block the activation of Akt, preventing it from phosphorylating FoxO members[55, 108-110]. Hypo-phosphorylation and increased abundance of FoxO members are critical for the transcriptional-dependent activation of the UPS and the ALS during muscle atrophy[35, 55]. In summary, glucocorticoids activate muscle protein breakdown through a network of transcriptional changes that ultimately leads to hyperactivation of the UPS and the ALS.

5.3 Glucocorticoid Receptor

With a few exceptions, the vast majority of glucocorticoids’ downstream effects are mediated by the glucocorticoid receptor (GR), an intracellular ligand-dependent transcription factor belonging to the nuclear-receptor superfamily[111]. Upon glucocorticoid binding, the GR moves to the nucleus and interacts with specific regulatory elements (e.g. glucocorticoid response elements or other regulatory proteins) of its targets genes to modulate their transcription[111]. Early cloning studies on human GR cDNA reveals the existence of two isoforms, hGRα and hGRβ[112]. These two forms of GR protein are generated by alternative splicing of exon-9, and they contain different amino acid sequences at their C-terminus[113]. hGRα functions as a classic steroid receptor that binds glucocorticoids and modulates target gene expression in a
hormone-dependent fashion[112]. Conversely, hGRβ does not respond to glucocorticoids and more importantly, exhibits a dominant negative effect on the activity of hGRα[114]. In this chapter, we will focus on the structure and function of hGRα (hereinafter referred to as GR), because its ability to bind glucocorticoids and exert regulatory effects on gene transcription is critical for glucocorticoid-induced muscle atrophy.

Consistent with other members of the nuclear-receptor superfamily, the structure of GR can be organized into three discrete functional regions: N-terminal domain (NTD), DNA binding domain (DBD), and ligand binding domain (LBD)[111]. The NTD of GR possesses a potent trans-activation region, in which insertions or deletions lead to mutants that can bind steroids with wild-type affinity and translocate to the nucleus, but fail to fully activate transcription[115]. This trans-activation region (AF-1), located between amino acids 77 and 262, functions independently of ligand-binding and is pivotal in the interaction of the GR with transcriptional coregulators, such as TATA-box binding protein (TBP) and TBP-associated factors[116]. The DBD of GR stretches from amino acids 420 to 480 and contains sequences that are highly conserved among members of the steroid receptor family[116]. Within this domain, two zinc finger motifs, which are formed through four cysteine residues tetrahedrally held together by a zinc ion, facilitates the binding of GR to the glucocorticoid response element (GRE) in the promoter region of target genes[117]. This specific recognition of GRE is primarily mediated by 3 amino acids, called the “P-box”, in the first zinc finger[117]. On the other hand, the second zinc finger is responsible for stabilizing the GR-DNA interaction, and 5 of its amino acids, termed the “D-box”, are important for the homodimerization of GR[117]. The LBD of GR resides within the C-terminus of the protein between amino acids 481 and 777[116]. This domain is critical for the recognition and binding of glucocorticoids, heat shock proteins, as well as
transcriptional co-activators[118, 119]. In addition, the LBD plays an important role in GR
dimerization and contains a second transactivation region (AF-2), whose activity is position-
independent but tightly regulated by ligand-binding[120].

In the absence of glucocorticoids, the GR is sequestered in the cytoplasm through the binding
of its LBD to a hetero-oligomeric complex that contains heat shock protein (HSP) 90, HSP70,
HSP40 and specific co-chaperones[6]. This interaction is critical for proper GR function, as the
heterocomplex not only regulates the nuclear localization of GR in a ligand-dependent manner,
but it also facilitates the folding of GR into a high-affinity ligand binding conformation[121].
Immediately following GR synthesis, the newly translated polypeptide is bound by both HSP40
and HSP70 to form the initial chaperone-receptor complex[122]. Next, the co-chaperone HSP-
organizing protein (HOP) mediates the transfer of GR from HSP70 to HSP90 by acting as a
physical bridge between the two chaperones[122]. HSP70 and HOP then dissociate from the
HSP90-GR complex, while the immunophilin FK506-binding protein (FKBP52) and the co-
chaperone p23 are recruited[122]. With the help of p23 and FKBP52, HSP90 uses energy from
ATP hydrolysis to open up the ligand-binding pocket of GR for access by glucocorticoids[121].
At the same time, the HSP90 complex also conceals the two nuclear localization sequences
(NLS) of GR, and thereby inhibiting its translocation to the nucleus[121]. Upon glucocorticoid
binding, the GR undergoes a conformational change that causes it to dissociate from the HSP90
complex and translocate to the nucleus where it can regulate target gene transcription[121]. In
the nucleus, ligand-bound GR can alter gene transcription via two different types of mechanisms.
Type I mechanism involves the formation of GR homodimers and their binding to GREs in the
promoter regions of target genes[123]. Once bound to GRE, the GR homodimer interacts with
transcriptional co-activators, through either direct physical contact or an intermediate “tethering”
factor, to facilitate the recruitment of basal transcriptional machinery, induce chromatin remodelling and activate gene transcription[123]. Furthermore, there are also negative GREs with the consensus sequence of CTCC(n)_{0-2}GGAGA, and in contrast to positive GREs, these are bound by two GR monomers and mediate glucocorticoid-dependent transrepression[124]. Alternatively, type II mechanism functions independently of GRE binding, and instead involves direct protein-protein interaction between monomeric GR and other transcription factors. These interactions, often referred to as “crosstalk”, can have either antagonistic (e.g. AP1 and NF-κB) or synergistic (e.g. Stat-5) effect on the transcriptional activities of both factors involved[125-127]. After the desired transactivation or transrepression is achieved, glucocorticoid dissociates from the GR, which induces a second conformational change in the receptor to release it from GRE[116]. Unbound-GR is then exported from the nucleus via the binding of calreticulin to its DBD and/or directly degraded by the ubiquitin-proteasome system[128, 129].

6. The Ubiquitin Proteasome System

The degradation of cellular proteins by the ubiquitin-proteasome system is an exceedingly complex, highly specific and tightly regulated process that plays important roles in many pathways essential for life and health. This proteolytic pathway can be separated into two discrete and sequential processes: (1) covalent attachment of multiple ubiquitin moieties to the target protein and (2) degradation of the ubiquitinated protein by the 26S proteasome complex[130](Figure II). In the first step, the conjugation of ubiquitin to the protein substrate is mediated by three classes of enzymes working in a cascade [130]. At the start, a ubiquitin-activating enzyme (E1) catalyzes the formation of a high-energy thioester bond between its active-site cysteine residue and the C-terminus of ubiquitin in an ATP-dependent manner [131].
Next, the activated ubiquitin moiety is transferred to a ubiquitin-conjugating enzyme (E2) via the formation of a new thioester bond [130]. Finally, the E2 enzyme transfers the ubiquitin onto the protein substrate with the help of a ubiquitin-ligase (E3) that is specific for the target[130]. The E3 is responsible for catalyzing the covalent attachment of ubiquitin to the substrate, usually on an internal lysine residue, or on the previously conjugated ubiquitin molecule[130]. Multiple repeats of this process result in the formation of a polyubiquitin chain, which is then recognized by the downstream 26S proteasome. Following this recognition and the targeting of substrate to the proteasome, the polyubiquitin chain is removed by deubiquitinating enzymes to release free and reusable ubiquitin.

Figure II: The Ubiquitin Proteasome System. The conjugation of ubiquitin to the substrate is mediated by three enzymes: E1 (ubiquitin activating enzyme) activates ubiquitin and transfers it onto a E2 (ubiquitin conjugating enzyme). Ubiquitin-loaded E2 cooperates with a E3 (ubiquitin ligase) to ubiquitinate the substrate, which targets it for proteasome degradation. The polyubiquitin chain can be cleaved by deubiquitinating enzymes.
6.1 Structure and Function of E1, E2 and E3 Enzymes.

The human genome encodes 2 E1s, roughly 35 E2s, and approximately 750 E3s[132]. E1 enzymes contain three major domains: an adenylation domain at the N-terminus, a catalytic Cys domain for thioester bond formation, and a C-terminal ubiquitin-fold domain for binding E2s[133]. To initiate the ubiquitination pathway, the E1-enzyme first binds ATP·Mg²⁺ and ubiquitin (#1) to catalyze the formation of an ubiquitin-AMP complex through C-terminal acyl-adenylation [133]. Next, a catalytic cysteine residue in the Cys domain attacks this complex, displacing the AMP leaving group and forming a covalent thioester bond between the E1 enzyme and the C-terminus of the ubiquitin[133]. Following this displacement reaction, the E1 enzyme catalyzes the adenylation of a second ubiquitin (#2) molecule and non-covalently attaches to it at the active site[133]. Finally, the asymmetrically loaded E1 undergoes a conformational change to reveal its E2 binding site for proper E1-E2 complex formation and subsequent ubiquitin (#1) transfer[134]. E2 enzymes are characterized by the presence of a ubiquitin conjugation catalytic (UBC) domain that is highly conserved among members of this protein family and required for their specific interactions with E3s as well as E1s[134]. Within this domain, a catalytic cysteine residue accepts the ubiquitin (#1) moiety from E1s through the formation of a new thioester bond[135]. Some E2 enzymes also have N-terminal and/or C-terminal extensions to their UBC domain, which can influence critical factors such as cellular localization, the stability of their interactions with E1s, the activity of their partner E3-ligases, and the type of linkages they use to form the polyubiquitin chains[135]. To complete ubiquitin conjugation and achieve target specificity, loaded E2s must cooperate with E3-ligases. Despite their large number and structural diversity, the vast majority of E3-ligases can be separated into two families based on their conserved domains and catalytic mechanisms[136]. The first family of E3-ligases contain a
signature Homologues to the E6AP Carboxyl Terminus (HECT) domain at the C-terminus, and during their catalytic cycle, an active-site cysteine in this HECT domain forms another thioester intermediate with ubiquitin prior to its final transfer onto the substrate[137]. The second family of E3 ligases are characterized by the Really Interesting New Genes (RING) or RING-like (e.g. U-box) domains in their structure[138]. In contrast to HECT domain E3-ligases, RING-containing E3-ligases do not form any intermediate with ubiquitin, but instead act as scaffolds and/or allosteric activators to catalyze the direct transfer of ubiquitin from E2 enzymes to target lysine residues[138].

6.2 The Proteasome

Polyubiquitinated proteins are degraded by the 26S proteasome, a 2.5 MDa multi-subunit complex designed to carry out efficient and selective proteolysis[139]. The structure of this highly sophisticated complex can be separated into two major components: the 20S catalytic core and the 19S regulatory particle[139]. The 20S catalytic core consists of 4 heptameric rings stacked on top of each other to form a barrel-like structure that is ~15nm long and ~11nm wide[140]. The inner channel of this “barrel” is lined by 3 distinct proteolytic active sites and only accessible to unfolded substrates, thus protecting native proteins from unwanted degradation[140]. The 19S regulatory particle, more commonly referred to as the “Cap”, is composed of a 10-subunit base and a 9-subunit lid[141]. It binds to either one or both ends of the catalytic “barrel”, and serves to recognize polyubiquitinated substrates and denature them for entry into the catalytic channel of the 20S core[141].

The four heptameric rings of the 20S catalytic core contain a total of 28 subunits, which can be classified into α and β family according to their sequence similarity to homologs initially discovered in archaeabacteria[142]. The number of distinct subunits in these two families varies
between species, ranging from 7 different α and 10 different β subunits in mammals to a single type of each in Thermoplasma acidophilum[143, 144]. Nevertheless, the barrel-shaped structure of the 20S core and the overall composition of each heptameric ring remain highly conserved throughout evolution. Its two outer rings at the ends are always made up of α-subunits, whereas the two inner rings in the middle are formed by β-subunits exclusively[144]. The two outer α-rings serve three important purposes: (1) they act as scaffolds for the assembly of inner β-rings[145], (2) the N-terminal tails of α-subunits keep the interior channel in a closed state to prevent substrate access [146], and (3) they provide docking surface for the 19S regulatory particle and other proteasome activators[141, 147]. On the other hand, the two inner β-rings form the actual catalytic channel, with each ring containing three distinct proteolytic sites that face towards the interior chamber[148]. Depending on their catalytic mechanism and substrate specificity, these three proteolytic sites located on β1, β2 and β5 are identified as peptidyl-glutamyl peptide-hydrolyzing, trypsin-like, and chymotrypsin-like respectively[148]. Once unfolded substrate enters this proteolytic chamber, it is degraded through a processive mechanism, releasing fragments of 3 to 25 amino acids with an average length of 7 to 8[149].

The 19S regulatory particle interacts with the two α-rings of the 20S core and carries out a series of biochemical processes to ensure the proper degradation of ubiquitin-tagged proteins. First, it recognizes and binds polyubiquitin chains on substrates that have been targeted for degradation[141]. Next, it is able to cleave off the polyubiquitin chains and recycle the ubiquitin monomers for subsequent uses[150, 151]. Following substrate recognition and de-ubiquitination, the 19S regulatory particle opens up the gated pore in the centre of the α-ring and unfolds the protein substrate to promote its entry into the catalytic channel[152]. These functions are primarily carried out by the 19 subunits, identified as either regulatory particle ATPase(Rpt) or
regulatory particle non-ATPase proteins (Rpn), that form the 19S regulatory particle’s lid and base subcomplexes. The lid is composed of nine subunits: Rpn3, Rpn5-9, Rpn12, Rpn15, and the deubiquitinating enzyme Rpn11, whose metalloisopeptidase activity is required for the efficient degradation of ubiquitin-tagged substrate[150, 153]. These subunits all have unique sequences and structures, but with the exception of Rpn11, their functions are not well understood as of yet[153]. The base subcomplex consists of Rpt1-6, Rpn1, Rpn2, Rpn10, and Rpn13[153]. The six ATPase subunits, Rpt1-6, belong to the ATPase associated with various cellular activities (AAA) family, and they form a hetero-hexameric ring in the pattern of Rpt1-Rpt2-Rpt6-Rpt3-Rpt4-Rpt5[154]. It is predicted that these ATPase subunits utilize energy from ATP binding and hydrolysis to unfold substrate proteins and translocate them into the catalytic channel of the 20S core[155]. Furthermore, in the presence of ATP, the C-terminal tails of these subunits interact with conserved residues on the α-ring of the 20S core, resulting in opening of the gated pore for easier substrate entry[152]. In addition to Rpt1-6, the other four non-ATPase subunits of the base also serve important regulatory purposes. Both Rpn10 and Rpn13 functions as ubiquitin receptors for substrate recognition, with Rpn10 also acting as a linker to stabilize the interaction between the lid and the base[141, 156, 157]. Furthermore, Rpn1 recruits ubiquitin shuttle proteins Rad23, Ddi1, and Dsk2, as well as the proteasome-interacting deubiquitinating enzyme Ubc6 to the base[158-160]. Together, the lid and the base of the 19S regulatory particle enable the 20S catalytic core, whose free form exhibits little to no protease activity, to carry out efficient and specific degradation of substrate proteins.

6.3 Deubiquitinating Enzymes.

In addition to E1s, E2s and E3s, the ubiquitination status of cellular proteins is also regulated by deubiquitinating enzymes (DUBs). The human genome encodes approximately 95 DUBs, and
based on the sequence homology of their catalytic domains, they can be divided into 58 ubiquitin-specific proteases (USPs), 4 ubiquitin C-terminal hydrolases (UCHs), 5 Machado-Joseph Disease protein domain proteases (MJDs), 14 ovarian tumor proteases (OTUs) and 14 JAMM motif proteases\[161\]. Furthermore, out of these five DUB families, the first four (USP, UCH, MJD, OTU) belong to the class of cysteine proteases, whereas members of the JAMM motif family are zinc metalloproteases\[161\]. As its name suggests, the proteolytic activity of cysteine proteases is mediated by an active site cysteine residue that forms a catalytic triad with its histidine and aspartate partners\[161\]. During catalysis, the histidine residue, which is already polarized by the aspartate, deprotonates the cysteine to facilitate its nucleophilic attack on the lysine-glycine isopeptide bond between ubiquitin and the tagged substrate\[162\]. This results in the release of the substrate as a leaving group and the formation of a ubiquitin-protease intermediate\[162\]. Then, a reaction between this intermediate and a water molecule causes the release of free ubiquitin from the enzyme\[162\]. In contrast to the cysteine proteases, the active site of JAMM motif protease coordinates with an essential Zn\(^{2+}\) ion through an aspartate and two histidine residues\[163\]. This Zn\(^{2+}\) binds a water molecule, polarizes it, and subsequently uses it to form a noncovalent intermediate with the ubiquitinated protein\[164\]. During this process, the water molecule becomes deprotonated to generate a charged hydroxyl group whose oxygen atom will react with the carbonyl at the C-terminal of ubiquitin\[164\]. Finally, this intermediate is broken down by proton transfer from a second water molecule, causing the release of ubiquitin, the DUB and the substrate\[164\].

With 58 members, the USP family represents the largest group of DUBs in humans. Structural studies on the catalytic core of different USPs reveal the existence of two highly conserved motifs, termed the Cys and His boxes, that contain all the catalytic triad residues\[165\].
In the absence of substrate, some of these DUBs are kept in an inactive configuration due to either the misalignment of their catalytic residues or the occlusion of their active site by a peptide loop[166]. Upon ubiquitin binding, the catalytic cleft undergoes dramatic and highly localized structural changes, switching the enzymes into an active conformation[166]. The four DUBS in the UCH family are small proteins that cleaves ubiquitin from substrates up to 20-30 amino acids in size. Their active site is located at the bottom of a narrow groove on the surface of the protein and shares similar structure with that of the USPs despite the lack of obvious sequence homology[167]. In their free form, UCHs have a disordered loop arching directly over their active site and obstructing substrate access[167]. Similar to ubiquitin-induced conformation changes in USPs, interaction between UCHs and ubiquitin causes the active site crossover loop to adapt an ordered open conformation for substrate entry[168]. However, the maximum diameter of this loop is no greater than 15Å, which restricts the size of potential substrates[168].

The OTU family of DUBs is identified based on their homology to the ovarian tumor gene in *Drosophila melanogaster*. The core domain of these DUBs includes 5 β-strands placed between two helical domains with varying sizes[169-171]. Furthermore, crystal structure of human otubain 2, an OTU family DUB, reveals a catalytic histidine stabilized by hydrogen bonding with an asparagine instead of the predicted aspartate[171]. OTU DUBs also exhibit inactive conformation in the absence of ubiquitin binding, for example, the catalytic histidine of free Otubain1 is not properly aligned with its partner cysteine, and otubain2 in the unbound form has a loop that spatially restricts its active site[171, 172]. The majority of the structural insights on the MJD family of DUBs comes from studies on Ataxin 3, a protein that causes Machado-Joseph Disease when its poly-glutamine stretch is extended[173]. The catalytic site of Ataxin-3 is located in its N-terminal Josephin domain, which also contains two binding sites for...
ubiquitin[174]. One of these site is in close proximity to the catalytic triad, and substrate binding appears to be regulated by a flexible helical hairpin[174]. The other ubiquitin-binding site is located far away from the catalytic core, but its existence explains the ability of Ataxin-3 to bind poly-ubiquitin chains and perform endo-type deubiquitination[175]. The structure of JAMM motif DUBs is elucidated by the crystal structure of AF2198 from *Archaeoglobolus fulgidus* and of Associated Molecule with the SH3 domain of STAM (AMSH)-like protease[163, 176]. In both studies, the two histidine and one aspartate residues responsible for coordinating with the Zn2+ are found in a central β-sheet and a flanking α-helix respectively. Moreover, a glutamic acid residue forms a hydrogen bond with the catalytic water molecule and functions as an acid/base during catalysis[163]. Interestingly, JAMM motif DUBs are often found as part of large protein complexes, such as Rpn11 (Yeast)/POH1 (Human) in the 19S regulatory particle, COP9 signalosome subunit 5, and BRCC36 of the BRCA1 A complex[166].

**6.4 Function of Deubiquitinating Enzymes.**

One of the most important functions of DUBs is their ability to regulate protein stability through either direct deubiquitination or indirect targeting of upstream regulators. Within the cell, protein degradation through both the proteasomal and the lysosomal pathways can be regulated by polyubiquitin chains of different linkages. Lys-63 linked chains on various membrane receptors target them for endocytosis and subsequent degradation by the lysosome[177]. For example, the epidermal growth factor receptor (EGFR) is rapidly ubiquitinated, endocytosed and degraded in the lysosome upon ligand binding[178]. However, this process is not only regulated by K-63 linked ubiquitination, but also by the activities of two DUBs, USP8 and AMSH, that exert opposite effects[179]. AMSH prevents the breakdown of ubiquitinated EGFR via direct removal of its K63-linked polyubiquitin chains, whereas USP8
associates and stabilizes endosomal sorting complex required for transport (ESCRT)-0, a component of the lysosomal sorting machinery responsible for binding ubiquitinated EGFR[180, 181]. In contrast to K-63 linked chains, K48-linked chains targets the substrate for proteasomal degradation. Examples of DUBs rescuing proteins from this proteolytic pathway can be found in a variety of processes involved in health and disease. Some prominent ones include USP2-mediated stabilization of fatty acid synthase, whose high levels are associated with poor prognosis in prostate cancer, and rescuing of the proto-oncogene MYC by USP28[182, 183]. Many DUBs can also regulate the ubiquitination status of specific substrates by interacting with E3-ligases. For instance, USP19 can control the level of p27Kip1 by stabilizing its E3-ligase KPC1, and likewise, USP8 can promote the degradation of ERBB3 through deubiquitinating the E3-ligase NRDP1[184, 185].

DUBs also play an important role in ubiquitin recycling and homeostasis. There are three DUBs associated with the 19S regulatory particle of the proteasome: POH1/Rpn11, UCH37, and USP14/UBP6. POH1/Rpn11 is predicted to be the DUB responsible for removing ubiquitin chains en bloc from the substrate and facilitating its degradation. Knockdown of POH1/Rpn11 leads to accumulation of ubiquitin-protein conjugates, impaired degradation of cellular proteins and compromised proteasome integrity[179]. Moreover, most mutations in the active site of POH1/Rpn11 have been reported to cause loss of cell viability, with the exception of D112A mutation in yeast Rpn11, which leads to a defect in protein degradation[150, 151]. Out of the three proteasome-associated DUBs, POH1/Rpn11 is the only one that cleaves at or near the proximal end of the polyubiquitin chain, and its activity appears to be coupled to the unfolding of substrate by 19S subunits Rpt1-6[179]. UCH37 and USP14/UBP6 shares three similarities: (1) they are activated upon direct interaction with the 19S regulatory particle, (2) their depletion
leads to accelerated protein degradation, and (3) they remove ubiquitin from the distal end of the polyubiquitin chains [160, 186-188]. However, while UCH37 inhibits substrate degradation by removing ubiquitin, USP14/UBP6 mediates the same effect independent of its catalytic activity [189]. UBP14/UBP6, and potentially UCH37, cooperate with HECT ubiquitin ligase 5 (Hul5) to perform polyubiquitin chain remodelling at the proteasome, which may function as an extra layer of control over the degradation of ubiquitinated substrates [190].

In addition to controlling protein degradation, DUBs also play important roles in cellular signalling. The activity of the major inflammatory mediator NF-κB is closely regulated by two DUBs, an UCH known as CYLD and an OTU containing protease called A20. CYLD negatively regulates NF-κB signaling by removing K63-linked polyubiquitin chains from TNF-receptor associated factor (TRAF) 2 and, to a lesser extent, TRAF 6 [191]. On these two signalling molecules, K63-linked chains interact with IKK and facilitate its phosphorylation/activation by recruiting its upstream activator TGF-β activated kinase-1 (TAK1) and TAK1 binding protein 2/3 (TAB2/3) [192]. Activated IKK phosphorylates the inhibitory IκB protein, promoting its K48-polyubiquitination and subsequent proteasomal degradation [85]. Similarly, A20 downregulates IKK activity by targeting K-63 linked polyubiquitin chains on TRAF6 and receptor-interacting protein 1 (RIP1) [193]. Upon TNF binding, RIP1 is subjected to K63-linked and M1-linked ubiquitination by TRAF2 and the linear ubiquitin chain assembly complex (LUBAC) respectively [194]. Ubiquitinated RIP1 catalyzes TAK1-mediated phosphorylation and activation of IKK by recruiting both factors to its ubiquitin chains [194]. Therefore, the removal of K63-linked ubiquitin chains from TRAF2, TRAF6 as well as RIP1 by CYLD and A20 leads to decreased IKK phosphorylation, which consequently prevents IκB breakdown and NF-κB signalling. More recently, a third DUB called Cezanne has been reported to also suppress NF-κB
activity upstream of IKK activation via blocking the build up of K63-polyubiquitinated RIP1 at activated TNF-receptors[195]. Intriguingly, the expression of CYLD is upregulated by NF-κB, and TNFα induces both A20 and Cezanne, suggesting that all three DUBs are functioning as negative feedback loops to regulate inflammatory responses[166, 195].

6.5 Regulation of Deubiquitinating Enzymes.

Similar to other cellular enzymes, the levels and activities of DUBs are under strict regulation. One of the mechanisms employed for this purpose is the aforementioned conformational changes upon substrate binding. The activity of most DUBs in vitro or in their apo-form is low due to their active site being maintained in an inactive conformation. Some DUBs, such as USP7, rely on substrate-induced conformational changes to align their catalytic residues into a productive conformation, while other DUBs like USP14 or USP8 have their active sites blocked by parts of their own structure in the absence of substrate binding[166]. These regulatory mechanisms, with some slight variations, are observed among members of all five DUB families and serve to prevent spurious deubiquitination as well as to increase specificity.

The second mechanism for regulating DUB activity relies on post-translational modifications, including phosphorylation, ubiquitination and sumoylation. For example, USP34 is phosphorylated by ataxia telangiectasia-mutated (ATM)/ataxia telangiectasia and rad3-related (ATR) kinases in response to DNA damage, and it subsequently regulates the repair process by stabilizing the E3-ligase RNF168, which is a core intermediate in DNA damage signal transduction[196, 197]. Other than USP34, phosphorylation is also observed on both A20 and CYLD, although it appears to have opposite effect on their ability to regulate NF-κB signalling. Phosphorylation of A20 by IKKβ is a negative feed back mechanism that enhances the
suppression of IKK activity and NF-κB signalling by this DUB, whereas phosphorylation of CYLD by IKKγ blocks its deubiquitination of TRAF2 and releases its inhibition on NF-κB activity[198, 199]. DUBs themselves are also subjected to ubiquitination and ubiquitin-like modifications. The deubiquitinating enzyme ataxin-3 is increasingly ubiquitinated under certain stressful conditions, and this leads to the activation of its catalytic activity[200]. In contrast, sumoylation of USP25 inhibits its capacity to bind and hydrolyze polyubiquitin chains by steric hindrance[201]. Furthermore, some DUBs can be inactivated by post-translational proteolytic cleavage. These include USP1 and A20, which undergo self- and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1)-mediated proteolysis respectively[202, 203].

The third level of control on DUB activity stems from their protein partners and subcellular localization. One great example of this type of regulation is the three proteasome-associated DUBs POH1/Rpn11, UCH37 and USP14/Ubp6 being kept in an inactive conformation until they bind to the proteasome’s 19S regulatory particle[186-188]. Similarly, the endosomal DUBs USP8 and AMSH are activated through their interactions with signal transducing adaptor molecule 2 (STAM2) [204, 205]. Conversely, associations with other factors can also have inhibitory effect on DUB activity: the INO80 chromatin remodelling complex reduces UCH37’s catalytic capability, and the yeast enzyme Doa4 is suppressed by the binding of Regulators of Free Ubiquitin chains 1 (Rfu1)[206, 207]. Various DUB-binding partners can function as adapter proteins to enhance substrate delivery, serve as scaffolds for anchoring DUBs to specific cellular structure, or act as allosteric regulators to directly control catalytic activity. Certain DUBs also contain structures that localize them to specific cellular organelles. Both USP19 and USP30 have transmembrane domains, which targets them to the endoplasmic-reticulum (ER) and mitochondria respectively. USP36 specifically accumulates in the nucleus to regulate nucleolar
structure as well as function, and USP8 contains a N-terminal Microtubule Interacting and Transporting (MIT) domain that is essential for its localization to the endosome[205, 208]. Overall, these regulatory mechanisms ensure substrates are delivered to the correct and active DUB in the right place and at the right time for proper cellular function.

6.6 The Ubiquitin Proteasome System in Muscle Atrophy.

The loss of muscle mass under a variety of catabolic conditions is associated with the induction or suppression of a common set of atrophy-related genes, referred to as atrogenes[30]. A large number of the strongly induced atrogenes encode components of the UPS, such as polyubiquitin, multiple subunits of the 26S proteasome holoenzyme, and the two atrophy-inducing E3-ligases MuRF1 and Atrogin-1/MAFbx[30]. Consistent with these transcriptional adaptations, atrophying muscles exhibit enhanced proteolysis that occurs concomitantly with increased levels of ubiquitin-protein conjugates, particularly in the myofibrillar protein compartment[209]. This activation of the UPS is critical in mediating muscle atrophy, as it facilitates the rapid degradation of normally long-lived myofibrillar proteins and inhibits myogenesis by specifically targeting myogenic regulatory factors[42].

The accelerated degradation of myofibrils is caused by the activities of several E3-ligases, each of which binds and ubiquitinates different myofibrillar proteins to target them for degradation. The muscle specific RING finger E3-ligase MuRF-1 is one of the most well-known atrogenes whose knockout in mice leads to protection against muscle atrophy[210]. In response to catabolic stimuli, MuRF-1 ubiquitinates several components of the thick filament, including myosin heavy chain (MyHC), myosin binding protein C (MyBP-C), myosin essential light chain (MyLC1) and myosin regulatory light chain (MyLC2)[41, 211]. Moreover, since MyHC is highly resistant to ubiquitination when existing as a part of the actomyosin complex or myofibril,
MyLC1, MyLC2 and MyBP-C may be selectively degraded in the early stages of myofibril breakdown to destabilize the structure of thick filaments and subsequently expose MyHC for MuRF1-mediated ubiquitination[211, 212]. On the other hand, thin filament proteins actin, tropomyosin, and troponins are ubiquitinated by TRIM 32, an E3-ligase that is expressed throughout the body[40]. In addition to thin filament proteins, TRIM 32 also targets α-actinin and desmin, which are two proteins associated with the Z-disk and essential for maintaining the integrity of the sarcomere[40]. Atrogin-1/MAFbx is a F-box protein with muscle specific expression, and it forms the Skp1-Cullin-F-box (SCF) E3-ligase complex with Skp1, Roc1 and Cul1[213]. Atrogin-1 is highly induced just before and during muscle atrophy, and similar to MuRF-1 inactivation, knockout of atrogin-1 in mice results in sparing of muscle mass in response to catabolic stimuli[210, 213]. In the context of myofibril disassembly, atrogin-1 has been shown to ubiquitinate desmin and the intermediate filament protein vimentin in cultured C2C12 myotubes upon myostatin treatment[214]. It is theorized that the loss of Z-disk and intermediate filament proteins disrupts the structural integrity of thin filaments, and thus increases the susceptibility of its components to ubiquitination/degradation[40].

The process of myogenesis is regulated by the family of basic helix-loop-helix (bHLH) myogenic regulatory factors: myogenic factor 5 (Myf5), muscle specific regulatory factor 4 (MRF4), myoblast determination protein (MyoD), and myogenin[215]. Expression of Myf5 and MyoD drives the proliferation of myoblasts, while myogenin and MRF4 are upregulated to promote the differentiation of myoblasts into multinucleated myotubes[215]. Recently, a study using murine cancer models and cachectic muscle biopsy specimens revealed the existence of impaired myoblast fusion in cancer cachexia[216]. More importantly, overexpression of MyoD and the resulting induction of its downstream myogenin rescued this defect in myoblast fusion.
and restored myofiber size in tumor-bearing mice, suggesting that myogenesis plays an important role in the maintenance of muscle mass under catabolic conditions[216]. Consistent with the observations made in that study, the E3-ligase Atrogin-1 has been shown to ubiquitinate both MyoD and myogenin in vitro[217, 218]. Furthermore, overexpression of ubiquitination-resistant MyoD mutant in mice TA muscle leads to protection against fasting-induced muscle atrophy[219]. Another E3-ligase that negatively regulates myogenesis is Trim72, and it acts through two different mechanisms[220, 221]. First, Trim72 interacts with insulin receptor substrate-1 and blocks its activation by IGF-1, leading to decreased phosphorylation of Akt[220]. Consequently, low Akt activity prevents the formation of MyoD-containing transcriptosome in myoblasts, which in turn, downregulates myogenin expression and myogenesis[220]. The second mechanism involves the ubiquitination of focal adhesion kinase (FAK) by Trim72 and its subsequent degradation through the proteasome[221]. FAK plays an essential role in skeletal myogenesis since: (1) its interaction with methyl-CpG-binding domain protein 2 (MDM2) promotes myogenin expression via heterochromatin remodelling[222], and (2) it upregulates the expression of profusion genes caveolin 3 and β1 integrin[223]. Therefore, the loss of FAK due to Trim72 activity impairs the process of myogenesis, particularly at the myoblast fusion/myotube formation stage. Interestingly, the expression of both Atrogin-1 and Trim72 are induced by high levels of myogenin, suggesting that they may be parts of a negative feedback loop designed to prevent excess skeletal muscle differentiation and growth[220, 224].

7. USP19

7.1 Structure of USP19

USP19 is a 150 kDa DUB whose expression is observed in a variety of tissues, including
testis, heart, kidney and skeletal muscles[1]. Within the structure of USP19 (Figure III), the N-terminus is primarily occupied by two CHORD/SGT1 (CS/P23) domains (amino acid 51-140 & 321-423) that are homologous to two co-chaperones (p23 and Sgt1) of HSP90 and may impart USP19 with its own co-chaperone activities[225]. Recently, the N-terminal region spanning amino acid 462-473 is identified to be an interaction motif for the E3-ligases seven in absentia homolog (SIAH) 1 and 2[226]. This motif is found in previously identified SIAH1/2 substrates and its deletion in USP19 results in a mutant whose stability is independent of SIAH1/2 levels[226]. Following the CS domains and the SIAH interaction motif is the catalytic core of USP19, which contains the highly conserved catalytic triad residues of cysteine, histidine and aspartic acid, as well the Cys and His box motifs. Interestingly, between its Cys and His boxes, USP19 also contains a ubiquitin-like (UBL) and a myeloid-Nervy-DEAF1 (MYND) zinc finger domain. The UBL domain is commonly found in other DUBs of the USP family as well, and it can regulate several key aspects of these DUBs’ function, including their catalytic activity, subcellular localization and substrate recognition[227]. On the other hand, MYND Zinc finger domains are found in transcriptional regulators, and they mainly serve as docking or recognition sites for interactions with other proteins[228]. Finally, alternative splicing of exon 27 and exon 28 leads to the generation of two major USP19 isoforms with variations at the very end of their C-terminus[229]. The isoform containing exon 27 is characterized by a transmembrane domain that localizes it to the endoplasmic reticulum (ER) membrane with its catalytic domain facing towards the cytoplasm[229]. In comparison, use of exon 28 creates an USP19 isoform with cytoplasmic localizon due to replacement of the transmembrane domain with a short terminal sequence[229].
**Figure III: Structure of USP19 Isoforms.** The structure of USP19’s two major isoforms, depicted with known structural domains. Amino acid positions are based on human USP19 and included for reference. Inclusion of exon 27 leads to the ER-localized isoform (USP19-ER) that contains a C-Terminal TMD. Inclusion of exon 28 generates the cytoplasmic isoform (USP19-CYT) lacking the TMD. p23/CS (CHORD and SGT1 domains), UBL (ubiquitin-like domain), MYND (myeloid-Nervy-DEAF1 domain), TMD (transmembrane domain). C, D and H are the catalytic triad of cysteine, aspartic acid and histidine.

### 7.2 Function of USP19

#### 7.2.1 Regulation of Cell Cycle.

USP19 is implicated in the regulation of a variety of cellular processes. First, USP19 has been shown to promote cell cycle progression by stabilizing Kip1 ubiquitination promoting complex 1 (KPC1), which is an E3-ligase capable of targeting the cyclin-dependent kinase (CDK) inhibitor p27\(^{Kip1}\)[184]. p27\(^{Kip1}\) plays a critical role in restricting cell proliferation, as its knockout in mice leads to enhanced growth, organ hyperplasia, and pituitary tumors[230]. Moreover, low levels of p27\(^{Kip1}\) in human cancer, specifically due to increased proteolysis, is associated with higher grade of malignancy and increased tumor aggressivity[231]. During G1 and early S phase of the cell cycle, p27\(^{Kip1}\) must be targeted for proteasomal degradation by the concerted actions of E3-ligases KPC1 and Skp2 in order to allow proper G1-S phase transition[232, 233]. Therefore, by deubiquitinating and stabilizing KPC1, USP19 is able to indirectly modulate the level of p27\(^{Kip1}\) and promote cell cycle progression. In rat myoblast and fibroblasts, RNAi mediated knockdown of USP19 results in inhibition of cell proliferation, slow
progression from G1 to S phase, and elevated levels of p27Kip1 even though there is no change in Skp2 abundance[184]. These defects can be rescued by either re-introducing USP19 through overexpression or using embryonic fibroblasts from p27-KO mice[184].

7.2.2 Stabilization of Hypoxia Inducible Factor-1α

Another role of USP19 is stabilizing hypoxia inducible factor-1α (HIF1α)[234]. In response to oxygen deprivation, commonly referred to as hypoxia, cells make a series of adaptations in an attempt to match oxygen supply with cellular demands. One of the key regulators of the transcriptional response to hypoxia is HIF1, a heterodimer consisting of a constitutively expressed β-subunit (HIF-1β) and an oxygen-level-dependent α-subunit (HIF-1α)[235]. HIF1α is expressed universally across all cell types, and together with HIF-1β, it regulates the transcription of genes essential for survival during hypoxic stress by recognizing and binding to hypoxia response elements in the genome[235]. However, overexpression of HIF-1α, due to either intratumoral hypoxia or genetic mutation, is associated with cancer progression, poor prognosis and resistance to therapy[236]. Therefore, under normal conditions, HIF-1α is maintained at a low level by O2-dependent phosphorylation of proline 402 and 564, which recruits the E3-ligase von Hippel-Lindau (VHL) to facilitate HIF-1α ubiquitination and degradation[237]. This regulatory pathway is inactive in hypoxia, leading to rapid accumulation of HIF-1α and activation of its transcriptional activity[237]. USP19 is shown to interact with HIF-1α and rescue it from proteasomal degradation[234]. Moreover, knockdown of USP19 with shRNA in HeLa cells impairs their ability to mount the proper transcriptional response when exposed to hypoxia[234]. Interestingly, the effect of USP19 on HIF-1α is independent of its catalytic activity and ER-localization, suggesting USP19 might be part of a large complex that regulates HIF-1α stability[234].
7.2.3 Stabilization of c-IAP1 and c-IAP2

Another set of factors regulated by USP19 is the cellular inhibitors of apoptosis-1 (c-IAP1) and 2 (c-IAP2). Apoptosis is an evolutionarily conserved cell suicide pathway mediated by a family of aspartic acid-specific cysteine proteases called caspases, whose activity can be blocked by the binding of inhibitors of apoptosis (IAP) proteins[238]. In response to apoptotic stimuli, the mitochondria release several cell death inducers, including cytochrome c and Second Mitochondria-derived Activator of Caspases (SMAC/DIABLO). Cytochrome c activates caspase 9, which serves as the initiator of the entire apoptotic protease cascade[239]. SMAC promotes this cytochrome c-dependent caspase activation by sequestering IAPs and thus eliminating their inhibitory activity[240]. However, instead of interacting with caspases like some other members of the IAP family, c-IAP1 and c-IAP2 function as E3 ligases to facilitate the ubiquitination and subsequent degradation of SMAC[241]. Furthermore, c-IAP1 and c-IAP2 have been shown to enhance NF-κB signaling. Through their constitutive interaction with TRAF-2, they are recruited to activated TNF-receptors, where they promote K63-linked ubiquitination of RIP1 to activate IKK[242]. The stability of c-IAP1 and c-IAP2 is regulated by self-ubiquitination, which can be induced by apoptotic stimuli or binding to SMAC and its mimetics[243, 244].

USP19 interacts with c-IAP1 as well as c-IAP2 and stabilizes them through a protease-independent mechanism[245]. shRNA mediated knockdown of USP19 results in a significant decrease in endogenous c-IAP levels, whereas its overexpression leads to marked increase in the amount of c-IAPs[245]. Moreover, USP19 knockdown cells display elevated levels of apoptosis and caspase activation, both of which can be reversed by overexpression of c-IAP1 and c-IAP2[245]. Lastly, as for HIF-1α, USP19 also seems to prevent the self-ubiquitination of c-IAP2 through a mechanism that is independent of catalytic activity, although the explanation for this
effect is still unknown[245].

7.2.4 Role in ERAD

Given the existence of an ER-localized USP19 isoform, it should be no surprise that USP19 participates in ER-associated degradation (ERAD). In eukaryotic cells, secretory proteins mature in the ER with the assistance of molecular chaperones and are subjected to ER quality control mechanisms. Proteins folded into their native conformation are targeted to their final destination, while misfolded and unfolded proteins will either undergo additional folding cycles or be degraded by the process known as ERAD[246]. Degradation of proteins via the ERAD pathway is primarily mediated by a group of ER-associated E3-ligases, such as Hrd1 and MARCH6, that facilitate the ubiquitination of ERAD substrates, leading to their retrotranslocation from the ER to the cytosol and degradation by the 26S proteasome[247].

Deubiquitinating enzymes can regulate this process in several different ways: (1) DUBs can counteract ERAD by deubiquitinating the substrates, (2) They are able to modulate the retrotranslocation of substrates into the cytosol, and (3) Some DUBs control the stability of E3-ligases involved in ERAD[248-250]. The role of ER-localized USP19 in ERAD is controversial. One study reported that USP19 rescues the ERAD substrate cystic fibrosis transmembrane conductance regulator (CFTR)ΔF508 and T-cell receptor-α (TCRα) from proteasomal degradation[251]. Co-immunoprecipitation experiments demonstrate that USP19 interacts with both proteins, and its ER-localization/transmembrane domain is essential for the stabilization effect[251]. However, rescuing of CFTRΔF508 requires the deubiquitinating activity of USP19, whereas a catalytically inactive USP19 mutant retains its effect on the level of TCRα[251]. USP19 has also been shown to interact and stabilize both MARCH 6 and Hrd 1 through K48-linked deubiquitination[250, 252]. Overexpression of USP19 results in increased levels of
endogenous MARCH 6 and Hrd1, while siRNA mediated knockdown of USP19 leads to lower expression of both targets[250, 252]. Furthermore, USP19 knockdown cells have increased amount of mutant bile salt export pump (ABCB11), which is a substrate known to be targeted for degradation by MARCH6[250]. However, in contrast to the above findings, another group reported that USP19 is predominantly localized to the cytosol despite the presence of a transmembrane domain in its structure[253]. Furthermore, siRNA-mediated knockdown of USP19 did not have significant effect on the stability of ERAD substrates NHK, TTR and TCRα, suggesting that USP19 is dispensable for ERAD[253].

7.2.5 Chaperone and Co-chaperone Activity.

Another protein quality control mechanism that involves ER-localized USP19 is an unconventional protein secretion pathway termed misfolded-associated protein secretion (MAPS)[254]. This pathway preferentially targets abnormal cytosolic proteins for secretion, and its activity is regulated by USP19 to provide short-term relief in the presence of proteasome dysfunction[254]. Si-RNA mediated knockdown and CRISPR knockout of USP19 in HEK293T cells reduce the secretion of misfolding-prone GFP mutants[254]. In contrast, normal cytosolic proteins, such as HSP90, Bag6 and Ubl4A, are not secreted regardless of USP19 level[254]. Moreover, both the ER-localization and catalytic activity of USP19 is required for its regulatory effects on MAPS, as either deletion of the transmembrane domain or mutation of the catalytic cysteine to a serine blocks the secretion of mutant GFP[254]. The catalytic domain of USP19 is also shown to have chaperone activity and capable of recognizing misfolded proteins. On the other hand, the transmembrane domain allows USP19 to recruit misfolded proteins to the ER, where they will eventually be secreted though late endosomes[254].

Recently, the cytoplasmic isoform of USP19 is identified to interact with HSP90 and control
the levels of polyglutamine (Poly-Q)-expanded proteins Ataxin-3 and huntingtin, whose aggregated forms are the primary cause of spinocerebellar ataxia type-3 (SCA3) and Huntington’s disease respectively[255]. The molecular basis of the interaction between USP19 and HSP90 is controversial, since one study reports that it is mediated by the catalytic domain of USP19, whereas another claims that it is dependent on the two N-terminal CS domains[253, 256]. Nevertheless, USP19 upregulates the level and promotes the aggregation of polyQ-expanded Ataxin-3 and Huntingtin-N552[256]. This potentially requires USP19 to interact with HSP90 through its CS domains, as mutations in these two domains of cytoplasmic USP19 significantly attenuated its binding to HSP90 and importantly, abolished its effect on the levels of Ataxin-3 and Huntingtin-N552[256]. Furthermore, in support of HSP90’s importance in this process, treatment of cells with the HSP90 inhibitor 17-AAG results in decreased levels of both soluble and aggregated polyQ-expanded proteins[256].

7.3 Myogenesis and Muscle Atrophy.

USP19 was initially identified as a DUB whose expression is upregulated in rat skeletal muscle under catabolic conditions, including fasting, streptozotocin-induced diabetes, dexamethasone treatment, and cancer[1]. The increase in USP19 mRNA expression in response to these catabolic stimuli ranges from 30% to 200% and inversely correlates with muscle mass[1]. In a later study carried out by our laboratory, lowering USP19 level by 70%-90% through si-RNA mediated knockdown in rat L6 myotubes results in increased expression of myofibrillar proteins MHC, actin, troponin T and tropomyosin[2]. This effect of USP19 on tropomyosin and MHC occurs at the transcriptional level and is dependent on the activity of myogenin[2]. Upon myogenin knockdown using si-RNA, USP19 depletion is no longer able to induce the expression of MHC and tropomyosin[2]. Together, these data indicate that the
induction of USP19 in response to catabolic stimuli promotes muscle atrophy by acting on specific substrates instead of regulating the ubiquitination status of the general protein pool.

The importance of USP19 in muscle cells is well characterized by two studies. In the first study, the ER-localized isoform of USP19 is shown to suppress myoblast fusion as well as the expression of myogenin and major myofibrillar proteins[257]. Both of these effects require USP19 ER-localization and its catalytic activity, since cytoplasmic or catalytically inactive USP19 is unable to induce the same effects in L6 muscle cells, and silencing of ER-localized USP19 but not its cytoplasmic isoform enhances fusion and the expression of myogenin as well as myofibrillar proteins[257]. It was subsequently revealed that USP19 downregulates these myogenic pathways by blocking differentiation-dependent unfolded protein response (UPR) signaling[257]. Overexpression of ER-USP19 attenuated the induction of the UPR target gene C/EBP homologous protein (CHOP), whereas silencing of USP19 increased the number of CHOP-positive cells[257]. Consistent with this idea, artificial induction of ER-stress by thapsigargin treatment and its downstream UPR activation reversed the USP19-ER mediated defect in myoblast fusion[257]. Finally, the effect of USP19 on muscle cell differentiation and myogenesis was confirmed in vivo, as USP19-KO mice display enhanced regeneration following cardiotoxin induced injury[257].

The second study focuses on the role of USP19 in muscle wasting through the use of USP19-KO mice. In response to dexamethasone treatment and denervation, USP19-KO mice lose significantly less muscle mass and retains more muscle strength than WT mice[3]. Moreover, food intake and the rate of protein synthesis remained similar between the two mice strains, suggesting that the protective effect of USP19 knockout is not due to changes in energy intake or protein synthesis, but instead is caused by altered protein degradation[3]. In support of this idea,
the upregulation of the E3-ligases MuRF-1 and MAFbx/Atrogin-1 as well as the autophagy genes Bnip3 and Atg4 is blunted in USP19-KO mice during both dexamethasone- and denervation-induced muscle atrophy[3]. The same study also examined USP19 expression in human skeletal muscles under catabolic conditions. In muscle biopsies obtained from patients with lung or gastrointestinal cancer, there is a significant correlation between USP19 expression and the mRNA levels of MuRF-1 and MAFbx/Atrogin-1[3]. Together, these observations suggest that USP19 plays a significant role in promoting muscle atrophy, and it can be an attractive target for treatment.

8. Objectives of This Thesis

The recent efforts in studying the mechanisms underlying muscle atrophy have primarily focused on the roles of E3-ligases and ubiquitin conjugation, whereas the potential function of DUBs and deubiquitination remains poorly understood. Previous work in our laboratory have identified USP19 as a DUB whose expression is upregulated in many common conditions of muscle wasting, including cancer, dexamethasone treatment, diabetes and fasting[1]. Silencing of USP19 in cells increases the expression of major myofibrillar proteins and its knockout in mice reduces the severity of muscle wasting in response to glucocorticoid, denervation and fasting[2, 3]. These observations suggest that USP19 promotes muscle wasting, but the mechanism of its action is unknown. The goal of my thesis is to identify the substrate(s) of USP19 that mediate its effects in atrophying muscles. C/EBPδ is capable of inducing muscle atrophy by upregulating myostatin, which is a well known negative regulator of muscle mass[5]. We initially observed lower protein levels of C/EBPδ in USP19-KO mice upon dexamethasone treatment. Therefore, I tested whether C/EBPδ is a substrate of USP19. In addition, we also observed decreased
glucocorticoid signalling and lower levels of GR in USP19-KO mice, thus I also examined if USP19 regulates the stability of GR. Lastly, since previous studies identified interaction between USP19 and HSP90[258], I tested whether USP19 can potentially function as a co-chaperone of HSP90 to influence GR activity.
II. Materials and Methods

1. Plasmids, Cell Culture and Transfection.

To generate a plasmid for expressing ER-localized USP19 (USP19-ER) with N-terminal 6×His and 3×Flag tags, oligonucleotides encoding these tags were inserted into USP19 containing Blue-script plasmids (L. Combaret et al, [1]) using the XbaI (5-prime) and SmeI (3-prime) restriction sites in the pBSKII vector backbone. For the cytoplasmic USP19, the transmembrane domain of USP19-ER was replaced with sequence from non-TMD region of rat USP19 (B. Wiles et al, [259]). The entire 6×His-3×Flag-USP19-ER or 6×His-3×Flag-USP19-CYT sequence was cut out from the Blue-script plasmid using XbaI and HindIII (blunt ended), and then inserted into the pcDNA 3.1A vector that had already been digested with XbaI and PmeI. The catalytically inactive mutant of USP19 (USP19-CA) is created by mutating the catalytic cysteine (amino acid 545) of USP19-ER into an alanine using a site-directed mutagenesis kit (Stratagene/Agilent). To generate the USP19 mutants with deletions at the N-terminus (USP19-ΔN1, USP19-ΔN2), region of interest in USP19-ER’s DNA sequence was amplified by PCR using the following primers:

USP19-ΔN1: Forward 5’ – GTCCGAATTTCCTGAAACCAGAAG – 3’

Reverse 5’ – CGTATCGATAAGCTTATGAT – 3’

USP19-ΔN2: Forward 5’ – GTCCGAATTCCACCAGACCAGGGATGAA – 3’

Reverse: 5’ – CGTATCGATAAGCTTATGAT – 3’

USP19-ΔN1 is missing the first CS domain, and it is generated by excluding the first 178 amino acids. USP19-ΔN2 is missing both CS domains due to the removal of amino acid 1-362. Wild-type USP19 was removed from blue script plasmid using EcoRI and HindIII, and the PCR products for USP19-ΔN1 or USP19-ΔN2 were inserted back using the same restriction sites.
Finally, 6×His-3×Flag-USP19-ΔN1 and 6×His-3×Flag-USP19-ΔN2 was cut out from the blue script plasmid using XbaI and HindIII, and then inserted into pcDNA 3.1A vector that had been digested with XbaI and PmeI. To generate the plasmid expressing the USP19 mutant that only has the CS domains (USP19-CS), 6×His-3×Flag-USP19-ER plasmid (pcDNA 3.1A backbone) was first digested with XbaI and AfeI. The resulting fragment, which contained nucleotide bases 1-1252 and the N-terminal tags, was cloned back into pcDNA 3.1A using the XbaI and PmeI sites in the vector backbone. Two plasmids were generated for expressing USP19-CYT. The plasmid expressing cytoplasmic USP19 with 6× His as well as 3× Flag tags fused to its N-terminus was used in the GR experiments. The other USP19-CYT plasmid was generated by first cutting out the DNA sequence of USP19-CYT from its blue script plasmid using EcoRI and then cloning it into the pGFP-C1 vector (GenBank #U55763) using the EcoRI site in this new backbone. The resulting USP19-CYT protein has a GFP tag attached to its N-terminus and this plasmid was used for C/EBPδ experiments. The plasmid for expressing mouse C/EBPδ was obtained from Addgene (plasmid #12559). To enable immunoprecipitation of C/EBPδ using a V5-antibody, a V5 epitope tag was fused to the N-terminal end of the protein using the EcoRI restriction site in the pcDNA 3.1(-) vector backbone. The plasmid for expressing GR with a C-terminal 3× Flag tag and 3×HA N-terminal tag was obtained from Dr. Jacques Drouin’s Laboratory at Institut de recherches cliniques de Montréal.

HEK293 cells and HEK293T-USP19KO cells (a gift from Dr. Yihong Ye’s laboratory at NIH[254]) were grown in DMEM (GIBCO), supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin, at 37°C with 5% CO2. Cells were transfected using jetPrime reagent (Polyplus-transfection). For C/EBPδ plasmid (1 μg/well) and USP19 plasmid (1 μg/well) transfection in 6-well plates, the jetPrime reagent (4 μl) and jetPrime Buffer
(200 μl) were mixed with the plasmids (typically 2 μg of total plasmid DNA for each well of a 6-well plate) and added to 2 ml of media containing 3×10⁵ HEK293 cells in suspension in the well. Twenty-four hours later, the adherent cells were at ~80% confluency and the transfection media was replaced with fresh media. Forty-eight hours post transfection, cells were washed with cold PBS twice and lysed with 250 μl of lysis buffer (2% SDS, 50 mM Tris, pH 7.5). For glucocorticoid receptor plasmid (0.4 μg/well) and USP19 plasmid (0.4 μg/well) transfection in 12-well plates, plasmids (0.8 μg of total plasmid DNA in each well of 12-well plate) were mixed with the jetPrime reagent (1.6 ul) and jetPrime Buffer (75 ul), and then added to 1ml of media containing 3.0×10⁵ HEK293T-USP19KO cells in suspension in the well. Twenty-four hours later, adherent cells were at ~80% confluency and the transfection media was replaced with fresh media. Forty-eight hours post transfection, cells were washed twice with cold PBS and lysed with 125 μl of lysis buffer.

2. Silencing in Rat L6 Cells.

Rat L6 myoblasts were cultured in alpha-MEM (GIBCO), supplemented with 10% FBS and 1% penicillin/streptomycin, at 37°C with 5% CO₂. For a typical experiment in 6-well plates, 1.5×10⁵ cells were grown in each well. Twenty-four hours later, when the cells were at ~ 70% confluency, oligonucleotide capable of targeting both USP19 isoforms or nonspecific control was transfected using Lipofectamine Plus reagent (Thermo Fisher Scientific) in the presence of Opti-MEM (Gibco) according to the manufacturer’s protocol. Twenty-four hours later, the cells were at ~95% to ~100% confluency and media was switched to differentiation media containing 2% FBS and 1% penicillin/streptomycin. Three days post the induction of differentiation, cells were washed with cold PBS twice and lysed with 250 μl of RIPA buffer. One of the nonspecific
control siRNA used was a universal negative control (DS NC1) obtained from Integrated DNA Technologies. The sequences of other oligonucleotides used were:

Non-specific control #1: 5’ – AAA CUC UAU CUG CAC GCU GAC – 3’

5’ – GUC AGC GUG CAG AUA GAG UUU – 3’

USP19 si-RNA #7: 5’ – AAG GGU GGU CUU CUA CAG UUG – 3’

5’ – CAA CUG UAG AAG ACC ACC CUU – 3’

3. Western Blot

Lysates were collected by scraping in lysis buffer and then passed through a 23G needle ~10 times to shear the DNA. Protein concentration for each sample was measured using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Protein (25 μg for C/EBPδ experiment, 10 μg for GR experiment) from each sample was loaded onto 7.5% or 8.5% SDS-PAGE gels respectively. Proteins were transferred onto 0.45 μm nitrocellulose membrane for western blotting. Membrane were probed with antibodies against USP19 (1:1000, our laboratory[184]), γ-tubulin (1:10000, Sigma Aldrich), C/EBPδ (1:5000, Acris), GR (1:1000, Santa Cruz), GR (1:1000, Cell signalling), USP19 (1:1000, Bethyl), HA (1:1000, F-7; Santa Cruz) or Flag (1:1000, M2; Sigma Aldrich). Bound primary antibodies were detected using horse radish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies and ECL chemiluminescence substrate (Bio-Rad). Signals were detected with a ChemiDoc™ MP Imaging System (Bio-Rad) and analyzed using the Image Lab software (Bio-Rad). Signal intensity of each protein was normalized with that of tubulin on the same blot to account for variations in sample loading and transfer.
4. Quantitative Real-time PCR (qPCR)

Cells were solubilized in 4 M guanidium isothiocyanate containing 7.5% β-mercaptoethanol, and the RNA was isolated using phenol-chloroform extraction[260]. The concentration of RNA was measured using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific). A High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem) was used to synthesize the cDNA from 1 μg of RNA. qPCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) and a ViiA7 qPCR machine (Applied Biosystem). The expression of USP19 was analyzed using the previously described ΔΔ-CT method[261]. The sequence of primers used for qPCR were:

USP19: Forward 5’ – GTAGTTTCATTTGGCGAGAC – 3’
Reverse 5’ – CCGATCATGCTCCGTAGTG- 3’

GAPDH: Forward 5’ – CACCATCTTCAGGAGCGCG – 3’
Reverse 5’ – CCTTCTCCATGGTGGTGAAGAC – 3’

5. Co-immunoprecipitation

HEK293 (2×10^6) cells were seeded into 100 mm plates 24 hours prior to transfection. For co-immunoprecipitating USP19 and C/EBPδ, 3.75 μg of plasmid expressing Flag-USP19-ER or GFP-USP19-CYT was transfected with 3.75 μg of plasmid expressing V5-C/EBPδ. For co-immunoprecipitating USP19 and GR, 3.75 μg of plasmid expressing Flag-USP19-ER or Flag-USP19-CYT was transfected with 11.25 μg of plasmid expressing HA-GR. For co-immunoprecipitating USP19 and HSP90, 3.75 μg of plasmid expressing Flag-USP19-ER or Flag-USP19-CYT was transfected. All transfections were carried out on adherent cells using jetPrime reagent according to the manufacturer’s protocol. Mixture containing the jetPrime
reagent (1:2 DNA to jetPRIME ratio, w/v), Jet Prime Buffer (500 μl) and plasmids were added to each plate along with 10 ml of DMEM. Twenty-four hours after transfection, cells were washed twice with ice cold PBS and lysed directly in the plate with IP lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 1 mM EDTA, protease inhibitor cocktail (Roche)]. Lysates were collected by scraping and cleared by centrifugation at 17000 g for 10 minutes at 4°C. Protein concentration was measured using Micro BCA Protein Assay Kit. Lysates were precleared with 20μl of protein-G Plus agarose beads (50/50 slurry, Santa Cruz) for 1 hour at 4°C. Protein (50 μg) from each pre-cleared lysate was taken out and mixed with loading buffer to be used as 10% input. Protein (500 μg) from each pre-cleared lysate was incubated with 5μg of anti-Flag antibody (M2, Sigma Aldrich), anti-HA antibody (F-7, Santa Cruz), anti-V5 antibody (E10/V4RR, Thermo Fisher), anti-GFP antibody (B-2, Santa Cruz) or nonspecific mouse IgG (Sigma) for twenty-four hours at 4°C. Following overnight incubation with antibody or mouse IgG, 20μl of protein G Plus agarose beads were added to each sample and incubated for another 4 hours at 4°C. Beads were collected by spinning down at 4000 g for 1 min and washed three times with 200 μl of IP buffer. Bound proteins were eluted by heating the beads with 40 μl of sample loading buffer at 95°C for 10 minutes.

6. CRISPR-CAS9

In an attempt to generate USP19 Knockout HeLa cells, two previously described guide RNA (gRNA) sequences were used to target two sequences near the translational start site of USP19 [254]. The two gRNA constructs were generated according to a published protocol[262], and the oligonucleotide sequences are:

Target 1: 5’ – caccgAGAGCAAGGATGGAGATCCT – 3’
5’ – aaacAGGATCTCCATCCTTGCTCTc – 3’

Target 2: 5’ – cacgcCTTCTGCTTCTTCTTACTAG – 3’

5’ – aaacCTAGTAAGAAGAAGCAGAAGc – 3’

Each pair of oligonucleotides (50 μM) in distilled water is annealed by incubating at 95°C for 5 minutes and then allowed to cool down to room temperature. The annealed oligonucleotides were inserted into the pSpCas9n(BB)-2A-GFP (Addgene #48140) plasmid. The two plasmids were co-transfected into HeLa cells. Transfection of CRISPR plasmids (1 μg/well) was done in a 6-well plate using jetPrime reagent. JetPRime reagent (4 μl), jetPrime Buffer (200 μl) were mixed with the plasmids (2 μg of total plasmid DNA/well) and added to 3×10^5 cells in suspension in the well. Twenty-four hours post-transfection, cells were diluted and seeded into 96-well plate at ~1 cell/well. Single cell clones were screened for USP19 knockout using quantitative real-time PCR (qPCR) and western blot.
III. Results:

1. Evaluation of C/EBPδ as a Potential Substrate of USP19.

C/EBPδ is a transcription factor whose expression can be induced by glucocorticoids or IL-6, and it has been shown to promote muscle atrophy by upregulating myostatin[4, 5]. Work in our laboratory suggested that the induction of C/EBPδ in response to glucocorticoid treatment is blunted in USP19 knockout (USP19-KO) mice. Moreover, we also observed decreased myostatin expression at the transcriptional level in our USP19-KO mice upon fasting (N.Bedard, unpublished data). Therefore, I tested whether C/EBPδ is a substrate of and stabilized by USP19. For this purpose, I overexpressed C/EBPδ with or without one of the USP19 isoforms in HEK293 cells and analyzed its protein levels using Western Blot. Contrary to our expectations, the presence of ER-localized USP19 (USP19-ER) induced a ~20% decrease in the level of transfected C/EBPδ (Figure 1A&1B). In contrast, the cytoplasmic USP19 (USP19-CYT) isoform had no significant effect on the protein level of C/EBPδ (Figure 1A, 1B). However, the ~20% decrease in C/EBPδ when co-expressed with USP19-ER is nearly undetectable within each individual experiment trials, and although statistically significant, the difference appears too small to be of important biological significance.

To better study the potential interaction between USP19 and C/EBPδ, I used si-RNA mediated silencing to knockdown USP19 in L6 muscle cells and looked for changes in the level of endogenous C/EBPδ. Knockdown of USP19 by approximately 60% did not produce any change in the level of endogenous C/EBPδ (Figure 1C, 1D). To further explore whether C/EBPδ might be a substrate of USP19, I attempted to co-immunoprecipitate C/EBPδ in HEK293 cells by expressing FLAG-tagged USP19 (Flag-USP19-ER or Flag-USP19-CYT) isoforms. I also carried out reciprocal co-IP experiments using V5-tagged C/EBPδ in HEK293 cells. Results from these
experiments showed that neither USP19-ER nor USP19-CYT appears to interact with C/EBPδ (Figure 1E–1H). Collectively, our data suggest that despite the ~20% decrease in C/EBPδ level when it is co-expressed with USP19-ER, C/EBPδ is unlikely to be substrate of USP19 under physiological conditions.
Figure 1

A

USP19

C/EBPδ

γ-tubulin

B

Relative C/EBPδ Protein Level

CTL  USP19-ER  USP19-CYT

C

USP19

C/EBPδ

γ-tubulin

D

Relative C/EBPδ Protein Level

Mock  nsp  NC1  USP19

siRNA
Figure 1: Evaluation of C/EBPδ as a Potential Substrate of USP19. Plasmids expressing C/EBPδ were transfected into HEK293 cells along with empty control vector or plasmids expressing USP19-ER or USP19-CYT. Cells were lysed 48 hours post-transfection and proteins level were analyzed by western blot. A) Representative image of C/EBPδ, USP19 and γ-tubulin levels in lysates. B) Quantitation of C/EBPδ, shown as mean±SEM (N≥3). ** P < 0.01 compared to empty vector control (Anova). L6 myoblasts were transfected with control (NSP/NC1) siRNA or siRNA targeting both isoforms of USP19. Cells were exposed to differentiation medium 24 hours post-transfection. After 72 hours in differentiation medium, cells were harvested and the levels of the indicated proteins were measured by Western blot. C) Representative image of C/EBPδ, USP19 and γ-tubulin levels in the lysates. D) Quantitation of C/EBPδ and USP19, shown as mean±SEM (N≥3). *P < 0.05 compared to non-specific control #1 (nsp) (Anova). HEK293 cells were transfected with plasmid expressing GFP-USP19-CYT or Flag-USP19-ER and plasmid expressing normal C/EBPδ or V5-C/EBPδ. Cells were lysed 24 hours post transfection and co-immunoprecipitation was carried out on lysate using the indicated antibodies. Sample were analyzed by SDS-Page and Western Blot. E&G) No interaction between Flag-USP19-ER or GFP-USP19-CYT and C/EBPδ. F&H) No interaction between V5-C/EBPδ and Flag-USP19-ER or GFP-USP19-CYT.
2. USP19 Regulates the Level of GR Independent of Its Catalytic Activity.

Hypergeometric Optimization of Motif EnRichment (HOMER) analysis as well as analysis of GR target genes expression indicated that USP19-KO mice have decreased glucocorticoid receptor signalling (E. Coyne, et al, submitted). Furthermore, the levels of the glucocorticoid receptor are lower in USP19-KO mice compared to wild-type mice, while its mRNA levels are unaffected (E. Coyne, unpublished data). Collectively, these observations suggest that USP19 can regulate the level of GR at the post-transcriptional level. Therefore, I asked whether GR is a substrate of USP19. To answer this question, I first compared the level of GR in wild-type and USP19-KO HEK293T cells. Consistent with our observation \textit{in vivo}, the depletion of USP19 in these cells leads to a \~40\% decrease in the protein level of GR (Figure 2A, 2B). Since previous studies by our laboratory have demonstrated that some functions of USP19 are dependent specifically on its ER-localization and catalytic activity, I asked whether the two isoforms of USP19 can have different effects on GR and whether the catalytic activity of USP19 is required to modulate GR levels. I overexpressed HA-GR with Flag-USP19-ER, Flag-USP19-CYT or a catalytically inactive USP19-ER (Flag-USP19-CA) mutant and analyzed the effects of the different USP19 isoforms on the protein level of transfected GR (Figure 2C-D). Both USP19-ER and USP19-CYT induced a \~50-70\% increase in GR protein levels compared to the control empty vector (Figure 2D, 2E). More interestingly, this effect of USP19 on GR is independent of its catalytic activity, as USP19-CA had a similar effect on GR levels as USP19-ER or USP19-CYT (Figure 2D, 2E). To further characterize the importance of USP19’s catalytic activity for its effect on GR, I analyzed the effect on GR stabilization of a USP19 mutant (USP19-CS) that is missing its catalytic domain (Figure 2C). From co-expression experiments, USP19-CS was shown to be incapable of stabilizing GR, in contrast to other USP19 isoforms (Figure 2D, 2E).
Together, our data indicates that USP19 is capable of stabilizing GR independent of its catalytic activity. However, the catalytic domain of USP19 is still required for this effect.
Figure 2

A

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>KO</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>USP19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-Tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

C

USP19-ER

USP19-CYT

USP19-CA

USP19-CS

Legend:
- C: Catalytic domain
- UBL: Ubiquitin-like domain
- MYND: MYND domain
- Zinc Finger: Zinc finger domain
- H: HD domain
- D: D Arm domain
- TMD: Transmembrane domain
Figure 2: USP19 Regulates the Level of GR Independent of Its Catalytic Activity. A) Representative blot of USP19, GR and γ-Tubulin in wild-type and USP19-KO HEK293T cells. B) Quantitation of GR, shown as mean ± SEM (N≥3). ** P < 0.01 compared to wild-type GR level (t-test). C) Structural diagrams of USP19 isoforms and mutants. USP19-KO HEK293T cells were transfected with plasmids expressing GR and either USP19-ER, USP19-CYT, USP19-CA, USP19-CS or a control empty vector. Cells were lysed 48 hours post-transfection and proteins level were analyzed by western blot. D) Representative blot of USP19 isoforms/mutants, GR and γ-Tubulin. E) Quantitation of GR, shown as mean ± SEM (N≥3). ** P < 0.01 and * P < 0.05 compared to empty vector CTL (ANOVA).
3. Heterozygous Knockout of USP19 Does Not Induce Change in GR Level.

Heterozygous USP19-KO mice showed similar protein levels of GR as wild-type mice (E. Coyne, unpublished data). To further study the effect of USP19 on GR in other relevant cells, we attempted to generate USP19 homozygous and heterozygous knockout HeLa cells using CRISPR-Cas9. Out of twenty-six single cell drived clones, only two clones (referred to as clone #1 and clone #2) showed significant decrease in USP19 mRNA and protein levels (Figure 3A-C). However, the ~75% decrease in USP19 mRNA and protein levels suggest that these two HeLa cell clones are most likely to be heterozygous USP19 KO. From comparing the protein levels of GR in these two heterozygous USP19-KO cells and wild-type cells, we observed that the ~75% decrease in USP19 did not lead to any significant change in the protein levels of GR, which is consistent with our data from heterozygous USP19-KO mice (Figure 3B, 3C). This indicates that although USP19 plays a role in the stabilization of GR, only a small fraction of it is required for this function under physiological conditions.
Figure 3: Heterozygous Knockout of USP19 Does Not Induce Change in GR Level. CRISPR-Cas9 constructs targeting the USP19 gene were transfected into HeLa cells. Clones derived from single cell were analyzed for their USP19 mRNA and protein levels. Two clones (referred to as clone #1 and clone #2) showed significant decrease in USP19 expression compared to wild-type control (CTL). A) Representative blot of USP19, GR and γ-Tubulin in wild-type and heterozygous USP19-KO clones. B) Quantitation of USP19 mRNA levels in wild-type (CTL) and heterozygous USP19-KO clones (C-#1 & C-#2), shown as mean ± SEM (N ≥ 3). ** P < 0.01 compared to wild-type CTL (Anova). C) Quantitation of USP19 and GR protein levels in wild-type and heterozygous USP19-KO clones, shown as mean ± SEM (N ≥ 3).
4. USP19 Interacts with GR and HSP90 Independent of Its Two CS Domains.

Our data suggest that USP19 can stabilize GR independent of its deubiquitinating activity, but the exact mechanism underlying this effect is unknown. Within the structure of USP19, the N-terminus contains two CS domains that are homologous to two co-chaperones of HSP90, SGT1 and p23, which may impart USP19 with co-chaperone activities[225]. Furthermore, interaction between HSP90 and USP19 has been reported[253, 256, 258]. However, the molecular basis of this interaction remains controversial, with one study reporting that it is mediated by the catalytic domain of USP19[253], whereas another claims that it is dependent on the two N-terminal CS domains[256]. Nevertheless, the ability of USP19 to interact with HSP90 may provide a potential explanation for its effect on GR, as GR is a well-known client of HSP90. Therefore, I asked whether USP19 forms a complex with GR and HSP90, and if it does, which part of USP19’s structure is required for this interaction. To answer these questions, we first tested if USP19-ER can interact with HSP90 and GR using co-IP assays. By pulling down FLAG-USP19-ER and HA-GR using the appropriate antibodies, USP19-ER is shown to interact with HSP90 and GR, which suggests that the three proteins may form a complex within the cell (Figure 4A, 4B). To determine which part of USP19’s structure is required for this interaction, we generated two USP19 mutants that are missing either the first (USP19-ΔN1) or both CS domains (USP19-ΔN2) (Figure 4C). Interestingly, the removal of CS domains did not affect the ability of the two USP19 mutants to pull down HSP90, indicating that these domains are disposable for the HSP90-USP19 interaction (Figure 4D, 4E). Next, we used the same USP19 mutants to test whether the removal of CS domains disrupts the interaction between USP19 and GR. Interestingly, the deletion of either one or both CS domains does not abolish the USP19-GR interaction, as HA-GR is able to pull down higher amount of both USP19 mutants in comparison
to non-specific mouse IgG control (Figure 4F, 4G). Therefore, the two CS domains of USP19 are unlikely to play a significant role in the HSP90-USP19-GR interaction. This interpretation is consistent with the data from our USP19-GR co-expression experiments, in which although USP19 is able to stabilize GR independent of its deubiquitinating activity, its catalytic domain is still required. USP19 may modulate GR protein through interacting with HSP90 through its catalytic domain.
Figure 4: USP19 Interacts with GR and HSP90 Independent of Its Two CS Domains. HEK293
cells were transfected with plasmids expressing FLAG-USP19 variants and HA-GR. Cells were lysed twenty-four hours post-transfection, and USP19 or GR were pulled down using mouse anti-FLAG or anti-HA antibodies respectively and non-specific mouse IgG was used as control. Samples were analyzed by SDS-PAGE and Western Blot. A) Interaction between FLAG-USP19-ER and endogenous HSP90. B) Interaction between FLAG-USP19-ER and HA-GR. C) Structure of USP19-∆N1 and USP19-∆N2. D) Interaction between FLAG-USP19-∆N1 and endogenous HSP90. E) Interaction between FLAG-USP19-∆N2 and endogenous HSP90. F) Interaction between HA-GR and FLAG-USP19-∆N1. G) Interaction between HA-GR and FLAG-USP19-∆N2.
IV. Discussion.

Recent studies by our laboratory identified USP19 as a DUB that plays a significant role in promoting muscle atrophy upon fasting, denervation and glucocorticoid treatment[1-3]. However, the exact mechanism and the substrate(s) of USP19 underlying its effects are unknown. In this thesis, I evaluated two proteins, C/EBPδ and GR, as potential substrates of USP19 in promoting muscle wasting.

C/EBPδ is a transcription factor that is induced in several conditions of muscle wasting, including the ones caused by exogenous glucocorticoids or elevated levels of IL-6, and it promotes the catabolic process by inducing the expression of myostatin[4, 5]. We initially considered C/EBPδ as a potential substrate because of our observations that the dexamethasone induced increase in C/EBPδ protein is absent in the muscle of USP19-KO mice and the expression of its downstream target myostatin is also markedly decreased. These two observations promoted us to hypothesize that USP19 may modulate C/EBPδ protein, at least in part, by deubiquitinating it and thereby stabilizing it. Contrary to our expectations, our data from over-expression, siRNA silencing and co-IP experiments argue that C/EBPδ is unlikely to be substrate of USP19. Interestingly, the ER-localized USP19 isoform (USP19-ER) appeared to induce instead a ~20% decrease in C/EBPδ protein level when they were co-expressed together in HEK293 cells. The biological significance of this small change is unclear, as it not only contradicted with our observations in USP19-KO mice, but it is also inconsistent with results from USP19 silencing experiments. Thus, we suspect that the decreased levels of C/EBPδ in USP19 KO muscle may instead be the consequence of decreased transcription and synthesis, which in turn are likely caused by the lowered glucocorticoid signaling described above (see also below). However, this remains to be further explored.
The lower levels of GR in our USP19-KO mice compared to WT despite identical levels of GR mRNA indicated that GR is being modulated at a post-transcriptional level and may be a direct substrate of USP19. Consistent with this possibility, I observed that USP19 can interact with GR in co-immunoprecipitation studies, and its overexpression or depletion can increase or decrease GR levels respectively. Although GR is found either in the cytoplasm (ligand free) or in the nucleus (ligand bound), the ER-localized USP19 isoform was equally effective as the cytoplasmic isoform in increasing levels of GR. This probably reflects the fact that the catalytic domain of the ER isoform of USP19 is located on the cytoplasmic face of the ER and therefore still accessible to GR.

Intriguingly though, this effect on GR levels was independent of USP19’s deubiquitinating activity, as expressing a catalytically inactive USP19 mutant retained the same effect on GR as expressing native USP19. However, the catalytic domain of USP19 is required as expression of the USP19-CS truncation, which contains USP19’s two N-terminal CS domains but lacks the catalytic domain, failed to induce a significant change in GR levels. Thus, USP19 may be regulating GR indirectly through interacting with an intermediate factor and the catalytic domain of USP19 may be required for facilitating this interaction. A possible intermediate factor for this is HSP90, as USP19 has previously been shown to interact with this chaperonin that is well known to bind and promote the folding and maturation of ligand free GR. Therefore, we have begun to determine whether HSP90 is the intermediate factor connecting USP19 to GR. While the USP19 and HSP90 interaction has been reported in several studies[253, 256, 258], the structural requirement for this interaction remains controversial with one group suggesting it is mediated by the N-terminal CS domains that are homologous to p23 and SGT1[256], two co-chaperones of Hsp90, whilst the other group reports that it is mediated by the catalytic
domain[253]. We demonstrated that the two N-terminal CS domains of USP19 alone are unable to interact with HSP90. Similarly, deletion of these two CS domains did not prevent USP19 from co-immunoprecipitating with GR. These data together with the above results support the idea that USP19 might be forming a complex with HSP90 through its catalytic domain. We have started to test the effects of USP19-ΔN1 and USP19-ΔN2 truncated forms, which lack the first and both CS domains respectively, on GR levels. We expect both of those mutants to induce an increase in GR level similar to wild-type USP19, but the data I currently have is too inconsistent to make a conclusion. Another member of our laboratory is in the process of generating V5-tagged GR. Once it becomes available, we can confirm our above co-IP results by performing the reciprocal immunoprecipitation study with V5-GR and detecting for the presence of the various Flag-tagged USP19 isoforms. Furthermore, we need to determine whether HSP90 activity is essential for USP19 to regulate GR. We plan to test the effect of USP19 on GR in the presence of a HSP90 inhibitor, geldanamycin. If USP19 is regulating GR stability through HSP90, adding the inhibitor should block the effect of USP19 on GR.

The observation that GR levels are normal in heterozygous USP19-KO mice and HeLa cells suggested that only a small fraction of USP19 is involved in or required for the regulation of GR. If USP19 does indeed modulate GR stability through interacting with HSP90 as a novel co-chaperone, other cellular factors may perform a similar function in the regulation of GR. Therefore, unless USP19 is completely knocked out, we will not be able to see an effect on GR level since only a small amount of USP19 is really needed. However, this is only speculation based on the normal levels of GR seen in heterozygous USP19-KO mice and our HeLa cells which we think are heterozygous knockout due to the significantly lower but still present USP19 expression. Interestingly, our cells showed a ~75% decrease in USP19 protein and mRNA levels
as opposed to the 50% one would usually expect from a heterozygous knockout. Thus, it is also possible that our samples are derived from cells that are not actually clonal, but contain mostly KO cells contaminated by small amounts of wild-type HeLa cells. We could exclude this by going through another round of single cell clonal expansion. Furthermore, we also plan to generate USP19 knockout L6 rat, C2C12 muscle cells and possibly primary mouse muscle cells using CRISPR-Cas9. Several gRNA constructs have been designed for this purpose, but our initial attempt in C2C12 cells was unsuccessful. We think this is most likely caused by poor transfection efficiency in C2C12 cells, and we plan to use a lentiviral CRISPR strategy moving forward.

While our data would be consistent with USP19 exerting its effects on GR through binding to HSP90, it remains possible that USP19 binds and acts directly on GR. Although overexpressing a catalytically inactive form of USP19 still exerted effects on GR levels, it is possible that the artificially introduced high amount of inactive DUB is just sequestering the substrate from or blocking its interaction with ubiquitin ligases. USP19 has previously been reported to stabilize HIF-1α, c-IAP1 and c-IAP2 in a manner that is also independent of its catalytic activity[234, 245]. As these results were also based on overexpressing an inactive enzyme, a prevention of interaction with the cognate ubiquitin ligases may also explain these findings. An inhibitor of endogenous USP19 enzymatic activity may be able to help us determine whether catalytic activity is indeed required for stabilization, but at the time of writing this thesis, no such inhibitor is commercially available.

The results in this thesis suggest that USP19 may indeed exerts its effects on muscle through GR. Glucocorticoids are one of the most common catabolic stimuli for muscle wasting[55, 101]. Activated GR downregulates muscle protein synthesis and upregulates muscle protein
degradation through a variety of mechanisms[75, 105, 107-110]. Therefore, by stabilizing GR, USP19 may increase glucocorticoid signalling and promote muscle atrophy. Indeed, other work indicates that GR plays an essential role in muscle wasting as adrenalectomy, GR antagonists or muscle specific deletion of GR is able to protect against muscle atrophy arising from many conditions[101, 102]. Finally, recent studies in our laboratory indicate that electroporating plasmids expressing GR into KO skeletal muscle can both restore GR levels and myofiber atrophy to levels seen in wild type mice. Collectively, all the above data would argue that GR is the substrate of USP19 that mediates its effects on muscle atrophy. To strengthen this conclusion, we need to demonstrate that USP19 can decrease the ubiquitination of GR and its rate of degradation. Such studies would be best carried out by studying these parameters with endogenous GR in normal and CRISPR/Cas9 mediated KO of USP19 in muscle cell lines or primary muscle cells. The different truncations of USP19 described in this thesis could then be expressed in the KO cells to determine which domains are required for restoring GR to normal levels. Such structure-function analyses would help us to ultimately define the interactions between USP19, HSP90 and GR that are required for these effects. This could lead to the identification of compounds which can inhibit these specific interactions of USP19. This would represent a novel approach to the prevention and treatment of muscle wasting and yield significant benefits in health, quality of life and economic cost to many patients, their families and society.
V. Reference


