Examining the Role of Caspase-2 in Skeletal Muscle Cell Differentiation

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Prior research has indicated a crucial role for apoptotic signaling in skeletal muscle cell differentiation. Although a number of caspases (-3, -8, -9) have been implicated in this process, few prior investigations have identified a role for the most enigmatic member of the caspase family, caspase-2. Due to its unique nuclear localization as well as its purported roles in cell cycle regulation and DNA damage response; caspase-2 is a likely candidate for regulating differentiation. In order to examine the role of caspase-2 in myocyte differentiation, we assessed enzyme activity throughout the time course of C2C12 differentiation. Additionally, we stably transfected C2C12 cells with caspase-2 shRNA to assess the impact of a caspase-2 knockdown on myocyte differentiation. Finally, we identified the subcellular localization of caspase-2 and p21 throughout the early stages of differentiation. Enzyme activity of caspase-2 transiently increased more than two-fold within 24 hours of differentiation induction, with levels returning to normal by day 7, indicating that the enzyme likely plays a role in the differentiation process. Furthermore, knockdown of caspase-2 dramatically impaired myotube formation and induction of cell cycle inhibitor p21 and myogenic regulatory factor myogenin. Caspase-3 activity was also ablated in the caspase-2 knockdown C2C12 cells. Finally, subcellular fractionation of C2C12 cells at early time points in differentiation revealed a nuclear retention of both caspase-2 and p21 throughout the process. Given the nuclear localization of caspase-2 and p21 as well as the impairment in p21 induction in caspase-2 KD cells, we propose that the role of caspase-2 in myocyte differentiation is to regulate p21 induction at the onset of differentiation. Collectively, the results of this study highlight a novel function for caspase-2 in regulating myocyte differentiation.

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Introduction

A complex multicellular organism is made up of a variety of tissues and cell types, each with their own distinct characteristics and functions. Every single cell in such an organism is derived from a single fertilized oocyte that must divide and transform into different cell populations (1). Cells that have the capacity to divide, self-maintain, and mature into specialized cell types are known as stem cells and this process of specialization is termed differentiation (reviewed in 1). Embryonic stem cells are nearly totipotent, meaning they have the ability to differentiate into almost all cell types in the human body. For some cell populations, such as lens epithelial cells, neurons, and muscle cells, differentiation takes place during embryogenesis, forming mature, functional tissues with very low rates of cell turnover in the mature organism (2). Other cell types, including keratinocytes, erythrocytes, and monocytes are continuously undergoing self-renewal and differentiation in adulthood (2). These cell types require the presence of undifferentiated precursor cell populations, ready to divide and differentiate as needed. As different tissues contain cells with specific and specialized morphologies and functions, each cell type has its own distinct differentiation program, and the program chosen for the stem cell is dependent on a number of intrinsic and environmental factors (3).

The Cell Cycle

In order for cells to enter into a differentiation program they must first arrest growth and withdraw from the cell cycle (4). The cell cycle consists of a series of highly
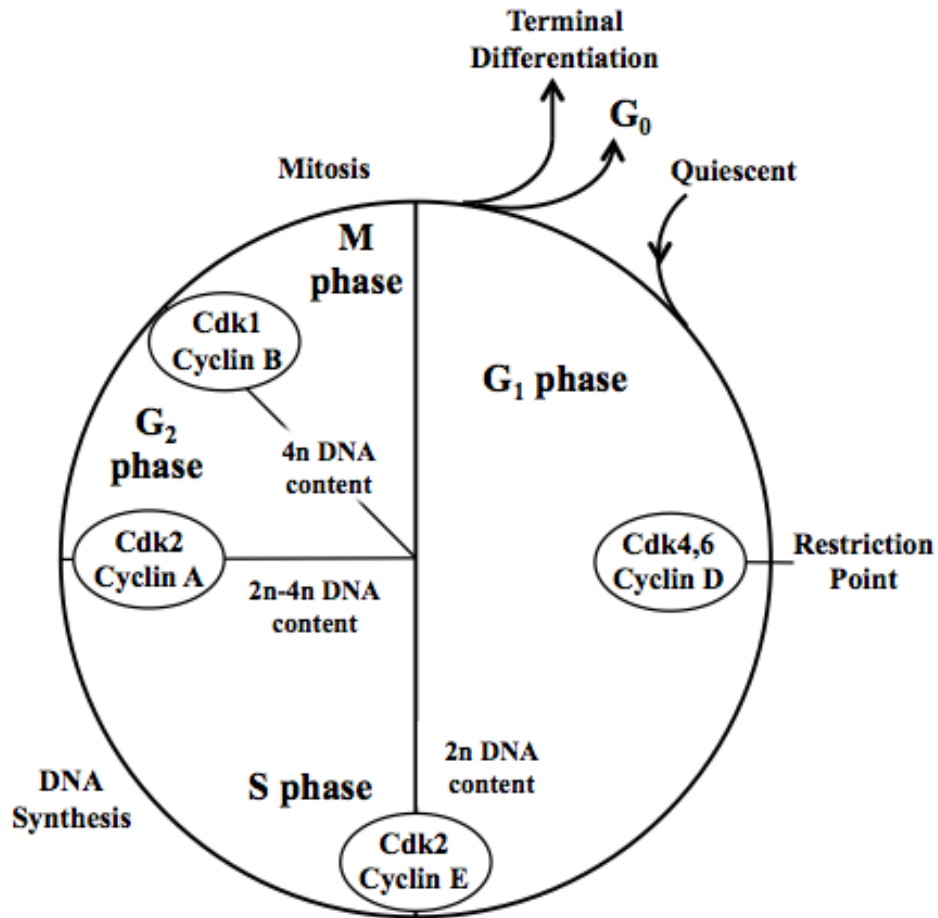
Figure 1: The stages of the cell cycle and sites of Cdk/Cyclin regulation. Adapted from Schafer, 1998 (5).

regulated events directing a cell through DNA duplication and eventual division into two daughter cells (reviewed in 5). The cell cycle can be broken down into four main phases: G_1, S, G_2 and M (Figure 1). Cells that are in the S (synthesis) phase are in the process of duplicating their DNA, and therefore will have between 2N and 4N DNA content (5). Cells that are in the M phase begin with 4N DNA and split into two daughter cells containing 2N DNA each (5). The G phases represent gaps in the cycle between the two main events of synthesis and division. Cells in the G_1 phase will be in the process of

preparing for DNA synthesis while cells in the G_2 phase are readying for mitosis (5). Rather than entering into the S phase, cells in G1 can also enter into a non-proliferative quiescent stage called G_0 (6).

A cell's progression through these cell cycle phases is dependent on the activity of cyclin-dependent kinases (cdks) and cyclin proteins (see Figure 1) (5). The cdk family of proteins are activated at different time points in the cell cycle and allow for progression through the cell cycle (5). Cdk activation is absolutely critical for progression of the cell cycle and their inhibition has been shown to prevent mitosis (5). While protein expression of cdks remains fairly stable throughout the cell cycle, expression of their activating proteins, cyclins, changes drastically at different phases (6). The changing levels of cyclin protein expression throughout the cell cycle allows for periodic activation of cdks (6). Full activation of the cdk-cyclin complexes requires further phosphorylation events at specific residues to induce conformational changes in the complexes (reviewed in 6).

Inhibition of these cell cycle activating proteins is carried out by CDK inhibitors (CKIs) which act to either directly bind and inhibit cdks or bind to cdk-cyclin complexes to achieve inhibition (6). CKIs consist of two main families of inhibitory proteins: INK4 proteins and Cip/Kip proteins (6). INK4 proteins, such as p15, p16, p18 and p19, bind directly to cdks, preventing their association with and activation by cyclins (6). Conversely, the Cip/Kip family of inhibitory proteins, including p21, p27, and p57, bind to and inhibit cdk-cyclin complexes (6). Cell cycle arrest can be achieved through upregulation of some of these inhibitory proteins. For example, p21 can be activated by p53 to inhibit DNA synthesis through the binding of proliferating cell nuclear antigen

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(PCNA) (6). Clearly, this process is tightly regulated by a number of proteins and complexes, and deregulation can lead to uncontrollable and unchecked cell division, such as in cancer.

Differentiation and the Cell Cycle

The onset of differentiation is coupled with a cell's terminal exit from the cell cycle. Differentiation-related factors function not only to induce gene expression of proteins required by the mature cell but also act to regulate the transition from proliferation to differentiation (7). In order for a cell to arrest growth and begin differentiating, cdks and cyclins must be downregulated while inhibitory CKIs must be upregulated (7). There is considerable crosstalk between cell cycle regulation and differentation and deregulation of the cell cycle can disrupt proper differentiation (8). Stimulation by growth factors prevents differentiation and encourages cell proliferation while removal of growth signals can induce differentiation (9). Thus, it is important to consider not only differentiation-related factors but also cell cycle regulatory factors when studying differentiation.

Myocyte Differentiation

In skeletal muscle, differentiation occurs during embryonic development when muscle precursor cells called myoblasts exit the cell cycle and fuse to form multinucleated myotubes, which further differentiate into muscle fibres (10). This process is tightly regulated by a variety of proteins, hormones, and transcription factors.
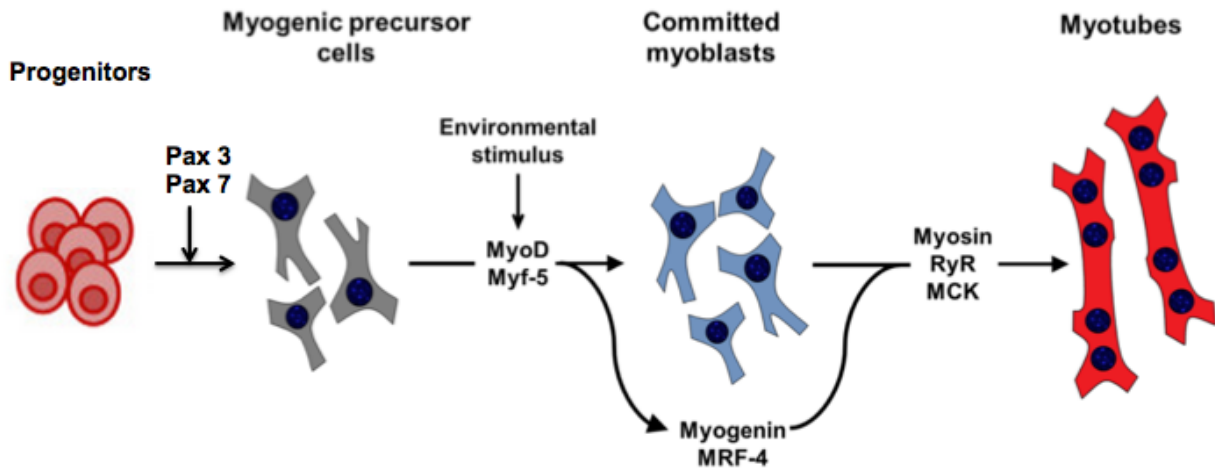
Figure 2: Regulation of myocyte differentiation. Pax3/Pax7 positive cells induce expression of MRFs Myf-5 and MyoD, committing these cells to the myogenic lineage. Secondary MRFs myogenin and MRF-4 are subsequently activated, resulting in transcription of muscle related genes such as myosin heavy chain. Adapted from Bloemberg, 2012 (94)

During embryogenesis, somite cells forming the dermomyotome (11) receive signals to express the transcription factors Pax3 and Pax7, specifying these cells as muscle cells (12) (see Figure 2). Pax3/Pax7-expressing progenitor cells initiate the expression of myogenic regulatory factors (MRFs) Myf5 and subsequently, MyoD, committing the cells to the myogenic program (13). Myf5 and MyoD expressing cells expand and proliferate to form myoblasts (10). At this point, MyoD expression halts cell proliferation (through enhancement of p21 expression) (14), and induces the expression of two other MRFs - myogenin and MRF4 (10). Expression of these regulatory factors results in formation of myocytes. Specifically, myogenin is necessary for the terminal differentiation of myocytes into multinucleated myotubes (15), which will express late differentiation markers such as myosin heavy chain (MHC). At this point, the muscle progenitor cells have fully differentiated to form functional, contractile, multinucleated myofibers.

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MRF expression and activity is regulated by inhibitor of differentiation (Id) proteins, that sequester E proteins from MyoD (16,17). E proteins regulate MyoD activity by forming activated complexes with MyoD, allowing for transcriptional upregulation of muscle-specific genes (17). Other inhibitors include Twist (18), Mist1 (19), MyoR (20) and Sharp-1 (21), which all act to inhibit MyoD transcriptional activity. The decline of these inhibitory proteins at the induction of differentiation allows for myogenesis to proceed.

Apoptosis

Apoptosis is a highly conserved physiological process that is crucial for the regulation of tissue development and disease. Like most biological processes, apoptotic signaling must be tightly regulated in order to maintain optimal function and health. For example, elevated levels of apoptosis have been observed in skeletal muscle during aging, as well as in many disease states leading to muscle atrophy, and dysfunction (22). Conversely, attenuated apoptotic signaling can lead to improper tissue development and cancer (23). Recent evidence has emerged suggesting that apoptotic signaling, a classical cell-death mechanism plays a crucial role in cell differentiation.

Caspases

Initiation and execution of apoptosis is carried out primarily by a family of proteolytic enzymes called caspases (cysteine-aspartic proteases). Caspases cleave a wide variety of specific proteins causing a cascade that results in the stereotypic morphological changes associated with apoptosis (DNA fragmentation, plasma membrane blebbing,

chromatin condensation) (24). Caspases can be divided into two groups: initiator caspases (caspase-8, caspase-9) and effector caspases (caspase-3, caspase-6, caspase-7). Both initiator and effector caspases are present in the cell as inactive pro-caspases but the method of enzyme activation differs between the two classes (25).

Initiator caspases are activated through dimerization with high molecular weight protein complexes (26). For example, the activation of initiator caspase-9 in response to DNA damage involves the release of cytochrome c from the mitochondria, which subsequently activates the apoptotic protease-activating factor-1 (APAF-1) apoptosome complex (27). This complex then recruits and activates caspase-9, which in turn directly activates caspase-3, an effector caspase, through cleavage of the zymogen form of the enzyme (27). Thus, in contrast to the initiator caspases, cleavage of the effector caspases by the initiator caspases into a small and large catalytic subunit is sufficient for their activation (25). The role of caspases in cell death is well defined; however, recent research has emerged suggesting that these classical apoptosis-related proteins have roles in other cellular processes.

Apoptotic Signaling in Differentiation

One of the cellular processes that apoptotic signaling has been implicated in is differentiation. The idea that apoptotic signaling may play a role in the differentiation process first arose when it was observed that nuclear degradation (a hallmark of apoptosis) is necessary for differentiation in keratinocytes (28), lens epithelial cells (29), and erythrocytes (30). Classical apoptotic events such as cytoskeletal reorganization (31), activity of matrix metalloproteinases (32), and exposure of extracellular

phosphatidylserine residues (33) are necessary for skeletal muscle differentiation. Due to the observed similarities between processes, it is likely that the molecules involved in one process also play a role in the other. Indeed, research has shown that certain caspases, historically cell death proteins, are also important for skeletal muscle differentiation.

In 2002, Fernando et al. first demonstrated that myocyte differentiation is dependent on caspase-3 activity, and that inhibiting caspase-3 drastically impairs the formation of myotubes (34). Since then, more recent studies have supported this finding (35), and have implicated initiator caspases in this process. Caspases-8 (36), -9 (37) and -12 (38,39) have been found to be important in skeletal muscle differentiation, albeit through activation of caspase-3.

The importance of caspase activity throughout differentiation has been further demonstrated through the identification of differentiation-related substrates for caspases. For example, Twist can be cleaved by caspase-3 (40), relieving the inhibition on MyoD and other pro-myogenic proteins (41). Furthermore, there is evidence to suggest that cleavage and activation of caspase-activated DNase (CAD) by caspase-3 is required for p21 induction and the subsequent cell cycle exit necessary prior to differentiation (42).

Whether a cell undergoes differentiation or death at the hands of caspases is likely dependent on the duration and intensity of the enzymatic activity. In skeletal muscle differentiation, caspase activation appears to be transient (34), and to a lesser degree than what is seen in apoptotic cell death (43,44). The tipping point between caspase activation leading to differentiation or apoptosis is also likely due to the increased expression of anti-apoptotic proteins early in the differentiation process (45). Consequently, caspases

are allowed to perform the remodeling required for differentiation but are inhibited before their activation can lead to apoptosis.

Caspase-2

Previous research in our lab suggests that caspase-2 may also play a role in the differentiation process, specifically in skeletal muscle (45). Caspase-2, the most physiologically conserved of the caspase enzymes, has proven to be enigmatic for researchers. Despite years of study, caspase-2 remains poorly understood in terms of modes of activation, target substrates, and physiological functions. Researchers have failed to definitively place this enzyme in the apoptotic cascade, as it shares functional similarities to both initiator and effector caspases. Similar to effector caspases, caspase-2 can cleave downstream cellular targets; however, like the initiator caspases, dimerization is required in addition to cleavage for full activation of the enzyme (46). Baliga et al tested the activity of an uncleavable form of caspase-2 as compared to a wild-type form of the enzyme and found that the cleavage defective form was able to retain approximately 20% of the enzymatic activity, as long as dimerization had occurred (47). Conversely, a cleaved, monomeric form of the enzyme showed no activity whatsoever (47). Therefore, similar to caspase-8 and -9, dimerization is a crucial initial step in caspase-2 activation.

Caspase-2 Activation

The most widely accepted method of caspase-2 activation involves its recruitment to a high molecular weight protein complex (48). Much of the initial work performed in

identifying the caspase-2 specific signaling complex was done by Jürg Tschopp and colleagues in the late 1990's.

Jürg Tschopp first coined the term "CARD" (caspase recruitment domain) when he discovered a conserved protein:protein interaction motif present in many caspases and caspase adaptor proteins (49). Similar to the other initiator caspases, caspase-2 consists of a long, CARD-containing prodomain, as well as a small and large catalytic subunit (50). The CARD allows for the recruitment of the enzyme to a larger signaling complex (51). In the case of caspase-2, the CARD of caspase-2 binds to the CARD in the adaptor molecule RAIDD (RIP-associated Ich-1/Ced-3-homologue protein with a death domain) which recruits the enzyme to the signaling platform (52).

Researchers in Tschopp's laboratory then identified the role of PIDD (p53 induced death domain containing protein) in this process (53). Interaction between caspase-2 and RAIDD allowed for the recruitment of caspase-2 to PIDD and subsequent activation of the apoptotic enzyme (53). PIDD, a p53 induced protein, contains a death domain (DD) at the carboxyl terminal as well as a series of leucine-rich repeats (LRRs) at the amino terminal (53). LRRs are also present in nucleotide-binding oligomerization domain-like (NOD-like) proteins and can function to recognize noxious stimuli resulting in inflammasome or NODosome assembly and subsequent caspase-1 or NFκB activation (54,55). It is hypothesized that PIDDosome assembly occurs in a similar manner; however, researchers have yet to identify molecules that can bind the LRRs in PIDD (56).

In response to a variety of cellular signals, including genotoxic stress, the DD of RAIDD binds to the DD of PIDD and induces caspase-2 activation (46). The PIDDosome complex consists of five PIDD DDs and seven RAIDD DDs forming two stacked rings,

which can then recruit seven caspase-2 molecules and engage the apoptotic pathway (56). It is generally well accepted that caspase-2 induces apoptosis through the cleavage of pro-apoptotic Bcl2 family member Bid into its active form tBid. tBid acts on the mitochondria to induce cytochrome c release, which in turn activates caspase-9 resulting in apoptosis (57).

Interestingly, other experiments have demonstrated caspase-2 activation in the absence of PIDD and RAIDD (58), suggesting that other modes of activation may exist. PIDD KO mice showed no impairment in caspase-2-mediated apoptosis (58) and heat shock appears to activate caspase-2 in a p53-independent (and therefore PIDDosome-independent) manner (59). Using T and B cell lines, researchers in Germany found caspase-2 docked to an alternative signaling complex, CD95 DISC (death-inducing signaling complex), most commonly associated with caspase-8 (60). These researchers did not, however, find measurable levels of RAIDD at the DISC, implying the presence of an unidentified adaptor protein with both a CARD and DD that can recruit caspase-2 to the DISC (60). It is interesting to note that the recruitment of caspase-2 to the DISC does not necessarily induce downstream apoptosis in the absence of caspase-8 (60). This would imply a redundant, amplification function of caspase-2 in conjunction with caspase-8 in inducing CD95-mediated apoptosis. Another possible mechanism of caspase-2 activation includes dimerization of caspase-2 molecules and subsequent autoproteolysis, triggered by the K+ efflux that is seen in response to bacterial pore-forming toxins (61). Collectively, this data shows that the precise mode of activation of caspase-2 is not fully understood, and future research would be beneficial in better defining these mechanisms.

Caspase-2 and Apoptosis

A variety of evidence has demonstrated a major role for caspase-2 in the apoptotic pathway. Kumar and colleagues showed that caspase-2 is highly expressed during embryonic development, a stage where widespread cell death occurs (62). Furthermore, it has been shown that caspase-2 is activated early in the apoptotic cascade (63,64), and that overexpression of caspase-2 induces cell death in mammalian cells (62,65).

The majority of research implicates caspase-2 in DNA-damage induced cell death (66). A number of researchers suggest a possible primary mechanism for caspase-2 apoptotic induction involving both the intrinsic and extrinsic pathways of apoptosis (52,60,67). In response to DNA damage, p53 induces activation of the Fas receptor (also referred to as CD95 receptor) on the cell membrane (66). Evidence for the activation of caspase-2 at this complex was discussed previously. FADD (Fas-associated protein with death domain) then recruits and activates caspase-8 and also caspase-2 (66). Both caspase-8 and caspase-2 are able to then cleave Bid into activated tBid, which induces mitochondrial outer membrane permeablization and cytochrome c release (66). Alternatively, a study by Sidi et al demonstrated the involvement of caspase-2 in an ATM/ATR-dependent cell death pathway (68). ATM (ataxia telangiectasia mutated) is a kinase that is activated by the presence of double-stranded breaks in DNA (68). This pathway appears to be activated in response to DNA damage in the absence of p53 by checkpoint kinase-1 (68). What is interesting about this mechanism is the lack of involvement of p53 and apoptotic molecules such as Bcl-2 or caspase-3 (68).

Caspase-2 in the Cell Cycle and DNA Damage Response

In contrast to the rest of the caspase family, which are exclusively found outside the nucleus, caspase-2 has been found to be present in both the cytosol and the nucleus of cells. Researchers discovered a nuclear localization signal in the long prodomain of caspase-2 that allowed for importin-mediated transport of caspase-2 to the nucleus (69). Studies using immunofluorescence of endogenous caspase-2 (70,71) and imaging of GFP-caspase-2 complexes (72) indicate that caspase-2 is primarily a nuclear protein. Conversely, a number of studies have also shown caspase-2 localized to the cytosol (73), Golgi (74), and mitochondria (75). Nonetheless, the nuclear localization of caspase-2 is consistent with recent research that has shown it likely plays a role in the DNA damage response, the cell cycle, and tumour suppression.

It has been proposed that a determining factor in the function of caspase-2 is the level of cellular damage that is present. As discussed above, when levels of DNA and cellular damage are high, caspase-2 is activated through the PIDDosome (or possibly other alternative mechanisms) to induce apoptosis (76). At lower levels of damage, however, it is likely that caspase-2 can function as a cell cycle checkpoint regulator and play a role in DNA repair.

A study by Mendelsohn et al has provided some evidence for a regulatory role of caspase-2 in the cell cycle (77). Researchers found a direct association between cyclin D3 and caspase-2 in that cyclin D3 expression increased the amount of active caspase-2 (77). The authors suggest that an interaction between cyclin D3 and caspase-2 may act to stabilize the protease, connecting cell cycle mechanisms to cell death mechanisms (77). Further research has demonstrated that phosphorylation of caspase-2 by cyclin-dependent

kinase 1 (CDK1)-cyclin B1 inhibits caspase-2 activation during normal mitosis, and protein phosphatase 1 (PP1) dephosphorylates the enzyme during interphase (78). Failure to inactivate caspase-2 through phosphorylation during mitosis results in the death of the cell (78). Low levels of PP1 activity are seen during normal mitosis; however, cellular insult such as DNA damage can induce activation of PP1, alleviating the suppression of caspase-2 and inducing cell death (78). Collectively, this data suggests that caspase-2 may function as a cell cycle checkpoint when low levels of damage occur and that caspase-2 plays an apoptotic role with higher levels of DNA damage.

Further evidence of a cell cycle regulatory role for caspase-2 is its proposed ability to positively regulate the cell cycle inhibitory protein, p21 (79). Researchers found that knocking down caspase-2 increased rates of cell proliferation and reduced p21 expression in HCT116 cells, while p53 levels remained unchanged (79). Furthermore, overexpressing caspase-2 resulted in increased p21 protein levels (79). The authors suggest that this p21 regulatory function of caspase-2 is actually the protease's primary function; however, the precise method with which caspase-2 is regulating p21 could not be elucidated (79).

In a study comparing wild type MEFs to caspase-2 deficient MEFs, researchers found evidence for a role of caspase-2 in the DNA damage response (80). Caspase-2 knockout MEFs were shown to proliferate faster than wild type MEFs and also avoid cellular senescence (the point at which a cell no longer divides (81)) (80). Due to the enhanced proliferation of the cells, researchers then considered the effect caspase-2 deficiency had on DNA damage by measuring the presence of micronuclei, which can arise due to DNA breaks or chromosomal fragments (80). Caspase-2 knockout cells had

consistently higher levels of micronuclei compared to their wild type counterparts (80). Furthermore, cells lacking caspase-2 showed delayed and reduced DNA damage repair, as a response to ionizing radiation treatment (80). Finally, cells deficient in caspase-2 showed enhanced chromosomal abnormalities including aneuploidy (an abnormal number of chromosomes) (80). Overall, this study points to an important role of caspase-2 in the DNA damage response.

Caspase-2 in Differentiation

Given these findings, it is possible that caspase-2 has a role in skeletal muscle cell differentiation. Indeed, previous work in our lab has shown transient upregulation of caspase-2 at the onset of skeletal muscle differentiation (45). Interestingly, this activation of caspase-2 appears to occur prior to the activation of caspase-3, which is essential for myogenesis. Caspase-2 has been shown to directly activate caspase-3 through binding of their pro-domains (82); thus, it is possible that upregulation of caspase-2 is contributing to caspase-3 activation in skeletal muscle differentiation. Furthermore, research using aged caspase-2 knockout mice found that loss of caspase-2 greatly reduced caspase-3 activation in the liver (83). Alternatively, the cell cycle regulatory properties of caspase-2 present an interesting and unique possible function for caspase-2 in regulating differentiation through moderating cell cycle exit.

Purpose

Previous research has demonstrated a role for caspases in myocyte differentiation. Given the findings from Bloemberg and Quadrilatero (94) showing caspase-2 activation at the onset of differentiation, as well as evidence that caspase-2 may function in cell cycle regulation, it is possible that caspase-2 activation early on in differentiation is required for cells to enter into the differentiation program, and that this activation may contribute either directly or indirectly to the previously observed caspase-3 activation in the early stages of myocyte differentation.

Therefore, the main objectives of my thesis project were to:

1) Characterize the activity of caspase-2 throughout skeletal muscle differentiation

2) Determine the effect of a lack of caspase-2 on caspase-3 activation and skeletal muscle differentiation

3) Determine the subcellular localization of caspase-2 throughout the differentiation process

Experiment 1: Caspase-2 activity throughout differentiation

An *in vitro* model was used to determine the activity of caspase-2 in skeletal muscle differentiation. C2C12 mouse skeletal myoblasts were maintained in an undifferentiated, proliferative state and subsequently induced to differentiate into mature myotubes via incubation in media containing low growth factor levels, as previously performed in our lab (45). Cells were collected at various time points throughout the differentiation process to be used for experimental analyses. Activity of caspases-2 and -3

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throughout the differentiation process were assessed using fluorometric assays. Cell cycle regulation across differentiation was assessed by immunoblotting for p21 and via flow cytometry cell cycle analyses. Protein levels of myogenin, MyoD, and Myosin were quantified along with fluorescent microscopy to analyze the degree of differentiation in these cells.

Experiment 2: Differentiation in the absence of caspase-2

To better elucidate the role of caspase-2 in the myocyte differentiation process, caspase-2 protein content was knocked down via shRNA. Western blotting for caspase-2 protein content was used to ensure efficient caspase-2 knockdown (KD) in cells. The impact of caspase-2 KD on cell cycle regulation was assessed by comparing flow cytometry cell cycle analyses between KD and controls as well as measuring p21 content throughout differentiation. The effect of caspase-2 KD on myocyte differentiation was assessed via immunoblotting for myogenic markers myogenin, MyoD, and Myosin in the caspase-2 KD cells. Finally, morphology of caspase-2 KD cells was analyzed using fluorescent microscopy.

Experiment 3: Subcellular Localization of caspase-2 during differentiation

In order to better determine the role of caspase-2 in differentiation, the subcellular localization of caspase-2 was assessed at various time points in differentiation. C2C12 cells were collected at early time points in differentiation, when caspase-2 is shown to be most active. Cells were processed into nuclear and extranuclear/nuclear-free fractions and caspase-2 content was measured in each compartment. Subcellular localization of p21

was also measured in the fractions in order to provide insight on the precise function of caspase-2 activation in differentiation.

Hypotheses

It was hypothesized that:

1) Caspase-2 activity would increase early in the differentiation process, prior to caspase-3 activation. This would be noted by:

    a. A transient spike in caspase-2 activity, occurring immediately prior to caspase-3 activity increase, measured by fluorometric assay

    b. Protein content of cell cycle regulator p21 would increase immediately upon induction of differentiation

2) Caspase-2 knockdown would impair cell cycle regulation and myocyte differentiation. This would be observed in the KD cells as:

    a. A decrease in p21 content

    b. Dysregulated cell cycle characteristics

    c. Lower levels of caspase-3 activation

    d. A decrease in differentiation markers: cell fusion, myosin, myogenin

3) Caspase-2 would be localized to the nucleus during differentiation

Methods

Cell Culture

      C2C12 mouse skeletal myoblasts (ATCC) were cultured in growth media (GM) consisting of low-glucose Dulbecco's Modified Eagles Medium (DMEM; Hyclone, ThermoScientific) containing 10% fetal bovine serum (FBS; Hyclone, ThermoScientific) with 1% penicillin/streptomycin (Hyclone, ThermoScientific) and plated in 100mm, 6-well and/or 12-well polystyrene cell culture dishes. Cells were seeded at a density of 650/cm^2. Media was aspirated, cells washed with 37°C phosphate buffered saline (DPBS) and fresh warmed GM was added every 1-2 days. As previously performed in our lab, cells were allowed to proliferate until they reached 80-90% confluence at which point differentiation was induced by switching from GM to a differentiation media (DM), consisting of DMEM containing 2% horse serum (Hyclone, ThermoScientific) and 1% penicillin/streptomycin (45).

Isolation and Determination of Total Protein Content

      Cells were isolated at various time points throughout differentiation, depending on the experiment being performed. The time points collected included: prior to differentiation (day 0; D0), and 6 hours (D0.25), 12 hours (D0.5), day 1 (D1), day 1.5 (D1.5), day 2 (D2), day 3 (D3), day 5 (D5), day 7 (D7) following induction of differentiation. Isolation of cells was done by aspirating the media, washing cells thoroughly with warmed PBS, and then trypsinizing (0.25% trypsin with 0.2g/L EDTA; ThermoScientific). Whole cell lysates were generated by adding lysis buffer (20mM HEPES, 10mM NaCl, 1.5mM MgCl, 1mM DTT, 20% glycerol, and 0.1% Triton-X100,

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pH 7.4) with protease inhibitors (Complete Cocktail; Roche Diagnostics) to cells and sonicating the mixture. Total protein content of cell lysates was measured by the BCA protein assay method.

Fluorometric Caspase Activity Assay

To assess activity of caspase-2 and caspase-3 during differentiation, the fluorogenic substrates Ac-VDTTD-AFC (21st Century Biochemicals) and Ac-DEVD-AMC (Enzo Life Sciences) were used, respectively. The uncleaved form of the substrates is weakly fluorescent; however, proteolytic cleavage by their respective caspase results in highly fluorescent products. Previous research regarding caspase-2 activity employed the use of the Ac-VDVAD-AMC substrate. This substrate has been shown to be cleaved by caspase-3 with almost equal efficiency. The Ac-VDTTD-AFC substrate used here is cleaved far more efficiently by caspase-2 than by caspase-3 and therefore provides an improved characterization of caspase-2 activity (84). Cells were isolated as previously described (using lysis buffer without protease inhibitors added) and incubated with the fluorogenic substrates in duplicate in black 96 well plates (Grenier Bio One). A Synergy H1 Microplate Reader (BioTek) was used to measure fluorescence. Caspase activity was normalized to total protein content, and is expressed as fluorescence intensity (arbitrary units)/mg protein.

Immunoblotting

As previously performed in our lab (85), equal amounts of protein were loaded and separated on 7-12% SDS-PAGE gels, and transferred to PVDF membranes.

Membranes were blocked for 1 hour at room temperature with 5% milk-Tris-buffered saline-Tween 20 (milk-TBS-T). Membranes were subsequently incubated overnight at 4°C with primary antibodies against myogenin, myosin (Developmental Studies Hybridoma Bank) MyoD, p21 (Santa Cruz Biotechnology), caspase-2, Histone H2B (Millipore), caspase-3, actin (Sigma-Aldrich), and MnSOD (Stressgen). After washing in TBS-T, membranes were incubated in the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 hour at room temperature. After washing with TBS-T, Clarity^{TM} Western Enhanced Chemiluminescence detection reagent (Bio Rad) was applied to the membranes for 1 minute and images were taken with the ChemiGenius 2 Bio-Imaging System (Syngene). The molecular weight of each protein was estimated using Precision Plus Protein^{TM} Kaleidoscope^{TM} Prestained Protein Standards (Bio-Rad Laboratories). To confirm equal loading and transfer, expression levels were normalized to actin as a loading control.

Fluorescent Microscopy

In order to assess cell morphology, fluorescent microscopy was used to visualize nuclei and expression of myosin. Cells were grown on glass coverslips in culture dishes and removed at various time points throughout differentiation. After washing 2 x 5min with PBS, cells were fixed for 10 minutes in 4% formaldehyde-PBS and washed. Cells were then permeablized with 0.5% Triton-X100 for 10 minutes, washed with PBS, and blocked with 10% goat serum in PBS for 30 minutes. Cells were then incubated in the primary antibody against myosin (MF20; Developmental Studies Hybridoma Bank) for 1 hour, washed with PBS, and then incubated in the appropriate fluorescent-conjugated

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secondary antibody (Alexa Fluor 488; Molecular Probes) for 1 hour. After washing with PBS, cells were counterstained with DAPI nuclear stain (Molecular Probes) for 5 minutes and washed with PBS. Coverslips were mounted with Prolong Gold Antifade Reagent (Molecular Probes) and visualized with an Axio Observer Z1 fluorescent microscope (Carl Zeiss).

Cell Fusion Index

Fluorescent microscopy images were used to assess the degree of fusion throughout differentiation. All nuclei within ten randomly chosen fields were counted. Cell fusion index was determined by the number of nuclei in multi-nucleated cells divided by the total number of nuclei, resulting in a fusion percentage per field.

Knockdown of caspase-2 via shRNA

Caspase-2 protein expression was knocked down in cultured myoblasts using shRNA. Caspase-2 shRNA plasmids were generously provided by Dr. Jonas Nilsson; University of Gothenburg (86). Plasmids were transfected into myoblasts at 50-60% confluence using Lipofectamine 2000 (Life Technologies) similar to previous work performed in our lab (87). Lipofectamine 2000 and plasmid shRNA were separately diluted in Opti-MEM (Gibco) and incubated at room temperature for 5 minutes. These mixtures were then added together and complexed at room temperature for 5 minutes. After washing cells twice with Opti-MEM, cells were incubated in the transfection mixture for 6 hours at 37°C. Transfection media was then aspirated and, after washing

twice with PBS, cells were incubated overnight in GM. Stable clones were obtained using puromycin. Caspase-2 knockdown was assessed via western blotting.

Flow Cytometry Analysis of Cell Cycle

In order to assess the impact of differentiation and caspase-2 knockdown on cell cycle properties, cells were collected at the following time points in differentiation: day -1 (D-1; approximately 50% confluent), day 0 (D0; just prior to induction of differentiation), day 0.5 (D0.5), day 1 (D1), and day 2 (D2) following induction of differentiation. Cells were harvested via trypsinization and centrifuged at 100g for 5 minutes. After removing the supernatant, the pellet was resuspended in 1mL of PBS and centrifuged a second time at 100g for 5 minutes. The supernatant was again removed and the cells were resuspended in 100μL of PBS. To fix the cells, 1mL of ice-cold 70% reagent-grade ethanol was added to each sample dropwise over the course of approximately one minute. Cells were stored at 4°C for at least 24 hours. To prepare the samples for flow cytometry, cells were centrifuged at 1000g for 5 minutes. After removing the supernatant, samples were washed twice with PBS. 100μL of RNAse was added followed by 400μL of propidium iodide (PI) solution (50μg/mL in 0.1% Triton-X PBS). Samples were then incubated at room temperature in the dark for 30 minutes at which point PI fluorescence was measured using flow cytometry (FACSCalibur, BD BioSciences). Resultant data was analyzed using Cell Quest Pro Software (BD Biosciences).

Fractionation

C2C12 cells were separated into nuclear and extra-nuclear fractions via differential centrifugation, as previously performed in our lab (45). Previous research regarding caspase-2 subcellular localization has shown that lysing of cells for experimental purposes artificially redistributes nuclear caspase-2 to the cytoplasm, thus preventing accurate localization information from these fractions (88). The same researchers found that pre-incubation of cells prior to harvesting with N-ethylmaleimide (NEM) prevented this redistribution and allowed for detection of nuclear caspase-2 via western blotting (88) (Appendix Figure 1). Thus, cells were treated for 10min with 7.5mM NEM as described previously (88). Cells were then washed with PBS, trypsinized for 5 minutes and centrifuged at 1000g for 5 minutes. After aspirating the supernatant, the resulting pellet was resuspended in lysis buffer as described above and incubated on ice/vortexed on and off for 10 minutes. Samples were centrifuged again at 1000g for 5 minutes. The supernatant was collected, centrifuged again at 1000g for 5 minutes and kept as the extra-nuclear fraction. The pellet was resuspended and centrifuged twice in lysis buffer at 1000g for 5 minutes and kept as the nuclear fraction. Fraction purity was assessed by immunoblotting for MnSOD (extra-nuclear) and Histone H2B (nuclear).

Statistical Analyses

All results are presented as means ± standard error of mean (SEM). Experiment 1 was analyzed by one-way ANOVA compared to time point D0, with the exception of cell cycle analyses, which were compared to time point D-1. Experiment 2 was analyzed by t-tests between groups at each time point. Experiment 3 was analyzed via t-tests between

nuclear and extra-nuclear fractions at each time point and one-way ANOVA across differentiation for changes in nuclear procaspase-2 expression. Statistical significance was set at $p < 0.05$ with $p < 0.10$ considered a trend. All statistical analyses were performed with Graph Pad Prism Statistical Software.

Results

Cell Cycle Regulation in Skeletal Muscle Cell Differentiation

Cell cycle characteristics throughout differentation were assessed by measuring PI fluorescence in cells harvested at time points representing rapidly dividing cells (D-1 time point), confluent cells (D0) and cells beginning to differentiate (D0.5 – D2). C2C12 cells will most rapidly divide when they are subconfluent and will slow down proliferation once they begin to reach confluence. At D-1, approximately 54.7% of cells were in the G_0/G_1 phase (Figure 3A & 3B). The remaining 43.3% of cells were in the S and G_2/M phases at this time point. (Figure 3A & 3B). As the cells began to reach confluence, cell cycle withdrawal was observed, as noted by a significant increase in G_0/G_1 cells ($p<0.05$, Figure 3A & 3B). At this point, approximately 69.2% of cells were in G_0/G_1 while 30.8% of cells were still in S or G_2/M (Figure 3A & 3B). Once differentiation was induced, the amount of cells in G_0/G_1 continued to significantly increase ($p<0.05$) and by D1, approximately 92.5% of cells were in this phase (Figure 3A & 3B). Cell cycle properties were further analyzed by measuring protein content of p21, a cell cycle regulator involved in cell cycle exit and growth arrest. Western blotting for p21 throughout differentiation revealed a 3-fold increase in p21 content at D1 and D2 ($p<0.05$) (Figure 3C & 3D). By D3, p21 content began to decrease but still showed a trend towards significance from day 0 ($p<0.10$, Figure 3C & 3D). When cells reached terminal differentation (D5 and D7), p21 content returned to day 0 levels (Figure 3C & 3D).
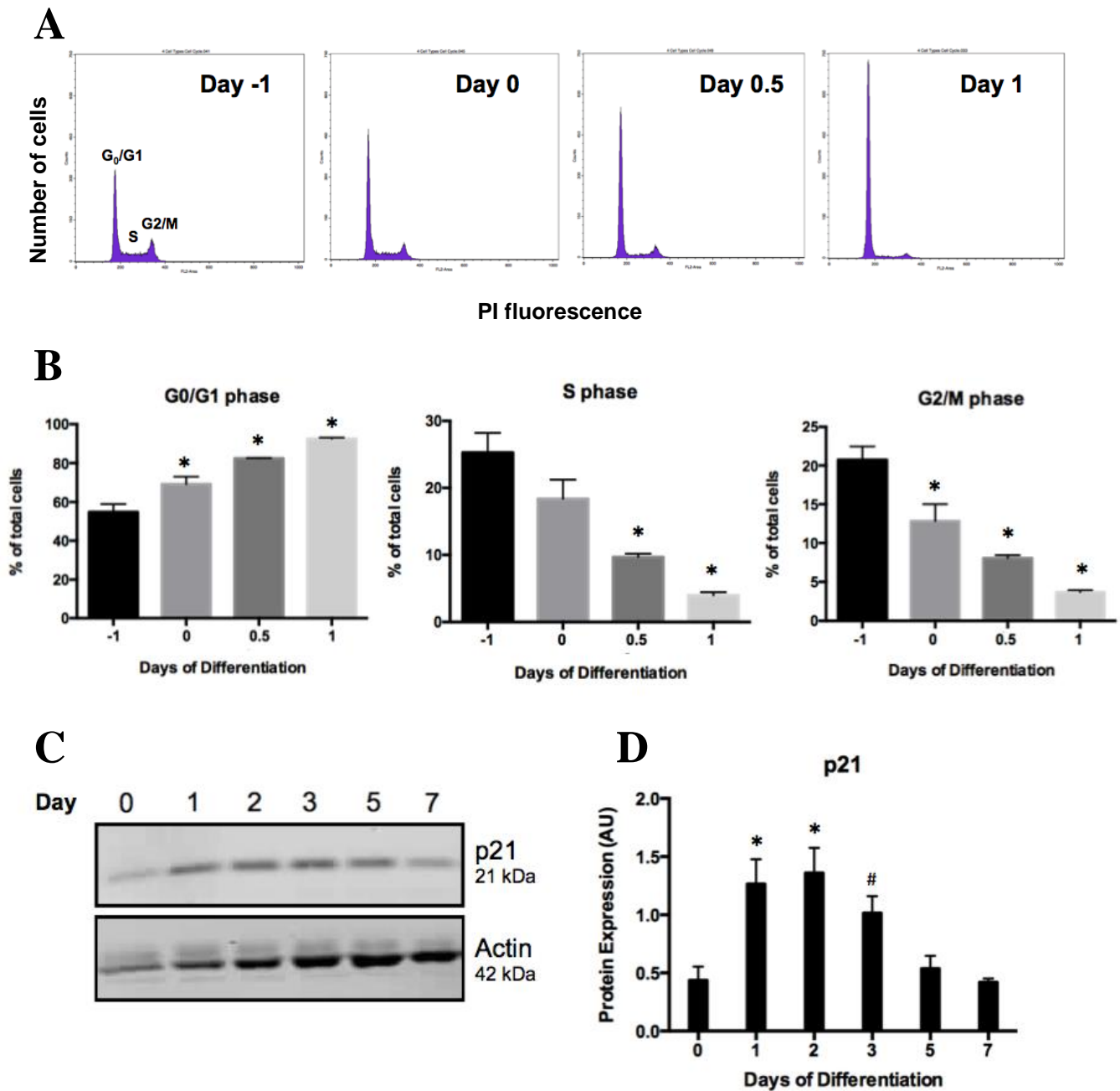
Figure 3: Cell cycle regulation in C2C12 myoblast differentiation.
A) Representative flow cytometry histograms of propidium iodide fluorescence, indicating cell cycle stage. **B)** Graphical representation of histograms shown in (A) highlighting observed differences in cell cycle phases as differentiation progresses. **C)** Representative immunoblot of whole-cell p21 protein expression across differentiation with actin loading control. **D)** Quantification of p21 protein expression levels normalized to actin loading control. *$p<0.05$ compared to day 0; #$p<0.10$ compared to day 0 (mean $\pm$ SEM, n=3 independent experiments).
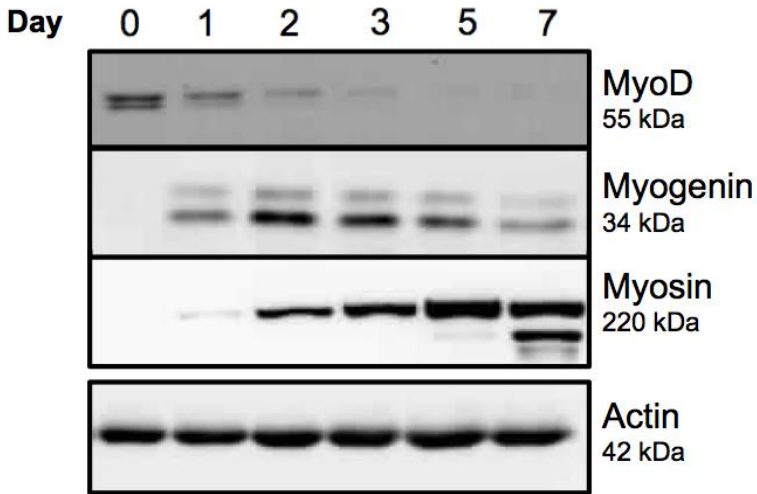
A

B

Skeletal Muscle Cell Differentiation Characteristics

As cells began to differentiate, MyoD protein decreased consistently from D0, with an 88% reduction by D7 ($p<0.05$, Figure 4). Expression of myogenin rapidly increased from D0 to D1, peaking at D2 with a nearly 60-fold increase from D0 ($p<0.05$, Figure 4). After this point, myogenin protein began to decrease and by D7, was no longer significantly different from D0. Expression of myosin protein, which is indicative of the degree of differentiation, was essentially negligible at D0 and was drastically increased by D7 ($p<0.05$, Figure 4). Similarly, fluorescent microscopy revealed a

A Day 0　　　　Day 2　　　　Day 5

B C2C12 Fusion Index

Fusion Index (% of total nuclei)

Days of Differentiation
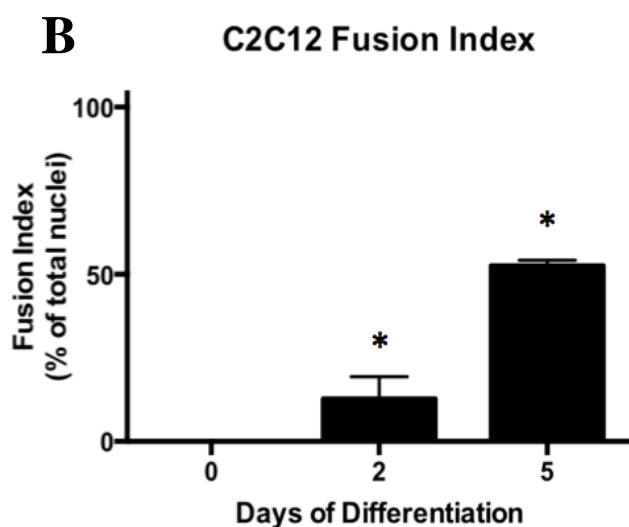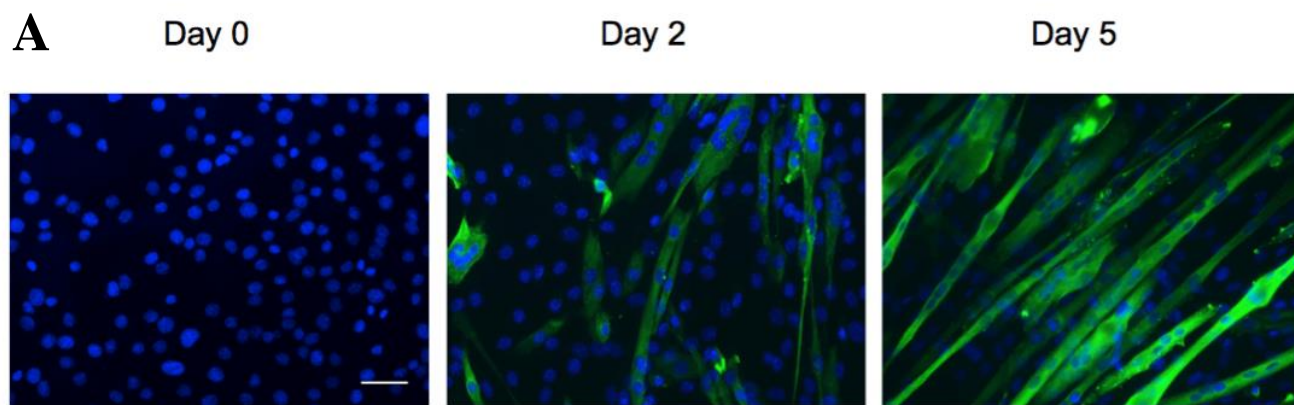
Figure 5: C2C12 cell morphology during myoblast differentiation. A) Fluorescent microscopy images depicting cell morphology at various stages of differentiation. Nuclei are stained blue (DAPI) and myosin heavy chain is stained green (Alexa Fluor 488). Bar represents 50μm. **B)** Quantification of fusion index calculated by dividing the number of nuclei in multi-nucleated fibres by the total number of nuclei in ten randomly chosen microscopic fields. *$p<0.05$ compared to day 0 (mean ± SEM, n=3 independent experiments**).**

complete lack of myosin positive cells at D0, with levels steadily increasing as myotubes developed ($p<0.05$, Figure 5). Quantification of the amount of nuclei within multinucleated cells revealed an increase in cell fusion as differentiation progressed ($p<0.05$, Figure 5). By D5, 53% of nuclei were contained in multinucleated cells (Figure 3).

Caspase Activity Throughout Differentiation

Activity of caspase-2 and caspase-3 throughout differentation was measured using fluorometric enzyme assays. Within 12 hours of inducing differentiation, caspase-2
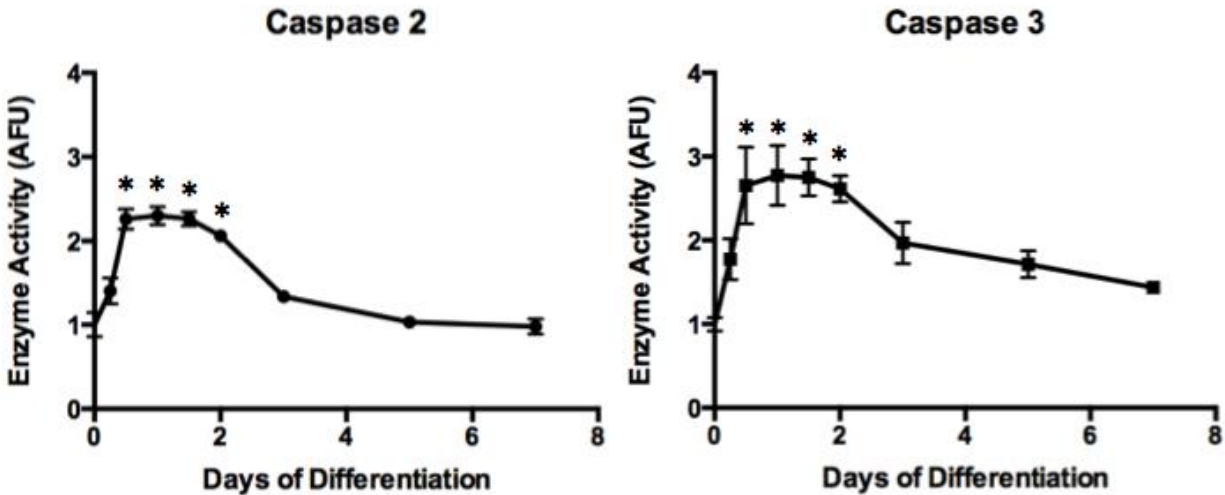
Figure 6: Caspase activity throughout differentiation. Caspase activity was assessed using fluorogenic substrates specific to each caspase. Activity is normalized to total protein content and expressed relative to day 0 levels. *$p < 0.05$ compared to day 0 (mean ± SEM, n=3 independent experiments).

activity more than doubled, with activity reaching a 2.3-fold increase by D1 ($p < 0.05$, Figure 6). Activity remained significantly elevated until D2 ($p < 0.05$) after which point it began to return to D0 levels (Figure 6). Similarly, caspase-3 activity rapidly increased upon induction of differentiation, reaching a 2.8-fold increase by D1 ($p < 0.05$, Figure 6). Like caspase-2, caspase-3 activity steadily tapered off after D2 and returned to near-D0 levels by D7 (Figure 6).

Knockdown of Caspase-2 in C2C12 cells

To elucidate the role of caspase-2 in skeletal muscle cell differentiation, C2C12 cells were stably transfected with caspase-2 shRNA (casp2 KD) or scramble shRNA

A

Procaspase-2
50 kDa

Actin
42 kDa

B

Procaspase-2

C
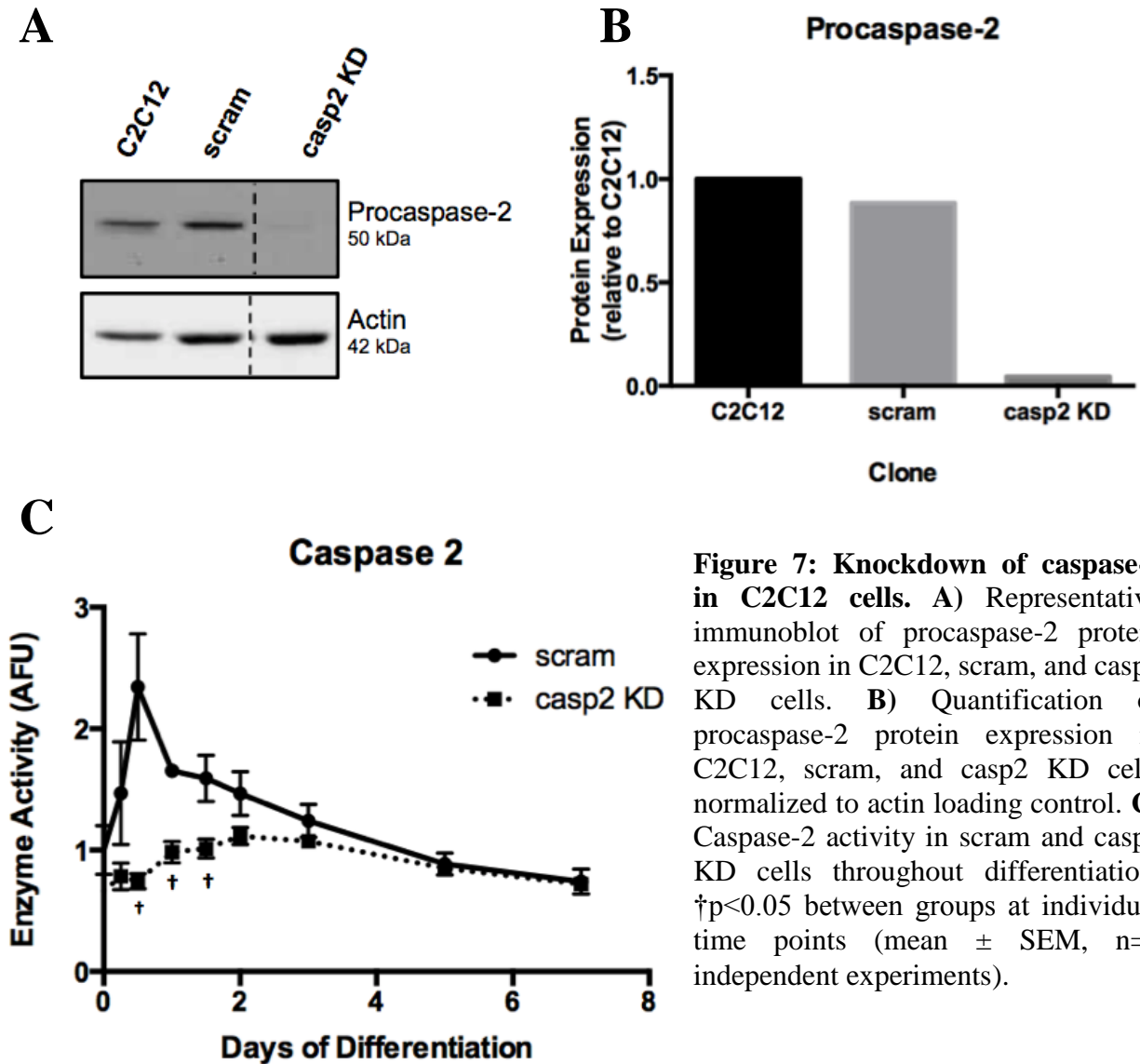
Caspase 2

Figure 7: Knockdown of caspase-2 in C2C12 cells. A) Representative immunoblot of procaspase-2 protein expression in C2C12, scram, and casp2 KD cells. **B)** Quantification of procaspase-2 protein expression in C2C12, scram, and casp2 KD cells normalized to actin loading control. **C)** Caspase-2 activity in scram and casp2 KD cells throughout differentiation. †$p<0.05$ between groups at individual time points (mean ± SEM, n=3 independent experiments).

(scram). Multiple casp2 KD clones were collected and tested for comparison. The clone used for all subsequent experiments was found to have similar cell cycle and differentiation characteristics as other casp2 KD clones (*data not shown*). Procaspase-2 was approximately 94% reduced in the casp2 KD cells compared to the scram cells (Figure 7A & 7B). Scram cells, conversely, were found to have similar procaspase-2 levels (Figure 7A & 7B) and exhibited similar cell cycle properties, differentiation

properties and morphology as control C2C12 cells, therefore, subsequent results will compare between scram cells and casp2 KD cells within time points. Consistent with a dramatic decrease in procaspase-2 content, activity of caspase-2 throughout differentiation was significantly reduced in casp2 KD cells compared to scram cells ($p < 0.05$, Figure 7C). Specifically, scram cells had significantly more caspase-2 activity than casp2 KD cells at D0.5, D1 and D1.5 (Figure 7C). In order to assess the impact of off-target effects of shRNA transfection on the cells' ability to differentiate, clones that had been transfected with the casp2 KD plasmid but did not exhibit protein knockdown were plated and induced to differentiate (Appendix figure 2A). By D6, visual observation of the cells revealed fused myotubes similar to control cells (Appendix figure 2B). Similarly, western blotting for myogenin and myosin revealed essentially normal differentiation characteristics (Appendix figure 2C). Collectively, these data suggest the subsequent effects of casp2 KD on differentiation are due to a lack of caspase-2 and this protein's critical role in skeletal muscle cell differentiation.

Cell Cycle Properties in the Absence of Caspase-2

Previous literature has suggested a role for caspase-2 in cell cycle regulation. Given these findings, along with the changes observed in cell cycle properties as differentiation progresses, we decided to measure cell cycle characteristics of caspase-2 KD cells at the induction of differentiation. Interestingly, casp2 KD cells showed higher percentages of cells in G_0/G_1 across all time points compared to scram cells ($p < 0.05$) and decreased levels of cells in S and G_2/M ($p < 0.05$, Figure 8A and 8B). These data would suggest an increase in growth arrest in the casp2 KD cells compared to the scram cells.
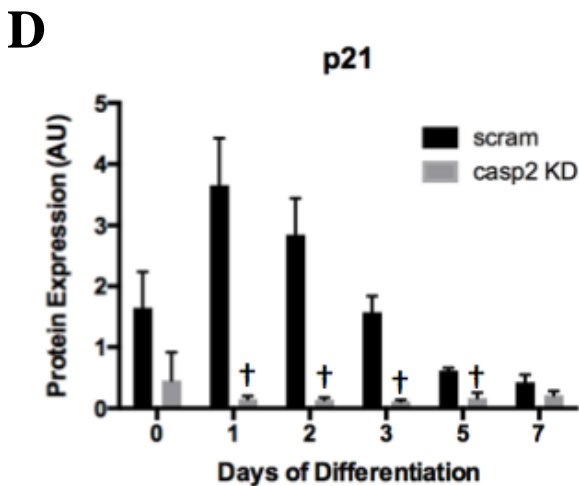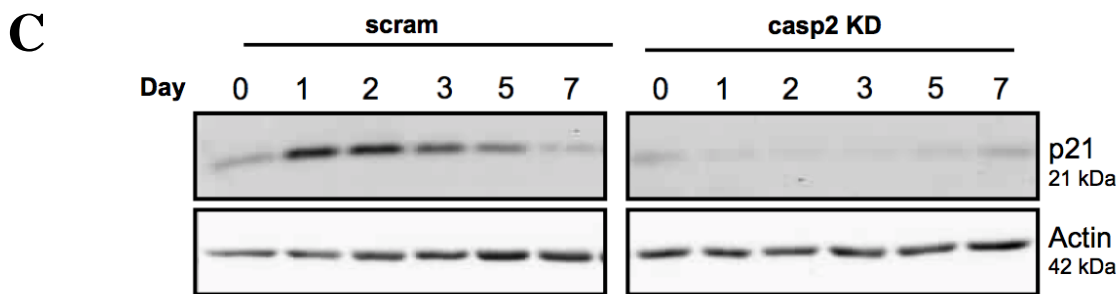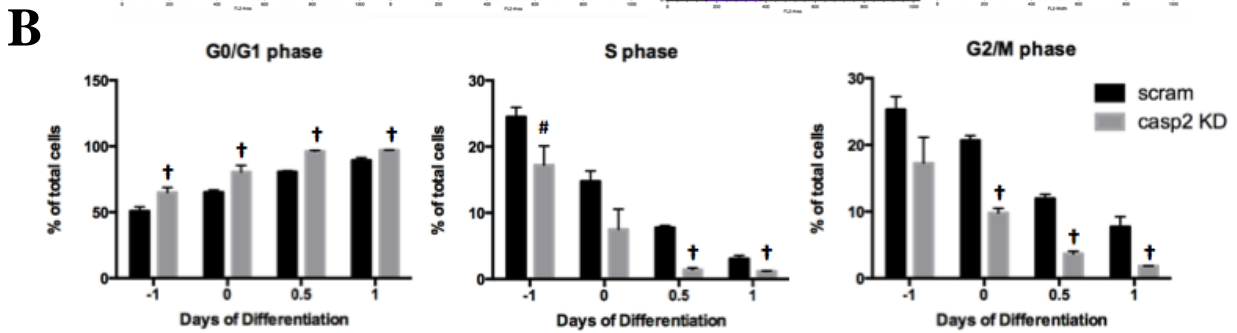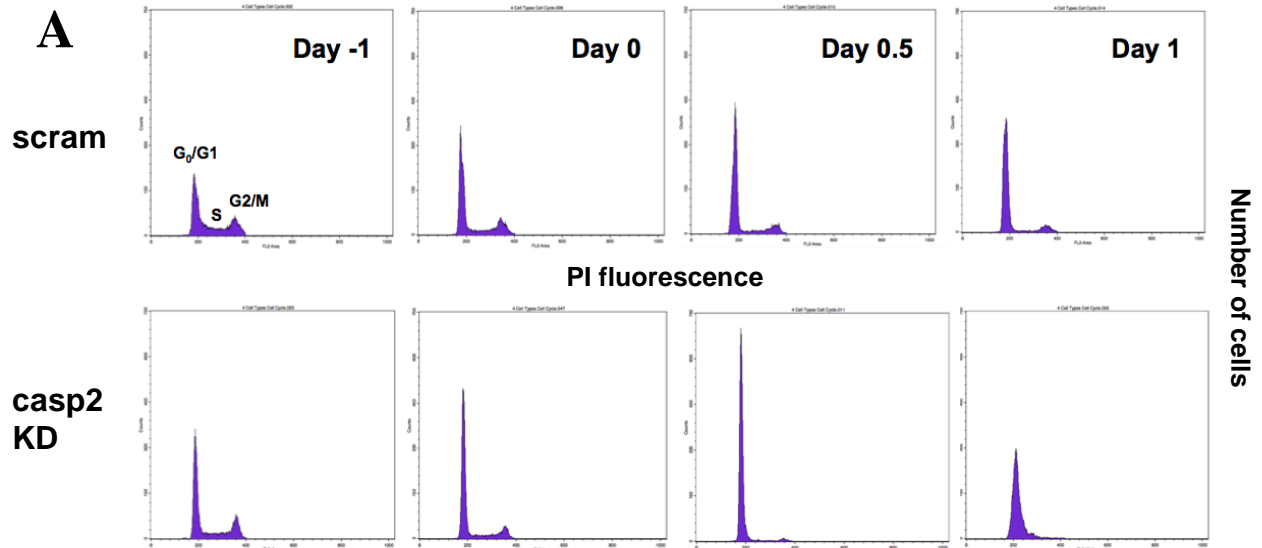
Figure 8: Cell cycle regulation in the absence of caspase-2. A) Representative histogram outputs of PI fluorescence, indicating cell cycle stage in scram and casp2 KD cells. **B)** Graphical representation of histograms shown in (A). **C)** Representative immunoblots of whole-cell p21 protein expression across differentiation in scram and casp2 KD cells with actin shown as a loading control. **D)** Quantification of p21 protein expression levels normalized to loading control. †p<0.05 compared to scram, #p<0.10 compared to scram (mean ± SEM, n=3 independent experiments).

Conversely, p21 protein expression was reduced 23-fold at D1 and remained significantly reduced until D5 in casp2 KD cells compared to scram ($p < 0.05$, Figure 8C & 8D). As p21 has been implicated in the cell cycle exit necessary for differentiation to begin, these data appear contradictory. Regardless, the overall cell cycle data show a deregulation of the cell cycle in the absence of caspase-2.
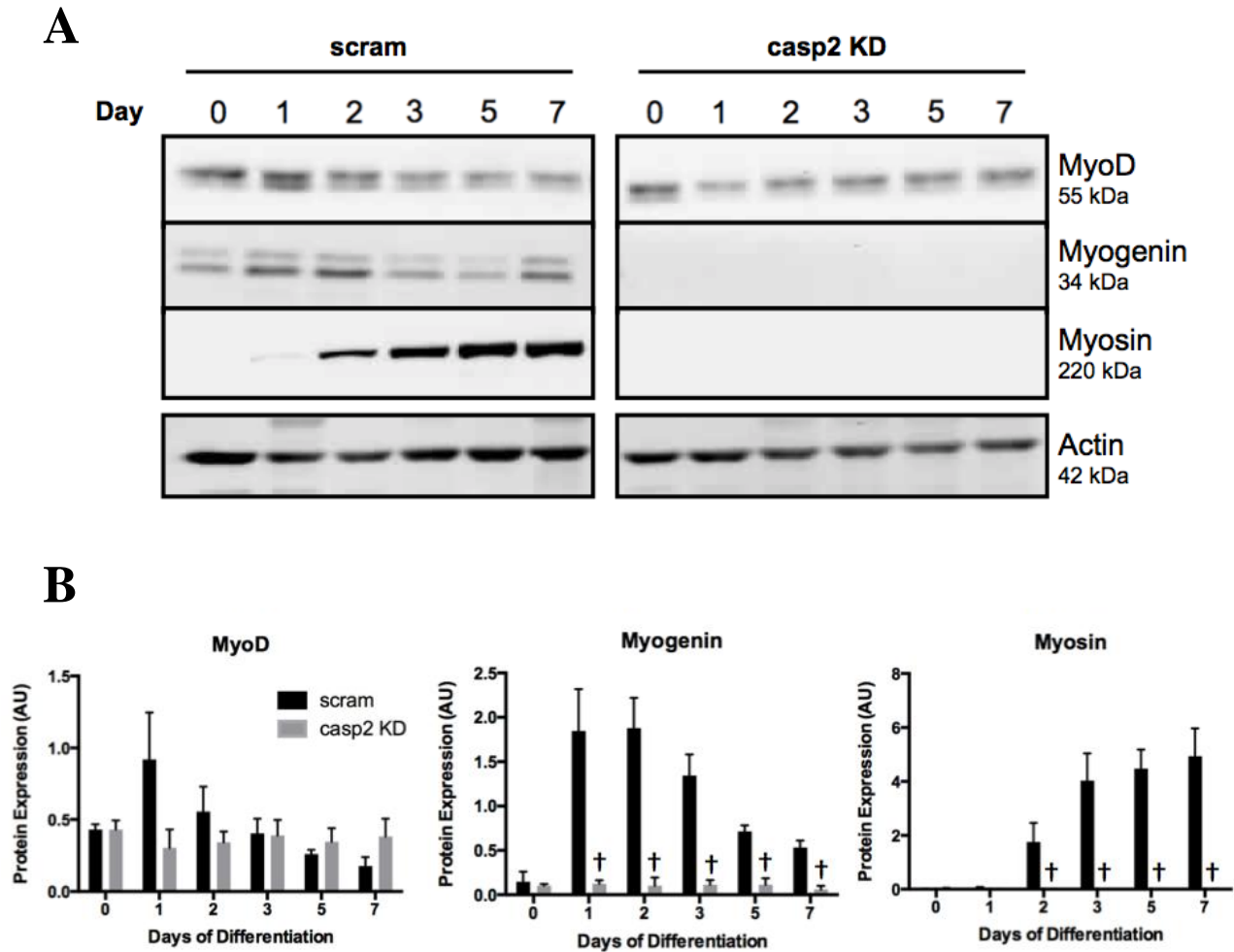
Figure 9: Expression of differentiation markers in scram and casp2 KD cells after inducing differentiation. A) Representative immunoblots of whole-cell MyoD, myogenin, and myosin expression in scram and casp2 KD cells with actin shown as a loading control. **B)** Quantification of MyoD, myogenin and myosin expression levels in scram and casp2 KD cells, normalized to loading control. †$p < 0.05$ compared to scram; (mean ± SEM, n=3 independent experiments).
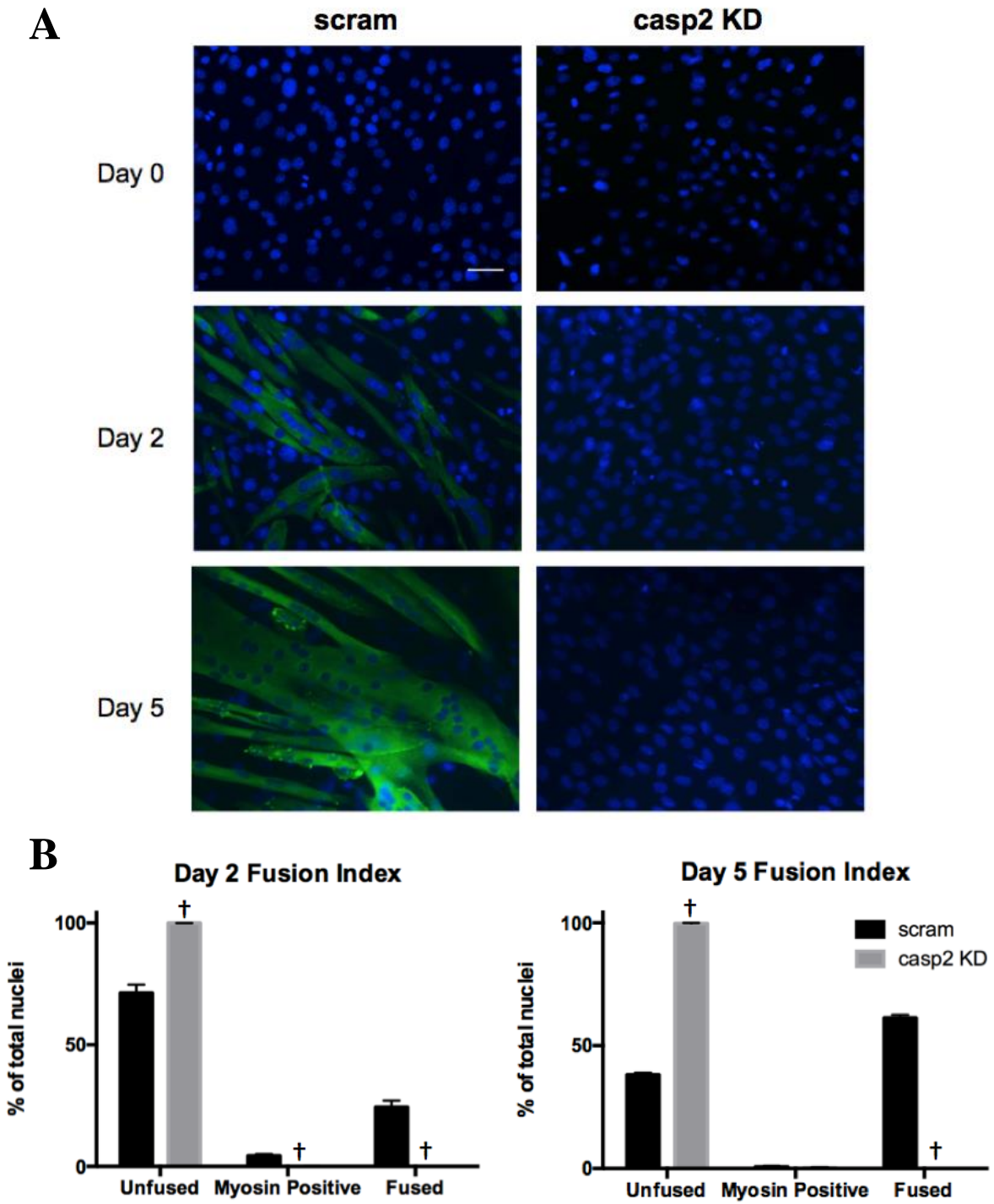
Figure 10: Morphology of scram and casp2 KD cells throughout differentiation. A) Fluorescent microscopy images depicting scram and casp2 KD cell morphology at various stages of differentiation. Nuclei are stained blue (DAPI) and myosin heavy chain is stained green (Alexa Fluor 488). Bar represents 50μm. **B)** Quantification of fusion index calculated by dividing the number of nuclei in multi-nucleated fibres by the total number of nuclei in ten randomly chosen microscopic fields. Casp2 KD cells exhibited severely impaired differentiation. †$p<0.05$ compared to scram (mean ± SEM, n=3 independent experiments).

Skeletal Muscle Cell Differentiation in the Absence of Caspase-2

In experiment 1, caspase-2 activity was shown to transiently increase early on in differentiation, suggesting a possible role for caspase-2 in this process. Using the casp2 KD cells and scram cells, we induced differentiation and measured differentiation markers in both groups. As discussed previously, scram cells were shown to exhibit similar characteristics as control C2C12 cells. Thus, in these experiments, comparisons were simply made between scram and caspase-2 KD cells at each time point. MyoD protein expression was not significantly different between groups (Figure 9A & 9B). However, myogenin expression was dramatically reduced in casp2 KD cells compared to scram at D1 and all time points thereafter ($p<0.05$, Figure 9A & 9B). Similarly, casp2 KD cells had virtually undetectable levels of myosin at all time points, which was significantly different between groups from D2 until D7 ($p<0.05$, Figure 9A & 9B). Consistent with these data, fluorescent microscopy revealed a dramatic increase in myosin expression in scram cells at D2 and D5 and no detectable myosin in casp2 KD cells at any time point (Figure 10A). By D5, 61.2% of nuclei in scram cells were located within multinucleated cells (Figure 10A & 10B). Conversely, at the same time point, 100% of nuclei in casp2 KD cells remained unfused and did not express myosin (Figure 10A & 10B).

Caspase-3 Activity in the Absence of Caspase-2

A transient spike in caspase-3 activity early on in differentiation has been consistently shown to be required for skeletal muscle cell differentiation. As we observed abrogated differentiation in the casp2 KD cells, we assessed the impact the

A Caspase 3

B scram / casp2 KD

Procaspase-3
34 kDa

Actin
42 kDa
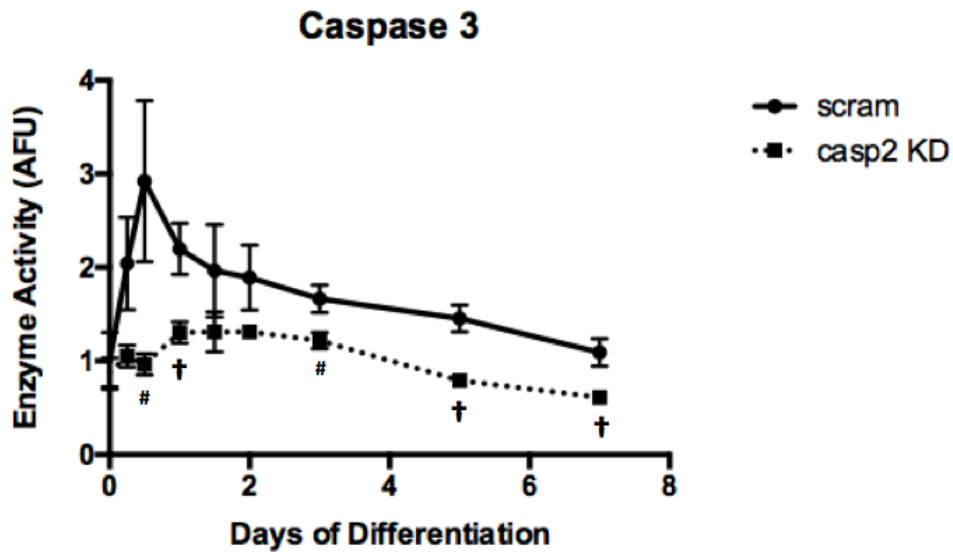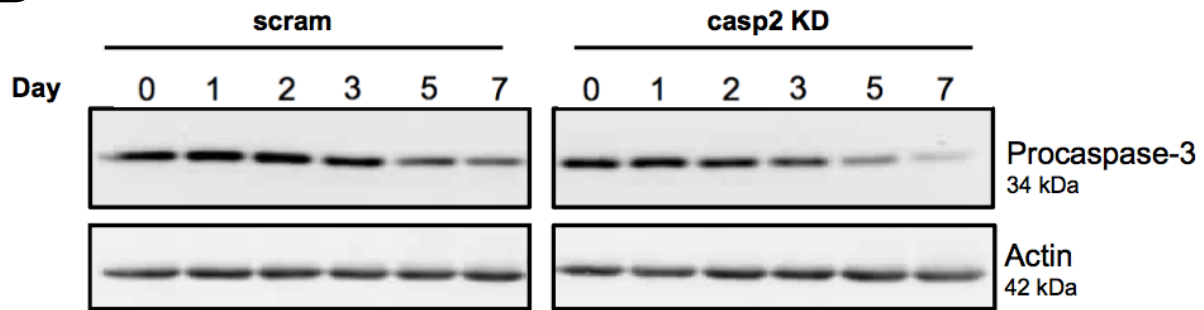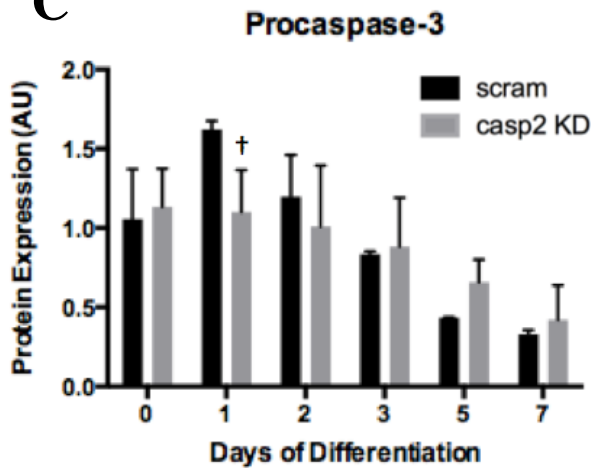
C Procaspase-3

Figure 11: Caspase-3 activity and protein expression in scram and casp2 KD cells throughout differentiation. A) Caspase-3 activity throughout differentiation in scram and casp2 KD cells. **B)** Representative immunoblots of procaspase-3 content throughout differentiation in scram and casp2 KD cells normalized to loading control. **C)** Quantification of procaspase-3 protein in scram and casp2 KD cells expressed relative to loading control. †p<0.05 compared to scram; #p<0.10 compared to scram (mean ± SEM, n=3 independent experiments).

knockdown had on caspase-3 activity. Caspase-3 enzyme activity was decreased by 40% at D1 in casp2 KD cells compared to scram cells (p<0.05, Figure 11A) and remained significantly decreased in casp2 KD cells at D5 and D7 (p<0.05, Figure 11A).

Furthermore, data from the D0.5 and D3 time points revealed a trend towards significance between the two groups ($p < 0.10$, Figure 11A). These effects were not explained by a difference in procaspase-3 protein content in the casp2 KD cells as there were few differences between groups (Figure 11B & 11C). Thus, caspase-3 activity but not content appear to be affected by a lack of caspase-2.

Subcellular Localization of Caspase-2 During Differentiation

In order to better elucidate the role of caspase-2 in differentiation, C2C12 cells were collected at early time points in differentiation, when caspase-2 has been shown to be most highly active. Cells were then processed into nuclear and extra-nuclear (or nuclear-free) fractions and immunoblotted for caspase-2 content. Expression of procaspase-2 was found almost exclusively in the nuclear fraction and was essentially absent in the extra-nuclear fraction at all time points ($p < 0.05$, Figure 12A & 12B) suggesting a nuclear localization of caspase-2 throughout differentiation. Interestingly, nuclear expression of procaspase-2 decreased significantly at D1 and D2 compared to D0 ($p < 0.05$, Figure 12A & 12B). According to previous caspase assay experiments, caspase-2 is most highly active from approximately 12-48 hours into differentiation. It was initially hypothesized that this decrease in nuclear procaspase-2 would result in a corresponding increase in extra-nuclear procaspase-2; however, this export was not observed. It is now hypothesized that the decrease in nuclear procaspase-2 is associated with a corresponding increase in nuclear cleaved caspase-2, thus suggesting that caspase-2 is being activated in the nucleus during differentiation rather than being exported into

38
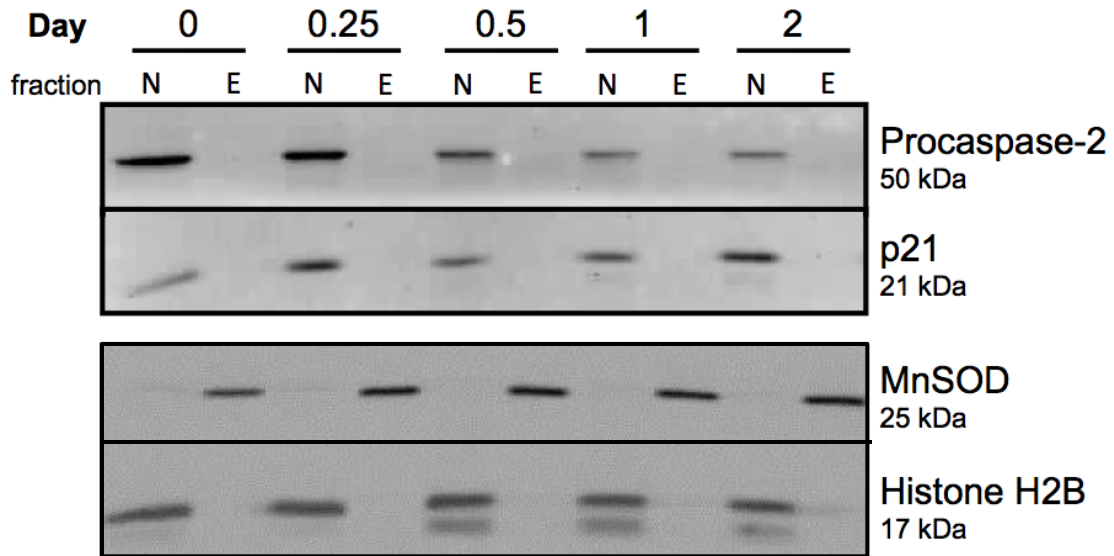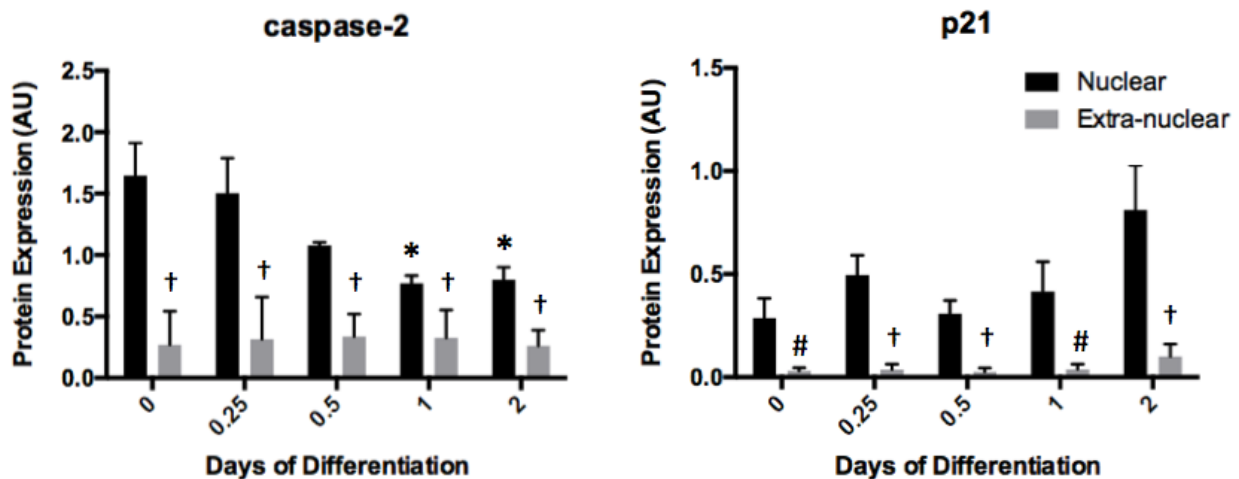
Figure 12: Subcellular localization of caspase-2 and p21 during differentiation.
A) Representative immunoblots of procaspase-2 and p21 in nuclear (N) and extra-nuclear (E) fractions during the early stages of differentiation. MnSOD and Histone H2B are used as purity controls as they are found exclusively in their respective fractions (MnSOD in extranuclear; Histone H2B in nuclear). **B)** Quantification of procaspase-2 and p21 protein expression in nuclear and extra-nuclear fractions during the early stages of differentiation. Both procaspase-2 and p21 are predominantly localized to the nucleus throughout this process. Expression of procaspase-2 decreases at day 1 and day 2 compared to day 0 levels. *$p<0.05$ compared to day 0; †$p<0.05$ compared to nuclear; #$p<0.10$ compared to nuclear (mean ± SEM, n=3 independent experiments).

the cytosol and activated. Preliminary experiments have supported this hypothesis (Appendix Figure 3).

As shown in experiment 1, immunoblotting for p21 expression throughout differentiation revealed a dramatic increase in p21 protein early on in differentiation as the cells are exiting the cell cycle and entering into the differentiation program. Furthermore, in experiment 2, p21 expression was dramatically reduced in the casp2 KD cells compared to scram. According to literature, caspase-2 plays a role in p21 activation; thus, it is possible that caspase-2 is exerting its role in differentiation through the activation of p21 early on in differentiation. Since we observed a nuclear localization of caspase-2 during differentiation, we determined if p21 was also localized to the nucleus during differentiation. Indeed, p21 expression was significantly higher in the nuclear fraction than in the extra-nuclear fraction at D0.25, D0.5 and D2 ($p < 0.05$, Figure 12A & 12B) and trended towards significance at D0 and D1 ($p < 0.10$, Figure 12A & 12B). Thus, p21 appears to remain in the nucleus throughout differentiation. Collectively, these data are in line with the hypothesis that caspase-2 may be inducing p21 expression early on in differentiation.

Discussion

The main focus of this study was to comprehensively examine the involvement of caspase-2 in skeletal muscle cell differentiation. As it stands, little is concretely known about caspase-2 in terms of functions, activation methods, and target substrates. Evidence exists for the interplay between apoptotic signaling mechanisms and differentiation with regards to caspase-3 (34), -8 (36), -9 (37), and -12 (39); however, only one study has previously identified a role for caspase-2 in this process. Previous work in our lab by Bloemberg & Quadrilatero (45) found that caspase-2 activity transiently increases early on in differentiation, appearing to increase even prior to caspase-3 activation. The present study aimed to better elucidate the role that caspase-2 may be playing in skeletal muscle differentiation. In order to test the hypothesis that caspase-2 activity is required for proper myocyte differentation, we measured caspase-2 activity in differentiating C2C12 murine myoblasts using a newer and more specific caspase-2 substrate. Furthermore, we produced stable caspase-2 KD clones and observed the differences in cell cycle and differentiation markers between the casp2 KD cells and scram control cells. Finally, we performed cellular fractionation to determine subcellular localization of caspase-2 throughout differentiation.

The results of this study demonstrate a critical role for caspase-2 in skeletal muscle cell differentiation. We confirmed the prior findings by Bloemberg and Quadrilatero (45) using a more specific substrate for caspase-2 activity. Furthermore, knock down of caspase-2 protein in C2C12 cells drastically impaired differentiation and resulted in a dysregulation of the cell cycle. Finally, we showed that caspase-2 remains

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localized to the nucleus throughout differentiation, alluding to its potential role in regulating differentiation via cell cycle exit.

Caspase-2 Activity Transiently Increases at the Onset of Differentiation

In order to lay the foundation for subsequent experiments, we aimed to confirm proper differentiation characteristics in the C2C12 cells. We assessed cell cycle profiles, myogenic regulatory factor expression levels and cell morphology and fusion events in differentiating C2C12 cells. Using flow cytometry detection of propidium iodide fluorescence, cell cycle stage was assessed as cells began to differentiate. Consistent with literature (4,7–9), induction of differentiation coincided with cell cycle exit as noted by an increase in the proportion of G_0/G_1 cells and a decrease of cells in the S and G_2/M phases (Figure 3A & 3B). Within 24 hours, approximately 92.5% of cells had entered into the nonproliferative G_0/G_1 phase of the cell cycle (Figure 3A & 3B). In accordance with cell cycle profiles, protein expression levels of p21 increased rapidly upon induction of differentiation (Figure 3C & 3D). As previously mentioned, p21 is a CDK inhibitor which can bind to and inhibit cdk-cyclin complexes 1 and 2 and plays a well established, required role in cell cycle exit prior to the onset of differentiation (14,89,90). Overall, our results are in accordance with the literature in regards to cell cycle characteristics at the onset of differentiation.

Protein expression levels of MyoD, myogenin, and myosin throughout differentiation were also consistent with the literature (reviewed in 10). At the onset of differentiation, MyoD levels decreased consistently from day 0 until day 7 (Figure 4A & 4B). As cells began to differentiate, myogenin protein levels increased rapidly, peaking at

day 2 and returning to near-basal levels by day 7 (Figure 4A & 4B). As cells reached terminal differentiation, myosin expression and cell fusion events both increased dramatically (Figure 4A & 4B; Figure 5A & 5B). Collectively, cell cycle, MRF, and microscopy data confirm proper differentiation in the C2C12 cells.

The vast majority of studies that have focused on the relationship between apoptotic signaling and myocyte differentiation have not measured caspase-2 activity, instead focusing on caspase-3 (34,91–93), -8 (36), and -9 (92,93). One study that did attempt to assess caspase-2 activation in differentiating C2C12 cells did not find measurable increases in the activated form of caspase-2; however, these researchers used only immunoblotting for the cleaved form of the enzyme and did not specify which antibody they used (37). Therefore, it is possible that the disparity in results between our study and the previously mentioned study are due to methodological differences. No prior studies have used the new, more specific caspase-2 substrate (VDTTD) to assess caspase-2 activation in differentiating cells. Consistent with previous findings in our lab (94) which used the former caspase-2 substrate (VDVAD), caspase-2 activity, measured with the VDTTD substrate, transiently increased very quickly upon induction of differentiation in C2C12 cells (Figure 6). The VDVAD substrate first arose in 1997 when researchers demonstrated efficient cleavage of VDVAD containing peptides by caspase-2; however, caspase-3 was shown to cleave the same peptide sequence with comparable kinetics (95). Other studies have had similar results (96,97), yet many researchers continue to use VDVAD substrates. The improved VDTTD substrate was developed by a group of researchers in Australia in 2014 (84). Using a yeast transcriptional reporter system, the authors were able to identify the minimal specificity of caspase-2 and employ

this data to create an appropriate fluorogenic substrate (84). In contrast to the oft-used VDVAD substrate, the VDTTD substrate has proven to be cleaved far more efficiently by caspase-2 than -3 (84). While this newly defined substrate is not absolutely caspase-2 specific, it is a far more promising tool than the commonly used VDVAD peptide sequence. Thus, the increased specificity of the VDTTD substrate adds credibility to our lab's previous findings with regards to caspase-2 activity in myocyte differentiation. In the present study, caspase-3 activity also increased rapidly and activity of both enzymes remained elevated until day 2, at which point activity levels began to decrease (Figure 6). By day 7, activity levels of caspase-2 and -3 were no longer statistically different from day 0 (Figure 6). Caspase activity in the C2C12 cells coincided with the onset of differentiation and as cells reached terminal differentiation, caspase activity returned to basal levels.

The most well-known and accepted function of caspase-2 is its role in apoptosis; its ability to cleave Bid to form tBid and cause subsequent release of pro-apoptotic factors from the mitochondria (57,98,99). Previous work from our lab, however, indicates that this mitochondrial-mediated apoptotic pathway is not involved in the differentiation process (45). Therefore, this classical apoptotic role of caspase-2 is likely not the means by which caspase-2 is affecting differentiation. In the present study, caspase-2 activity appeared to increase around the same time as caspase-3 activity (Figure 6). This is in contrast to Bloemberg & Quadrilatero's findings that caspase-2 activity increased just prior to caspase-3 activation (94). However, our results indicated that caspase-2 appears to peak and plateau by 12 hours (Figure 6). In contrast, caspase-3 activity continues to rise after 12 hours, peaking between 24 and 36 hours into differentiation (Figure 6). Thus,

due to the rapid and transient nature of enzymatic activity, it is possible that caspase-2 is indeed becoming active prior to caspase-3. In order to better examine the temporal profiles of caspase-2 and -3 at the onset of differentiation, future studies should collect more frequent time points between 0 and 48 hours to determine precise activation times.

If caspase-2 activity is in fact increasing prior to caspase-3 activation, it could be suggested that caspase-2 may be responsible for activating caspase-3 in differentiation. Indeed, previous work has provided evidence for caspase-2's ability to activate caspase-3 directly through binding of the prodomain (82). The cell cycle regulatory function of caspase-2 may suggest, however, that caspase-2 is playing a different role in differentiation, one that is more upstream of caspase-3 activation.

Caspase-2 KD Cells Display Dysregulated Cell Cycle Profiles and Impaired Differentiation

In order to explore the precise role of caspase-2 in differentiation, we produced shRNA-mediated caspase-2 KD clones in C2C12 cells. Cells transfected with casp2 shRNA plasmids displayed dramatically reduced caspase-2 content, measured via immunoblotting (Figure 7A & 7B), and caspase-2 activity, measured via fluorogenic enzyme assays (Figure 7C). The enzyme assay results from differentiating C2C12 cells suggested a requirement for caspase-2 activation in skeletal muscle differentiation. We further investigated this theory by comparing cell cycle profiles and differentiation characteristics between casp2 KD cells and scram control cells.

Due to the abundance of evidence demonstrating a role for caspase-2 in cell cycle regulation, we first compared cell cycle properties in casp2 KD cells to our scram control cells. Interestingly, flow cytometry data revealed a significant difference in the

proportion of cells in G_0/G_1 phase at all time points between groups in that casp2 KD cells had a higher proportion of cells in the G_0/G_1 phase (Figure 8A & 8B). Correspondingly, casp2 KD cells had a smaller proportion of cells in S and G_2/M phase at almost every time point (Figure 8A & 8B). These results would imply that casp2 KD cells are arresting earlier than scram cells, which appears to be in contrast to literature showing that caspase-2 knockout MEFs proliferate faster than wild-type MEFs and avoid cellular senescence (80); however, the difference in cell line used may contribute to the disparity in results that was observed. A role for caspase-2 in cell cycle control has been shown in previous literature, implicating the protease in G_2/M checkpoint control (80,100), a time when DNA is assessed for mutations and cells are prevented from undergoing mitosis if significant mutations are present (6). In one study, caspase-2 knockout MEFs were able to overcome G_2 arrest much more easily than wild type MEFs in response to infrared radiation (80). Similarly, we observed a decrease in proportion of cells in G_2/M phase in the casp2 KD condition compared to the scram condition (Figure 8A & 8B), suggesting that in the absence of caspase-2, cells may be moving through the G_2/M phase unchecked and unregulated. Aberrations in cell cycle checkpoint control leading to accumulations of damaged DNA has been shown to result in aneuploidy (abnormal number of chromosomes) and genomic instability (101,102); an effect that has been observed in caspase-2 deficient MEFs (80). Examination of our cell cycle data from day 1 revealed that casp2 KD cells had a more heterogeneous distribution of cells in G_0/G_1 compared to scram (Figure 8A). According to previous literature, G_0/G_1 peaks with wide coefficients of variation often contain aneuploid cell populations (103); thus, it is

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possible that the wider G_0/G_1 peak observed in the casp2 KD cells at day 1 is caused by aberrant DNA checkpoint control resulting in abnormal DNA content and aneuploidy.

While we did observe an increased proportion of casp2 KD cells in the G_0/G_1 phase, immunoblotting also revealed a lack of p21 induction in casp2 KD cells, which is typically needed for cell cycle exit. Protein levels of p21 were dramatically reduced in casp2 KD cells compared to scram at almost all time points after the onset of differentiation (Figure 8C & 8D). This is not particularly surprising, given the evidence that caspase-2 is required for p21 expression (79); however, it does appear to be in contrast with our cell cycle data. Nonetheless, it is clear that casp2 KD cells demonstrate irregularities in cell cycle control.

Given the interplay that exists between cell cycle regulation and differentiation, it is not surprising that differentiation was dramatically impaired in casp2 KD cells. Fluorescent microscopy revealed a complete lack of myotube formation at any time point in the casp2 KD cells, while scram cells appeared to differentiate normally (Figure 10). Likewise, myosin protein increased dramatically until day 7 in scram cells while casp2 KD cells displayed virtually undetectable levels of myosin at any time point (Figure 9A & 9B). Previous work in our lab has also demonstrated impaired C2C12 differentiation with a lack of caspase-2 activity (94). Treatment of cells with caspase-2 inhibitor (VDVAD-CHO) decreased myosin expression and cell fusion events in a dose-dependent manner (94). Chemical inhibition also resulted in a reduction of caspase-3 activity (94). Similarly, we observed a dramatic abrogation of caspase-3 activation, while protein levels of procaspase-3 remained unchanged (Figure 11), indicating that caspase-2 knockdown affected caspase-3 processing and activation. There is evidence to suggest

that caspase-2 can directly activate caspase-3 (82); however, we are unable to conclude with our results whether caspase-3 activity repression is directly due to the knockdown or instead due a lack of upstream differentiation events that are prevented by the caspase-2 knockdown. Nevertheless, caspase-3 has been shown to be critical for myocyte differentiation; therefore, it is not surprising that we observed a dramatic impairment in this step of the pathway in our KD cells.

Furthermore, Bloemberg et al found that while lower doses (30μm) of caspase-2 inhibitor did not appear to impact myogenin induction, higher doses (75μm) significantly inhibited myogenin in differentiation-induced C2C12 cells (94). In the present study, we also observed a dramatic impairment in myogenin protein expression in the casp2 KD cells. While scram cells displayed normal myogenin induction at the onset of differentiation, casp2 KD cells had very little myogenin protein content and showed no increase early on in differentiation (Figure 9A & 9B). Perhaps unsurprisingly, MyoD protein content was not affected by the protein knockdown, as there were no significant differences in MyoD content between groups at any time point (Figure 9A & 9B). This would suggest that MyoD is upstream of caspase-2 activation in differentiation. Previous research has implicated MyoD in inducing p21 expression in myoblasts in order to cause cell cycle exit (4,8,14,89); however, given that MyoD expression was not affected by casp2 KD but p21 expression was, it is possible that caspase-2 is responsible for inducing p21 expression independently of MyoD. Indeed, previous work has shown a need for caspase-2 in p21 expression in response to DNA damage (79). Furthermore, p21 expression has been shown to be crucial for proper myocyte differentiation (90,104). In 1999, Zhang and colleagues observed profound defects in skeletal muscle differentiation

in p21/p57 KO mice and posited that these CKI proteins are controlling differentiation at the myogenin step (i.e. downstream of MyoD; 101). More recently, researchers have demonstrated that p21 KO mice had greatly impaired muscle regeneration in response to bupivacaine-induced muscle injury (90). Exactly where p21 fits into the temporal landscape of myocyte differentiation is difficult to establish. Some researchers place p21 activation downstream of myogenin (105); some believe it is a parallel occurrence to myogenin induction (104), and others suggest that myogenin is translocated to the nucleus only after proliferation has ceased, putting myogenin activation after p21 activation (106). Thus, at this point it is difficult to determine if and how caspase-2 may be regulating myogenin during differentiation. Collectively, these results suggest that caspase-2 is likely acting downstream of MyoD and upstream of p21, myogenin, and caspase-3 during myocyte differentiation.

Caspase-2 Remains Localized to the Nucleus Throughout Differentiation

In order to better understand the precise role that caspase-2 is playing in differentiation, we assessed the subcellular localization of both caspase-2 and p21 throughout differentiation. We posited that knowing the location of these proteins might help to shed light on where caspase-2 is acting in this complex process. Our results indicated that caspase-2 retained its nuclear localization throughout differentiation, with no apparent cytosolic translocation (Figure 12A & 12B). Interestingly, expression levels of procaspase-2 decreased in the nuclear fraction around 12 hours – 2 days of differentiation (Figure 12A & 12B). As this is the timeframe that caspase-2 is most active, a decrease in expression levels initially appears counter-intuitive. A corresponding

increase in extra-nuclear expression levels would explain this nuclear decrease; however, this export from the nucleus was not observed (Figure 12A & 12B). Intriguingly, preliminary work from our lab indicated a corresponding increase in nuclear cleaved caspase-2 levels (Appendix Figure 3), indicating activation of caspase-2 occurring within the nucleus itself. Subcellular localization of caspase-2 has been widely debated, with some researchers suggesting it is primarily cytosolic, mitochondrial, or nuclear (69-75). Our work suggests that caspase-2 is primarily nuclear in C2C12 myocytes and it remains in the nucleus as differentiation proceeds (Figure 12A & 12B; Appendix Figure 3). That being said, subcellular localization of the enzyme could very well differ between cell types and processes. The enzyme's localization provides intriguing information as to caspase-2's role in differentiation. If caspase-2 were located in the cytosol, it is likely that it would be playing a similar role to the other apoptotic proteases (caspase-3, -8, -9) in differentiation. For example, research has implicated a need for caspase-3-mediated cleavage-induced activation of promyogenic kinase MST1 (34). Furthermore, Twist, an inhibitor of differentiation, has been identified as a substrate for cleavage-induced inactivation by caspase-3 (40). Initiator caspases -8 (36) and -9 (37) have also been implicated in skeletal muscle cell differentiation, albeit through activation of the executioner caspase-3. In contrast to these other caspases, caspase-2 is uniquely found in the nucleus suggesting that it plays a myogenic role quite distinct to the other members of its family; its nuclear localization would suggest a cell cycle regulatory role, as we have hypothesized.

Furthermore, we also found p21 localized to the nucleus throughout differentiation. Human p21 is primarily found in the nucleus however; it has been

reported in other cellular compartments (107). The localization of p21 is predominantly regulated through post-translational modifications such as phosphorylation and ubiquitination (reviewed in 104). As previously discussed, research has shown that caspase-2 is required for p21 expression; however, investigators were unable to definitively determine how regulation was occurring, but speculated that it may be through inhibition of microRNAs that are known to prevent p21 translation (79). Alternatively, it is possible that caspase-2 is indirectly impacting the activation of p21 by affecting its degradation. A variety of studies have shown that nuclear p21 can be targeted for proteasomal degradation by the E3 ubiquitin ligase Mdm2 (108,109). Interestingly, additional research has shown that caspase-2 can cleave and inactivate Mdm2, specifically in the context of Mdm2-mediated degradation of p53 (110); however, a similar mechanism could be at play with p21 (i.e. caspase-2 inactivates Mdm2, preventing Mdm2 from targeting p21 for degradation). Indeed, our immunoblotting results for p21 expression in scram cells and casp2 KD cells could be explained by a increased degradation of p21 in the absence of caspase-2 (Figure 8C & 8D). Due to the fact that Sohn et al (79) could not elucidate the mechanism with which caspase-2 was contributing to p21 expression, it is possible that caspase-2 is acting indirectly on p21 via Mdm2 inhibition and, consequently, decreased protein degradation.

As previously mentioned in the context of C2C12 differentiation, evidence suggests that myogenin is localized to the cytoplasm in proliferating cells and is translocated to the nucleus only after proliferation has ceased (106). As p21 has been shown to be required for cell cycle exit in differentiation, this would place myogenin translocation after p21 induction. Our results indicated a dramatic impairment of protein

expression for both p21 and myogenin in casp2 KD cells, both of which would have significant effects on myocyte differentiation (Figure 8C & 8D; Figure 9). As the literature is inconclusive with respect to the timeline of p21 and myogenin activation, the signaling cascade of events is currently unclear. However, if myogenin is indeed translocated to the nucleus only after cells have arrested proliferation, then p21 is the more likely primary target of caspase-2 in this process, and the observed impairment in myogenin induction is probably an indirect effect. Taken together, the co-localization of both caspase-2 and p21 in the nucleus throughout differentiation along with the dramatic decrease in p21 expression in casp2 KD cells suggests that caspase-2 is contributing to the induction of p21expression during differentiation.

Summary and Conclusions

For years, researchers have struggled to pin down a singular, primary role for the enigmatic protease caspase-2. Unlike its family members, caspase-2 is difficult to fit into the classical apoptotic pathway and its unique nuclear localization is consistent with findings that it functions in cell cycle regulation, DNA damage response, and tumour suppression. It is clear that caspase-2 is a multifaceted enzyme with a variety of cellular roles. The main goal of this study was to investigate the role that caspase-2 might be playing in cellular differentiation. The results of this study highlight three main points: 1) caspase-2 is temporarily activated early on during myoblast differentiation, 2) myoblasts lacking caspase-2 do not differentiate, and 3) caspase-2 remains localized to the nucleus throughout myoblast differentiation. Given the findings that p21 expression is dramatically reduced in casp2 KD cells, as well as the suggestion that the primary role of caspase-2 is likely its ability to regulate p21, we propose that the role of caspase-2 in differentiation is to contribute to the activation of p21 to allow for cell cycle exit and subsequent differentiation. While this theory requires further investigation, the results of this study indicate a novel, critical role for caspase-2 in skeletal muscle cell differentiation.

Limitations

shRNA transfection as a means of knocking down protein expression is widely used in literature; however, stably transfecting a plasmid into a cell line requires the integration of a piece of foreign DNA into the cell's genome, which can result in off target effects depending on where the plasmid has been incorporated (111). As a means of controlling for these potential effects, scramble cell clones were transfected, isolated and passaged in the same way as KD clones; however, it is still possible that some of the observed effects of caspase-2 KD on differentation were due to the transfection process and not the protein knockdown. To further attempt to control for this possibility, clones that were transfected with the casp2 KD plasmid but did not display measurable protein KD were induced to differentiate. These "non-KD" clones appeared to differentiate normally and showed normal myogenin and myosin protein expression (Appendix figure 2). Furthermore, a separate casp2 KD clone displayed similar differentiation impairment and cell cycle dysregulation (data not shown). Therefore, while the possibility still exists that some of the observed changes in differentiation are due to off-target effects, we are confident that our overall results are indicative of the importance of caspase-2 protein expression in myocyte differentiation.

A major barrier to caspase-2 research is the lack of caspase-2 specific tools to monitor enzymatic activity. VDVAD containing peptides are efficiently cleaved by caspase-2 but caspase-3 is shown to also cleave the peptide sequence with comparable kinetics (95). Other studies into caspase-2 substrates have found similar results (96,97), yet many researchers continue to use VDVAD substrates, claiming they are caspase-2 specific. It is clear that there is a need for more selective tools for caspase-2 activity and

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inhibition; substrates that can be cleaved effectively by caspase-2 only. More recently, researchers were able to develop a better caspase-2 substrate. In contrast to the commonly used VDVAD-derived substrates, Ac-VDTTD-AFC has been shown to be cleaved far more efficiently by caspase-2 than -3 (84). Thus, we employed the use of the VDTTD substrate in this study. While this newly defined substrate is not absolutely caspase-2 specific (84), it is a far more promising tool than the commonly used VDVAD peptide sequence. Therefore, although we cannot definitively say that the observed fluorescence is due to caspase-2 activity, we can be more confident that we are measuring caspase-2 and not caspase-3 activity with this new substrate.

Future Directions

Although the results of this study indicate that caspase-2 is critically important for C2C12 differentiation, further is required to elucidate the precise activation methods for caspase-2 in this process. The nuclear localization of caspase-2 does provide some insight into how it may be activated (i.e. likely not through the PIDDosome). Knocking down some key potential activators of caspase-2 (Cyclin D3, p53 etc.) via shRNA or CRISPR and observing how these knockdowns impact caspase-2 activation in differentiation may aid in better understanding this important pathway.

The results of this study suggest that caspase-2 may be exerting its effect on differentiation through contributing to the activation of p21 early on in differentiation. In order to definitively provide causal evidence for this activation, future studies should attempt to rescue the observed phenotype in the caspase-2 KD cells. If reintroducing p21 expression and activation into the KD cells attenuates the effects of the KD, this potential link between caspase-2 and p21 would be even more compelling. Increasing p21 expression has been identified as a possible method of arresting proliferation in cancer cells (107). For example, histone deacetylase (HDAC) inhibitors are being employed in studies to de-repress p21 (112,113) and proteasome inhibitors are being tested as a means of reducing protein degradation of p21 (114). It would be of interest to test out drugs in the casp2 KD cells and assess if any rescuing of the phenotype is observed. Furthermore, if caspase-2 is in fact contributing to p21 activation via cleavage and inactivation of Mdm2, it would be interesting to determine protein levels of Mdm2 in the absence of caspase-2, and further investigate how this may be impacting p21 expression. It is

possible that inhibiting Mdm2 activity would prevent degradation of p21 and improve differentiation in the absence of caspase-2; however, this remains to be determined.

This study provides evidence for a critical role of caspase-2 in C2C12 differentiation *in vitro*. Future studies should aim to replicate these results *in vivo*. While caspase-2 knockout (KO) mice appear to develop muscle normally, it is possible that compensatory mechanisms are at play in embryonic development. It would be interesting to employ a caspase-2 transgenic KO mouse line and induce skeletal muscle injury to determine if caspase-2 is important in muscle cell regeneration via satellite cell differentiation. Another possible approach to circumventing the effects of compensatory mechanisms *in vivo* would be to use a tamoxifen inducible caspase-2 KO mouse line to introduce protein KO in the adult animal.

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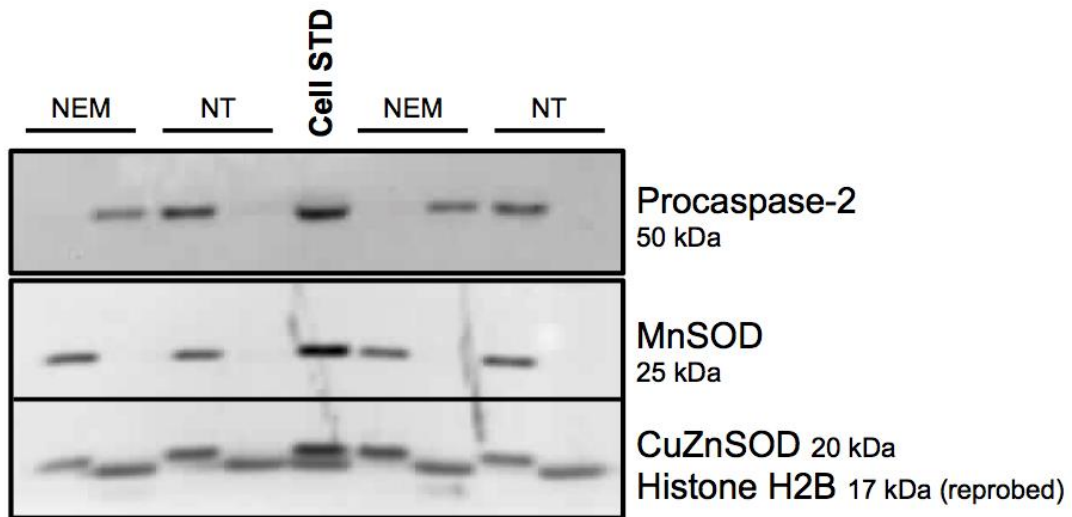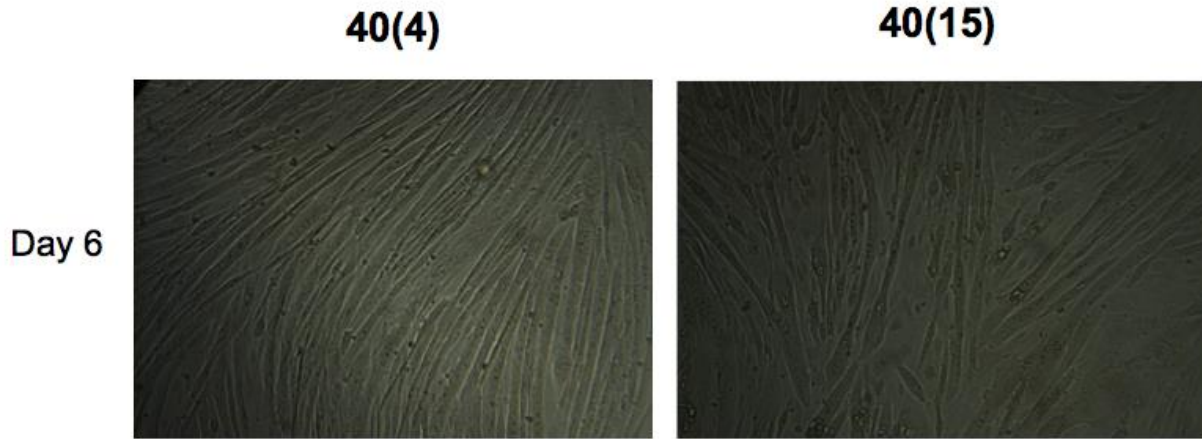
Appendix

Appendix Figure 1: Validating NEM pretreatment for fractionation. Cells were either pretreated for 10 minutes with n-ethylmaleimide (NEM) or not pretreated (NT) prior to harvesting for fractionation. NT cells expressed exclusively extra-nuclear procaspase-2 while NEM treatment prevented this artifactual redistribution in the pretreated cells. MnSOD and CuZnSOD are located exclusively in the extra-nuclear fraction while Histone H2B is found only in the nuclear fraction. Fraction purity was confirmed with these control proteins.

A
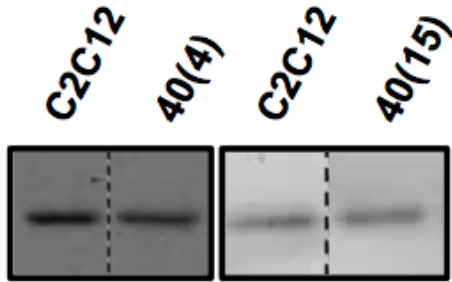
Appendix Figure 2: Differentiation testing of nonKD clones. Cells that were transfected with caspase-2 KD plasmid but did not exhibit knockdown appear to differentiate normally. **A)** Immunoblots of caspase-2 content in C2C12 cells and nonKD clones. **B)** Light microscope images of nonKD clones at day 6 of differentiation. **C)** Immunoblots of myogenin and myosin protein expression at various stages of differentiation in nonKD clones.

B

C
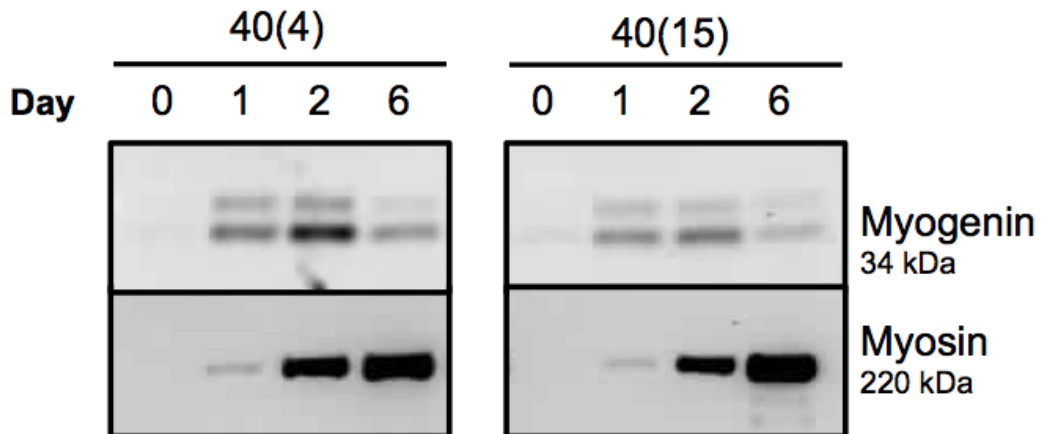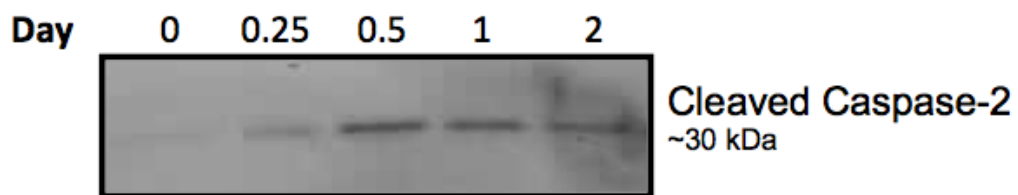
Appendix Figure 3: Expression of nuclear cleaved caspase-2 during the early stages of differentiation. Protein expression of cleaved caspase-2 appears to be increased at days 0.5, 1 and 2 compared to day 0. This increase seems to correspond with the decrease in nuclear procaspase-2 observed in experiment 3.