Investigating the structure-function relationship of the USP19 deubiquitinating enzyme in muscle cell differentiation *in vitro*

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ABSTRACT

Muscle wasting is a common complication of aging and a number of chronic and degenerative diseases. Previous work has demonstrated that the deubiquitinating enzyme (DUB) USP19 is induced at the mRNA level in atrophying rat skeletal muscle under various conditions of catabolism [1]. To further explore USP19's role in muscle, loss of function approaches were employed. siRNA-mediated silencing of USP19 in L6 muscle cells enhanced the expression of a panel of major myofibrillar proteins and the myogenic regulatory factor myogenin that regulates muscle differentiation [2]. The enhanced expression of MHC and tropomyosin upon USP19 depletion was found to be dependent on USP19's regulation of myogenin. The effects observed upon USP19 depletion may be due to an effect on muscle cell differentiation. USP19 is expressed as two major isoforms, one with and the other without a C-terminal transmembrane domain (TMD) that confers ER localization. Therefore, I characterized the mechanism by which USP19 modulates muscle cell differentiation and examined the structure-function relationship of USP19 in regulation of this process. The negative role of USP19 in muscle cell differentiation was confirmed as adenovirus-mediated overexpression of wild type USP19 in C2C12 muscle cells suppressed the protein levels of MHC, tropomyosin, and myogenin at the molecular level and inhibited myotube fusion morphologically. These effects are dependent on USP19's DUB catalytic activity and localization to the ER as overexpression of a catalytically inactive mutant or the non-ER localized USP19 isoform abolished these molecular and

morphological effects. USP19 N-terminal domains appear to be important for suppression of myotube fusion, as overexpression of USP19 mutants lacking various N-terminal regions weakened the fusion defect observed in wild type USP19 expressing cells, but did not affect myogenin expression. The inhibition of myoblast fusion upon overexpression of wild type USP19 coincided with a decrease in the induction of ER stress required for myogenesis as assessed by the number of CHOP positive cells and ER stress treatment of wild type USP19 expressing cells recovered the fusion defect, suggesting a differentiation-dependent role for USP19 at the ER. The regulation of USP19 was investigated to observe if USP19 isoforms are uniquely regulated during myogenesis. Total USP19 protein expression in C2C12 cells increased ~ 1.6 fold over six days of differentiation, while the mRNA levels of the USP19ΔTMD and USP19TMD isoforms increased ~ 2.5 fold and ~ 1.4 fold, respectively. USP19 was also demonstrated to interact with USP9X and suppress IGF1/PI3K/mTOR/Akt growth signaling. Collectively, these results implicate USP19 as a negative regulator of muscle cell differentiation dependent on its DUB catalytic activity and ER localization. Dysregulated or suppressed myogenesis contributes to muscle atrophy as well as a delayed recovery following injury, identifying USP19 inhibition as a novel therapeutic intervention for the treatment of muscle atrophy.

RÉSUMÉ

La perte musculaire est une complication fréquente du vieillissement et d'un grand nombre de maladies chroniques et dégénératives. Des travaux antérieurs ont démontré que l'enzyme de déubiquitination (DUB) USP19 est induite au niveau de l'ARNm durant l'atrophie du muscle squelettique de rat dans diverses conditions cataboliques [1]. Pour explorer davantage le rôle de USP19 dans les muscles, des approches de perte de fonction ont été employées. En utilisant le silençage génique médié par des siRNA contre USP19 dans des cellules musculaires L6, une augmentation de l'expression de plusieurs protéines myofibrillaires ainsi que de la myogénine, un facteur de régulation de la différenciation musculaire, a été observée [2]. L'expression accrue de la MHC et de la tropomyosine suite à l'inhibition de USP19 s'est révélée être dépendante de la régulation de la myogénine par USP19. Les effets observés suite a l'appauvrissement de USP19 pourrait être dû à un effet sur la différenciation des cellules musculaires. USP19 est exprimée sous forme de deux isoformes majeures, l'une avec et l'autre sans domaine transmembranaire C-terminal (TMD) qui confère la localization dans le réticulum endoplasmique (ER). Par conséquent, j'ai caractérisé le mécanisme par lequel USP19 module la différenciation des cellules musculaires et examiné la relation structure-fonction de USP19 dans la régulation de ce processus. Le rôle négatif de USP19 dans la différenciation des cellules musculaires a été confirmé par la surexpression de USP19 de type WT en utilisant des adénovirus dans les cellules musculaires C2C12. La surexpression de USP19 a provoqué la suppression des niveaux de

protéines de la MHC, de la tropomyosine et de la myogénine et inhibé morphologiquement la fusion des myotubes. Ces effets dépendent de l'activité catalytique et de la localisation de USP19 dans le ER puisque la surexpression d'un mutant catalytiquement inactif ou d'un isoforme non-ER localisé aboli ces effets moléculaires et morphologiques. Les domaines N-terminaux de USP19 semblent aussi être importants pour la suppression de la fusion des myotubes, étant donné que la surexpression de mutants USP19 dépourvus de diverses régions N-terminales atténue l'effet de la fusion cellulaire observée dans les cellules exprimant le USP19 de type WT, mais n'a pas d'incidence sur l'expression de la myogénine. L'inhibition de la fusion des myoblastes exprimant le USP19 de type WT coïncide avec une diminution de l'induction du stress relié au réticulum endoplasmique démontré par le nombre de cellules positives pour CHOP qui est nécessaire à la myogenèse. Le traitement de cellules exprimant USP19 de type WT avec un inducteur du stress relié au ER récupère le défaut de fusion, ce qui suggère un rôle pour USP19 dans la differentiation cellulaire relié au stress du ER. La régulation de USP19 a été étudiée pour observer si les isoformes USP19 sont modulés uniquement pendant la myogenèse. L'expression protéique globale de USP19 dans des cellules C2C12 est augmentée ~1,6 fois sur six jours de différenciation, tandis que les taux d'ARNm de ΔTMD et TMD sont augmentés ~2,5 et ~1,4 fois, respectivement. Il a été également démontré que USP19 peut interagir avec USP9X et peut supprimer la voie de signalisation de la croissance IGF1/PI3K/mTOR/Akt. Collectivement, ces résultats impliquent USP19 comme un régulateur négatif de la différenciation

cellulaire du muscle, dépendant de son activité catalytique et de sa localisation dans le ER. Une myogénèse mal régulée ou supprimée contribue à l'atrophie musculaire ainsi qu'à une convalescence prolongée suite à une blessure, identifiant ainsi l'inhibition de USP19 comme une intervention thérapeutique potentielle pour le traitement de l'atrophie musculaire.

LIST OF ABBREVIATIONS

CTL: control

CA: USP19 catalytically inactive mutant

ΔTMD: USP19 non-transmembrane domain isoform

DUB: Deubiquitinating enzyme

E1: Ubiquitin activating enzyme

E2: Ubiquitin conjugating enzyme

E3: Ubiquitin ligase

MAFbx/Atrogin1: Muscle atrophy F-box/Atrogin1

MuRF: Muscle-specific ring finger

MHC: Myosin heavy chain

MRF: Muscle regulatory factor

Ub: Ubiquitin

UPS: Ubiquitin proteasome system

ALS: Autophagy lysosome system

Tg: Thapsigargin

I. INTRODUCTION

In this thesis, I describe and characterize a role for the deubiquitinating enzyme USP19 in modulating muscle cell differentiation. Mechanistically, USP19 appears to suppress the endoplasmic reticulum stress response. The regulation of muscle cell differentiation is a critical mechanism for muscle growth and is suppressed or dysregulated in conditions of muscle atrophy. Thus, this introduction will cover the relevant information from a broad range of topics required for the understanding of the experimental work presented herein.

1. SKELTAL MUSCLE

Skeletal muscle comprises nearly half of body mass and is the largest reservoir of proteins in the body, as well as a major site of metabolic activity. Skeletal muscles are the locomotor organs of the body, consuming energy to produce contraction and locomotion. The elemental unit forming striated muscle is the sarcomere; comprised of the highly organized actin and myosin contractile filaments as well as regulatory, scaffolding, and cytoskeletal proteins [3]. Sarcomeres are constructed from an antiparallel arrangement of actin (thin) and myosin (thick) filaments that are interlinked through flexible connector proteins titin and alpha-actinin and assembled into large multi-sarcomere myofibrils. These alternating thick and thin filaments impart muscle with its characteristic striation [4] (Figure *I*). Skeletal muscle possesses four myosin isoforms: type I, IIa, IIb, and IIx, with muscle fibers containing predominantly one myosin isoform. As such, muscles can be characterized by determining the fiber type composition.

Type II fibers, also known as fast-twitch fibers, contain greater ATPase activity than type I slow-twitch fibers [5]. To generate locomotion, forces are amplified along parallel arrangements of alternating thick and thin filament contractile units in an ATP-dependent manner. Sarcomeres contain a variety of regulatory and structural proteins such as the troponin-tropomyosin complex and α-actinin, myomesin, and titin. Some of these proteins can control transcription and protein turnover, often exerting their functions through relocalization to specific compartments of the sarcomere [6]. For example, the kinase domain of the actin-myosin cross-linker protein titin modulates muscle gene expression and protein turnover through sequestering the ubiquitin-associated p62/SQSTM1 and E3 ligase MuRF2 to sarcomeres [7].

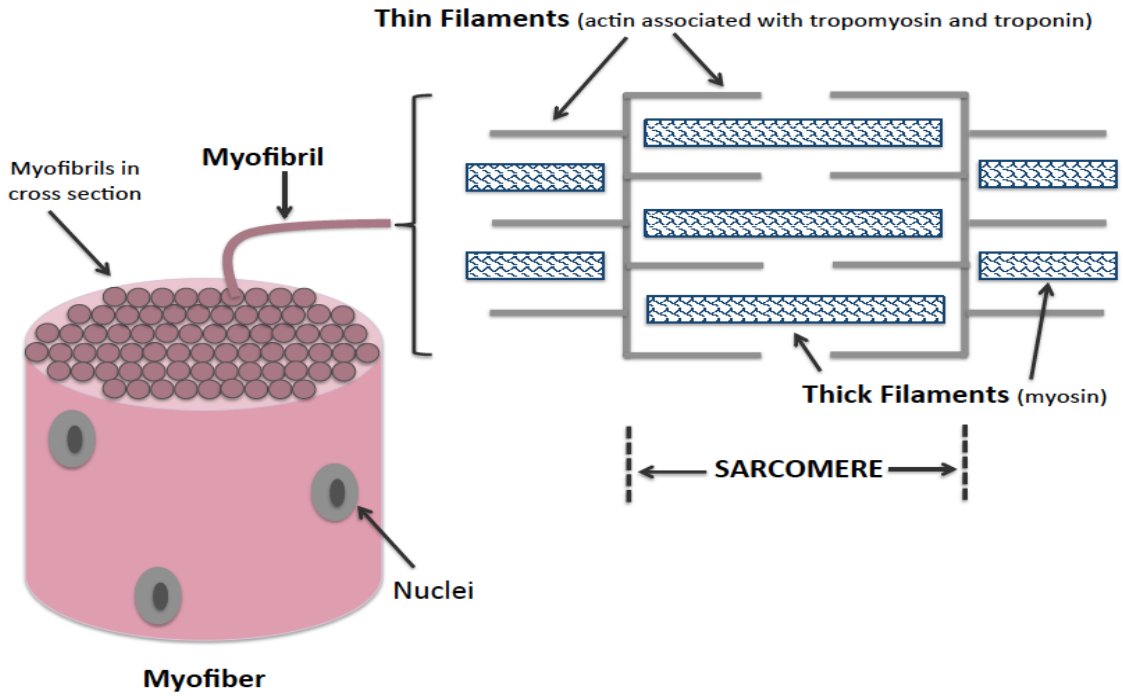


Figure I: The structural components of skeletal muscle. Skeletal muscle is comprised of multinucleated myofibers. Each myofiber consists of many individual myofibrils that are formed by repeating sarcomere. The sarcomere is the contractile unit of muscle where actin thin filaments and myosin thick filaments overlap with each other, forming the striations of skeletal muscle. The myosin thick filament is composed of 2 heavy chains and 4 light chains.

1.1 MYOGENESIS

In vertebrates, striated muscle cells are found in both skeletal and cardiac tissues, although they arise from distinct embryonic cell lineages. Muscle cells first arise in vertebrates during midgestation from three separate areas within the developing embryo: the segmented paraxial mesoderm, the unsegmented cranial paraxial mesoderm, and the prechordal mesoderm that are precursors for muscles of the trunk and limbs, the head muscles, and the extraocular muscles controlling eye movement, respectively [8]. At these sites the development of myoblast cells and their differentiation into skeletal muscle is highly directed by a specialized family of transcription factors, termed myogenic regulatory factors (MRFs): myogenic factor 5 (MYF5), muscle-specific regulatory factor 4 (MRF4 or MYF6), myoblast determination protein (MYOD) and myogenin [4]. Although all muscle precursor cells express the core set of MRFs required for myogenic differentiation, their regulation and expression patterns differ among muscle groups in the developing embryo. In somite cells destined to become limb muscles, sine oculis homeobox homologue (SIX) and eyes absent (EYA) proteins regulate the expression of paired box 3 (PAX3), which controls proliferating myogenic cells [9] [10]. Limb muscle somite cells become induced to differentiate through a signaling cascade whereby either MYF5 or MRF4 becomes activated first followed by MYOD and myogenin [11]. In contrast, trunk muscle somite cells require the action of all four MRFs: MYF5 and MRF4 can activate MYOD and myogenin in parallel and PAX3 can increase MYOD levels independent of the MRFs [12].

MYOD and MYF5 act as cell lineage determination genes transcriptionally directing myogenic differentiation at the onset of muscle development [13] [14]. MYOD and myogenin can be distinguished based on their differential modulation of cell cycle progression; whereas MYOD directly activates genes involved in cell cycle progression leading to myoblast proliferation, myogenin activates genes that inhibit cell cycle progression leading to cell cycle exit and triggering myoblast differentiation [15]. Moreover, myogenin is critical for the terminal differentiation of committed myoblasts into differentiated myotubes, as targeted mutation of myogenin in mice causes muscle deficiency and neonatal death [16]. MRF4 plays a dual role in muscle cell proliferation and differentiation, as it is expressed in both undifferentiated proliferating and differentiated postmitotic cells [17]. MRF4 can direct normal muscle cell lineage determination and terminal differentiation of myotubes in mice lacking both MYF5 and MYOD, supporting a dual role of MRF4 in these two processes [18]. MRFs exert their function as transcription factors through the formation of heterodimers with E-proteins [19] and Myocyte Enhancer Factor 2 (MEF2) [20]. These heterocomplexes are able to recognize and bind conserved sequences in the promoter regions of muscle-specific genes, called E-box sequences (CANNTG sequences) [21]. For example, MYOD and MYF5 can bind E-box elements to activate the myosin heavy chain genes during differentiation of muscle cells [22]. During embryonic development, muscle growth is primarily driven by the proliferation and differentiation of myoblasts. However, in the postnatal stage when muscle fibers have formed

alterations in muscle are predominantly driven by growth and remodeling of existing fibers.

Skeletal muscle possesses an ability to regenerate following damage, injury or inactivity, through the activation of a subset of muscle progenitor cells called satellite cells [23] [24]. Muscle satellite cells are a population of quiescent, undifferentiated myogenic precursor cells that reside along the myofiber basal lamina adjacent to the plasma membrane [25]. Satellite cells can be activated by physiological events to proliferate and differentiate, ultimately generating new myofibers to replenish damaged muscle [26] [27]. In an early experiment using neotoxin-induced damage of rat soleus muscle, activated satellite cells were capable of generating large numbers of new myofibers in only a few days [26]. Although satellite cells were originally identified manually by their anatomical location, these cells can be identified by their expression of distinctive markers that denote both cell type and lineage commitment. Quiescent and activated satellite cells express the transcription factor paired box 7 (PAX7) [28]. This transcription factor is critical for the lineage specification and survival of satellite cells, as PAX7-null mice display reduced growth and severe muscle wasting coupled with complete lack of functional satellite cells and poor muscle regeneration following injury [29]. When satellite cells become activated, they coexpress PAX7 with the myogenic regulatory factors MYOD and MYF5 and undergo one of two fates: most will proliferate, downregulate PAX7 and differentiate to form myotubes while a smaller population will maintain PAX7

expression, but lose MYOD and return to quiescence to replenish the satellite cell pool [28]. Despite this stem cell activity, adult muscle is predominantly a terminally differentiated tissue. Therefore alterations in muscle size occur primarily though growth or atrophy of myofibers, accomplished mainly by assembly of new myofibrillar structural proteins or breakdown of existing ones. When muscle protein breakdown outpaces assembly, atrophy occurs resulting in adverse effects on muscle strength.

1.2 SKELETAL MUSCLE ATROPHY IN DISEASE

Muscle wasting occurs during inactivity and is also a common complication of both aging (sarcopenia) and a number of chronic and degenerative diseases (cachexia) including cancer, HIV/AIDS, diabetes and sepsis, as well as heart, lung and renal conditions [30]. Sustained muscle atrophy in disease, or cachexia, is characterized by an ongoing loss of skeletal muscle mass, with or without the loss of fat mass, which cannot be reversed by increased nutritional intake and is generally defined as greater than five percent weight loss [31]. Moreover, chronic muscle wasting, such as in cancer cachexia, is associated with poor prognosis and increased morbidity and mortality [32] [33] [34]. In a clinical study of patients with metastatic breast cancer, cachectic patients displayed increased tumor progression and drug toxicity compared to non-cachectic disease patients [35] and cachexia predicts mortality in patients with congestive heart failure independent of other disease factors [32]. Simply, muscle atrophy is the shrinkage of muscle fibers due to a net loss of structural

and cellular proteins, cytoplasm, and cellular organelles. Conversely, muscular dystrophies are characterized by the progressive weakening and degeneration of skeletal muscle primarily caused by mutations in genes encoding critical muscle cytoskeletal proteins. Given the genetic defects underlying muscular dystrophies, these likely occur due to mechanisms distinct from those causing atrophy and as such have been omitted here.

Skeletal muscle protein turnover occurs naturally in order to efficiently remove misfolded or damaged proteins. Additionally, muscle protein can be broken down to provide free amino acids which can be oxidized to generate ATP in times of nutrient deprivation. Protein turnover in muscle is directed by a number of dynamic and interconnected signaling systems, which adapt to the metabolic and contractile requirements of the muscle. These systems help sustain fiber size through strict regulation of protein turnover, maintaining a well-regulated balance between the rates of protein synthesis and degradation [36]. Clinical studies have shown that atrophic muscle from disease patients display elevated activities of catabolic systems often in conjunction with reduced activities of anabolic systems [37], suggesting muscle atrophy is caused by an imbalance in the processes of protein synthesis and degradation.

1.3 PROTEIN TURNOVER IN MUSCLE ATROPHY

Muscle atrophy is a problem of negative protein balance regulated by anabolic and catabolic stiumuli. Under catabolic conditions in muscle either a

decrease in protein synthesis and/or an increase in protein degradation is observed. Growth factors, such as insulin and IGF-1 are physiological regulators that promote muscle hypertrophy through the induction of protein synthesis and suppression of protein breakdown. Many catabolic stimuli, such as cytokines and glucocorticoids can both enhance protein degradation and suppress protein synthesis [36]. For example, glucocorticoid treatment both in cell culture and in mice models induces atrogin-1 and MuRF1 expression and muscle wasting as well as inhibits protein synthesis via the inhibition of mTOR (mammalian target of rapamycin), a major growth signaling molecule [38] [39] [40]. Specifically, upon stimulation of the glucocorticoid receptor it can translocate to the nucleus where it activates the expression of REDD1 and KLF15. Both REDD1 and KLF15 can inhibit mTOR activity via distinct mechanisms decreasing protein synthesis [41]. Suppressed protein synthesis rates were observed in an early study of human muscle during disuse atrophy [42]. Recently, net protein synthesis was shown to be upregulated in muscle in an mTOR-dependent manner following denervation, suggesting a possible adaptive response to certain catabolic conditions [43].

Accelerated protein breakdown in muscle under catabolic conditions reflects the activation of primarily two cellular systems: the ubiquitin proteasome system (UPS) and the autophagy-lysosome system (ALS) [44]. These two systems appear to play distinct roles within the proteolytic process. The UPS degrades nuclear and cytosolic cellular proteins, and myofibrillar proteins, which constitute the major structural components of muscle. In contrast, the ALS

degrades primarily extracellular proteins such as cellular membrane receptors, foreign material engulfed by the cell, as well as proteins and organelles degraded through autophagic processes [44]. A number of additional cellular systems have been implicated in the control of muscle atrophy. These include the calpain family of Ca^{2+}-dependent cytosolic cysteine proteases, which respond to contractile activity changes and cysteine dependent proteases (caspases) that are highly involved in programmed cell death [45]. Muscle-specific overexpression of the calpain inhibitor calpstatin reduces muscle wasting following disuse atrophy, suggesting calpains can also play a role in atrophy [46]. The UPS and the ALS are coordinately regulated during atrophy through the action of FOXO3 transcription factors [47], however only the UPS inhibition via potent inhibition of the proteasome appears to be able to reverse the enhanced proteolysis observed across a variety of atrophy models [48] [49]. These findings argue the UPS plays a dominant role in regulation of overall protein turnover in muscle and is responsible for the muscle loss seen in atrophying skeletal muscle.

1.3.1 The Ubiquitin Proteasome System

The control over protein turnover by the UPS arises due to its ability to post-translationally modify target proteins by conjugating them with ubiquitin, an 8-kDa protein found in all eukaryotic cells. Target proteins are covalently conjugated to ubiquitin chains through an isopeptide bond formation between the ε-amino group of the side chain of a lysine reside in the target protein and the C-terminal glycine of ubiquitin [50] [51]. Ubiquitination is mediated by the sequential

enzymatic action of three classes of enzymes: ubiquitin-activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3) [52] [53] [54] [55] (Figure *II*). Two mammalian genes encode two E1 enzymes: the dominant E1 enzyme in most tissues UBE1 (UBA1 in yeast) and UBA6 that is predominately expressed in the testis and appears to be required for charging UBA6-specfic E2s [56] [57]. E1 enzymes hydrolyze ATP to activate ubiquitin to supply the larger family of E2 enzymes [58]. Mammalian cells possess roughly 30 genes that encode E2 enzymes [55]. Individual E2s appear to cooperate with specific E3 enzymes. The E2 plays a role in determining the types of ubiquitin chain linkage formed while the E3 recognizes specific substrates or families of substrates [59]. Substrate specificity arises due to the diversity of the ~ 700 E3 ubiquitin ligases encoded in the human genome. E3s are divided into two major categories. Approximately 90 C-terminal Homologous to E6-AP Carboxy-Terminus (HECT) domain containing ligases are distinguished by their ability to accept the ubiquitin moiety from an E2 onto an internal cysteine residue before conjugating the ubiquitin to the substrate [60]. The larger category of RING finger domain containing ligases, of which there are over 600 genes in humans, function by approximating both the substrate and the E2 followed by activation of the conjugating activity of the E2 [61] [62]. Ligases are capable of functioning as singular proteins or as part of larger polypeptide complexes containing distinct substrate recognition and E2 binding domains, as occurs in the family of cullin-RING ligases [63].

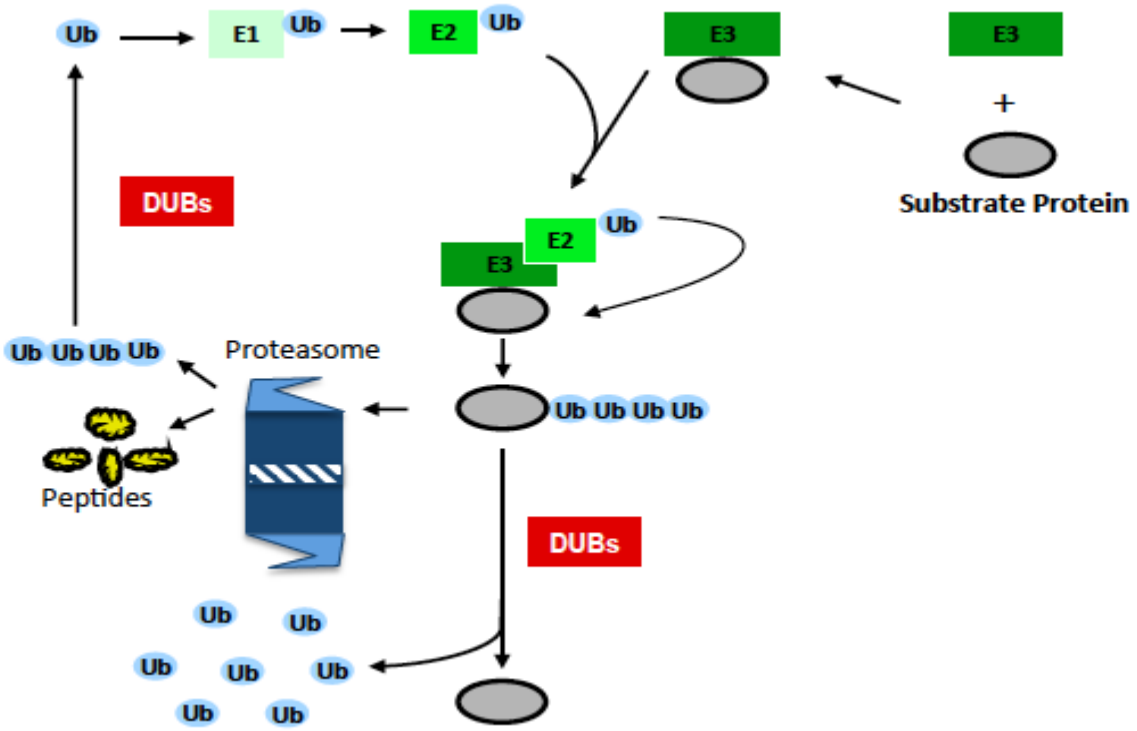


Figure II: The Ubiquitin Proteasome System (UPS). Three enzymatic steps mediate Ubiquitin conjugation: E1 (ubiquitin activating enzyme) activates and transfers ubiquitin to E2 (ubiquitin conjugating enzyme), which then functions with a E3 (ubiquitin ligase) to mediate ubiquitination. The E3 provides substrate recognition and specificity. The linkage of ubiquitin can be reversed through the catalytic action of deubiquitinating enzymes (DUBs).

Additional ubiquitin moieties can be attached to the initiating ubiquitin also via isopeptide bonds to produce a polyubiquitin chain. Given that each ubiquitin possesses seven internal lysine residues, different conformations of chains can be formed depending on the lysines linked to form the chain [64]. A proteomics analysis of *Saccharomyces cerevisiae* demonstrated that all seven ubiquitin lysine residues are employed in chain formation yielding a variety of chain types [65], which have been characterized to elicit various cellular functions (Table *1*). Polyubiquitin-linked chains of lysine-48 (K48) type are the best understood and

trigger the recognition and degradation of the target protein by the 26S proteasome [66] [67]. The 26S proteasome is a highly abundant macromolecular complex that is comprised of a 20S cylindrical core capped at both ends by 19S cap structures [68]. The 19S cap regulatory particle recognizes proteasomal substrates and catalyzes substrate entry into the 20S core where they are acted upon by its proteolytic active sites. The proteasome links ATP hydrolysis to protein degradation; each 19S regulatory particle contains a 6-ATPase ring structure that binds the substrate's polyubiquitin chain and uses ATP hydrolysis to sequentially unfold and translocate the protein into the 20S core. Accompanying this process is the recognition and removal of ubiquitin from substrates, accomplished by 19S cap subunits containing polyubiquitin binding domains and three distinct proteasome-associated DUBs: RPN11 [69], UCH37 [70], and USP14 [71] [72]. The DUB RPN11 is an integral component of the 19S cap that cleaves at the base of the ubiquitin chain where it is linked to the substrate, thereby performing a coupling of deubiquitination of the substrate with its degradation [73] [74]. The two other DUBs, UCH37 and USP14, associate with the 19S cap and appear to mediate a stepwise removal of ubiquitin by disassembling the chain from its distal end [75] [76] [74]. This stepwise removal of ubiquitin serves an editing function to antagonize protein degradation and promote substrate disassociation from the proteasome, as cells treated with USP14 inhibitors display enhanced proteasome mediated degradation [74].

Table 1: Ubiquitin chain linkage types and their cellular consequences

Ubiquitin chain linkage type	Consequence of protein ubiquitination
Polyubiquitination (K-48)	Canonical ubiquitin signal, Proteasomal degradation
Polyubiquitination (K-63)	DNA damage response/repair, cytokine & NF-κB signaling, endocytosis
Polyubiquitination (K-29, K-33, K-27)	Proteasomal degradation, associated with HECT E3 ligases, kinase signaling
Polyubiquitination (K-6)	Currently unclear, predicted DNA damage repair
Polyubiquitination (K-11)	Proteasomal degradation, cell cycle regulation, ERAD
Linear chains, monoubiquitination, multiple monoubiquitination	NF-κB signaling, endocytosis, DNA replication, viral budding

Early evidence implicating the UPS in muscle protein breakdown was provided by the observation of increased ubiquitin conjugation in atrophying skeletal muscles under a variety of *in vivo* models of atrophy including fasting and denervation [77], cancer cachexia [78] [79], and sepsis [80]. Supporting the activation of UPS-mediated proteolysis in muscle atrophy are the findings that a variety of players in the system are induced under catabolic conditions, including E2 enzymes [81, 82] [83], several subunits of the 20S core proteasome [84] [85] [86] [87], and ubiquitin itself [88] [84] [89]. Gene expression profiling across various models of muscle atrophy identified two upregulated muscle-specific E3 ubiquitin ligases termed Muscle Atrophy F-box protein (MAFbx; also known as

atrogin-1) and Muscle-specific Ring Finger protein-1 (MuRF1) [90] [38] [91]. Loss of either atrogin-1 or MuRF1 in muscle is protective against atrophy, imparting resistance to muscle wasting induced by denervation [90] [38]. Atrogin-1-null mice are also resistant to wasting during fasting [92], whereas MuRF1-null mice show a resistance to wasting induced by dexamethasone treatment [93]. This protective effect against wasting is also observed in cultured myotubes, whereby silencing of MuRF1 and atrogin-1 individually or in combination could prevent atrophy following dexamethasone treatment [94]. Interestingly in this study, silencing of either MuRF1 or atrogin-1 produced a reciprocal upregulation of the other E3 ligase, suggesting a possible compensatory mechanism of regulation.

Despite the clear role of these E3 ligases in muscle wasting, only a few of their potential substrates have been identified to date. Atrogin-1 can trigger the degradation of the muscle transcription factor MYOD [95] and the protein synthesis activator eIF3f [96], whereas MuRF1 appears to target muscle structural proteins for degradation including troponin I [97], myosin heavy chain subtypes [40], and myosin light chain [98]. More recently, additional UPS components have been described as promoting the catabolism of muscle proteins. The E3 ligase TRIM32 is required for fasting induced muscle atrophy and the degradation of a variety of muscle structural proteins including thin filaments proteins, actinin, and desmin [99]. During muscle atrophy, the UPS appears to also be regulated at the level of ubiquitin deconjugation. To date, two DUBs have been shown to be induced in atrophying skeletal muscle. USP14, a

proteasome-associated DUB, likely regenerates free ubiquitin from substrates targeted for degradation at the site of the proteasome in order to replenish the pool of ubiquitin in atrophying muscle, a condition where ubiquitin need is elevated [100]. USP19 is the other DUB upregulated in atrophying muscle [1], however its precise function in atrophy remains unclear. The increased expression of DUBs under conditions of elevated ubiquitin conjugation suggests they act on specific substrates rather than modulating overall levels of ubiquitination. Collectively, these findings strongly support a role for the UPS in driving muscle proteolysis under catabolic conditions.

Ultimately, skeletal muscle atrophy is driven by an imbalance between the rates of protein synthesis and degradation and the heightened action of proteolytic systems in muscle is a driving force behind atrophy. Investigation of the cellular signaling mechanisms implicated in regulating muscle atrophy has established a complex network of signaling cascades that may provide insights regarding clinical interventions for the treatment of cachexia and other types of muscle wasting.

1.4 CELLULAR SIGNALLING SYSTEMS REGULATING MUSCLE ATROPHY

An intricate network of anabolic and catabolic signaling mechanisms regulate overall myofiber size [36]. Described below are some of the major signaling pathways that play a role in these processes. These signaling pathways

modulate growth or atrophy of muscle by regulating protein synthesis, degradation, or both [45].

1.4.1 IGF1-PI3K-Akt-mTOR and IGF1-Akt-FoxO

The role of the IGF1 signaling cascade in muscle growth has been well characterized by several gain and loss of function studies both *in vitro* and *in vivo* [101]. Inhibition of IGF1 and insulin signaling via inhibiting PI3K or expressing dominant negative Akt reduces myotube diameter in culture and mice lacking Akt display smaller muscles than wild type littermates [102]. Conversely, treatment of myotubes with IGF-1 prevented the myotube atrophy and protein degradation triggered by glucocorticoids [103], while genetic activation of Akt in mice prevented muscle loss following hindlimb denervation [104]. Moreover, IGF1 can completely suppress the induction of MuRF1 and atrogin-1 ubiquitin ligases under a variety of catabolic conditions, including the muscles of IGF1 transgenic mice under angiotensin treatment [105] and via local IGF1 injection, which can block disuse atrophy [106].

Insulin-like growth factor 1 (IGF1) is a circulating growth factor but can also be produced by skeletal muscle and so can act through both endocrine and paracrine mechanism. IGF1 first binds its cell surface receptor to activate intracellular kinase activity and phosphorylate phosphoinositide 3-kinase (PI3K), which can then phosphorylate and activate Akt (protein kinase B). Akt controls both protein synthesis and degradation, via its activation of mammalian target of

rapamycin (mTOR) and through its phosphorylation of the forkhead box (FoxO) family of transcription factors, respectively. mTOR is part of two larger multi-subunit complexes, mTORC1 and mTORC2. Of these two complexes, primarily mTORC1 controls protein synthesis through phosphorylation of S6 kinase and 4EBP1 that results in activation of eIF4B [107]. In addition, the downstream functions of FoxO transcription factors (FoxO1, FoxO3, and FoxO4) are blocked by activated Akt, which phosphorylates three conserved FoxO residues leading to sequestering of p-FoxOs in the cytoplasm away from target genes [108] [109]. Dephosphorylation of FoxOs permits their translocation to the nucleus whereby they activate target genes involved primarily in atrophy [110] [47] including the E3 ligases MuRF1 [111] and atrogin-1 [39], as well as genes promoting autophagy [112].

FoxO transcription factors themselves are induced under conditions of catabolism in muscle and are suppressed or returned to normal levels under anabolic conditions, as is observed following fasting and re-feeding [113]. In support of the importance of FoxOs, loss and gain of function experiments display marked changes in muscle size. Expression of FoxO mutants unable to be phosphorylated (constitutively active) cause significant atrophy of myotubes and myofibers concomitant with induction of E3 ubiquitin ligase atrogenes, while RNAi-knockdown in myotubes or expression of a dominant negative (inhibitory) form of FoxO in myofibers abolished atrogene induction and prevented denervation-induced muscle atrophy [106] [39]. Thus, IGF1 signaling acts

through Akt to promote anabolic processes and suppress catabolic processes in tandem through activation of mTOR and inactivation of FoxO transcription factors, respectively.

1.4.2 Myostatin

Myostatin (also known as GDF8) is a member of the transforming growth factor beta (TGFβ) family of transcription factors that is produced by skeletal muscle and acts as a major negative regulator of muscle growth [114]. Genetic abolition or mutations of the myostatin gene in a variety of mammalian species leads to significant hypertrophy of skeletal muscle, such as in double-muscled cattle that lack myostatin [115] or the Belgian Blue and Piedmontese cattle who harbor a natural autosomal recessive myostatin mutation [116]. Loss of function mutations in human myostatin genes have also been observed, resulting in increased muscle mass [117]. The increased muscle mass arising from loss or mutation of myostatin reflects both myofiber hyperplasia and hypertrophy, or an increase in overall number and size of myofibers. Injection of myostatin-expressing transgenic CHO cells into the muscle of adult mice caused marked wasting [118]. In differentiated myotubes *in vitro*, purified myostatin is capable of reducing myotube size and number, inhibiting protein synthesis, and upregulating MuRF1 and atrogin-1 [119] [120]. *In vivo* myostatin injection could also reduce myofiber size and number, upregulate atrogene expression, and antagonize the IGF1/PI3K/Akt hypertrophy pathway through inhibiting Akt phosphorylation and increasing the levels of active (dephosphorylated) FoxOs [120]. This suggests

that the catabolic effect of myostatin signaling is coupled with a suppression of anabolic signals. Consistent with a catabolic role of myostatin, it has recently been identified as a novel tumoral cachectic factor abundantly secreted by C26 colon cancer cells [121]. Treatment of C2C12 myotubes with C26 conditioned media resulted in atrophy, as demonstrated by inhibited myotube formation, induction of MuRF1 and atrogin-1, increased activity of the UPS, activated ActRIIB/Smad and NF-κB signaling, suppressed IGF-1/PI3K/Akt signaling, and elevated flux through autophagy [121]. These finding support the catabolic effect of myostatin signaling and suggest myostatin as a tumor-secreted factor that may contribute to cachexia.

Myostatin, along with other TGF family members, bind with high affinity to the activin type II receptor (ActRIIB) that form heterodimeric complexes with a type I receptor, activin receptor-like kinase 4 or 5 (ALK4 or ALK5). These receptor complexes possess serine-threonine kinase activity and mediate the downstream phosphorylation of mothers against decapentaplegic homolog (Smad) transcription factors Smad2 and Smad3, triggering the degradation of muscle sarcomeric proteins [122] [123]. Smad2 and Smad3 first interact with Smad4 to form active transcription factor complexes, however specific targets of activated Smads are not currently known. Given the simple DNA recognition sequence for Smads and their low affinity for it, activated Smads likely partner with a variety of accessory cofactors to elicit their effects [124]. Interestingly, myostatin signaling selectively suppresses the IGF1/PI3K/Akt growth-signaling

cascade *in vitro* [125] and *in vivo* [126], further evidence of crosstalk between catabolic and anabolic pathways in muscle. Moreover, recent evidence has implicated bone morphogenic protein (BMP) signaling in control of muscle mass as a potent hypertrophic signal counteracting catabolic myostatin-Smad2/3/4 signals [127]. Specifically, BMP activates Smads1/5/8, which are also capable of forming a complex with Smad4 and thereby compete with Smad2 and Smad3 for Smad4 availability. This novel BMP-Smad1/5/8 signal cascade negatively regulates the expression of a novel gene encoding a ubiquitin ligase required for muscle atrophy, termed muscle ubiquitin ligase of the SCF complex in atrophy-1 (MUSA1) [127].

1.4.3 Inflammatory cytokines and NF-κB

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factors are the major downstream effectors of inflammatory stimuli and are expressed in skeletal muscle where they are activated by inflammatory cytokines binding to their cell surface receptors, particularly receptors for tumor necrosis factor-α (TNFα) [128] and TNF-related weak inducer of apoptosis (TWEAK) [129]. The role of inflammatory cytokines and NF-κB in muscle atrophy and cancer cachexia is well characterized [130]. NF-κB is maintained in an inactivated state in the cytoplasm through its association with its inhibitory partner IκBα. In response to TNFα and other inflammatory cytokines, IκB kinase (IKKβ) complex phosphorylates IκBα triggering its ubiquitination and proteasomal degradation. The freed NF-κB is then able to translocate to the nucleus where it activates target gene transcription.

Transgenic mice overexpressing IKKβ in skeletal muscle display marked muscle wasting mediated specifically by MuRF1 and activation of the UPS, while mice overexpressing a dominant negative form of IκBα that prevents NF-κB activation display attenuated wasting under denervation or cancer cachexia [131]. The attenuation of atrophy observed through NF-κB inhibition may arise due to crosstalk with the IGF1/PI3K/Akt pathway, as IKKβ conditional knockout mice are resistant to muscle atrophy and display Akt hyper-phosphorylation [132]. This suggests that NF-κB signaling may enhance protein degradation and suppress synthesis in muscle. In both cultured muscle cells treated with TWEAK and TWEAK expressing transgenic-mice, a significant decrease in myofiber size was observed [129] and TWEAK knockout out mice display less atrophy than wild type littermates following hindlimb denervation [133]. In this study, TWEAK mediated signaling through its cell surface receptor, fibroblast growth factor-inducible 14 (Fn14), was required for NF-κB activation and muscle atrophy. The Fn14-NF-κB signaling cascade is mediated through TRAF6, as TRAF6 ablation prevented fasting induced muscle atrophy [134], as well as Fn-14 upregulation, FoxO activation, and the induction of the UPS and ALS proteolytic systems following fasting [135]. Collectively, these results illustrate the importance of inflammatory cytokines and NF-κB signaling in driving muscle wasting.

2. DEUBIQUITINATING ENZYMES

Research in the muscle atrophy field has focused primarily on the role of ubiquitin ligases, but less is known regarding the role(s) of DUBs in this process. These enzymes are likely to be involved in the regulation of muscle growth given their ability to dynamically modulate the ubiquitination status of proteins.

2.1 STRUCTURES AND MECHANISMS OF DEUBIQUITINATING ENZYMES

Approximately 90 DUBs are responsible for the reversal of ubiquitination in a variety of cellular processes. There are five families of DUBs categorized mainly by the sequence homology of their catalytic domains. The ubiquitin specific proteases (USPs), ubiquitin COOH-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), and Josephins families are all cysteine proteases, whereas the JAB1/MPN/MOV34 (JAMM) family DUBs are metalloproteases [136]. Cysteine proteases rely on a trio of conserved residues that come together through tertiary structure forming a catalytic triad active site architecture, such as the Cys223, His464, and Asp481 of HAUSP/USP7 [137]. The active site cysteine attacks the peptide bond, forming a tetrahedral transition state with the ubiquitin moiety of the substrate. The proximal histidine serves to prime the cysteine for nucleophilic attack and the third residue (aspartic acid or asparagine) facilitates the alignment and/or polarization of the histidine. A reaction between this DUB intermediate and a water molecule leads to the release of free ubiquitin. JAMM-family DUBs are zinc (Zn^{2+}) metalloproteases, whereby their active site residues and a water molecule coordinate a zinc ion required for catalysis. The water

molecule becomes deprotonated forming a charged hydroxyl ion capable of attacking the substrate-ubiquitin linkage, and the release of free ubiquitin is accomplished upon the reaction of the hydroxyl with the carboxyl terminal of the ubiquitin moiety.

The USP subclass is the largest family of DUBs in humans, comprised of approximately 60 DUBs. A number of USP crystal structures have been solved, including HAUSP/USP7 [137], USP14 [72], USP8 [138], USP2 catalytic domain [139], USP21 [140], and USP4 [141]. These depict a conserved catalytic core architecture and an active site conformational change upon interaction with ubiquitin. Two conserved motifs are integral to the catalytic function of the USP family of DUBs, the Cys and His boxes containing the cysteine and histidine residues of the catalytic triad. A number of USPs also possess a ZnF-UBP binding domain that plays a role in recognizing the COOH terminus of ubiquitin [142]. The majority of USP family DUBs also have been shown to possess ubiquitin-like (UBL) domains, which appear to play different roles on different USPs [143]. The N-terminal UBL domain in USP14 plays a critical functional role in its recruitment to the proteasome [72], whereas the UBL domains in USP4 and USP7/HAUSP negatively or positively modulate their DUB catalytic activity, respectively. The N-terminal UBL domain in USP4 competes with ubiquitin for binding, thereby inhibiting catalytic activity [141], while the five tandem C-terminal UBL domains in USP7/HAUSP are required for full catalytic activity [144].

2.2 FUNCTIONS OF DEUBIQUITINATING ENZYMES

In general, DUB catalytic activity can be employed to maintain free ubiquitin levels in the cell, rescue proteins from proteasomal degradation, or alter the activity or localization of substrates whose ubiquitination does not result in degradation [145]. Ubiquitin is transcribed as a ubiquitin precursor. In humans, four genes encode ubiquitin: two genes, *Ubb* and *Ubc*, encoding polyubiquitin chains [146] [147] and two genes, *Uba52* and *Rps27a*, encoding ubiquitin-ribosomal fusion proteins to the L40 or S27a subunits of the 60S ribosomal complex, respectively [148] [149]. From these precursors, free monoubiquitin is generated via the catalytic action of DUBs. This processing occurs rapidly in vivo and can occur even co-translationally as the precursors are generated. Generation of free ubiquitin is also accomplished by the proteasome-associated DUBs RPN11 [69], UCH37 [70], and USP14 [71] [72]. Ubiquitin removal by RPN11 promotes substrate degradation and occurs at the base of the ubiquitin chain, but presumably only upon the proteasome committing to degradation [73]. In contrast, UCH37 and USP14 cleave ubiquitin from the distal end of the polyubiquitin chain [75] [76] [74]. Since efficient binding of polyubiquitinated substrates to the proteasome requires a polyubiquitin signal of at least four ubiquitin moieties [150], these two DUBs can serve editing functions by removing the ubiquitin signal and promoting disassociation from the proteasome, rescuing proteins with relatively short chains from degradation [74].

Although proteasome associated DUBs may have fairly broad substrate specificity, the abundance of DUBs encoded in the human genome and their diversity in structure suggest that many have specific functions and substrates. Indeed, the stabilization of target proteins by DUBs has been implicated in a variety of cell processes including cell cycle checkpoints, tumorgenesis, the DNA damage response, as well as neurological disorders [136]. During the cell cycle, cyclin-dependent kinases (CDKs) phosphorylate target proteins to control progression through sequential phases of the cycle. The Cdc25A phosphatase activates CDKs to promote cell cycle progression and Cdc25A levels are carefully restricted in order to prevent oncogenic transformation. This fine regulation of Cdc25A is accomplished by the ubiquitin hydrolase Dub3, which deubiquitinates and stabilizes Cdc25A [151]. *In vitro* Dub3 knockdown increased Cdc25A ubiquitination and degradation causing reduced CDK/Cyclin activity and cell cycle arrest. Conversely, Dub3 overexpression brought about oncogenic transformation characterized by accumulation in S phase and DNA damage [151]. The tumor suppressor gene p53 prevents tumorgenesis by activating cell cycle arrest and apoptosis [152]. p53 is a short-lived protein that is rapidly ubiquitinated by the E3 ligase MDM2 and degraded under normal conditions. When p53 escapes this regulation it allows cells to survive and proliferate [153] [154]. The DUB HAUSP/USP7 can stabilize p53 by direct interaction and deubiquitination [155]. Additionally, a number of USP and OUT family DUBs have been linked to DNA damage repair pathways. Repair protein recruitment to sites of DNA damage requires ubiquitination of the surrounding chromatin by the E3 ligases

RNF8 and RNF168 [156]. However this response is strongly opposed by the histone/chromatin deubiquitinating action of at least three known DUBs: OTUB1 [157], USP3 [158], and USP44 [159].

DUBs located in the brain have also been characterized as playing a role in progressive and inherited neurodegenerative disorders. The UCH subclass DUB UCHL1 is one of the most abundant proteins in the brain, and studies have shown that altered levels of UCHL1 contribute to a variety of neurodegenerative disorders including Parkinson's disease (PD) and Alzheimer's disease [160] [161]. A homozygous mutation in humans within the ubiquitin binding domain of UCHL1 (GLU7ALA) results in early onset progressive neurodegeneration and directly affects the affinity of UCHL1 for ubiquitin [161]. UCHL1 is also predicted to inhibit the turnover of alpha-synuclein leading to its abnormal accumulation, a characteristic associated with the progression of PD.

2.3 REGULATION OF DEUBIQUITINATING ENZYMES

DUB activity is regulated by multiple mechanisms, including allosteric interactions, transcriptional regulation, post-translational modifications, and subcellular localization. Some DUBs, such as the proteasome-associated DUB USP14, have been shown to require association into larger complexes in order to be fully catalytically active [72]. Another example of this assembly-dependent allosteric regulation of catalytic activity is USP22, which requires association with the SAGA co-activator complex for DUB activity in order to facilitate chromatin

access for transcription factors [162]. Other DUBs require interaction with their own internal motifs; USP7/HAUSP contains five tandem ubiquitin-like (UBL) domains, which are required for full catalytic activity. USP7/HAUSP substrate GMPS binds to the first three UBL domains stabilizing the interaction of the final two UBL domains with USP7/HAUSP catalytic domain and activating its DUB activity ~100-fold [144]. Recent experimental work *in vitro* demonstrated that USP19 catalytic activity could be inhibited through an association between its catalytic site and C-terminal transmembrane domain [163]. DUB activity can also be regulated at the level of mRNA transcription. Recently, Tiruppathi and colleagues demonstrated that the transcription factors DREAM and USF1 are both capable of binding the promoter of the A20 DUB gene, which functions normally to suppress NF-κB signaling. The occupation of the A20 promoter by DREAM prevented A20 expression and increased NF-κB signaling, while USF1 binding activated A20 expression in response to inflammatory stimuli. DREAM deficient mice also display unabated A20 expression [164].

A wide variety of DUBs can also be modulated by post-translational modification such as phosphorylation and ubiquitination [165]. Phosphorylation at a specific serine residue in USP1 is critical for its activation and subsequent interaction with its activator UAF1 [166]. Ataxin-3 is a DUB previously implicated in regulating protein stability in quality control pathways and is regulated by ubiquitination at lysine-117 that serves to enhance its DUB activity *in vitro* [167]. DUBs possess differential activity throughout the cell cycle, mainly through

specifically timed modifications. USP37 is activated by phosphorylation by the cell cycle protein CDK2 in order to antagonize the ubiquitination of CDH1 by APC/C and promote entry into S phase [168], whereas USP8 becomes phosphorylated and activated during M-phase of the cell cycle [169]. DUB activity is also modulated through re-localization within the cell to specific cellular sub-compartments. USP4 undergoes nuclear to cytoplasmic translocation to become fully active following phosphorylation by Akt and deubiquitinates the TGF-beta type I receptor raising its levels at the plasma membrane and leading to elevated TGF-beta signaling [170].

3. ENDOPLASMIC RETICULUM STRESS

The endoplasmic reticulum (ER) is an expansive cellular organelle responsible for protein folding and quality control, maturation of newly synthesized proteins, and trafficking, as well as a site of calcium (Ca^{2+}) homeostasis. Under conditions of cellular stress, such as increased protein folding load or accumulation of newly synthesized proteins, the ER activates a specific signaling network called the unfolded protein response (UPR) [171]. The UPR signaling arms intersect a number of cellular stress signaling systems, interconnecting ER stress to inflammatory, apoptotic, autophagic, and oxidative stress signaling [172].

3.1 ER STRESS REGULATION AND THE UNFOLDED PROTEIN RESPONSE

The ER and its associated stress signaling pathways act as cellular stress sensors to respond to challenges facing the cell. Three integral ER membrane proteins monitor cellular stress levels and the ER lumen protein-folding load: PKR-like eukaryotic initiation factor 2α (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). These three proteins comprise the three canonical arms of the UPR. Each arm acts in parallel possessing a distinct mechanism, but collectively they modulate the expression of genes that help relieve the stress or induce the apoptotic signaling cascade if the stress becomes severe or prolonged [171]. Under normal cellular conditions, the monitoring proteins are rendered inactive through the interaction of their ER luminal domains with the ER resident chaperone protein BiP/GRP78 [173] [174].

Additional layers or mechanisms of interaction may also be involved, as disrupting this inhibitory interaction with BiP does not always result in UPR activation [175]. Under conditions in which ER homeostasis becomes challenged, most often through increased unfolded proteins within the ER lumen, BiP binds to the unfolded proteins and is therefore sequestered away from the UPR sensors permitting the oligomerization and activation of PERK and IRE1 [176] and the unmasking of Golgi localization signals within ATF6 [177]. For full activation, ATF6 requires translocation to the Golgi apparatus where it is cleaved by two serine proteases, S1P and S2P, to liberate its N-terminal domain and produce an active transcription factor [178] [179].

Overall, ER stress involves at least three rapid responses: reducing the protein folding load through decreased translation, upregulation of ER chaperones to increase its folding capacity and ER-associated degradation (ERAD) proteins to degrade misfolded proteins through the UPS, and induction of anti-apoptotic and inflammatory signaling through NF-κB [171]. These downstream responses of the UPR are evoked through each arm's activation of a transcription factor with specific gene targets. These b-ZIP transcription factors act either alone or cooperatively to induce UPR target genes. b-ZIP factors have been implicated in ER stress due to their ability to respond to canonical ER stress signals, to activate known targets of the UPR, and by their activity at UPR response elements [180]. Activated (oligomerized, auto-phosphorylated) IRE1 cleaves the mRNA encoding X-box binding protein-1 (XBP1) via its ribonuclease

activity to remove a 26 base-pair region allowing translation of this active (spliced) form of the XBP1 transcription factor [181], although unspliced XBP1 mRNA expression can also be induced by the ATF6 arm of the UPR [182] [183]. XBP1s can induce gene expression to elevate the folding and processing capacity of the ER, such as increasing the production of chaperones and proteins required for ER biogenesis, endoplasmic reticulum associated degradation (ERAD), and protein secretion. PERK activation results in the dimerization and auto-phosphorylation of its ER lumen domains, which in turn phosphorylates and inactivates the translation initiation factor eIF2α inhibiting mRNA translation and diminishing the ER protein-folding load [184]. The inhibition of eIF2α leads to preferential translation of mRNAs containing short open reading frames in their 5'-UTRs, of which one of these encodes the transcription factor activating transcription factor-4 (ATF4) [185]. ATF4 targets two critical ER stress response genes, transcription factor C/EBP homologous protein (CHOP; also known as DNA damage-inducible gene (GADD)-153) and growth arrest DNA damage-inducible protein-34 (GADD34). CHOP plays a critical role in ER stress induced apoptosis as CHOP-null mice display a reduced level of apoptosis in response to ER stress and delayed ER-stress mediated diabetes [186]. GADD34 acts as a negative feedback signal for ER stress by counteracting PERK via dephosphorylation of eIF2α [187]. The proteolytic cleavage of ATF6 upon translocation to the Golgi apparatus releases the N-terminal cytosolic fragment to produce an active transcription factor, termed ATF6(N). This transcription factor can translocate to the nucleus and activate selected UPR target genes, primarily

ER-resident proteins involved in protein folding such as BiP, GRP94, and XBP1, and CHOP/GADD34 [178] [188].

When the ER stress response is prolonged and severely impairs ER function, ER-stress mediated apoptosis is triggered to remove damaged cells [186]. One of the main components of the ER-stress apoptotic cascade is CHOP, a member of the CCAAT/enhancer binding proteins (C/EBP) family of transcription factors. CHOP serves as a dominant negative inhibitor of other C/EBPs through heterodimerizing with other C/EBPs that strongly prefer homodimerization inhibiting their DNA binding ability [189]. CHOP is ubiquitiously expressed in the cytoplasm at low levels, however under cellular stress it is strongly induced and accumulates in the nucleus [189]. Expression of CHOP is primarily modulated at the level of transcription, with its promoter region containing recognition sites for both ER-stress transcription factors ATF6 and ATF4 [188] [190]. Although overexpression of active ATF6 can induce CHOP expression [188], the other signaling arms of the UPR can also induce CHOP and maximum activation of CHOP occurs only in the presence of all UPR signaling arms [191]. This ER stress-mediated apoptotic signaling is transmitted to classical caspase-mediated apoptosis through the activation of the ER-associated caspase-12 [192] [193]. When activated, caspase-12 moves to the cytosol where it cleaves caspase-9, which can then cleave caspase-3 to activate the canonical apoptotic pathway.

3.2 ROLE OF ER STRESS IN SKELETAL MUSCLE AND MYOGENESIS

Although the role of ER stress has been extensively studied in other metabolic organs, much less focus has been given to its roles in skeletal muscle. Skeletal muscle contains an extensive matrix of specialized ER, called sarcoplasmic reticulum, responsible for calcium signaling and homeostasis required during contraction. Evidence for a role of ER stress in muscle was first demonstrated in myopathies, with muscle samples from myopathic patients presenting elevated levels of the ER-stress induced chaperones GRP94 and calreticulin [194]. Under conditions of atrophy in skeletal muscle, the role of ER stress has been studied only under disuse atrophy and fasting. The expression of ER stress components is upregulated following disuse atrophy due to hindlimb unloading [195]. Food deprivation in mice for 2 or 3 days did not alter CHOP or eIF2α expression in the tibialis anterior or soleus muscle, but BiP expression was decreased in the tibialis anterior only following 2 or 3 days of fasting [196]. In addition to the characterized downstream effects of ER stress, this response in muscle also triggers autophagy through the ER-stress sensor PKCθ, which is upregulated in muscle cells following ER stress treatment and when inhibited can prevent ER-stress induced autophagic puncta formation [197]. Given that few studies have evaluated the role of ER stress in muscle atrophy and the few published *in vivo* studies evaluated only a small number of ER stress markers, further investigation is needed.

Apoptosis and ER stress/CHOP induction have been suggested as normal process required for proper growth and differentiation of cells. Indeed, upregulation of CHOP is observed in differentiation phases in various cells such as B cells [185], erythroids and keratinocytes [198], T cells [199], and adipocytes [200]. In skeletal muscle, myoblasts fuse to form multinucleated myotubes that differentiate into myofibers. Under normal myoblast growth and differentiation, apoptosis is activated with at least part of this apoptotic induction arising from the activation of the ER-stress caspase cascade, as caspase-12 is induced in differentiating myoblasts *in vitro* and *in vivo* along with caspase-9 and -3 [201]. Differentiating and apoptotic muscle cells display induction of the ER-stress specific proteins BiP and CHOP, which occurs specifically through the activation of the ATF6 signaling arm of the UPR [201]. ER stress induction, as shown by CHOP expression, occurs concomitantly with the differentiation-dependent appearance of MHC [201]. Inhibition of apoptosis prevented the formation of myotubes [201], while pretreatment of myoblasts with ER stress inducers thapsigargin or tunicamycin enhanced apoptosis and the formation of myotubes [202]. Collectively, these results suggest ER stress and the UPR are required for proper myogenesis and tissue maintenance in skeletal muscle.

4. UBIQUITIN SPECIFIC PROTEASE – 19 (USP19)

4.1 USP19 STRUCTURE

USP19 is a 150-kDa protein expressed in a variety of tissues, including skeletal and cardiac muscle, testis, kidney and adipose tissue [1]. USP19 was first identified as a DUB that is upregulated in atrophying rat skeletal muscle [1]. In addition to its conserved USP catalytic core, USP19 contains a variety of interesting structural domains (Figure *III*). Those include myeloid translocation protein 8, Nervy and Deaf (MYND) [203] and CHORD/SGT1 (CS/p23) domains [204] [205], which may mediate protein-protein interactions or suggest a possible chaperone role for USP19, respectively. Although ubiquitin-specific proteases often contain ubiquitin-like (UBL) domains, USP19 is unique in that it exhibits a UBL domain within its catalytic core [206]. UBL domains in this class of DUBs have been shown to have a number of functions including modulating DUB catalytic activity, imparting recognition for a substrate or partner, or localization to a specific subcellular localization [143]. However, the function of USP19's UBL domain is not known. Recently, USP19 was shown to contain a unique N-terminal interaction domain spanning amino acids 462-473, which is conserved among previously identified substrates of the seven in absentia homolog (SIAH) 1 and 2 E3 ubiquitin ligases [207]. SIAH1 and SIAH2 can indeed target USP19 and modulate its stability, as ubiquitination and degradation of USP19 was impaired in cells expressing either catalytically inactive SIAH 1 or 2, or a USP19 mutant lacking its SIAH interaction motif. Employing a bioinformatics approach to screen for DUBs containing putative transmembrane domains (TMD), an extreme

C-terminal TMD was discovered within USP19 in two of its seven transcript variants [208]. This TMD was demonstrated to impart ER localization, whereby USP19 is tail-anchored into the ER membrane with its catalytic domains facing the cytosol.

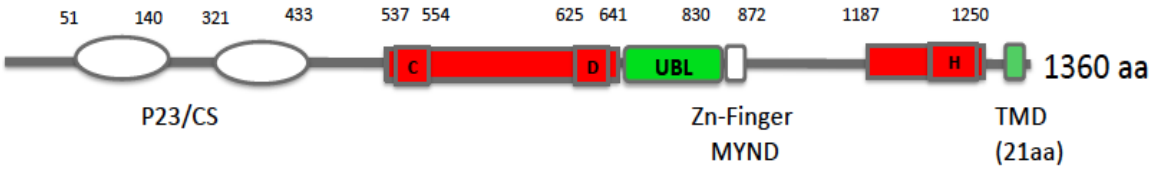


Figure III: USP19 structural and functional domains. USP19 protein is depicted with known structural domains. Amino acid number positions are indicated for reference, with total number of amino acids for the full-length TMD containing isoform. The non-ER localized isoform has a divergent final exon and lacks the transmembrane domain (TMD). p23/CS (CHORD and SGT1 domains), UBL (ubiquitin-like domain), MYND (myeloid translocation protein 8, Nervy and Deaf1 domain), TMD (transmembrane domain). The C, D, and H denote USP19's catalytic triad residues cysteine, aspartate, and histidine, respectively.

4.2 USP19 FUNCTIONS

4.2.1 Regulation of the Cell Cycle

Progression through the cell cycle is tightly regulated through the cyclic synthesis and degradation of a variety of molecules called cyclin-dependent kinases (CDKs). The rise and fall of specific CDK levels and activities are mediated by CDK inhibitors and post-translational modifications such as phosphorylation and ubiquitination to manage progression through a variety of cell cycle checkpoints and ensure proper cell division [209]. The CDK inhibitor p27Kip1 restricts cell proliferation, as demonstrated by the increased organ and tissue weights in p27Kip1 knockout mice [210] [211]. Skp2, a SKP1-CUL1-F-box (SCF) protein ligase, plays a critical role in regulating the transition from G1 to S phase by ubiquitinating the CDK inhibitor p27Kip1 in early S phase and targeting

it for proteasomal degradation thereby allowing progression through S phase [212] [213]. Additionally, another E3 ligase termed Kip1 ubiquitination promoting complex 1 (KPC1) targets p27Kip1 for proteasomal degradation in G1 phase [214]. USP19 was identified as the first DUB to regulate the stability of a CDK inhibitor by interacting with and stabilizing KPC1, thereby modulating p27Kip1 levels and supporting cell cycle progression [215]. RNAi-mediated knockdown of USP19 in rat myoblasts and fibroblasts inhibited cell proliferation, slowed progression from G1 to S phase, and accumulated levels of p27Kip1. The elevated p27Kip1 levels were associated with normal levels of Skp2, but reduced levels of KPC1. Growth defects observed upon depletion of USP19 were recovered upon overexpression of KPC1 or use of $p27^{-/-}$ mouse embryonic fibroblasts (MEFs) and USP19 stabilized KPC1 in a DUB activity-dependent manner [215].

4.2.2 Stability of Hypoxia Inducible Factor-1α (HIF-1α)

Cells respond to conditions of low oxygen, or hypoxia, by mounting a response to mitigate negative effects on the cell. This response is dependent on the induction of hypoxia inducible factors (HIF) transcription factors of which there are three human isoforms HIF-1α, 2α, and 3α [216]. HIF-1α directs the acute response to hypoxia driving the transcription of hypoxia response element (HRE)-containing genes essential for adaptation and survival [217] [218]. Moreover, dysregulated HIF-1α is also involved in a number of human diseases including cancer and inflammatory conditions [219]. The UPS tightly regulates

HIF-1α to prevent aberrant activation of its downstream effects. At normoxia HIF-1α is maintained at scarce levels by efficient proteasomal degradation, but in response to hypoxic conditions rapid degradation is inhibited, allowing accumulation of HIF-1α to promote its transcriptional response [220]. USP19 was demonstrated in HEK293T cells to bind and stabilize HIF-1α, namely by deubiquitinating HIF-1α and rescuing it from proteasomal degradation [221]. Co-immunoprecipitation revealed USP19 interacts with HIF-1α specifically, and not the 2α or 3α isoforms. shRNA-mediated knock down of USP19 impaired the cells' ability to mount an appropriate response to hypoxic conditions as shown by attenuated induction of known HIF-1α downstream targets. Interestingly, stabilization of HIF-1α was independent of USP19 DUB catalytic activity and ER localization, as cells transfected with a catalytically inactive USP19 mutant or a mutant lacking its ER localization sequence maintained the ability to stabilize HIF-1α and to mount an appropriate response to hypoxic conditions. This suggests that USP19 may have developed a non-catalytic stabilization of HIF-1α, whereby USP19 is proposed to interact in such a way as to prevent poly-ubiquitination rather than to direct deubiquitination.

4.2.3 Stability of Cellular Inhibitors of Apoptosis (cIAPs)

Inhibitors of apoptosis (IAPs) prevent apoptotic induction through binding and inhibiting caspases [222]. The well-characterized IAPs are the cellular IAP1 (cIAP1) and IAP2 (cIAP2) as well as the X-chromosome linked IAP (XIAP). Upon apoptotic stimuli second mitochondrial-derived activator of caspases (SMAC)

proteins become activated, whereby they are released from mitochondria and sequester IAPs thereby promoting apoptosis [223]. Subsequent studies have identified cIAP1 and cIAP2 as positive regulators of NF-κB signaling pathways and tumor necrosis factor (TNF) receptor signaling [224]. Many IAPs are ring domain containing ubiquitin ligases whose stability is affected by self-ubiquitination and/or by their interacting proteins and subsequent proteasomal degradation [225]. cIAP1 and cIAP2 directly interact with USP19 as shown by co-immunoprecipitation and USP19 appears to stabilize cIAPs in HEK293 cells. shRNA-mediated knockdown of USP19 decreased cIAP levels while USP19 overexpression markedly increased cIAP levels [226]. Mechanistically, USP19 was shown to effectively remove ubiquitin from cIAPs *in vitro* and also to stabilize cIAPs *in vivo* but through a DUB activity-independent mechanism. Moreover, USP19 knock down enhanced TNF-induced caspase activation and apoptosis in a cIAP-dependent manner. These results suggest that USP19 stabilizes cIAPs through inhibiting cIAP self-ubiquitination. Recently, cIAPs have been shown to inhibit myogenesis and regulate myoblast fusion in primary muscle cells through the non-canonical NF-κB and TNFα signaling [227]. It is tempting to hypothesis that USP19 may stabilize cIAPs to promote muscle atrophy through induction of the TNFα/NF-κB signaling pathways. However, to date we have not been able to demonstrate an interaction between USP19 and cIAPs in muscle cells.

4.2.4 ERAD and ER Localization

ER localized USP19 appears to be upregulated under ER stress induced unfolded protein response (UPR) and rescues ERAD substrates from proteasomal degradation through its DUB activity [208]. These studies suggest that USP19 may participate in late stage protein quality control machinery and/or cellular responses to stress. Recent work also in non-muscle cells to characterize USP19's functions in ERAD disputes a role for USP19 in this process, as depletion of USP19 had no effect on the levels of ERAD substrates [163]. Moreover, Lee and colleagues suggest that TMD-containing USP19 is primarily cytosolic and its TMD domain inhibits USP19 DUB activity; incubation of purified TMD-deficient USP19 or USP19 catalytic core domain only with its synthetic TMD fragment inhibited its catalytic ability to cleave di-ubiquitin *in vitro.* This suggests that USP19's TMD is partially stabilized in the cytosol through an interaction with its own catalytic domain auto-inhibiting its own DUB activity. However, this work evaluated total USP19 levels in without evaluating levels of individual isoforms. Further work is required to determine what role such regulation may play in the cellular functions of USP19 as well as if such regulatory mechanisms are active in muscle.

4.2.5 Myogenesis and Muscle Atrophy

Current evidence implicates an important role for the UPS in muscle atrophy. Although ongoing research invokes important roles for E3 ubiquitin conjugating enzymes, little is know regarding the function of DUBs in this process. To evaluate the contribution of DUBs to muscle wasting, our laboratory sought to

identify DUBs expressed in muscle. Four DUBs were identified, but only USP19 was regulated and was induced under a variety of conditions of muscle catabolism in rat skeletal muscle, including fasting, streptozoticin-induced diabetes, dexamethasone treatment, and cancer cachexia [1]. Expression of USP19 mRNA increased approximately 30-200 percent in atrophying rat skeletal muscle and inversely correlated with muscle mass, suggesting an important role in atrophy [1]. The induction of a DUB under heightened conditions of ubiquitin conjugation suggests that USP19 may be acting on specific substrates as opposed to modulating the overall levels of ubiquitination. To explore a role for USP19 in muscle atrophy, siRNA-mediated depletion of the enzyme was carried out in L6 rat muscle cells. Depletion of USP19 increased the expression of a panel of major myofibrillar proteins including myosin heavy chain (MHC), actin, troponin T, and tropomyosin as well as the myogenic regulatory factor myogenin [2]. USP19 ability to modulate myofibrillar proteins was subsequently shown to be dependent on its ability to modulate myogenin, as silencing myogenin in USP19-silenced muscle cells abolished USP19's modulation of myofibrillar proteins. In addition, silencing of USP19 could reverse the loss of MHC induced by dexamethasone [2]. Collectively, these results suggest USP19 promotes muscle catabolism. Our laboratory has recently generated USP19 knockout (KO) mice as an *in vivo* model to further investigate the role of USP19 in muscle wasting and development. These mice are grossly normal, but display attenuated muscle atrophy under certain models of muscle proteolysis (unpublished data). This suggests that USP19 is required for the full catabolic response in muscle.

5. OBJECTIVES OF THIS THESIS

Previous loss of function experiments carried out in our laboratory showed silencing USP19 in L6 rat myotubes increased the expression of a panel of major myofibrillar proteins which was dependent on USP19's ability to modulate the expression of the myogenic regulatory factor myogenin [2]. Moreover, silencing of USP19 enhanced myotube fusion as shown by increased formation of multinucleated myotubes (unpublished results). These results in addition to the observation that USP19 levels are regulated over a time course of muscle cell differentiation suggested that USP19 might suppress myogenesis. The goals of my thesis were to identify structural elements in USP19 that are required for these functions. Since USP19 is expressed as two major isoforms, one with and the other without a C-terminal transmembrane domain (TMD) that confers ER localization [208], I wanted to determine if USP19's ER localization is essential for its ability to regulate myogenic proteins and muscle cell differentiation *in vitro*. Since the unconserved sequences in USP enzymes located N-terminal to the conserved catalytic core domain have been invoked to play roles in specificity of substrate recognition and function, I have also tested whether N-terminal regions of USP19 are required for these myogenic functions. Previous studies indicated that some functions of USP19 are not dependent on catalytic activity. Therefore, I also tested if catalytic activity is required. As described below, USP19's ER localization was required for these effects. As a result, I explored whether USP19 modulates the ER stress response that occurs during myogenesis.

II. MATERIALS AND METHODS

1. Cell culture and transfection

L6 rat myoblasts (Dr. Amira Klip, University of Toronto) or C2C12 mouse myoblasts (ATCC) were cultured in alpha-MEM (GIBCO) or DMEM (GIBCO), respectively, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (growth medium, GM) at 37°C with 5% CO_2. Cells were differentiated in alpha-MEM or DMEM supplemented with 2% FBS and 1% penicillin/streptomycin (differentiation medium, DM). For adenovirus transduction, C2C12 cells were seeded in 6-well plates at a density of $1.5E10^5$ cells per well for protein or RNA (qPCR) analysis and incubated overnight in growth medium. The next day cells were approximately 75-80% confluent and used for experimental procedures as described. For siRNA transfection, L6 Klip cells were seeded in 6-well plates at a density of $1.5E10^5$ cells per well. The next day cells were approximately 75-80% confluent and were transfected with 25 nM oligonucleotides (IDT) targeting USP19 (both isoforms), USP19TMD, USP19ΔTMD or nonspecific control (CTL) using Lipofectamine and PLUS reagent (Invitrogen). First, separate mixtures containing both OPTI-MEM (490 µL per well) and Lipofectamine (10 µL per well) (*MIX A*) or containing siRNA (to final volume of 25 nM per well), Plus reagent (10 µL per well), and OPTI-MEM (to final volume of 500 µL per well) (*MIX B*) were prepared. After 15 minutes, 500 µL (per well) of MIX A was added to MIX B and incubated for 15 minutes (transfection mix). Each well was first washed with 1 mL of PBS, then overlaid with 500 µL serum/antibiotic free alpha-MEM and 500 µL transfection mix. Three hours

following transfection, the transfection mixture was supplemented with an equal volume of alpha-MEM containing 20% FBS and incubated overnight at 37°C with 5% CO_2. 24 hours later, the media was replaced with DM and samples were harvested at indicated time points beginning from day 0 (the day of induction of differentiation) up to day 4 post differentiation, with the DM replaced every other day. siRNA sequences used for transfection were as follows:

Nonspecific control (NSP)	5'-AAA CUC UAU CUG CAC GCU GAC-3' and 5'-GUC AGC GUG CAG AUA GAG UUU-3'
USP19 #7	5'-AAG GGU GGU CUU CUA CAG UUG-3' and 5'-CAA CUG UAG AAG ACC ACC CUU-3'
USP19TMD #1	5'-CGU AUU CUA UCC UCU GGU AUC UCA G-3' and 5'-AAC AAG UCU CCC ACC AGA UAC AUA CAC-3'
USP19TMD #2	5'-GUA UGU AUC UGG UGG GAG ACU UGT T-3' and 5'-CAC GGU ACC CAG GAC AAA GUA UCG GAG-3'
USP19ΔTMD #1	5'-UGC UAA UCG ACC UCC UCC AUG UUG CUG-3' and 5'-GCA ACA UGG AGG AGG UCG AUU AGC A-3'
USP19ΔTMD #2	5'-CUG GCU CUA CCA AUG GGA AUU UG-3' and 5'-CCC AAA UUC CCA UUG GUA GAG CCA GUG-3'

2. Western Blotting

Cells were washed once in PBS and lysed directly in the well in 250 µL of 50 mM Tris-HCl, pH 7.5, containing 2% SDS followed by scraping to collect the lysate. DNA in the lysate was sheared by passing 3 times through a 1 mL syringe with a 23G needle and lysates were cleared on a table-top microcentrifuge (Beckman) at 12,500 rpm for 15 minutes. Sample protein concentration was quantified by BCA Micro Protein Assay (Thermo Fisher Scientific). 20 µg to 40 µg protein from each sample was subjected to SDS-PAGE and the proteins were transferred onto 0.45µm nitrocellulose or PVDF membranes for Western blotting. Membranes were probed with antibodies against: myosin heavy chain (MHC; MF20, Developmental Studies Hybridoma Bank), γ-tubulin (Sigma Aldrich), tropomyosin (CH1; Developmental Studies Hybridoma Bank), USP19 [215], USP19 (Santa Cruz Biotechnology), GFP (Sigma Aldrich), myogenin (F5D; Santa Cruz Biotechnology), FLAG (M2; Sigma Aldrich), USP9X (Cedarlane), AKT (Cell Signaling), pAKT(Ser478) (Cell Signaling), CHOP (GADD153; Santa Cruz Biotechnology). Bound primary antibodies were detected using either horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL (Thermo Fisher Scientific) and imaged using the Versadoc cooled digital camera (BioRad) or with an iodinated secondary antibody followed by exposure to a phosphorimager screen and analysis with a Typhoon phosphorimager (GE Healthcare). The latter was used to avoid signal saturation during fine quantitation. Protein signals were normalized to tubulin on the same blot to control for loading and membrane transfer variations.

3. RNA analysis by quantitative real-time PCR (qPCR)

RNA was isolated by solubilizing the cells in 4 M guanidium isothiocyanate followed by phenol-chloroform extraction, as described previously [228] and then RNA concentration was quantitated using a NanoDrop LITE spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized using 1 µg of RNA a High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems) and qPCR analysis was performed using SyberGreen Reagents and a ViiA7 qPCR machine (Applied Biosystems). Differential quantitation of target gene expression was analyzed using the delta-CT method, as previously described [229]. The qPCR primer sequences were used were:

USP19	Forward 5'- GTA GTT TCA TTT GGC GAG AC -3' Reverse 5'- CCG ATC ATG CCT CCG TAG TG -3'
USP19TMD:	Forward 5'- GCC CTA CCA CAC CAG ACG AG -3' Reverse 5'- GAC CTC ATC TCC AGC GAC TC -3'
USP19ΔTMD	Forward 5'- CAC TAC GGA GGC ATG ATC GG -3' Reverse 5'- GCC TGG CCA GGG CCT AGT C -3'
CHOP (GADD153)	Forward 5'- TAG CTG AAG AGA ACG AGC GC-3' Reverse 5'- CTG ATG CCC ACT GTT CAT GC-3'
BiP (GRP78)	Forward 5'- ATA CTG GCC GAG ACA ACA C-3' Reverse 5'- GAG GAG ACA CGA AGC AGA C-3'
ATF4	Forward 5'- GCC TAA GCC ATG GCG CTC TT -3' Reverse 5'- GGT CAT GTT GTG GGG CTT TGC -3'

XBP1	Forward 5'- AAC AGA GTA GCA GCG CAG ACT -3' Reverse 5'- AGG CAA CAG TGT CAG AGT CCA -3'
GAPDH	Forward 5'- CAC CAT CTT CCA GGA GCG CG -3' Reverse 5'- CCT TCT CCA TGG TGG TGA AGA C –3'
PBGD	Forward 5'- GAA GTG GAC CTG GTT GTT C -3' Reverse 5'- GTT TTC CCG TTT GCA GAT GG -3'

4. Adenovirus cloning, generation, and infection

cDNA was prepared from 2.5 ug of rat testis extract RNA using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). The non-transmembrane domain (ΔTMD) region of USP19 was amplified from the cDNA using the sequence specific primers shown below, which also insert an AccI restriction site at the C-terminal end. The amplified fragment was then cloned into pGEM-t vector (Promega).

Forward *USP19TMDF2:*	5'- CAC TAC GGA GGC ATG ATC GG -3'
Reverse *USP19noTMDAccR2*:	5'- GAT TCT GCA GTT TGT CTA CGG ACC TGC TAA TCG ACC -3'

The amplified fragment was digested with AccI and ligated into pBluescript (Stratgene) containing rat USP19, which had also been digested with AccI in order to remove its TMD domain. Recombinant adenovirus vector plasmids expressing Flag-tagged USP19ΔTMD were then generated using AdEasy XL

adenoviral vector system (Stratgene), as per manufacturer's protocol. GFP and USP19 WT and C545A mutant expressing adenoviral plasmids were previously generated using the AdEasy XL system. Viruses were amplified in AD293 cells and collected as instructed in the manufacturer's protocol. Viral titer was measured using the AdEasy viral titer kit (Stratagene) according to the manufacturer's protocol. For infection experiments, the same cell culture and seeding procedures were used as outlined above. The following day after seeding in 6-well plates, cells were infected with 1 mL growth medium containing the MOIs of ~ 80-120 for each viral type (infection medium) to achieve similar levels of overexpression. After 2 hours, an additional 1 mL of growth medium was added to each well and the cells were incubated for a further 18 hours. The infection medium was then replaced with differentiation medium (day 0), and subsequently changed every other day. Media and virus volumes were scaled to account for changes in plate well sized and cell number. Cells were collected as described on the indicated days for protein or RNA analysis.

5. Fusion Index determination

Cells were washed twice with PBS and fixed in 95% ethanol/5% acetic acid for 15 minutes. The cells were rinsed once in PBS and stained in 0.2% Trypan blue solution (Sigma Aldrich) for 15 minutes. The cells were then washed twice in PBS and covered with ultra pure 100% glycerol. Stained cells were visualized using a light microscope at 10X magnification. Nuclei were counted manually from three randomly chosen fields per experimental group (a total of

2500-3500 nuclei per experimental group) and the fusion index was determined by calculating the percentage of total nuclei found in myotubes. A myotube is defined as having two or more nuclei indicating myoblast fusion.

6. Immunofluorescence

C2C12 myoblasts were seeded in 24-well plates on glass cover slips at a density of $6E10^4$ in growth medium and incubated overnight. The next day cells were infected with adenovirus as outlined previously. The cells were induced to differentiate in differentiation medium. On the indicated days of differentiation, cells were rinsed once in DMEM containing 10mM HEPES and then fixed in 4% paraformaldehyde for 20 minutes. The cells were then rinsed twice as above followed by a 1 hour incubation in DMEM containing 10 mM HEPES, 2% FBS, 0.1% Triton X-100 and primary antibody against CHOP (1:50, Santa Cruz Biotechnology). Cells were washed three times (10, 5, and 5 minutes) in DMEM containing 10 mM HEPES prior to 1 hour incubation in DMEM containing 10 mM HEPES, 2% FBS and fluorescently labeled goat anti-rabbit (GAR) Alexa-Flor 568 (Invitrogen) secondary antibody. The cells were washed three times as above, incubated for 10 minutes with DAPI in PBS (Invitrogen), rinsed two times with distilled water, and mounted on glass slides using ProLong Gold anti-fade reagent (Invitrogen). Slides were visualized as described using an Axio florescent microscope (40X magnification; Zeiss) and MetaMorph imaging software.

7. Anti-Flag USP19 coimmunoprecipitation and identification of USP19 interacting proteins by mass spectrometry

C2C12 myoblasts were seeded in two 100 mm plates per virus type (GFP and USP19-WT, -CA, -ΔTMD) at a density of $9.5E10^5$ cells per plate and incubated overnight in growth medium. The next day cells were approximately 75% confluent and were infected with 5 mL growth medium containing MOIs 80-120 for each viral type (infection medium). After 2 hours, an additional 5 mL of growth medium was added to each plate and the cells were incubated for a further 20 hours. The infection medium was then replaced with differentiation medium (day 0) and cells were harvested 24 hours later. Cells from duplicate plates were collected and pooled by scraping and centrifugation, washed once in PBS, and lysed in 1 mL of ice-cold immunoprecipitation (IP) lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2% Triton X-100 plus Protease Inhibitor Cocktail (Roche)]. All subsequent steps were carried out at 4°C. Samples were lysed on ice for 30 minutes with vortexing every 10 minutes, and lysates were then cleared in a tabletop microcentrifuge (Beckman) for 20 minutes (13,500rpm). Lysates were pre-cleared with 30 µL of Protein G-plus sepharose (50/50; Santa Cruz Biotechnology) for 1 hour, before incubation with 30 µL of pre-washed anti-Flag (M2) magnetic beads (50/50; Sigma Aldrich) for 3 hours (rotating). Beads were collected using the DynaMag-2 magnet (Sigma Aldrich) and washed 3 times with 20 packed bead volumes of ice-cold IP wash buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl plus Protease Inhibitor Cocktail (Roche)]. Bound complexes were eluted twice by gentle mixing over 30 minutes in 5 packed bead volumes of ice-cold TBS [50 mM Tris-HCl, pH 7.4, 150 mM NaCl]

containing 200 ng/µL 3X Flag peptide (Sigma Aldrich). Eluates were pooled and concentrated by lyophilization followed by suspension in nanopure water and 3X SDS-PAGE loading buffer. Triplicate eluates were submitted for mass spectrometry analysis. Samples were resolved by SDS-PAGE, sliced into 10 identical slices and pooled between triplicate samples, and analyzed using a UHPLC/Agilent Q-TOF 6550 LC-MSMS system. Peptide results were probed for interacting proteins using Scaffold analysis software.

8. Muscle cell differentiation ER-stress treatment

C2C12 myoblasts were seeded in 24-well plates pre coated with 0.2 mg/mL gelatin at a density of $3E10^4$ cells in growth medium and incubated overnight. The next day cells were infected with adenovirus as outlined previously. The following day cells were fully confluent. The infection media was removed, cells were washed once in PBS and then treated with 0.1µM thapsigargin (Tg) in growth media for 30 minutes to induce ER stress. Media was downshifted to DMEM supplemented with 2% horse serum and 1 µg/mL insulin. On the indicated days of differentiation, cells were analyzed for fusion index by fixing and staining cells on the indicated days as outlined previously and imaged under a light microscope (10X).

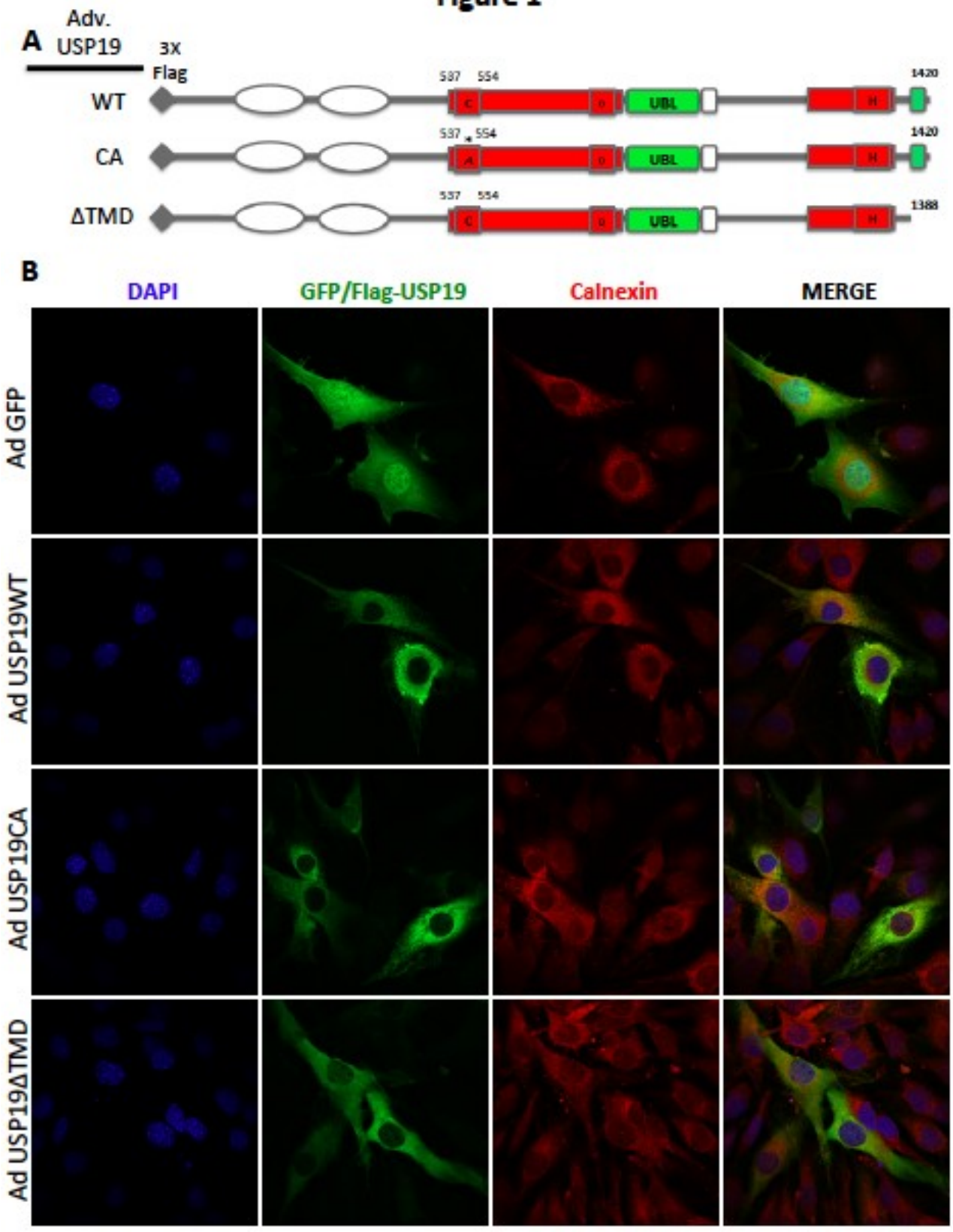
III. RESULTS

1. USP19 catalytic activity and ER localization are required for modulation of major myofibrillar proteins and myogenin in C2C12 myoblasts.

Our previous studies have shown that USP19 depletion increased the expression of a panel of major myofibrillar proteins as well as myogenin, a master regulator of muscle cell differentiation (M. Miao, unpublished data). Work from our group and others characterizing other USP19 functions has demonstrated USP19 functions can be dependent or independent of its DUB catalytic activity [215] [226] [230] [208] [221] as well as requiring or not requiring ER localization [208] [221]. I therefore tested whether overexpressing USP19 in muscle cells would produce the opposite effects in muscle cell differentiation and on muscle specific protein expression and, if so, investigated whether USP19`s ability to modulate muscle cell differentiation was dependent on its DUB catalytic activity, ER localization, or both. To do so, I transduced C2C12 myoblasts with adenovirus expressing Flag-tagged wild type (WT) USP19, a catalytically inactive mutant USP19 (cysteine to alanine mutation of its active site cysteine residue (CA)), a non-ER localized USP19 (replacement of TMD exon sequence with non-TMD exon sequence (ΔTMD)), or GFP control (Figure *1A*). The expected subcellular localization (cytoplasmic or ER localized) of these adenovirus constructs was confirmed by immunofluorescence (IF) and confocal microscopy co-staining for Flag and calnexin, an ER marker (Figure *1B*). As expected, both GFP and non-ER localized USP19ΔTMD staining was diffuse throughout the cytoplasm, whereas the TMD containing, ER-localized USP19 WT and CA displayed a strong mesh-like staining pattern consistent with the ER and co-localized with the

ER marker calnexin (Figure *1B*). Work form our laboratory revealed that L6 muscle cells overexpressing WT USP19 in L6 muscle cells suppresses levels of the major myofibrillar proteins myosin heavy chain (MHC) and tropomyosin (Trpm), suppressed induction of the myogenic regulatory factor myogenin as well as impaired muscle cell fusion in L6 muscle cells (M. Miao, unpublished data). I transduced C2C12 cells as these cells tolerated higher MOI than L6 cells as well as to evaluate whether the effect of USP19 on muscle cell differentiation was cell-type specific. I therefore tested whether these various isoforms would modulate expression of myogenin and myofibrillar proteins. Protein levels of MHC, tropomyosin (Figure *1D & 1F*) as well as the transcriptional regulator, myogenin (Figure *1C & 1E*) were decreased by approximately 50% upon expression of WT USP19 compared to GFP control. Interestingly, overexpression of either USP19 CA or ΔTMD variant abolished the ability to modulate the expression levels of these proteins (Figure *1C-F)*. These results indicate that USP19 requires its catalytic activity and ER localization to suppress myogenic differentiation *in vitro.*

Figure 1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Adv.
USP19
3X
Flag
WT
CA
ΔTMD
537
554
1420
1388
UBL
B
DAPI
GFP/Flag-USP19
Calnexin
MERGE
Ad GFP
Ad USP19WT
Ad USP19CA
Ad USP19ΔTMD

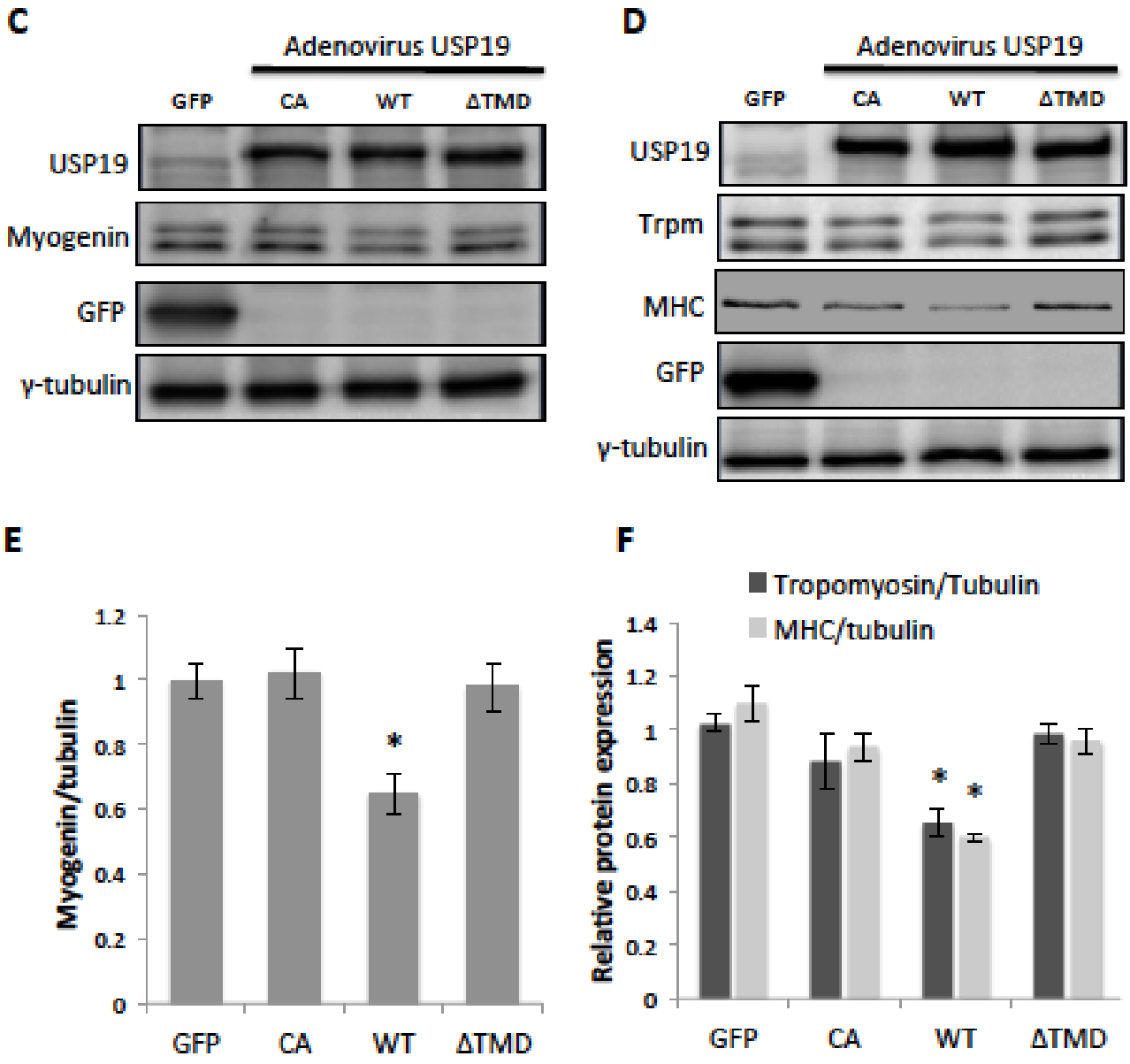

C
Adenovirus USP19
GFP
CA
WT
ΔTMD
USP19
Myogenin
GFP
γ-tubulin
D
Adenovirus USP19
GFP
CA
WT
ΔTMD
USP19
Trpm
MHC
GFP
γ-tubulin
E
Myogenin/tubulin
1.2
1
0.8
0.6
0.4
0.2
0
*
GFP
CA
WT
ΔTMD
F
Tropomyosin/Tubulin
MHC/tubulin
Relative protein expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
GFP
CA
WT
ΔTMD

Figure 1: USP19 catalytic activity and ER localization are required for modulation of myogenin and major myofibrillar proteins. C2C12 myoblasts were infected with adenovirus expressing GFP control or USP19 WT, CA, or ΔTMD to yield similar levels of overexpression. *(A):* Depiction of USP19 protein isoforms overexpressed using adenovirus showing N-terminal 3X Flag tag, catalytic cysteine residue (C540) and C-terminal transmembrane domain (TMD). (*B*): The cells were differentiated for 24 hours and then fixed, probed for Flag (green), the ER-marker calnexin (red) and DAPI (blue) using indirect immunofluorescence and visualized using a confocal microscope (63X-oil magnification). Representative images of triplicate samples shown in (*B*). Cell lysates were prepared 24 hours *(C, E)* or 48 hours *(D, F)* after shifting the cells into differentiation medium and then analyzed by western blotting with the indicated antibodies. *(E):* quantitation of myogenin, *(F):* MHC and tropomyosin protein levels ($n \geq 4$). Quantitation displayed as mean ± SE. Means that are statistically significant compared to GFP with * $P < 0.01$ (ANOVA).

2. USP19 catalytic activity and ER localization are required for modulation of C2C12 muscle cell fusion.

The ability of USP19 to modulate the levels of multiple myofibrillar proteins and the myogenic regulatory factor myogenin suggested that USP19 might modulate muscle cell differentiation. Indeed, previous work silencing USP19 in L6 cells enhanced muscle cell differentiation as shown by increased formation of multinucleated myotubes (M. Miao, unpublished data). Therefore, I evaluated whether overexpressing USP19 could have the opposite effect and inhibit myotube fusion. Moreover, given the observed requirement of USP19 catalytic activity and ER localization to modulate the expression of major myofibrillar proteins and the myogenic regulatory factor myogenin, I also asked whether these attributes of USP19 are required for its ability to modulate muscle cell fusion. As was seen with L6 cells, overexpressing WT USP19 in C2C12 cells delayed myotube fusion denoted by impaired formation of multinucleated myotubes (Figure *2A* & *2B*). Cells infected with adenovirus expressing GFP were ~ 55% fused after 72 hours of differentiation whereas USP19 overexpressed cells were only ~ 20% fused. This difference continued at five days of differentiation, when cells infected with adenovirus expressing GFP were >90% fused compared to only ~45% for cells infected with adenovirus expressing WT USP19 (Figure *2A* & *2B)*. Interestingly, the loss of either USP19 catalytic activity or the transmembrane domain prevented the defect in myotube fusion, with both variants showing similar cell differentiation and fusion index as the GFP control at all time points (Figure *2A* & *2B).* These findings confirm that the ability of USP19

to modulate muscle cell differentiation is dependent on its catalytic activity and ER localization.

Figure 2

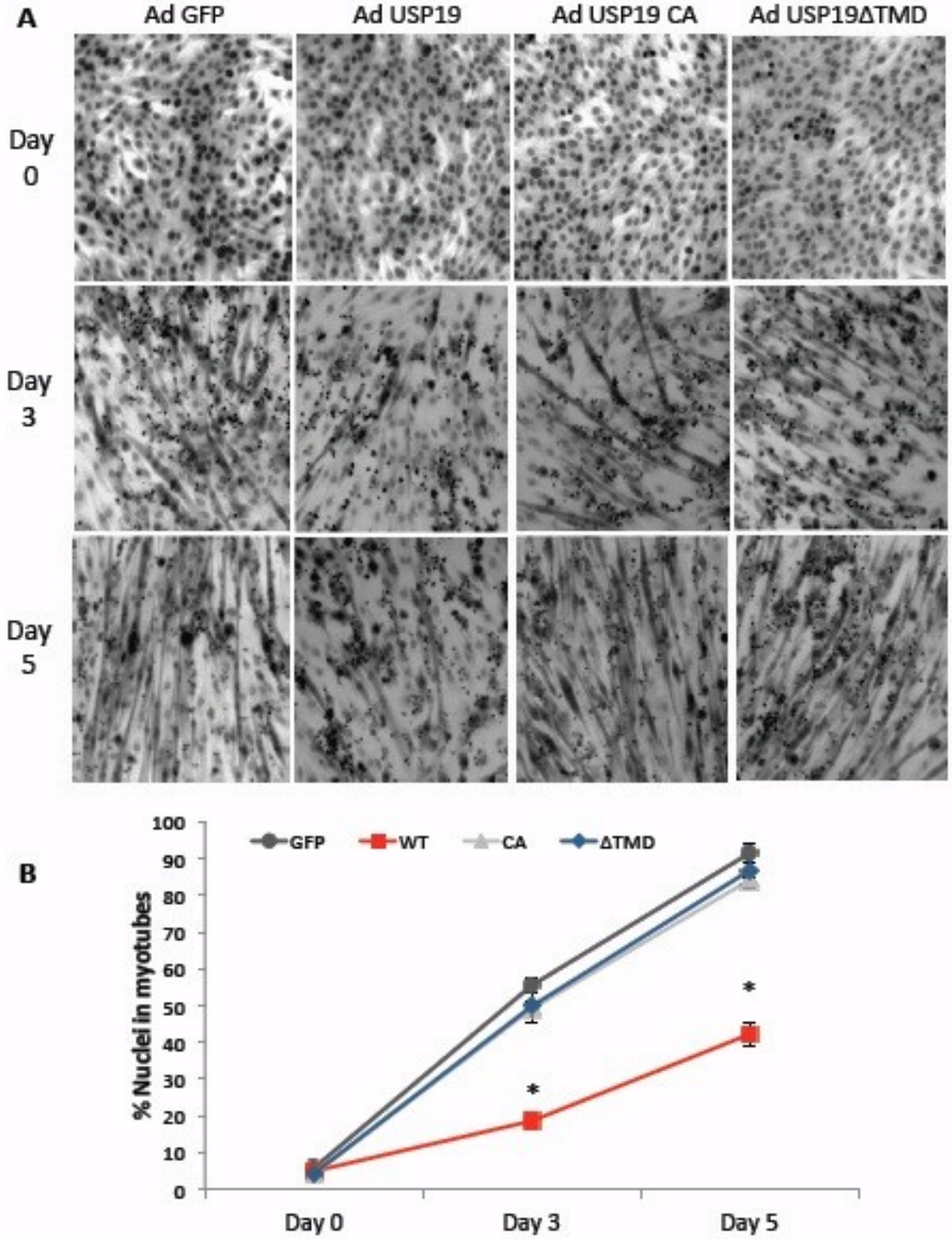

A
Ad GFP
Ad USP19
Ad USP19 CA
Ad USP19ΔTMD
Day 0
Day 3
Day 5
B
GFP
WT
CA
ΔTMD
% Nuclei in myotubes
100
90
80
70
60
50
40
30
20
10
0
Day 0
Day 3
Day 5
*
*

Figure 2: USP19 catalytic activity and ER localization are required for modulation of C2C12 muscle cell fusion. C2C12 myoblasts were infected with adenovirus expressing GFP control or USP19 WT, CA, or ΔTMD to yield similar levels of overexpression and allowed to differentiate for five days. The cells were fixed, stained with trypan blue, and visualized under a light microscope (10X) on the indicated days. Representative samples of cell morphology on day 0, 3 and 5 of differentiation are shown in (*A*). (*B*) The fusion index was determined by counting the nuclei from three randomly selected images per group per day and calculating the proportion of nuclei in myotubes (defined as cells with ≥ 2 nuclei). Means that are statistically significant compared to GFP with * $P < 0.05$ (ANOVA).

3. Isoform specific silencing of USP19TMD but not USP19ΔTMD increases expression of myofibrillar proteins and myogenin.

USP19 is expressed as two isoforms, one containing an extreme C-terminal TMD that confers ER localization and another that is expressed in the cytoplasm, due to the inclusion of either exon 27 or 26, respectively [208]. The USP19 mRNAs encoding these isoforms also possess unique 3'-UTRs. Previous studies demonstrated that USP19 depletion using siRNA oligonucleotides common to both isoforms could increase major myofibrillar protein and myogenin levels, and increase myotube fusion. To confirm the specific requirement of USP19's ER localization for its ability to modulate myofibrillar proteins, myogenin, and myotube fusion, I tested whether specific knockdown of the USP19TMD isoform specifically could reproduce the phenotype observed upon depletion of all USP19 isoforms. Therefore, I transfected L6 myoblasts with a nonspecific control (CTL) siRNA oligonucleotide, or oligonucleotides directed towards the specific C-termini of the TMD or ΔTMD isoforms, or an oligonucleotide targeting both isoforms and induced the transfected cells to differentiate for 48 (Figure *3A, 3B, & 3D)* or 72 hours (Figure *3C & 3E*). Since all available USP19 antibodies recognize both isoforms, the specificity of the TMD and ΔTMD siRNA oligonucleotides towards their intended USP19 isoform(s) were evaluated by qPCR using primers directed towards the 3' end sequence specific for the TMD or ΔTMD isoform (Figure *3A*). Cells transfected with siRNA oligonucleotides common to both USP19 isoforms could lower the transcript level of both isoforms by more than 80% (Figure *3A*). Two independent USP19TMD-specific oligonucleotides specifically lowered the mRNA level of the USP19TMD transcript by ~ 80% without significantly altering

USP19ΔTMD transcript levels, while two independent USP19ΔTMD-specific oligonucleotides specifically lowered the mRNA level of the USP19ΔTMD transcript by ~ 80% and ~ 60% without significantly altering USP19TMD transcript levels confirming the respective oligonucleotide specificities (Figure *3A*). Quantification of total USP19 by western blot revealed a greater than 80% suppression of total USP19 levels when targeting both isoforms or the USP19ΔTMD isoform specifically, and ~ 10-20% knockdown when targeting the USP19TMD isoform specifically, indicating that the major portion of USP19 is located in the cytoplasm. Depletion of all-isoforms of USP19 increased myogenin protein levels by ~ 2 fold (Figure *3B & 3D*) and tropomyosin and MHC protein levels by ~ 3 fold (Figure *3C & 3E*). Indeed, silencing of the TMD containing USP19 isoform could reproduce these effects (Figure *3B, 3C, 3D & 3E*). In contrast, silencing of the USP19ΔTMD isoform by more than 80% at the protein level did not significantly alter the levels of myogenin (Figure *3B & 3D),* tropomyosin or MHC (Figure *3C & 3E*). These results strongly implicate specifically the USP19TMD isoform in suppressing muscle cell differentiation and confirm that the ER localization of USP19 is critical for its functions in muscle cell differentiation.

Figure 3

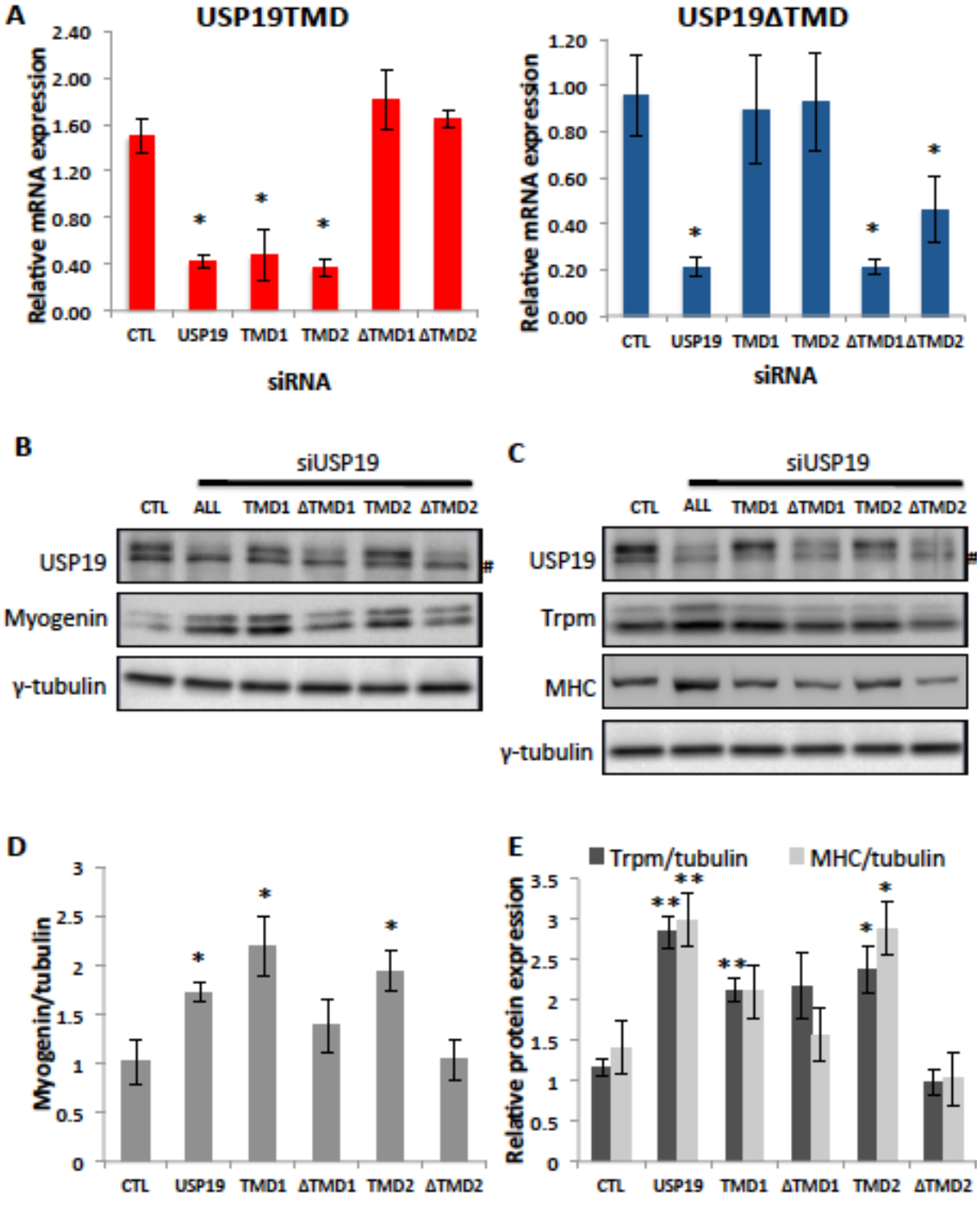

A
USP19TMD
Relative mRNA expression
2.40
2.00
1.60
1.20
0.80
0.40
0.00
CTL
USP19
TMD1
TMD2
ΔTMD1
ΔTMD2
siRNA
USP19ΔTMD
Relative mRNA expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
CTL
USP19
TMD1
TMD2
ΔTMD1
ΔTMD2
siRNA
B
siUSP19
CTL
ALL
TMD1
ΔTMD1
TMD2
ΔTMD2
USP19
Myogenin
γ-tubulin
#
C
siUSP19
CTL
ALL
TMD1
ΔTMD1
TMD2
ΔTMD2
USP19
Trpm
MHC
γ-tubulin
#
D
Myogenin/tubulin
3
2.5
2
1.5
1
0.5
0
CTL
USP19
TMD1
ΔTMD1
TMD2
ΔTMD2
E
Trpm/tubulin
MHC/tubulin
Relative protein expression
3.5
3
2.5
2
1.5
1
0.5
0
CTL
USP19
TMD1
ΔTMD1
TMD2
ΔTMD2

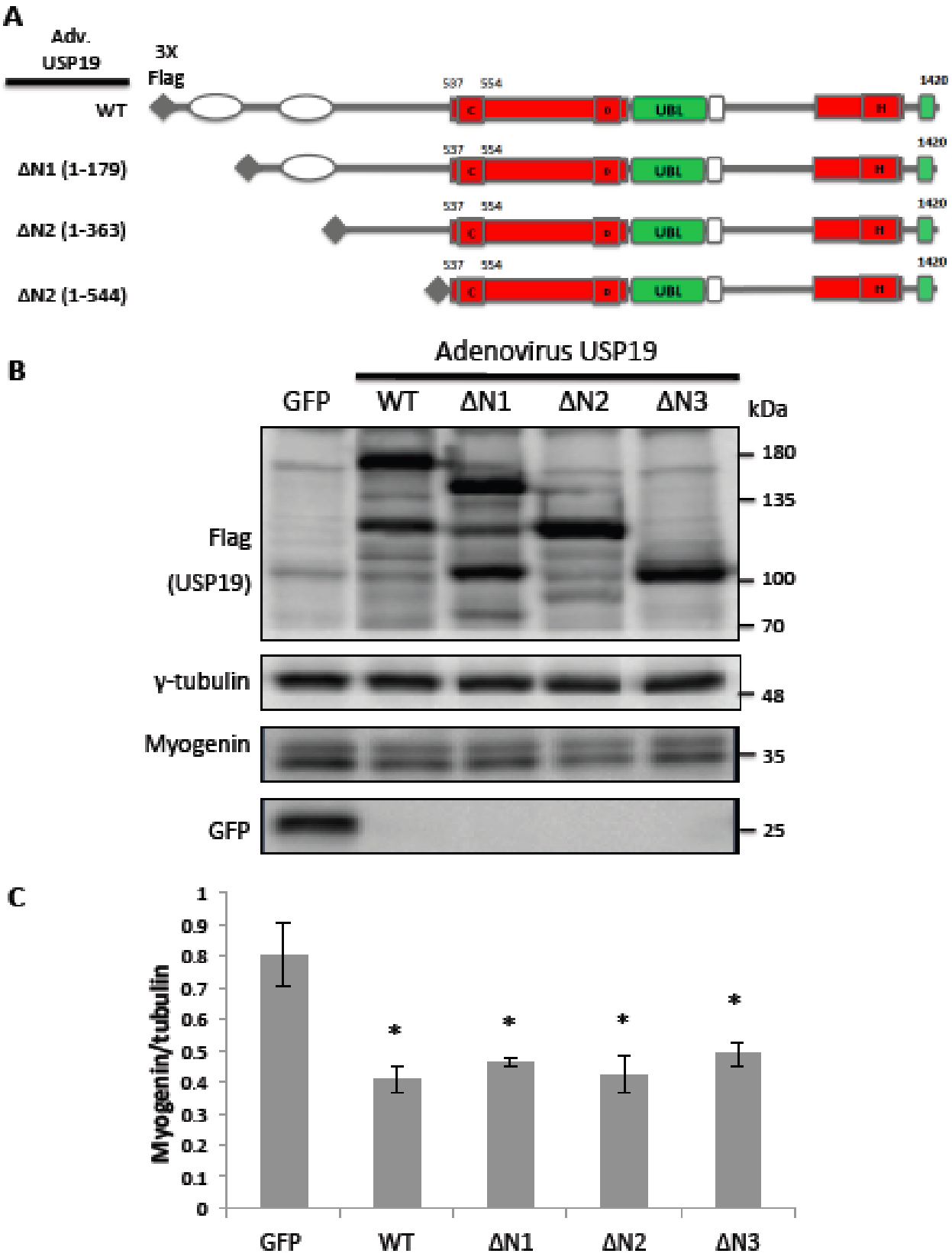
Figure 3: Isoform specific siRNA silencing of USP19TMD but not USP19ΔTMD increases expression of myofibrillar proteins and myogenin. L6 myoblasts were transfected with control (CTL) siRNA oligonucleotides or oligonucleotides towards both isoforms of USP19 (ALL), the TMD isoform, or the ΔTMD isoform and differentiated for the indicated days. (*A*): RNA was harvested after 48 hours differentiation and the relative mRNA expression for each USP19 isoform was measured by qPCR to assess siRNA specificity. Cell lysates were prepared 48 hours *(B, D)* or 72 hours *(C, E)* after shifting the cells into differentiation medium and then analyzed by western blotting with the indicated antibodies. # denotes non-specific band. *(D):* quantitation of myogenin, *(E):* MHC and tropomyosin protein levels. Quantitation displayed as mean ± SE ($n \geq 3$). Means that are statistically significant to CTL with * $P < 0.05$, ** $P < 0.01$ (t-test).

4. USP19 N-terminal structural domains are dispensable for modulating myogenin, but important for inhibiting muscle cell fusion.

USP19 possesses a catalytic core conserved among USP-family DUBs, suggesting this domain likely does not confer substrate specificity. A number of structural domains have been identified N-terminal to the catalytic core domain, including two tandem p23/CS homology domains [208] and an SIAH recognition sequence that [207]. However, the requirement of these and other unidentified N-terminal domains in mediating USP19's effects on myogenesis has not been studied. I therefore transduced myoblasts with adenovirus expressing various USP19 N-terminal deletion mutants to observe whether these would abolish USP19's effects on muscle cell differentiation (Figure *4A*). Interestingly, overexpression of any of the USP19 N-terminal deletion mutants could still suppress myogenin protein to similar levels as WT USP19 expressing cells (Figure *4B & 4C*). I also evaluated whether these various isoforms would modulate the formation of multinucleated myotubes by evaluating the fusion index. As shown above, overexpression of full-length WT USP19 significantly delayed myotube fusion (Figure *5A & 5B*). Cells expressing GFP adenovirus were ~ 50% fused whereas USP19 overexpressed cells were only ~ 15% fused after 72 hours of differentiation. This effect continued at five days of differentiation, where cells infected with adenovirus expressing GFP were ~ 90% fused compared to ~ 40% for cells infected with adenovirus expressing WT USP19 (Figure *5A* & *5B*). Cells expressing USP19 N-terminal deletion mutants showed less inhibition of fusion compared to WT USP19 expressing cells (Figure *5A* & *5B*). At 72 hours of differentiation, cells expressing ΔN1, ΔN2, and ΔN3

USP19 displayed a fusion of ~ 20%, ~ 30%, and ~ 35%, respectively and by five days of differentiation this difference was maintained with fusion indices of ~ 45%, ~ 60%, and ~ 70%, respectively (Figure *5A* & *5B*). These results demonstrate that USP19 N-terminal domains are dispensable for USP19's ability to regulate myogenin expression, but are required for its ability to modulate muscle cell fusion.

Figure 4


A
Adv.
USP19
3X
Flag
WT
ΔN1 (1-179)
ΔN2 (1-363)
ΔN2 (1-544)
537
554
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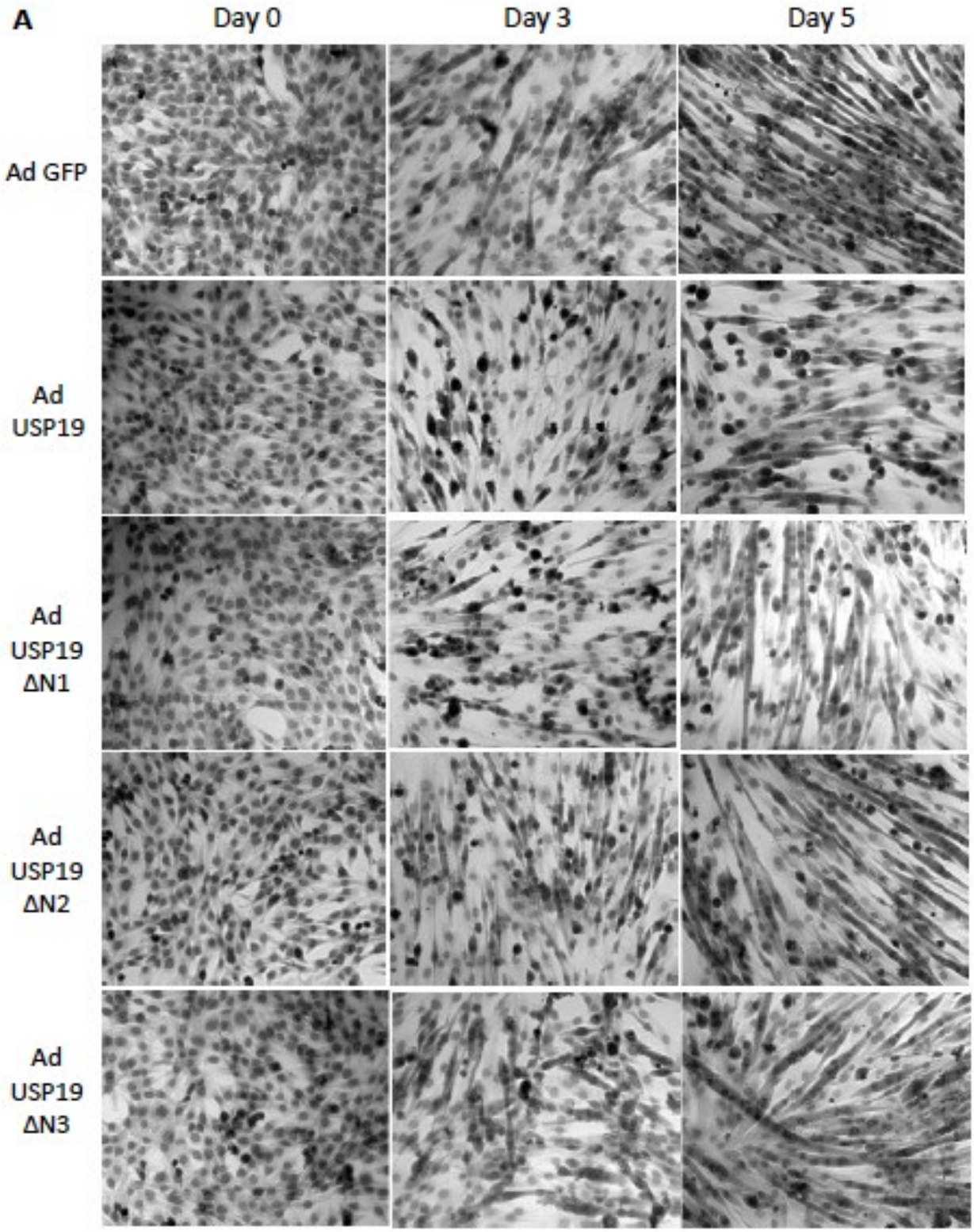
C
D
UBL
H
B
Adenovirus USP19
GFP
WT
ΔN1
ΔN2
ΔN3
kDa
180
135
100
70
48
35
25
Flag
(USP19)
γ-tubulin
Myogenin
GFP
C
Myogenin/tubulin
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
GFP
WT
ΔN1
ΔN2
ΔN3

Figure 4: USP19 N-terminal structural domains are dispensable in modulating myogenin. C2C12 myoblasts were infected with adenovirus expressing GFP control or USP19 WT, ΔN1, ΔN2, or ΔN3 to yield similar levels of overexpression. *(A):* Depiction of USP19 protein WT and N-terminal deletion mutants overexpressed using adenovirus showing N-terminal 3X Flag tag, catalytic cysteine residue (C540) and C-terminal transmembrane domain (TMD). (*B*): Cell lysates were prepared 24 hours*)* after shifting the cells into differentiation medium and then analyzed by western blotting with the indicated antibodies. *(C):* quantitation of myogenin protein levels (*n*=3). Quantitation displayed as mean ± SE. Means that are statistically significant compared to GFP with * $P < 0.05$ (t-test).

Figure 5


A
Day 0
Day 3
Day 5
Ad GFP
Ad USP19
Ad USP19 ΔN1
Ad USP19 ΔN2
Ad USP19 ΔN3

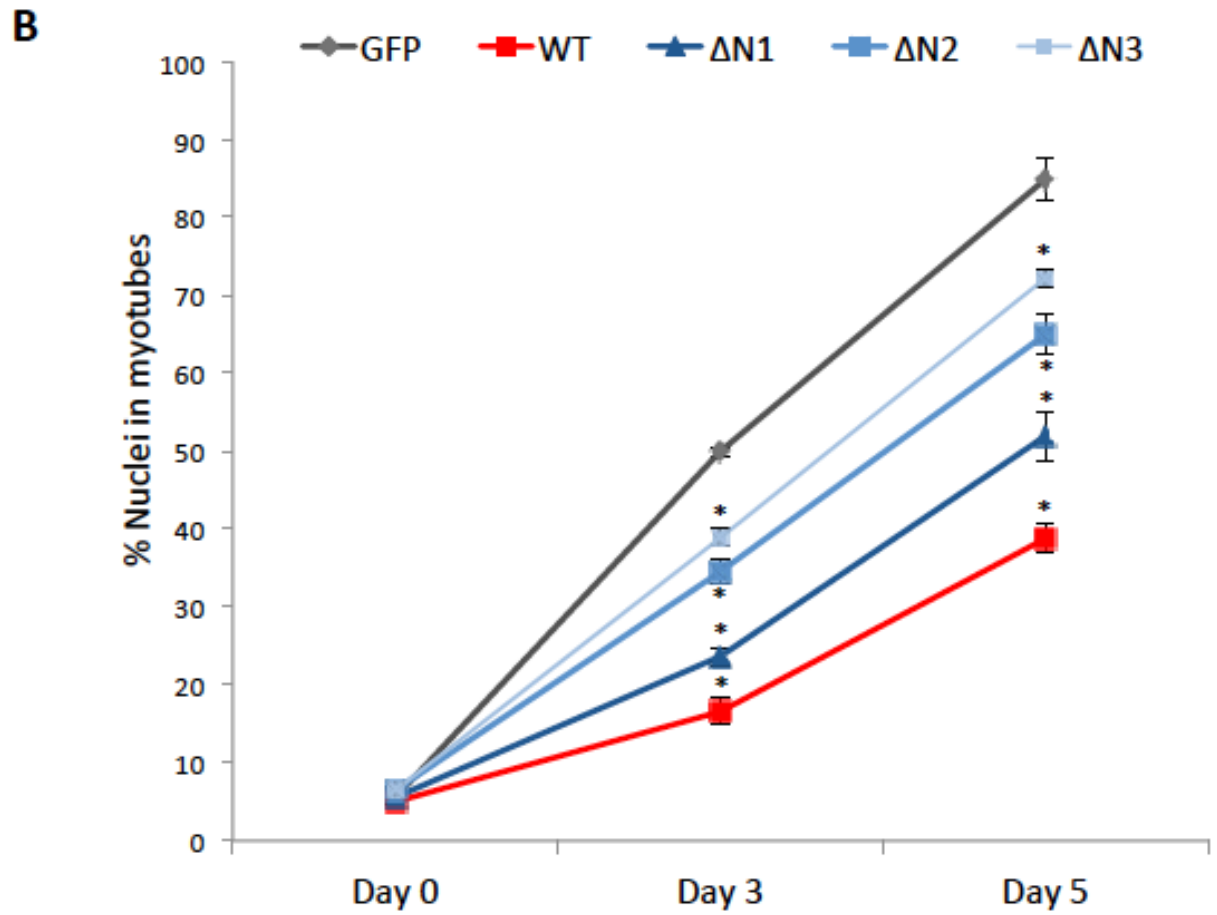


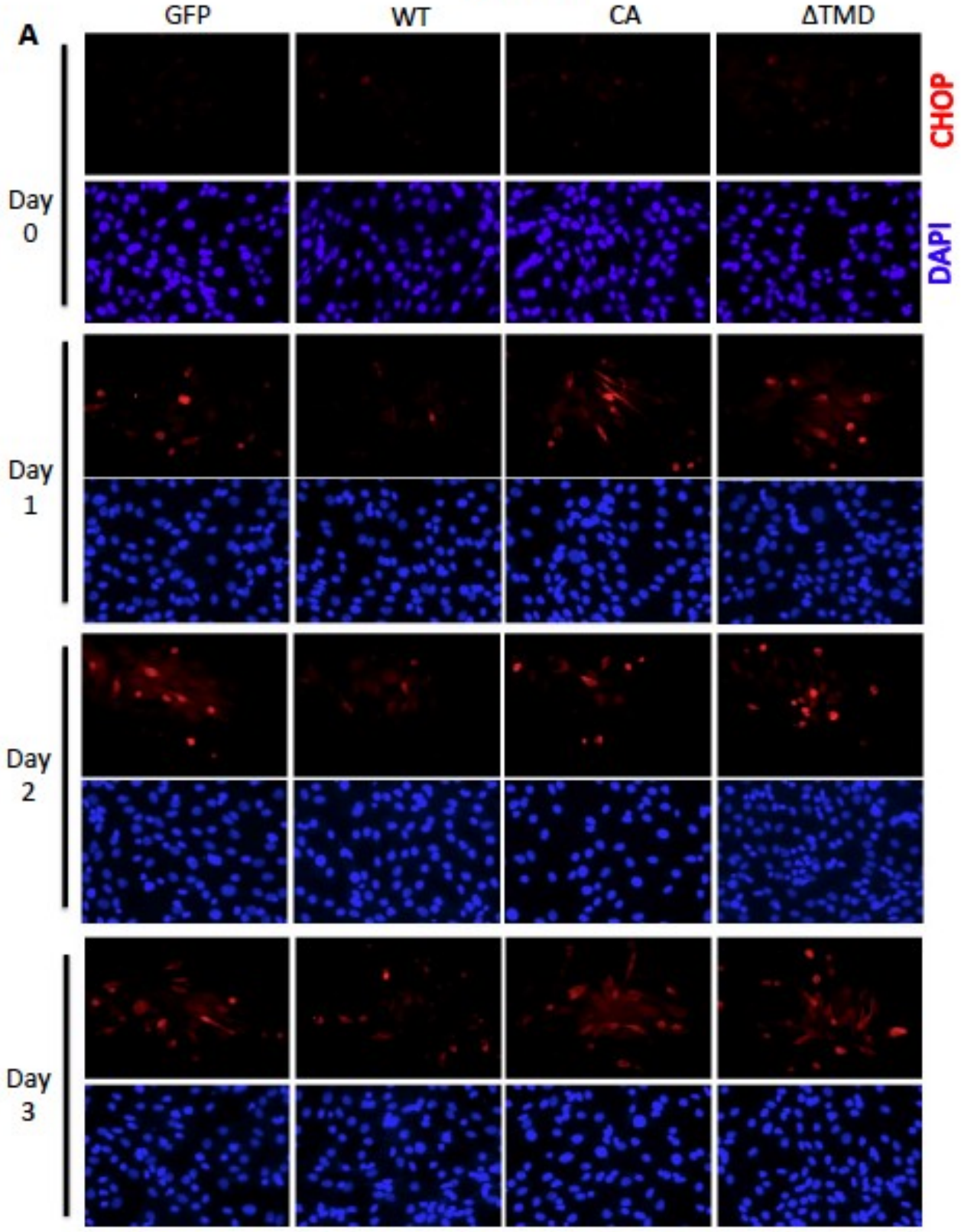
Figure 5: Deletion of USP19 N-terminal domains diminishes its ability to modulate muscle cell fusion. C2C12 myoblasts were infected with adenovirus expressing GFP control or USP19 WT, ΔN1, ΔN2, or ΔN3 to yield similar levels of overexpression and induced to differentiate for five days. The cells were fixed, stained and visualized under a light microscope (10X) on the indicated days. Representative samples of cell morphology on day 0, 3 and 5 of differentiation are shown in *(A)*. *(B)* The fusion index was determined by counting the nuclei from three randomly selected images per group per day and calculating the proportion of nuclei in myotubes (defined as cells with ≥ 2 nuclei). Means that are statistically significant compared to GFP on the same day with * $P < 0.001$ (2-way ANOVA).

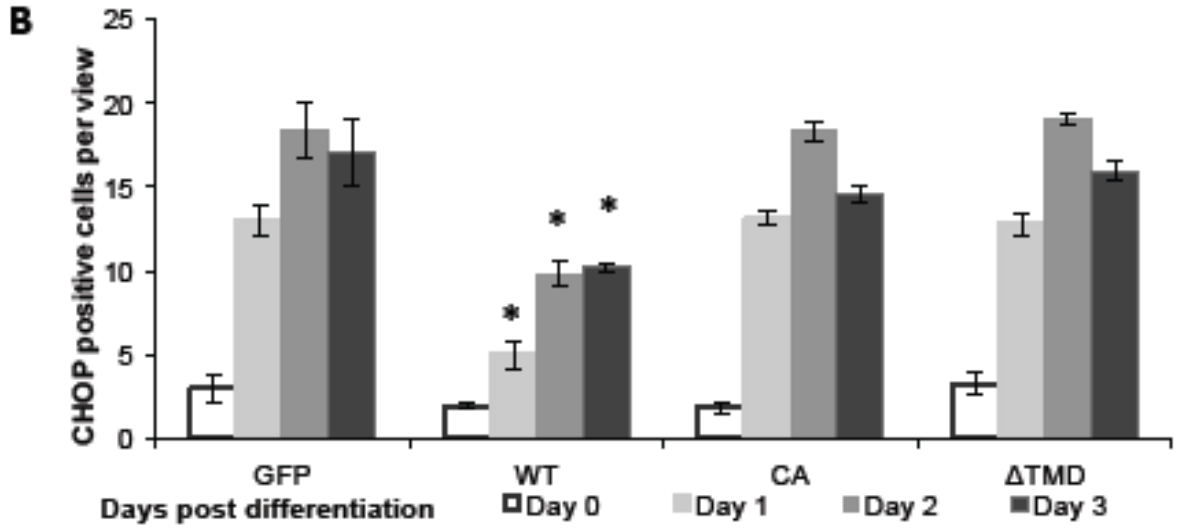
5. USP19 suppresses differentiation-dependent ER stress induction required for myogenesis.

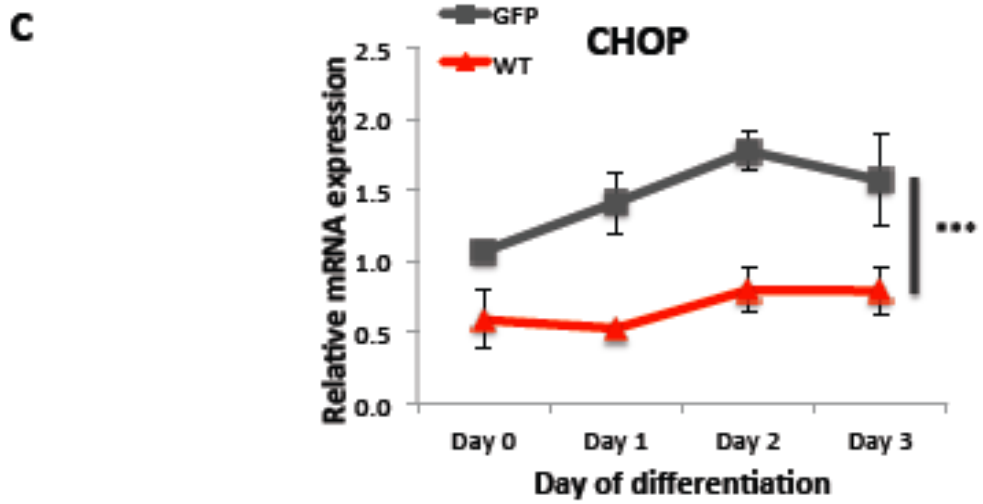
The requirement of USP19 ER localization for its myogenic functions suggested a functional role for USP19 at the ER. Previous work from other groups has characterized a transient induction of ER stress coincident with the differentiation dependent appearance of MHC, as indicated by the induction of the downstream ER stress transcription factor CHOP [201]. This induction of ER stress in a sub-population of myoblasts during the early stages of differentiation, as measured by appearance of the downstream transcription factor CHOP, results in ER stress-induced apoptosis and is required for differentiation [201] [202]. The transient CHOP induction was confirmed in our hands by immunofluorescence in C2C12 cells from day 0 to day 5 of differentiation and the ER stress response was transcriptionally induced in differentiating C2C12 cells from day 0 to day 3 of differentiation as shown by the upregulation of downstream ER stress protein ATF4 and the ER stress sensor BiP/GRP78 at the mRNA level (data not shown). I therefore tested whether USP19 could modulate this differentiation-dependent CHOP induction in an isoform dependent manner. C2C12 cells expressing WT USP19 had a suppressed induction of CHOP from day 0 to day 3 of differentiation compared to GFP expressing control cells (Figure *6A, 6B*). The ability of USP19 to regulate this ER stress induction was in a DUB and ER localization-dependent manner, as cells expressing either a catalytically inactive (CA) USP19 mutant or the non-ER localized USP19 isoform (ΔTMD) showed an induction of CHOP that was similar to that of GFP expressing control cells

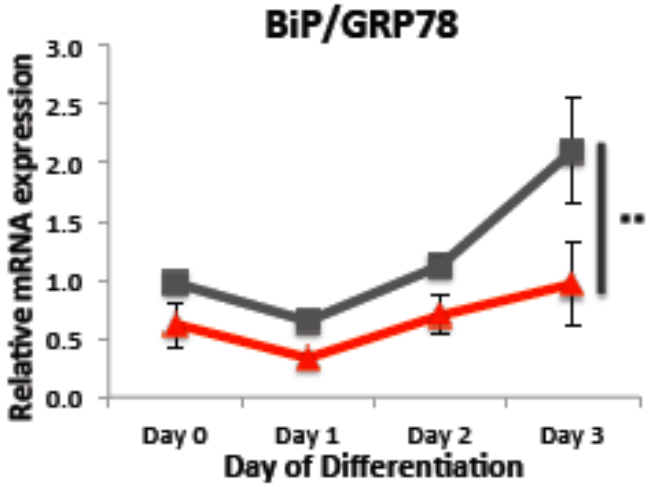
(Figure *6A & 6B*). USP19's suppression of ER stress did not appear limited to suppression of CHOP, as the transcriptional ER stress response in early differentiation (from day 0 to day 3) was suppressed in WT expressing cells (Figure *6C*). WT USP19 expressing cells showed significantly blunted mRNA levels of CHOP, ATF4, and BiP/GRP78 over the early days of muscle cell differentiation compared to GFP expressing control cells (Figure *6C*).

Figure 6




B
CHOP positive cells per view
25
20
15
10
5
0
GFP
WT
CA
ΔTMD
Days post differentiation
Day 0
Day 1
Day 2
Day 3


C
GFP
WT
CHOP
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
***
Day 0
Day 1
Day 2
Day 3
Day of differentiation


BiP/GRP78
Relative mRNA expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
Day 0
Day 1
Day 2
Day 3
Day of Differentiation

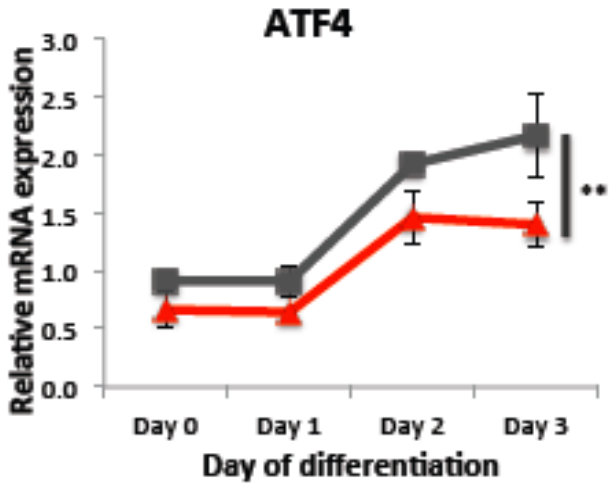
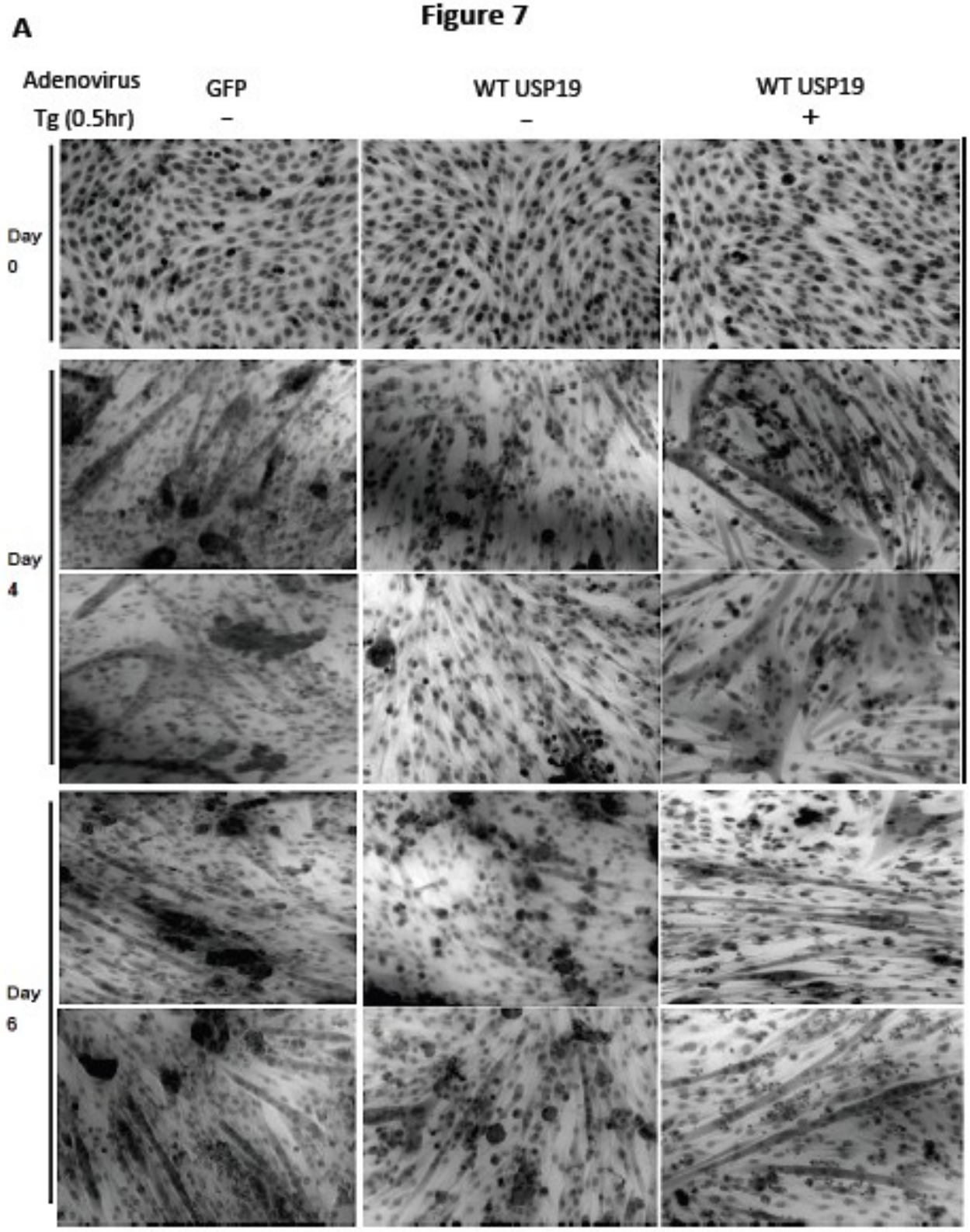

ATF4
Relative mRNA expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
Day 0
Day 1
Day 2
Day 3
Day of differentiation

Figure 6: USP19 suppresses differentiation-dependent endoplasmic reticulum stress induction required for myogenesis. C2C12 myoblasts were infected with adenovirus expressing GFP control or USP19 WT, CA or ΔTMD to yield similar levels of overexpression and induced to differentiate for the three days. The cells were fixed, probed for CHOP expression using indirect immunofluorescence, and visualized using a florescence microscope (40X magnification) on the indicated days of differentiation. *(A):* Representative images of Indirect immunofluorescence for visualization of CHOP (red) or DAPI (blue) from *day 0* to *day 3* of differentiation. *(B):* Quantification of CHOP positive cells per view; displayed as average count from 5 images per group (n=3). Shown are means ± SE. Means that are statistically significant compared to GFP with * $P < 0.01$ (2-way ANOVA). (*C*): RNA was harvested on the indicated days of differentiation and the relative mRNA expression for the indicated genes was measured by qPCR. Means that are statistically significant compared to GFP with ** $P < 0.01$ and *** $P < 0.001$ (2-way ANOVA).

6. ER-stress treatment in USP19 overexpressing muscle cells rescues the defect in myotube fusion.

These results suggest USP19 inhibits muscle cell differentiation by suppressing the differentiation-dependent induction of the ER stress response required for myogenesis. To test this possibility, I evaluated whether the fusion defect observed in WT USP19 expressing muscle cells could be reversed by treatment with low concentrations of chemical ER stress inducers, such as thapsigargin (Tg, inhibitor of an ER-specific calcium ATPase). As seen previously, overexpressing WT USP19 in C2C12 cells significantly delayed myotube fusion denoted by impaired formation of multinucleated myotubes (Figure *7A & 7B*). Cells infected with adenovirus expressing GFP were ~ 80% fused after 4 days of differentiation whereas USP19 overexpressed cells were only ~50% fused (Figure *7A* & *7B)*. Both of these fusion indices were higher than seen in previous experiments because of growth on gelatin coated plates, which appears to enhance the rate of fusion. Interestingly, treating WT USP19 expressing cells with low concentrations of Tg reversed the fusion defect. WT USP19 Tg treated cells were > 75% fused at 4 days of differentiation whereas WT USP19 untreated cells were only < 50% fused (Figure *7A & 7B*). After 6 days of differentiation WT USP19 Tg treated cells maintained significantly enhanced myotube fusion compared to WT USP19 untreated cells (Figure *7A & 7B*). Moreover, the myotubes formed by the untreated WT USP19 expressing cells were thinner, shorter and less sheet-like (Figure *7A*). Thus, the ability of USP19 to modulate fusion appears to be via its effects on ER stress.

Figure 7



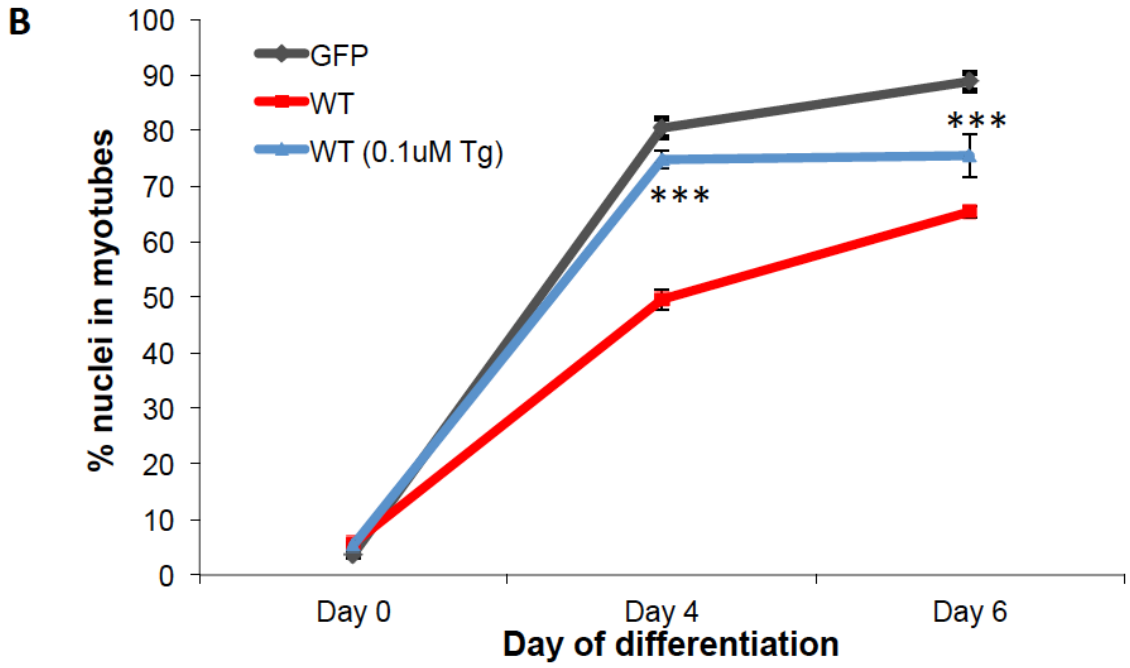


Figure 7: ER-stress treatment in myoblasts overexpressing USP19 recovers the defect in myotube fusion. C2C12 myoblasts were seeded on gelatin-coated plates (0.2 mg/mL) and infected with adenovirus expressing either GFP control or USP19 WT to yield similar levels of overexpression. Following infection cells were treated with 0.1μM thapsigargin (*Tg*) for 30 minutes and differentiated for the indicated days. (*A*): The cells were fixed, stained and visualized under a light microscope (10X) on the indicated days. Representative samples of cell morphology on day 0, 4 and 6 of differentiation are shown. (*C*): The fusion index was determined by counting the nuclei from three randomly selected images per group per day and determining the proportion of nuclei in myotubes (defined as cells with ≥ 2 nuclei). Means that are statistically significant between WT and WT (Tg) on same day with *** $P < 0.001$ (2-way ANOVA).

7. USP19 mRNA and protein expression are regulated during muscle cell differentiation in an isoform specific manner.

The modulation of myogenin expression and cell fusion by USP19 suggested that it likely plays a role in overall regulation of muscle differentiation. Given these observations, I asked whether USP19 mRNA and protein levels are regulated during C2C12 differentiation. Previous work from our laboratory revealed a regulation of USP19 mRNA and protein during differentiation in L6 muscle cells (M. Miao, unpublished data). Specifically, USP19 mRNA and protein were upregulated approximately four fold and 1.5 fold, respectively, from day 0 to day 5 of differentiation. Since the ER localized isoform is critical in modulating both myotube fusion and regulating expression of myofibrillar proteins as well as the use of C2C12 cells for USP19-adenovirus studies, I explored whether USP19 isoforms are differentially regulated during differentiation in C2C12 cells. Since available USP19 antibodies cannot discriminate between the individual isoforms, I profiled total USP19 protein levels by western blot and the isoform-specific expression of USP19 mRNAs by qPCR over day 0 to day 6 of differentiation in C2C12 cells. USP19 protein expression was increased ~1.6 fold over differentiation, similar to what was observed in L6 cells (Figure *8A* & *8B).* Using isoform-specific primers directed towards the unique C-termini of the USP19 isoforms, the non-ER localized isoform displayed a more robust upregulation over differentiation compared to the TMD-containing isoform increasing ~ 2.5 fold and ~ 1.4 fold, respectively (Figure *8C).* These results suggest the non-ER localized isoform of USP19 is preferentially induced during muscle cell differentiation.

Figure 8

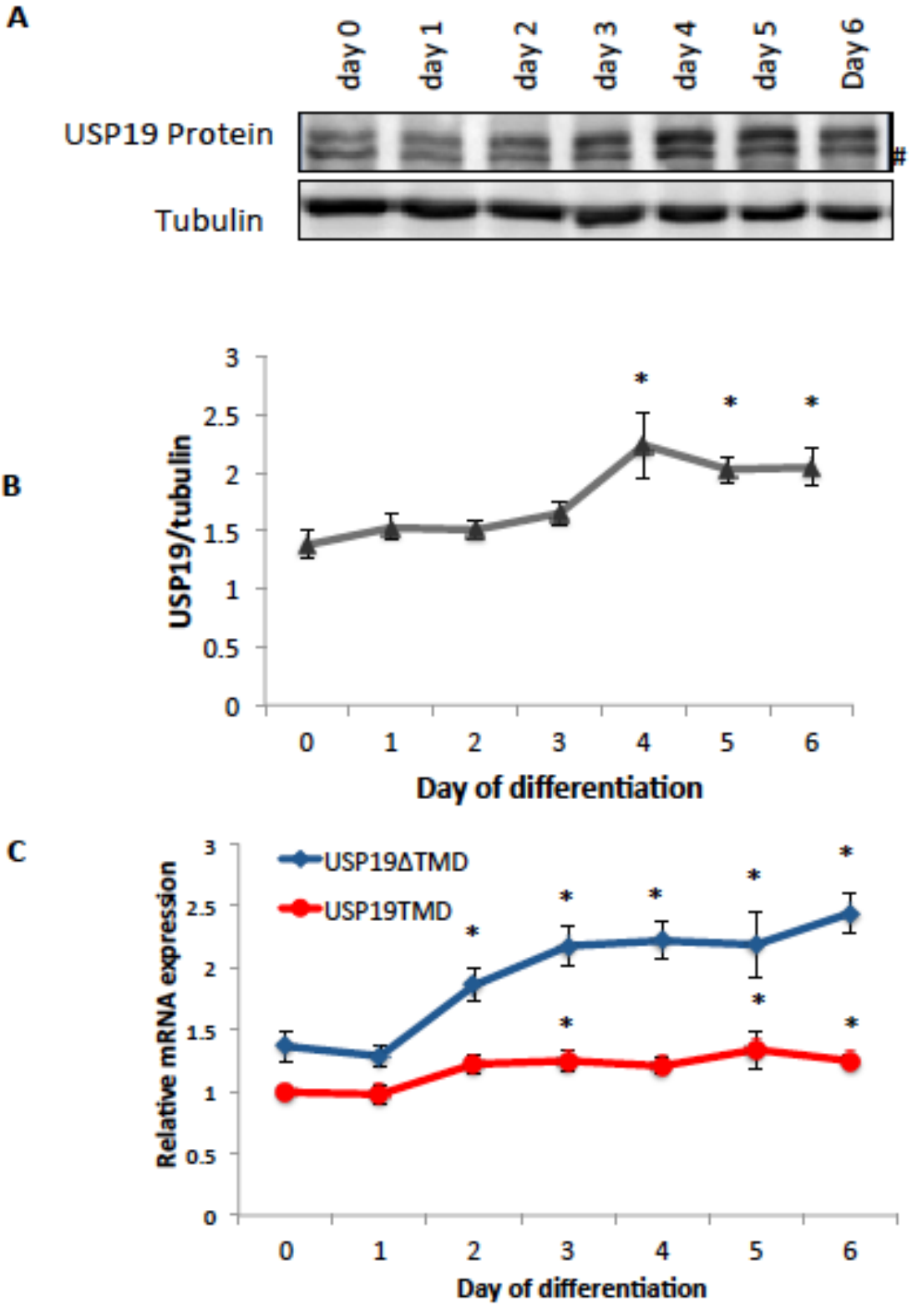

A
day 0
day 1
day 2
day 3
day 4
day 5
Day 6
USP19 Protein
Tubulin
#
B
USP19/tubulin
3
2.5
2
1.5
1
0.5
0
0 1 2 3 4 5 6
Day of differentiation
C
USP19ΔTMD
USP19TMD
Relative mRNA expression
3
2.5
2
1.5
1
0.5
0
0 1 2 3 4 5 6
Day of differentiation

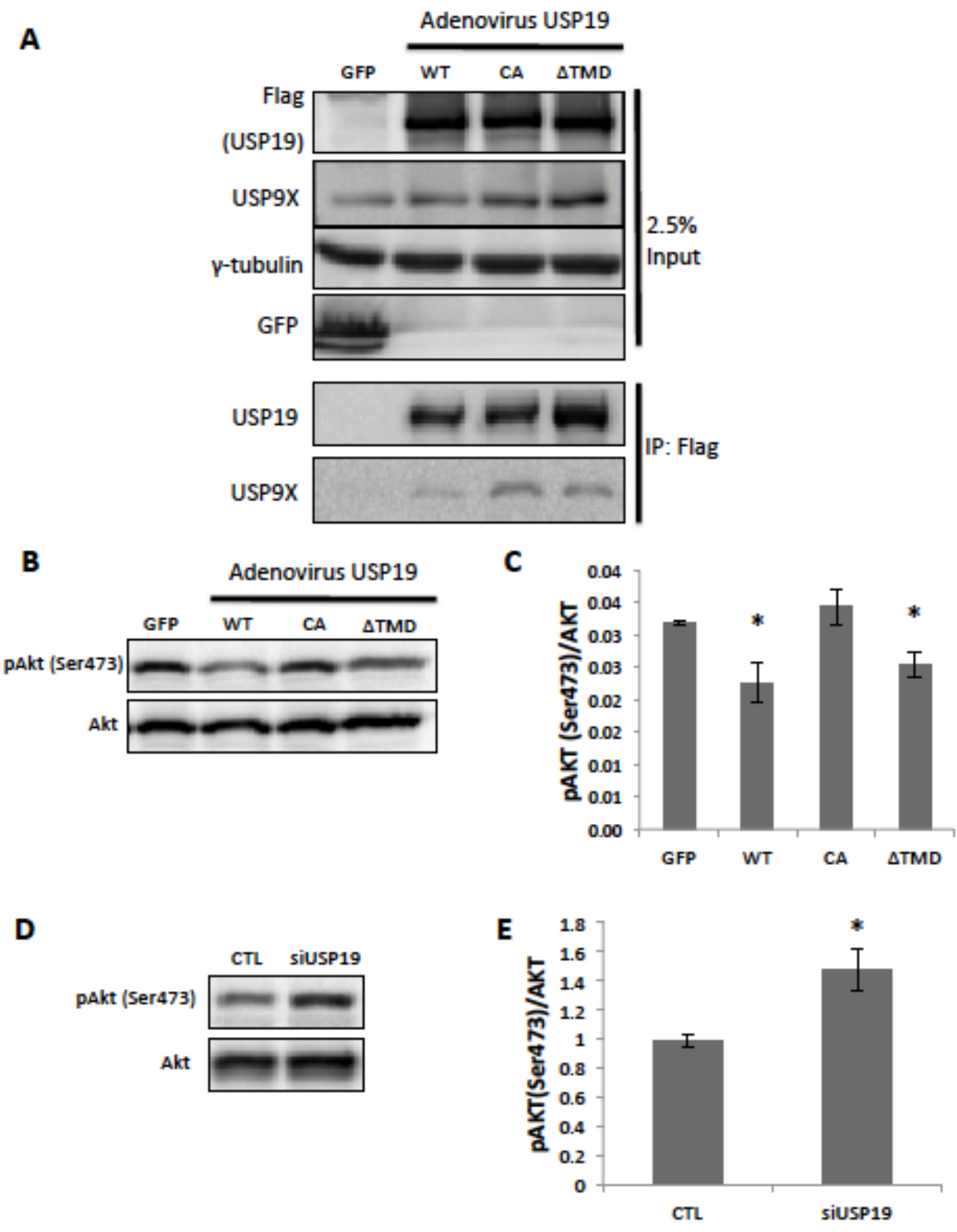
Figure 8: USP19 mRNA and protein expression are regulated during muscle cell differentiation in an isoform specific manner. C2C12 myoblasts were plated and induced to differentiate for six days. mRNA and protein from the samples were extracted fro the cells on the indicated days. (*A*): Representative western blot of USP19 protein from *day 0* to *day 6* of differentiation. # denotes non-specific band. (*B*): Quantitation of USP19 protein levels in (*A*) (*n*=6). Shown are means ± SE. Means that are statistically significant compared to day 0 with * $P < 0.01$ (ANOVA). (*C*): Quantitative PCR for USP19 isoforms from *day 0* to *day 6* of differentiation (*n*=6). Shown are means ± SE. Means that are statistically significant compared to day 0 with * $P < 0.05$ (ANOVA).

8. USP19 suppresses phosphorylated Akt levels *in vitro* requiring its DUB catalytic activity but independent of ER localization.

To better understand the function of USP19 in regulating muscle cell differentiation and muscle atrophy, it is necessary to identify its substrates. To this end, I identified proteins that can interact with the USP19 isoforms. The CA mutation may serve to trap substrates and our studies show an important role for ER localization. Therefore, we were particularly interested in proteins that would preferentially associate with the CA mutant and would be less abundant in the ΔTMD eluate. I performed an vitro co-immunoprecipitation (IP) from C2C12 cells gently overexpressing Flag-tagged USP19 WT, CA, or ΔTMD variant or GFP control followed by mass spectrometry of the eluates. Indeed, a number of interesting candidate proteins were identified (data not shown). One intriguing candidate substrate was the USP-family DUB USP9X, which was stabilized in CA USP19 expressing cells compared to both WT and ΔTMD expressing cells. USP9X suppresses the IGF1/PI3K/mTOR/Akt signaling cascade through inhibition of mTOR, resulting in lower levels of activated (phosphorylated) Akt (pAkt(Ser473)), suppressed myogenin and MHC expression concomitant with inhibition of myotube fusion [231], a phenotype strikingly similar to WT USP19 overexpressing cells (Figure *1B-D,* Figure *2A-B*). Given the suppression of myotube fusion by USP19, I hypothesized that USP19 may stabilize USP9X resulting in the inhibition of mTOR inhibition and suppression of myotube fusion. I first evaluated the interaction by co-IP in cells expressing GFP control or flag-tagged USP19 variants, confirming the interaction of USP9X with all USP19 isoforms compared to control cells (Figure *9A*). I then asked whether modulation

of USP19 levels by overexpression or silencing could modulate the levels of pAkt(Ser473). Overexpression of either WT (ER localized) or ΔTMD (non-ER localized) USP19 in C2C12 muscle cells suppressed pAkt(Ser473) levels (Figure *9B-C)*. This suppression of pAkt(Ser473) is dependent on its catalytic activity, as pAkt(Ser473) levels were unchanged in muscle cells expressing CA USP19 compared to control GPF expressing cells. siRNA-mediated USP19 depletion in L6 muscle cells caused increased pAkt(Ser473) levels confirming a role for USP19. These results suggest that USP19 may bind and stabilize USP9X through its DUB catalytic activity leading to inhibition of IGF1/PI3K/mTOR/Akt growth signaling in muscle.

Figure 9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WT
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(USP19)
USP9X
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GFP
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IP: Flag
B
Adenovirus USP19
GFP
WT
CA
ΔTMD
pAkt (Ser473)
Akt
C
pAKT (Ser473)/AKT
0.04
0.04
0.03
0.03
0.02
0.02
0.01
0.01
0.00
*
*
GFP
WT
CA
ΔTMD
D
CTL
siUSP19
pAkt (Ser473)
Akt
E
pAKT(Ser473)/AKT
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
CTL
siUSP19

Figure 9: USP19 modulates phosphorylated AKT (Ser478) levels *in vitro* requiring its DUB catalytic activity but independent of ER localization. *(A):* C2C12 myoblasts were infected with adenovirus expressing either GFP control or USP19 WT, CA or ΔTMD to yield approximately five fold overexpression and induced to differentiate for 24 hours. Protein levels in input and following co-IP analyzed by western blot. (*B*): C2C12 myoblasts were infected with adenovirus expressing either GFP control or USP19 WT, CA or ΔTMD and differentiated for 48 hours. Lysates were analyzed for phosphorylated-Akt (Ser473) and total Akt levels by western blot. (*C):* Quantitation of protein levels in (*B*) (*n*=3). Shown are means ± SE. Means that are statistically significant compared to GFP with * $P < 0.05$ (ANOVA). *(D):* Cells were transfected with control (CTL) siRNA oligonucleotides or oligonucleotides targeting USP19 and differentiated for 72 hours. Lysates were analyzed for phosphorylated-Akt (Ser473) and total Akt levels by western blot. (*E):* Quantitation of protein levels in (*D*). Shown are means ± SE (*n*=3). Means that are statistically significant compared to compared to CTL with * $P < 0.05$ (t-test).

IV. DISCUSSION

Previous work from our laboratory has demonstrated a role for the USP19 deubiquitinating enzyme in skeletal muscle atrophy. We first observed that USP19 is induced in rat skeletal muscle atrophying in response to multiple catabolic conditions [1]. More recently, our laboratory has generated mice lacking USP19 and found that these mice lose less muscle mass in response to fasting, glucocorticoids, or denervation (manuscript in preparation) confirming an important role for USP19 in muscle wasting. In addition, we had previously reported that USP19 depletion in muscle cells increases the expression of a panel of major myofibrillar proteins and that it does so by inducing the expression of the myogenic regulatory factor myogenin [2]. Furthermore, silencing USP19 in these cells also prevented glucocorticoid induced myofibrillar protein catabolism. Although these findings would be consistent with the sparing of muscle loss seen in the knockout mice, the effects on myogenin also raised the possibility that USP19 modulates muscle cell differentiation. Indeed, using both overexpression and loss of function approaches, I have demonstrated in this thesis that USP19 can indeed modulate differentiation of muscle cells in vitro as reflected by changes in expression of myogenin, myofibrillar proteins as well as in the rate of myoblast fusion. These observations were seen in both rat L6 muscle cells as well as mouse C2C12 muscle cells indicating that these effects are not cell line dependent.

Although this regulation of muscle cell differentiation may be viewed as distinct from its effects on muscle size observed in our KO mice, recent work

from other groups has suggested that suppressed or dysregulated myogenesis can contribute to skeletal muscle atrophy [232] as well as a delay in recovery following muscle injury [233] [234]. Thus, it is possible that the USP19 induction *in vivo* under atrophic conditions may result in impaired myoblast fusion in the muscle and thereby contributes to muscle atrophy. These results provides further support for the hypothesis that targeting USP19 therapeutically may be an effective approach to prevent or treat muscle wasting or to enhance the rate of recovery of muscle mass following injury.

In addition to characterizing a novel function for the USP19 deubiquitinating enzyme, I have identified structural requirements for this function. Specifically, I have demonstrated for the first time that USP19's myogenic functions are isoform-specific and dependent on its catalytic activity. Although a requirement for catalytic activity may not be surprising, other researchers have shown that USP19 can stabilize the levels of substrates such as HIF-1α [221] or cIAP1 and 2 [226] in the absence of catalytic activity. These conclusions were based on the observations that overexpressing both active and catalytically inactive USP19 was equally effective in preventing degradation of the substrates. These observations may be related to overexpression of the enzyme whereby the artificial high levels of the inactive DUB simply sequesters the substrate away from interaction with its ubiquitin ligase. In the case of USP19 modulation of muscle cell differentiation, deubiquitination of some presently unidentified substrates appears essential for this effect. USP19 is expressed as two major

isoforms arising from alternative splicing of the last exon – one isoform that is localized to the ER and the other cytoplasmically localized. Our studies demonstrate clearly using both overexpression and silencing of the specific isoforms that only the ER localized isoform is capable of modulating muscle cell differentiation, thereby suggesting that USP19 deubiquitinates and likely stabilizes a substrate at the ER. The relative contribution of USP19's specific isoforms in driving muscle atrophy *in vivo* remains to be seen, as our KO mice are deficient in all isoforms of USP19. Recently, we have observed that electroporation of plasmids expressing shRNAs against USP19 can protect the transduced myofibers from denervation atrophy (N. Bedard, unpublished data). Thus, it will be of interest to see if targeting specific isoforms can mimic this effect. Overexpression of distinct USP19 isoforms in KO mice muscle via electroporation of plasmid constructs has been attempted, but we have not been able to achieve any significant expression of the catalytically active forms.

Like many other USPs, USP19 contains a conserved core catalytic domain at its C-terminal end, suggesting that the divergent sequences N-terminal to the catalytic domain play important roles in substrate specificity. Indeed, I demonstrated in this thesis that USP19 does require its N-terminus in order to suppress muscle cell differentiation. Surprisingly though, the N-terminus was not required for its regulation of myogenin expression indicating that the catalytic core domain is capable of recognizing the substrate(s) involved in modulating myogenin. Although this core domain is conserved, there are divergent

sequences in this region, which could play roles in substrate specificity and indeed proteins such as Hsp90 have been shown to be recognized by USP19 through its core catalytic domain [163]. The divergent effects of N-terminal deletions on myoblast fusion and myogenin expression raise the possibility that USP19 regulates these two elements through different mechanisms. Alternatively, myogenin levels may become suppressed only upon a robust inhibition of cell fusion not achieved by USP19 N-terminal deletions. The latter possibility could be ruled out by overexpressing WT USP19 in muscle cells at a variety of reduced levels than employed in these studies and observing if the supersession of myogenin expression is reversed concomitant with decreased inhibition of cell fusion. Further work is needed to define more precisely the specific USP19 N-terminal domains critical for each of these myogenic functions. The most obvious domains to evaluate would be the CS domains and the MYND domains, as both are known to function as protein-protein interaction domains. More precise deletions and/or mutations of specific amino acids in these regions would be required to test these possibilities.

The molecular mechanism by which USP19 regulates muscle cell fusion remains to be elucidated. The requirement of USP19's TMD for its modulation of myogenin and myotube fusion suggests that the enzyme exerts a function at the ER. In this thesis I demonstrate the novel finding that USP19 regulates the differentiation dependent ER-stress response in muscle cells. Previous work from other groups has characterized a transient induction of ER stress, as indicated by

the induction of the downstream ER stress transcription factor CHOP, in the early days of muscle cell differentiation [201] [202]. I observed that WT USP19 suppressed this transient induction of CHOP in a DUB activity and ER localization-dependent manner. In addition, other components of the ER stress response such as ATF4 and BiP/GRP78 were also downregulated by USP19 confirming that ER stress was indeed being modulated. Finally but importantly, inducing ER stress with thapsigargin treatment in WT USP19 expressing muscle cells could reverse the morphological fusion defect induced by USP19 arguing that the inhibition of ER stress plays an essential role in the enzyme's effect on myoblast fusion. It is unlikely that USP19 targets CHOP directly, as an interacting partner of USP19 would be stabilized rather than suppressed, indicating USP19 is likely modulating upstream signaling players in ER stress. CHOP is transcriptionally induced predominantly by ATF4, but also through all signaling arms of the UPR (ER stress response) providing a large number of candidates for regulation by USP19. In addition, the stability of CHOP can also be modulated. Recent work in pancreatic beta cells characterized a role of the E3 ubiquitin ligase cIAP1 in promoting the ubiquitination and degradation of CHOP [235] and USP19 was shown to stabilize cIAP1 in non-muscle cells [226]. An interesting hypothesis is that USP19 suppresses CHOP in muscle differentiation by stabilizing cIAP1. However work in our laboratory to date has failed to detect an interaction of USP19 with cIAPs in muscle cells. It would be interesting to explore this further by testing if silencing of cIAP1 in USP19 overexpressing muscle cells

could reverse the CHOP suppression and fusion defects observed in USP19 overexpressing cells.

The molecular mechanism by which USP19 controls myogenin during differentiation is not currently known. Myogenin is also unlikely to be a direct target of USP19, as USP19 would be expected to deubiquitinate and thereby stabilize it rather than lower its levels. Previous work from our laboratory demonstrated that USP19 could modulate myogenin at the transcriptional level [2]. The most straightforward mechanism is one in which USP19 stabilizes a known negative transcriptional modulator of myogenin, such as Id1 [236], MITR [237], Dach2 [238], MSY-3 [239], Hey1 [240] and TSHZ3 [241], or other unknown myogenin transcriptional suppressors. Another possibility is that USP19 modulates signaling pathways that modulate myogenin transcription such as the IGF-1/PI3K/mTOR/Akt pathway. In my studies, I employed a substrate-trapping approach in C2C12 muscle cells to identify interacting partners of USP19. One such protein detected was the USP family DUB USP9X which appeared to be more abundant in the immunoprecipitate of catalytically inactive USP19 compared to WT. USP9X has previously been shown to suppress the IGF1 signaling cascade through inhibition of mTOR, resulting in lower levels of activated (phosphorylated) Akt (pAkt(Ser473)), suppressed levels of myogenin and MHC expression, and impaired myotube fusion [231], a phenotype strikingly similar to WT USP19 overexpressing cells. In this thesis, I observed a novel role for USP19 in suppressing mTOR/pAkt(Ser473) signals, as USP19 depletion in L6

muscle cells increased pAkt(Ser473) levels and overexpression of either WT (ER localized) or ΔTMD (non-ER localized) USP19 in C2C12 muscle cells suppressed pAkt(Ser473) levels. USP19's suppression of pAkt(Ser473) was dependent on its catalytic activity, as pAkt(Ser473) levels were unchanged in muscle cells expressing CA USP19 compared to control GPF expressing cells. Thus, an attractive hypothesis is one in which USP19 modulates myogenin and muscle differentiation through stabilizing USP9X to suppress IGF1/PI3K/mTOR/Akt signaling. To my knowledge, this would be the first demonstration of a DUB regulating the stability of another DUB. Further cell-based assays, such as endogenous and reciprocal immunoprecipitation as well as silencing of USP9X in USP19 overexpressing cells, are required to confirm the direct USP19-USP9X interaction to modulate pAkt levels.

Data presented here is consistent with previous work from our laboratory demonstrating that USP19 expression is regulated during muscle cell differentiation *in vitro.* Total USP19 protein level was upregulated ~50% over muscle cell differentiation in L6 cells, while total USP19 mRNA increased dramatically by 4 fold over the same differentiation time course (M. Miao unpublished data). I have demonstrated for the first time that USP19 mRNA levels are regulated in an isoform-specific manner. Evaluating the expression profile in C2C12 cells, USP19 protein was upregulated by ~60% over six days of differentiation. The increase in USP19 mRNA over six days of differentiation appears to be primarily due to the upregulation of the ΔTMD isoform, increasing

~ 2.5 fold compared to ~ 1.4 fold for the TMD isoform, suggesting that USP19 isoforms are differentially regulated during myogenesis. USP19 is expressed at low levels during early differentiation when myogenic signals are becoming activated, but increased at both the mRNA and protein level at late stages of differentiation when the majority of myoblasts have fused, forming multinucleated myotubes. This indicates that this upregulation of USP19 may inhibit differentiation and act as a fusion termination signal to create an optimal degree of myoblast fusion for myofiber formation. USP19 modulation in muscle cells does not impact the overall levels of cellular ubiquitination, strongly suggesting that USP19 is acting on specific substrates (data not shown). As the TMD isoform appears to be primarily responsible for modulating myogenesis and the mRNA levels of USP19TMD increase only modestly during this process, it is possible that USP19TMD is activated during myogenesis through post-translational modifications or interaction with a substrate whose levels are regulated over myogenesis.

The discrepancy between the degrees of induction of USP19 at the mRNA and protein level strongly suggest that USP19 is post-transcriptionally regulated to reduce its translation, possibly through lessening USP19 mRNA availability, enhanced USP19 protein degradation, or targeting of USP19 mRNA by a myogenic differentiation-induced microRNAs (miRNAs). The emerging roles of miRNAs in regulating myogenesis have received increased focus recently [242], with several miRNAs induced during various stages in myogenesis. Myogenic

miRNAs can be induced directly by MRFs during differentiation and appear to control proliferation by targeting regulators of the cell cycle and alter myogenic signals such as the IGF1 pathway. Moreover, the isoform-specific regulation of USP19 during differentiation by distinct miRNAs is an interesting possibility given their distinct regulation during differentiation and the unique 3'UTRs contained in the TMD containing and non-TMD containing USP19 isoforms. It would be interesting to evaluate if USP19 is modulated by miRNAs in a common or isoform-specific mechanism by evaluating their divergent 3'UTRs for any common or unique sites that are complementary to any known miRNAs modulated during myogenesis.

Collectively, the studies in this thesis present a number of novel USP19 functions, including regulation of muscle cell differentiation, suppression of ER stress during this differentiation, and modulation of IGF-1/PI3K/mTOR/Akt signaling. Moreover, these studies reinforce the importance of DUBs in controlling cellular ubiquitin signals through dynamically regulating the ubiquitination status of cellular proteins. Future studies are required to definitively identify and characterize USP19 substrates, such as detailed analysis of the protein interacting partners discovered using substrate trapping (data not shown). In addition, it is important to delineate the contribution of USP19's effects on myogenesis to the muscle wasting process.

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