Interplay between Autophagy and Apoptosis During Skeletal Muscle Differentiation

by

Fatemeh Keyvani

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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. I understand that my thesis may be made electronically available to the public.

Abstract

Our previous studies showed that autophagy regulates apoptosis and is required for proper skeletal muscle differentiation. Previously, we inhibited autophagy in C2C12 cells using 3MA (3-methyladenine) treatment or shRNA against ATG7, both of which resulted in elevated apoptotic signaling and impaired skeletal muscle differentiation. In the present study, we treated C2C12 cells with ad-DN-ATG5 (adenovirus expressing the dominant negative form of the autophagy related protein 5) to interrupt autophagosome formation and inhibit autophagy. The ad-DN-ATG5 treated C2C12 cells displayed elevated apoptosis (increased CASP3 (caspase3) activation) as well as lower MYH (myosin heavy chain) expression and impaired myoblast fusion and differentiation. The increased CASP3 activation in ad-DN-ATG5 treated C2C12 cells was accompanied by significantly reduced BECN1 (beclin 1) levels. Studies in other cell types and contexts have indicated that CASP3 could cleave BECN1 and inactivate BECN1 autophagic function. Therefore, we investigated whether STS (staurosporine)-induced CASP3 activation is also accompanied by lower BECN1 levels. STS treatment also resulted in reduced BECN1 levels in differentiating C2C12 cells. Next, we investigated the importance of BECN1 regulator; AMBRA1 (activating molecule in BECN1 regulated autophagy protein 1), during C2C12 differentiation. Silencing AMBRA1 expression in C2C12 cells using an AMBRA1 siRNA, lead to reduced levels of anti-apoptotic protein BCL2 (BCL2 apoptosis regulator) as well as significantly lower MYH expression. Collectively, the result of this study i) confirm the previously observed protective role of autophagy against apoptosis during skeletal muscle differentiation, ii) introduce BECN1 as a potential factor contributing in the interplay

between autophagy and apoptosis, and iii) indicate an important role for AMBRA1 in regulating apoptotic signaling and skeletal muscle differentiation.

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I never can thank my family enough for all the support they have given me during this journey and before. Dad, thank you for seeding and nurturing the love of science in me. Mom, thank you for always believing the best in me. My brothers for always being so supportive, and Mahdi for all your supports throughout this journey especially, patiently listening to my complaints about experiments not working.

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List of Abbreviations

3MA, 3-methyladenine

ADP, adenosine di-phosphate

AIFM1/AIF, apoptosis-inducing factor, mitochondrion-associated 1

AMBRA1, activating molecule in BECN1 regulated autophagy protein 1

AMPK, AMP-activated protein kinase

APAF, apoptotic protease activating factor 1

ATG, autophagy related

BAD, BCL2 associated agonist of cell death

BA/F3, mouse tumor cell

BAK, BCL2 antagonist/killer

BAX, BCL2-associated X, apoptosis regulator

BCL2, BCL2 apoptosis regulator

BCL2L/BCLXL, B cell leukemia/lymphoma 2

BECN1, Beclin1

BH, BCL2 homology

bHLH Twist, twist basic helix-loop-helix transcription factor 1

BID, BH3 interacting domain death agonist

BNIP3, BCL2/adenovirus E1B interacting protein 3

BNIP3L/NIX, BCL2/Adenovirus E1B 19 KDa Protein-Interacting Protein 3-Like

C2C12, murine myoblast cell line

CASP, caspase

CASP3, caspase 3

CASP8, caspase 8

CASP9, caspase 9

CDK, cyclin dependent kinase

c-MYC, MYC proto-oncogene, bHLH transcription factor

CYCS, cytochrome C

DIABLO/SMAC, diablo IAP-binding mitochondrial protein

DM, differentiation media

DMEM, Dulbecco's modified Eagle's medium

DYNC1LI1/DLC, dynein cytoplasmic 1 light intermediate chain 1

ER, endoplasmic reticulum

FOXO, forkhead box O

FIP200, FAK family interacting protein of 200 kD

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

GFP, green fluorescent protein

GM, growth media

JNK, c-Jun N-terminal kinase

LIR, LC3-interacting region

MAP1LC3B/LC3B, microtubule-associated protein 1 light chain 3 beta

MAPK, mitogen-activated protein kinases

MCF7, breast cancer cell line

MOI, multiplicity of infection

mPTP, mitochondrial permeability transition pore

MRF, myogenic regulatory factor

MST1, mammalian sterile twenty-like kinase

MuSC, muscle stem cell

MYF5, myogenic factor 5

MYH, myosin heavy chain

MYOD, myogenic differentiation 1

MYOG, myogenin

OMM, outer mitochondrial membrane

P21, cyclin dependent kinase inhibitor 1

PARL, protease presenilin-associated rhomboid- like protein

PE, phosphatidyl ethanolamine

PI3P, phosphatidylinositol 3-phosphate

PIK3C3/VPS34, phosphatidylinositol 3-kinase catalytic subunit type 3

PIK3R4/VPS15, phosphoinositide-3-kinase regulatory subunit 4

PINK1, PTEN induced putative kinase 1

PRKN, parkin RBR E3 ubiquitin protein ligase

RAB, RAS-related in brain

RB1CC1/FIP200, RB1 inducible coiled-coil 1

RHEB, Ras homologue enriched in brain

ROS, reactive oxygen species

SH-SY5Y, human neural cell line

SMAC, second mitochondria-derived activator

SNARE, soluble N–ethylmaleimide sensitive factor (NSF) attachment protein receptor

SOD1, superoxide dismutase 1, soluble

SOD2, superoxide dismutase 2, mitochondrial

SQSTM1/P62, sequestosome 1

STS, staurosporine

TIM23, translocase of the inner mitochondrial membrane 23

TNF, tumor necrosis factor

TRAF6, TNF receptor-associated factor 6

UBA, ubiquitin-associated domain

ULK1, unc-51 like autophagy activating kinase 1

VDAC1, voltage-dependent anion channel 1

WT, wild type

Chapter 1 Introduction and Literature Review

1.1 Background

During differentiation and maturation of skeletal muscle stem cells, specific cellular reprogramming must occur to generate functional cells. Cysteine aspartate proteases (caspases) are involved in this reprogramming given their roles in regulating the cell cycle, activating pro-myogenic factors, and reorganizing cytoskeletons. However, caspases (CASPs) are also well-defined apoptosis inducers, so their activity should remain below a certain intensity threshold; otherwise, they cause apoptotic cell death and impaired skeletal muscle differentiation. In differentiating myoblasts, autophagy regulates CASP (caspase) activation, thereby assuring proper skeletal muscle differentiation. In this work, we first illustrated the interplay between autophagy and apoptosis during skeletal muscle differentiation using a new treatment and then introduced BECN1 (beclin 1) and AMBRA1 (activating molecule in BECN1 regulated autophagy protein 1) as potential factors contributing to this interplay.

1.2 Autophagy

Autophagy is a degradative pathway responsible for the intracellular breakdown of proteins and organelles. It also acts as an energy provider for cells at times of starvation by sacrificing cellular components to provide enough metabolic substrate [1-3]. Autophagy is additionally involved in cellular remodelling, as well as the removal of damaged and long-lived proteins and organelles [1-3]. Briefly, autophagy begins with the formation of double-membranes (isolation membrane), that are filled with cellular cargos, which then fuse with lysosomes

and ultimately conclude with the cargo degradation [1-3]. Autophagy regulated (ATG) proteins comprise the core machinery that regulates different stages of autophagy. ATG proteins were first identified in yeast; however, their mammalian homologues are now extensively characterized [4]. Other factors also contribute to regulate autophagy, which together with ATGs can be grouped based on their functions at the key stages of autophagy which includes; initiation, elongation, maturation, and fusion to the lysosome (Figure 2).

1.2.1 Initiation of autophagosome formation

Two main phosphorylation platforms are required for the initiation of autophagosome formation. The first phosphorylation platform that is activated following the induction of autophagy is the ULK (UNC-51-like kinase) complex, which contains ULK1/2 (unc-51 like autophagy activating kinase 1) proteins, ATG13 (autophagy related 13), and FIP200 (FAK family interacting protein of 200 kD) [1, 5, 6]. Normally, this complex is hyperphosphorylated and inhibited, but starvation (or excretion of other autophagy inducers) leads to hypo-phosphorylation of ULK1/2 [5, 6]. This activates ULK1/2, promoting its autophosphorylation and phosphorylation of distinct residues on ATG13 and FIP200 [1, 5, 6]. These phosphorylation events induce ULK complex translocation to the site of pre-autophagosome production as well as activation of the BECN1 complex; the second phosphorylation platform required for pre-autophagosome formation (Figure 1).

The BECN1 complex acts as a class-III phosphatidylinositol 3-kinase (PI3K) and is responsible for the production of phosphatidylinositol 3-phosphate (PI3P), required for the assembly and activity of autophagosomes [6]. It consists of the core proteins of PIK3C3/VPS34 (phosphatidylinositol 3-kinase catalytic subunit type 3), which is a

phosphatidylinositol 3 kinase, PIK3R4/VPS15 (phosphoinositide-3-kinase regulatory subunit 4), ATG14 (autophagy related 14), AMBRA1, and BECN1 [7]. The BECN1 complex synthesizes PI3P, thereby regulating the nucleation of the double membrane [7]. Studies suggest that the source of phosphatidylinositol (PI) is from cellular membranes, including the plasma, mitochondria, and endoplasmic reticulum (ER) [8, 9]. Therefore, the BECN1 complex converges to these membranes and phosphorylates the phosphatidylinositol by its PIK3C3 kinase [8-10]. Ultimately, PI3P, the product of BECN1 complex, mediates the recruitment of other adaptor proteins, which promote the elongation of autophagosome [1].

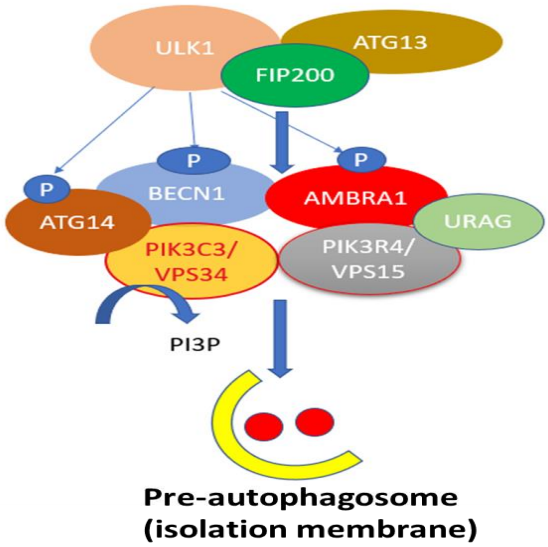

Figure 1: Two phosphorylation platforms of ULK- and BECN1-complex are involved in the initiation of autophagosome formation. The first complex that is activated following autophagy induction is the ULK1-ATG13-FIP200 complex, which in turn activates BECN1 complex by phosphorylating its BECN1, ATG14, and AMBRA1 members. The activated BECN1 complex then produces PI3P and initiates autophagosome formation.

1.2.2 Elongation of the double membrane

The pre-autophagosome membrane elongates with the aid of two ubiquitin-like conjugation systems. In the first system, ATG12 (autophagy related 12) is activated by ATG7 (autophagy related 7) (that acts as an E1 ubiquitin-conjugated enzyme), then transferred to ATG10 (autophagy related 10) (which acts as an E2 like ubiquitin-conjugated enzyme), and finally covalently linked by its C-terminal domain to internal lysine residues of ATG5 (autophagy related 5). The ATG12-ATG5 then forms a conjugate with ATG16, resulting in an 800-kDa complex [1, 9]. This complex is essential in the elongation of the pre-autophagosome membrane and is later dissociated from the final complete autophagosome membrane [1, 11]. In the second conjugated system, MAP1LC3B/LC3 (microtubule-associated protein 1 light chain 3 beta) is synthesized in a precursor form, which is cleaved by ATG4B (autophagy related 4B), producing the cytosolic isoform, LC3-I. LC3-I is then conjugated with the phospholipid phosphatidylethanolamine (PE) in a reaction mediated by ATG7 (E1-like) and ATG3 (E2-like), producing LC3-II. LC3-II then contributes to the elongating autophagosome membrane and exists both on the internal and external sides of the membrane [1]. Unlike the ATG12-ATG5-ATG16 complex, LC3-II remains on the autophagosome membrane until autophagosome-lysosome fusion and the subsequent autophagosome degradation [1].

1.2.3 Fusion and degradation of autophagosome

Autophagy concludes by autophagosome-lysosome fusion and the subsequent degradation of autophagosome by lysosomal enzymes [1]. First, the complete autophagosome migrates toward microtubule-organizing centers, the sites that are rich in lysosomes [1]. Next, the outer membrane of autophagosome fuses to the membrane of the lysosome in a process

mediated by SNARE [soluble N–ethylmaleimide sensitive factor (NSF) attachment protein receptor) and RAB (RAS-related in brain] family of proteins [1, 12]. Then, lysosomal hydrolases break the autophagosome cargo to their building blocks. Finally, hydrolyzed materials are released into the cytosol, where they can be used for energy production, protein synthesis, and other tasks [13].

General (or bulk) autophagy is responsible for the degradation of non-specific cytosolic sequestered portions. SQSTM1/P62 (sequestosome 1) protein is the most well-known mediator of lysosome-ubiquitinated-cargo recognition [13, 14]. Damaged and misfolded proteins can be tagged by ubiquitin through the Ubiquitin-Proteasome System (UPS) [13, 14]. SQSTM1 can identify ubiquitinated proteins and bind to them via its ubiquitin-associated domain (UBA) [13-15]. SQSTM1 can also bind to LC3 since it possesses an LC3-interacting region (LIR), thereby facilitating the identification and degradation of ubiquitinated substrates by autophagosome [14].

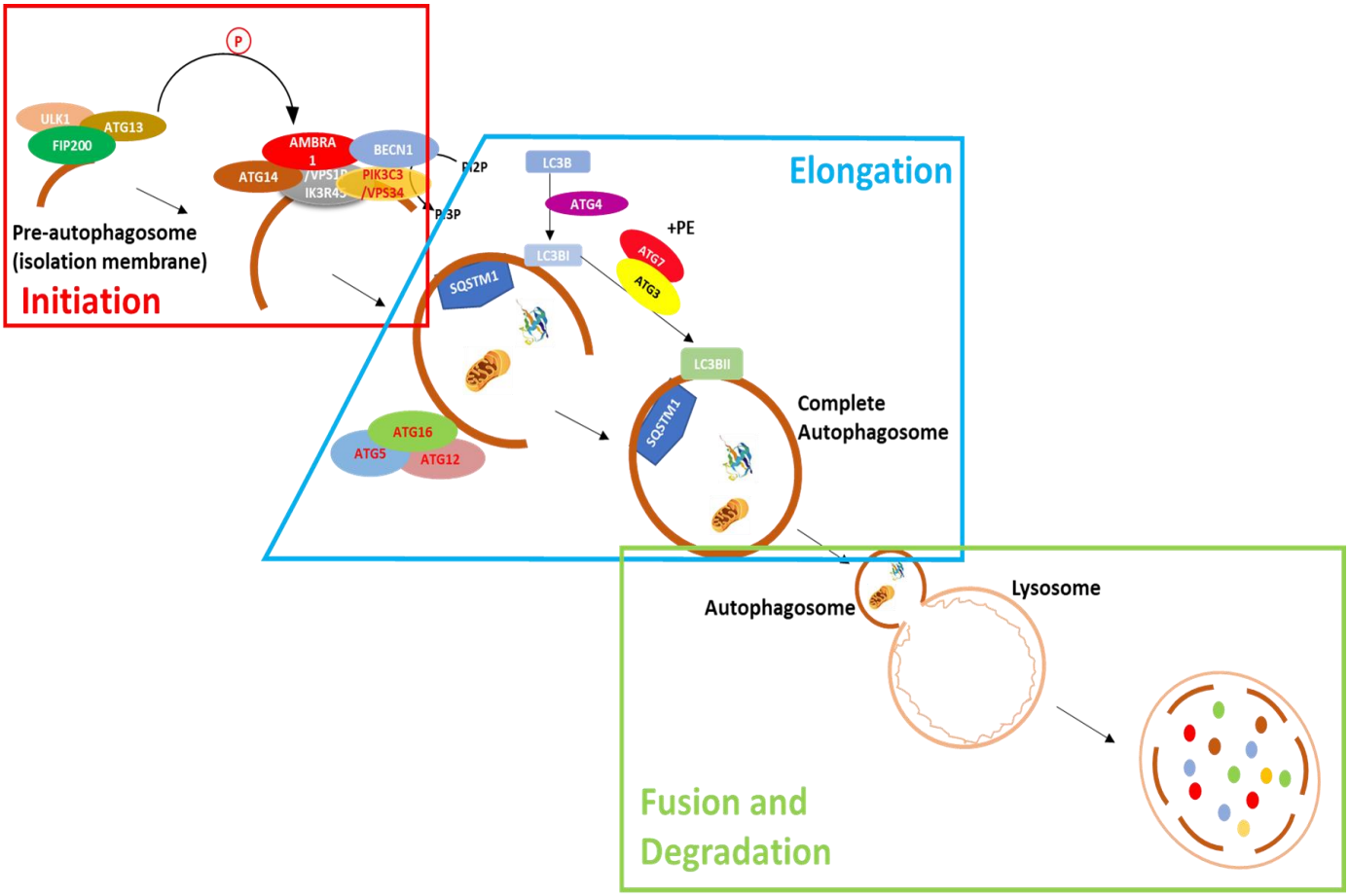

Figure 2: Autophagy steps. The autophagy process is accomplished in three main steps. The first step is the formation of a double membrane (pre-autophagosome structure) and is mediated by ULK- and BECN1-complexes. In the second step, the pre-autophagosome structure elongates with the aid of ATG12-ATG5-ATG16 and LC3BII protein conjugates. Finally, the complete autophagosome fuses with lysosome where lysosomal enzymes degrade autophagosomes.

1.3 Apoptosis

Apoptosis is a form of programmed cell death responsible for the removal of abnormal, damaged, and/or unnecessary cells. Apoptosis is characterized by morphological changes of cell shrinkage, nuclear condensation, membrane blebbing and fragmentation into membrane-bound apoptotic bodies that are eventually removed by macrophages (Figure 3) [16].

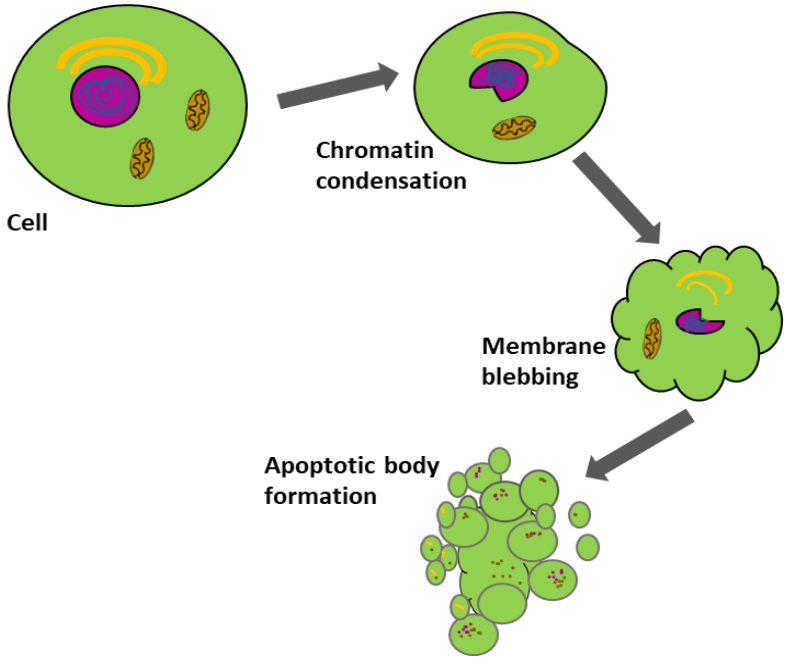

Figure 3: Apoptotic morphological changes. Apoptosis is characterized by a series of typical morphological features such as cell shrinkage, chromatin condensation, membrane blebbing and fragmentation into membrane-bound apoptotic bodies.

1.3.1 Caspase

Cysteine aspartic acid proteases (caspases; CASPs) are the primary executioners of apoptotic cell death by functioning to cleave substrates between cysteine and aspartic acid residues [17, 18]. Generally, CASPs consist of two broad categories: initiators (e.g. CASP8 (caspase8), CASP9 (caspase9)) and effectors (e.g. CASP3 (caspase3)). Both exist in an inactive procaspase form, but upon apoptosis induction, they become activated [18]. CASPs can cleave over 400 cellular proteins, which result in cellular degradation, DNA fragmentation, and blebbing, typical phenotypes of cell death [18].

Several intra- and/or extra-cellular mechanisms could initiate apoptotic cell death [18]. Apoptosis execution is then followed by a cascade of protein activation inside the cell,

which eventually leads to the activation of an initiator CASP like CASP8 or CASP9 (Figure 4). Initiator CASPs then cleavage-activate effector CASPs like CASP3 to execute the rest of the cell death program [18]. For instance, intracellular cell death is induced when stressors converge on mitochondria and permeabilize the mitochondrial membrane leading to the release of several apoptosis-inducing factors such as; CYCS (cytochrome C), which in turn activate initiator CASP9 and its downstream effector CASP3 [17]. The inactive 30-kDa CASP3 is composed of an N-terminal pro-domain, a large 17-kDa subunit (p17), and a smaller 12-kDa C-terminal subunit (p12) [19]. CASP9 cleaves CASP3 at its interdomain linker between p17 and p12, thereby imposing a conformational change in CASP3 that exposes its active sites [19].

1.3.2 BCL2 family and apoptosis

Another important set of apoptosis regulating proteins are those belonging to the BCL2 (BCL2 apoptosis regulator) family that are broadly grouped as anti-apoptotic or pro-apoptotic [17]. BCL2 family members have one or more BH (BCL2 homology) domains that allow their interaction with other BCL2 members [17]. The anti-apoptotic members BCL2 and BCLXL (B cell leukemia/lymphoma 2) contain four BH domains, whereas the pro-apoptotic members either have the first three BH domains (like BAX (BCL2-associated X, apoptosis regulator) and BAK (BCL2 antagonist/killer)) or contain only the third BH domain (like BID (BH3 interacting domain death agonist) and BNIP3 (BCL2/adenovirus E1B interacting protein 3)) (Figure 5 a) [17, 18]. The anti-apoptotic members usually inhibit apoptosis by binding to and inactivating their pro-apoptotic counterparts, while the

pro-apoptotic members usually induce apoptosis by triggering mitochondrial outer membrane permeabilization and the subsequent caspase activations [20].

For example, the anti-apoptotic protein BCL2 binds to the pro-apoptotic proteins BAX and BAK, thus inhibiting their apoptosis-inducing effect [17, 21]. However, when apoptosis is induced, BCL2 dissociates from BAX leading to BAX oligomerization in the mitochondrial outer membrane, which results in loss of mitochondrial membrane potential, CYCS release from mitochondria to cytosol, CASP activation, and apoptotic cell death (Figure 5 b) [17, 22].

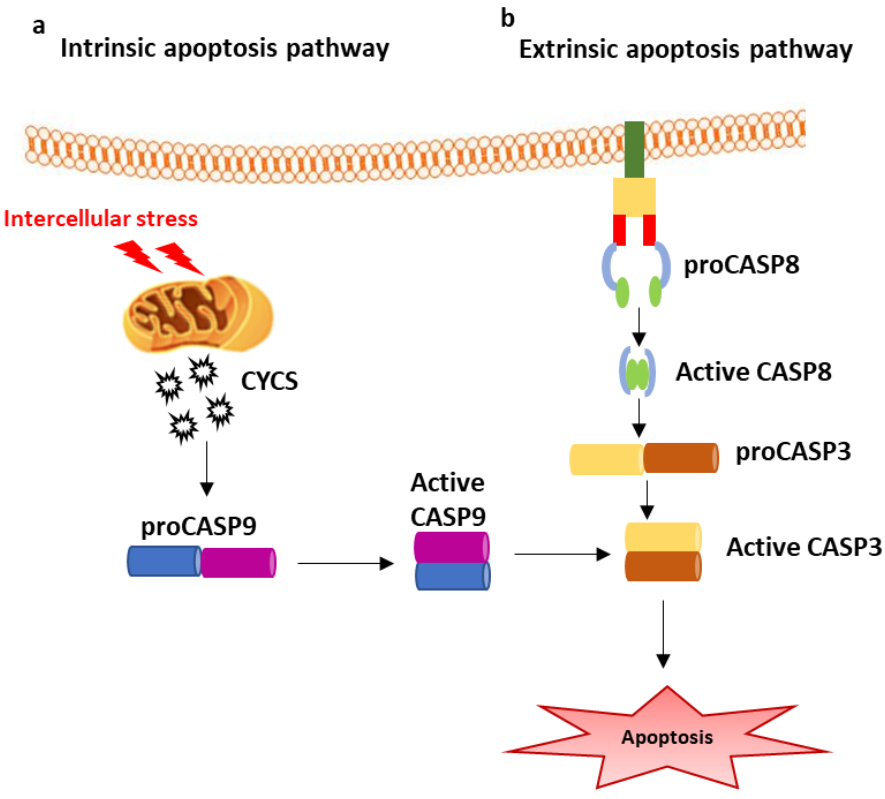

Figure 4 : Intrinsic and extrinsic apoptosis pathways. In the intrinsic apoptosis pathway, intercellular stress leads to initiator CASP9 activation (a), while in the extrinsic apoptosis, an extra-cellular stimuli trigger initiator CASP8 activation (b). The activated initiator CASP8 and CASP9 then cleavage-activate effector CASP3, which in turn executes the apoptotic cell death **[23]**.

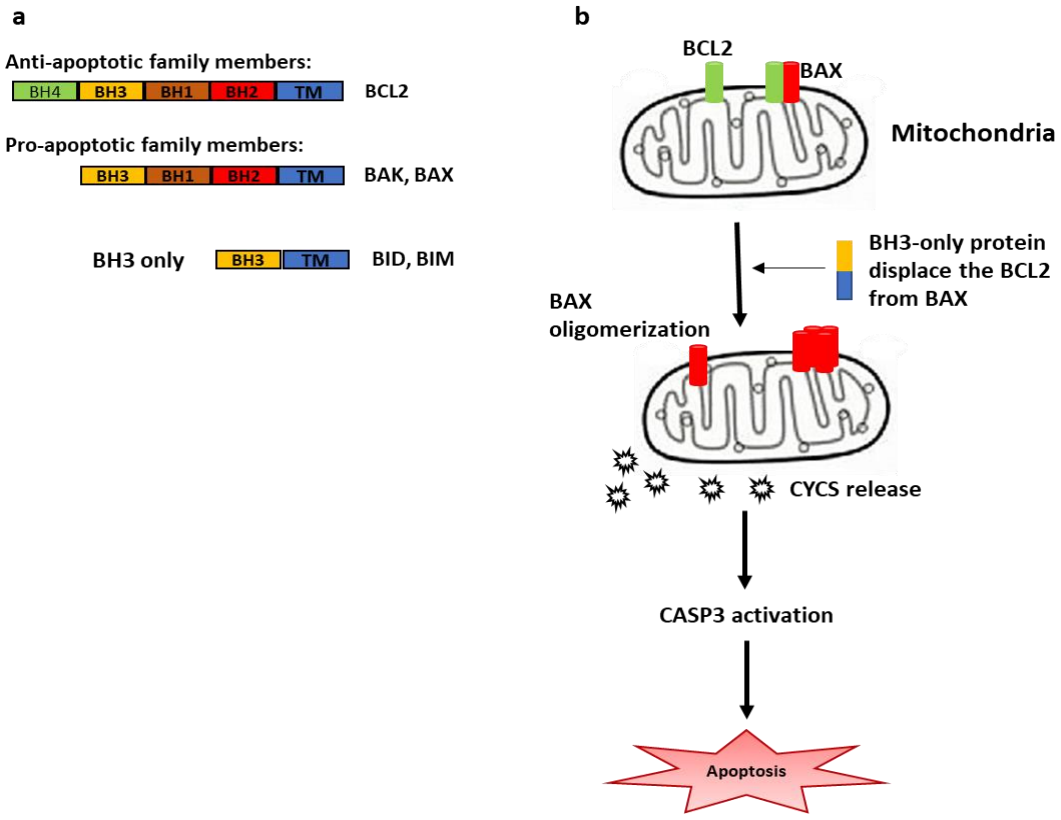

Figure 5 : Apoptosis regulation via BCL2 family of proteins. Anti-apoptotic BCL2 family members contain 4 BH domain, while the pro-apoptotic members have 3 or 1 BH domain (a). In the absence of apoptosis, the anti-apoptotic BCL2 inhibits apoptosis by binding to BAX; however, when apoptosis is induced, BCL2 releases its inhibition from BAX, leading to BAX oligomerization in mitochondrial outer membrane, CYCS release, CASP3 activation, and apoptosis (b).

1.4 BECN1 Protein

Autophagy starts with the formation of a double membrane (pre autophagosome structure), continues with elongation of that double membrane to an autophagosome, and ends with lysosomal degradation of the autophagosome. As discussed above, the BECN1 complex, consisting of BECN1 and PIK3C3/VPS34 core proteins, initiates autophagosome formation by creating a pool of PI3P from phosphatidylinositol [9, 23]. Normally, BECN1 autophagic activity is inhibited by BCL2; however, upon autophagic induction, BCL2 alleviates its

inhibition from BECN1, allowing it to bind to PIK3C3/VPS34, the lipid kinase responsible for adding phosphates to phosphatidylinositol [24]. Once the BECN1-PI3KC3/VPS34 core complex is formed, BECN1 recruits other members of the BECN1complex [24].

BECN1 plays a critical role in fine-tuning the autophagy and apoptosis pathways. For instance, anti-apoptotic proteins BCL2 and BCLXL bind to the BH3 domain of BECN1 and restrict autophagy induction [25], while BNIP3 and BNIP3L disrupt the BECN1-BCL2 complex thereby promoting autophagy and inhibiting apoptosis in hypoxia conditions [26]. BECN1 inhibition leads to increased apoptosis, yet its activation protects cells against apoptosis in various cells and conditions. For example, AMPK-associated phosphorylation of BCL2 and the subsequent dissociation of BCL2-BECN1 protects cardiomyocytes against apoptosis in diabetic patients [27]. Likewise, BECN1 inhibition in pancreatic cancer cells is accompanied by elevated apoptosis [28]. Interestingly, several studies reported that elevated CASP3 activity during apoptosis leads to BECN1 cleavage at multiple sites and subsequent autophagic inactivation [29-31]. Moreover, the C-terminal fragment of cleaved BECN1 could localize on mitochondria, induce CYCS release, and further induce apoptosis [30]. Overall, these findings identify BECN1 as a critical regulator of autophagy that protects cells against apoptosis.

1.4.1 BECN1 and AMBRA1

AMBRA1 is a member of the BECN1 complex that positively regulates BECN1 pro-autophagic function [32]. AMBRA1 facilitates BECN1-associated autophagy by i) binding to BECN1, thereby increasing BECN1 interaction with the PIK3C3/VPS34 lipid kinase and ii) competing with BCL2 to bind to BECN1 leading to dissociation of BCL2

inhibition from BECN1 [33]. AMBRA1 is also attached to microtubules DLC1 (dynein cytoplasmic 1 light intermediate chain 1), where it sequesters BECN1 and PIK3C3/VPS34 [32]. Upon autophagy induction, ULK1 phosphorylates AMBRA1 disrupting the interaction between AMBRA1 and the DLC1 leading to translocation of AMBRA1 with the BECN1 complex to the ER and mitochondria, the potential sites of PI3P [34]. Once AMBRA1 translocates the BECN1 complex to the ER membrane, it is phosphorylated by ULK1 and dissociates from the BECN1 complex [34]. AMBRA1 can also regulate autophagy by ubiquitinating ULK1 via TRAF6 E3 ligase (TNF receptor-associated factor 6), thereby promoting ULK1 self-association and function [34]. Altogether, AMBRA1 seems to be an important autophagy regulator mainly through its BECN1 regulatory function.

1.5 The Crosstalk between Myogenesis, Autophagy, and Apoptosis

Skeletal muscle cells or multi-nucleated myofibers are post-mitotic terminally differentiated cells [35]. To achieve this, multipotent skeletal muscle stem cells, known as satellite cells, aid in muscle function, maintenance, and regeneration [35]. Satellite cells are typically quiescent, but if the need for muscle regeneration arises, they can become activated, proliferate to maintain a pool of satellite cells, withdraw from the cell cycle to become terminally differentiated myocytes, and fuse together to form new fibers (Figure 6) [35]. Differentiation of satellite cells to myotubes starts with the upregulation of MYOD (myogenic differentiation 1) and MYF5 (myogenic factor 5), the basic helix-loop-helix transcriptional activators of the myogenic regulatory factor family (MRF) [35, 36]. These proliferative MYOD and/or MYF5 positive myogenic cells are termed myoblasts. Proliferating myoblasts then withdraw from the cell cycle to become terminally differentiated myocytes that express the “late” MRFs,

MYOG (myogenin) and MRF4 (myogenic regulatory factor 4), and subsequently muscle-specific proteins such as myosin heavy chain (MYH) and muscle creatine kinase (MCK) [35-37]. Mononucleated myocytes then fuse to each other or to the existing fibers to form a multi-nucleated myofiber, which eventually matures into contracting muscle fibers (Figure 6).

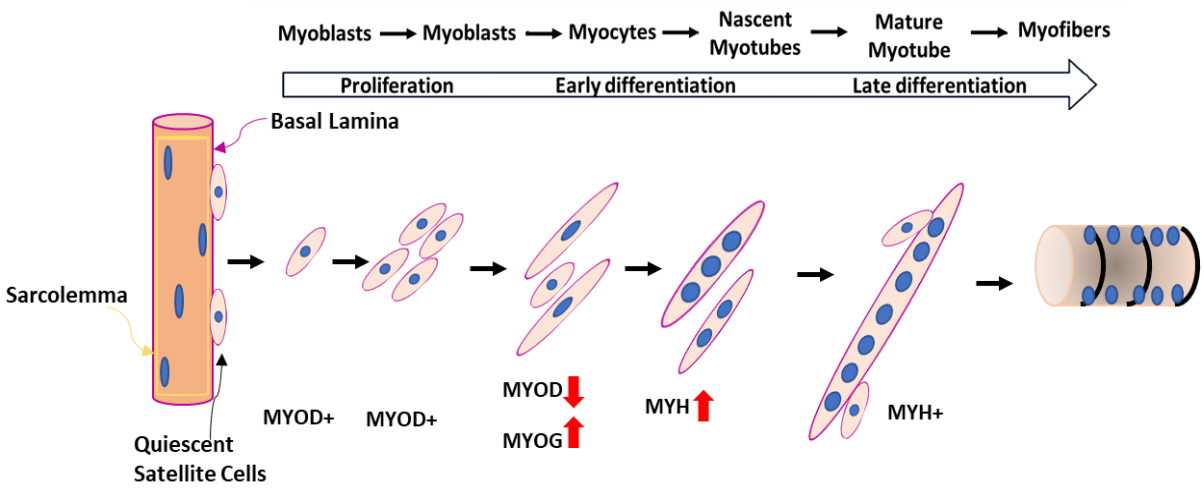

Figure 6 : Regulation of satellite cell differentiation. When the need for skeletal muscle regeneration arises, quiescent satellite cells that are located between the sarcolemma and basal lamina of terminally differentiation myofibers, become activated and proliferate. Proliferating satellite cells are called myoblasts, which then withdraw from the cell cycle and become terminally differentiated myocytes. Myocytes then fuse together or to existing fibers and form multi-nucleated myotubes. The myotubes then integrate with each other and form the contractile myofibers.

Skeletal muscle differentiation is also accompanied by transient CASP2 (caspase2) and CASP3 activation at early differentiation, events that are critical for proper skeletal muscle differentiation [38, 39]. CASPs are important in facilitating skeletal muscle differentiation by regulating the cell cycle and promoting the expression of promyogenic proteins [38, 39] . For instance, CASP2 positively regulates P21 (cyclin dependent kinase

inhibitor 1) expression, thus allowing myocytes to exit from the cell cycle and proceed with the differentiation program [38]. CASP3 also can inhibit myoblasts proliferation and facilitate their differentiation by cleavage-activating a major inhibitor of cell proliferation called MST1 (mammalian sterile twenty-like kinase) [39]. Further, CASP3 could cleave the inhibitor protein bHLH Twist (twist basic helix-loop-helix transcription factor 1), thereby alleviating its inhibition from promyogenic factor MYOD and promoting its expression [40]. Nevertheless, if caspase activation exceeds a certain level, it proceeds with the apoptosis pathway causing cell death and impaired differentiation [41]. Therefore, a regulatory system should exist during skeletal muscle differentiation to maintain caspase activation below the apoptosis-inducing threshold.

Autophagy is activated during skeletal muscle differentiation and allows the differentiating cells to adapt to the rapid changes in their microenvironment as it can degrade pre-existing structures, signaling proteins, and organelles [42]. Autophagy also regulates apoptosis during skeletal muscle differentiation [42]. Generally, in response to cellular stress, autophagy acts as the first line of defense by eliminating the source of stress, which otherwise accumulates, exceeds a certain intensity level, and causes apoptotic cell death [43]. However, if stress reaches the point of triggering apoptosis, the elevated apoptosis inactivates autophagy, partly through caspase-mediated cleavage of autophagy proteins [44-46].

Apoptosis and autophagy mechanisms share several regulator proteins, which achieve the balance between apoptosis and autophagy [44-47]. For instance, anti-apoptotic proteins BCL2 and BCLXL could bind to and inhibit BECN1 autophagic activity while pro-apoptotic protein BAD could phosphorylate BCLXL, displace it from BECN1 and promotes autophagy

[25]. Further, BAX induced apoptosis enhances caspase-mediated cleavage of BECN1 and disables BECN1-associated autophagy [46, 47]. Interestingly, autophagy inhibition has been shown to sensitize cells to apoptosis in various cells and settings [44, 48, 49]. For example, in our lab, we observed that ATG7-deficient myoblasts displayed significantly decreased autophagic responses, elevated CASP3 activation, and severely impaired myogenesis [41]. We also observed that ATG7-deficient myoblasts displayed CYCS and AIFM1 (apoptosis-inducing factor, mitochondrion-associated 1) release from mitochondria, which are the hallmarks of mitochondrial-associated stress signaling and apoptosis [50]. Thus, autophagy plays an apoptosis regulatory role during skeletal muscle differentiation.

Chapter 2 Purpose

Previous research in our lab has demonstrated an important role for autophagy in regulating apoptosis during skeletal muscle differentiation [41]. We also found that BECN1 plays an important role in regulating autophagy during C2C12 differentiation since: i) BECN1 expression increases early during differentiation, ii) inhibition of BECN1 complex via 3MA treatment impairs autophagy and C2C12 differentiation, and iii) BECN1 association with its inhibitor, BCL2, decreases in early differentiation [41]. Interestingly, BECN1 also plays a role in the interplay between autophagy and apoptosis in different cells and contexts [25, 30, 31]. Further, AMBRA1 (activating molecule in BECN regulated autophagy protein 1) is shown to positively regulate BECN1-associated autophagy [32, 51].

Therefore, the objectives of this thesis were to:

1. Investigate apoptosis and skeletal muscle differentiation in ad-DN-ATG5 treated C2C12 cells as a new autophagy-deficient C2C12 model.
2. Investigate BECN1 levels following elevated CASP3 activation in early differentiation.
3. Investigate AMBRA1 expression during C2C12 differentiation and its regulation on differentiation and apoptotic signaling.

Experiment 1: Effect of ad-DN-ATG5 treatment on autophagy, CASP3 activity, and C2C12 differentiation

C2C12 cells were transduced with either ad-GFP (as a control) or ad-DN-ATG5 and maintained in a proliferative state and subsequently induced to differentiate. Cells were collected before differentiation induction (D0) and up to five day (D1-D5) of differentiation.

Autophagy-related proteins, cleaved CASP3, and MYH expression were analyzed via immunoblotting, and skeletal muscle differentiation and fusion index were analyzed by florescent imaging.

Experiment 2: Effect of STS treatment on BECN1 levels

C2C12 cells were treated with DMSO vehicle (CTRL), 125 nM STS for 8 hours, or 2 μM STS for 3 hours. Cells were collected at early differentiation and analyzed for proCASP3, cleaved CASP3, and BECN1 levels.

Experiment 3: Changes in AMBRA1 levels throughout skeletal muscle differentiation and the effects of its inhibition on skeletal muscle differentiation and apoptotic signaling

C2C12 myoblasts were maintained in a proliferative state and subsequently induced to differentiate. Cells were collected throughout differentiation and analyzed for AMBRA1 levels via immunoblotting. To investigate the role AMBRA1 plays in skeletal muscle differentiation, C2C12 cells were transfected with CTRL or AMBRA1 siRNA and differentiated. AMBRA1 knockdown was confirmed by immunoblotting, while MYH and BCL2 levels also were analyzed via immunoblotting.

2.1 Hypotheses

It was hypothesized that:

1) ad-DN-ATG5 treatment would reduce autophagy, increase CASP3 activity, and impair skeletal muscle differentiation.
2) BECN1 levels would decrease in STS treated cells.
3) AMBRA1 expression would increase at early differentiation, and AMBRA1 knockdown would impair skeletal muscle differentiation.

Chapter 3 Methods

3.1 Cell Culture and Treatments

C2C12 mouse skeletal myoblast (ATCC) cells were cultured on polystrene culture dishes (BD Bioscience) in growth media (GM) consisting of low-glucose Dulbecco's Modified Eagels Medium (DMEM; Sigma-Aldrich, D6046) containing 5% fetal bovine serum (FBS; Sigma-Aldrich, 14009C), 5% Serum Plus II (Sigma-Aldrich, 14009C) , and 1% penicilin/streptomycin (Sigma-Aldrich; P0781) and incubated at 37°C in 5% CO2. At 24 hours intervals, culture dishes were aspirated of GM, washed with warmed PBS (Sigma-Aldrich; D1408), and fresh GM was added. At 80–90% confluence, differentiation was induced by replacing the GM with DM (differentiation medium) consisting of DMEM supplemented with 2% horse serum (Sigma-Aldrich; H1270) and 1% penicillin/streptomycin (Sigma-Aldrich; P0781). Cells were collected as sub-confluent myoblasts before DM addition D0 (day 0) as well as at 24 hours (D1), 48 hours (D2), 72 hours (D3) and 120 hours (D5) following the addition of DM. Cells were removed from culture dishes by trypsinization (0.25% trypsin with 0.2g/l EDTA; Sigma-Aldrich, T4049), collected by centrifugation (1000 x g for 5 min), resuspended /washed in PBS and frozen at -80°C until further analysis.

3.2 Gene Knockdown, Cell Transfection, and STS Treatment

Cells were incubated with adenoviruses expressing GFP (ad-GFP) or dominant-negative ATG5 in which the lysin 130 is replaced with arginine [ad-DN-ATG5 (ATG5K130R); MOI = 100] (kindly provided by Dr. Gökhan S. Hotamisligil, Harvard School of Public Health, Boston, MA [52]) for 24 hours prior to differentiation, washed, and provided fresh GM. Cells

were then collected (D0) or induced to differentiate by replacing GM with DM. Adenoviruses were amplified using HEK293 cells and viral titer was determined using the Adeno-X Rapid Titer Procedure (Clontech Laboratories, Inc.).

Elevation in CASP3 activity was achieved by treating cells with staurosporine (STS; Alexis Biochemicals 380-014-C100) 125 nM or 2 μM for 8 hours and 3 hours, respectively or vehicle (equal volume of DMSO) during differentiation, with media/drug replaced every 24 hours.

To downregulate AMBRA1 expression, C2C12 cells were transfected with AMBRA1 siRNA (sc-141039) or CTRL siRNA (sc-37007) using jetPRIME Transfection Reagent (Polyplus-transfection; 114-07), optimized according to the manufacturer's instructions. Briefly, appropriate siRNA concentrations were diluted in jetPRIME transfection buffer and subsequently incubated with jetPRIME transfection reagent for 10 mins at room temperature, added to 50-60% confluent cells containing low-glucose DMEM, and incubated for 24 hours at 37°C and 5% CO_2, after which regular growth media was replaced. Differentiation was induced 48 hours following siRNA transfection.

3.3 Immunoblotting

For immunoblotting, cells were lysed in ice cold lysis buffer [20 mM Hepes, 10 mM NaCl, 1.5 mM MgCl, 1 mM DTT, 20% glycerol and 0.1% Triton X-100 (pH 7.4)] with protease (Roche Applied Sciences, 04693116001) and phosphatase (Thermo Scientific; 78420) inhibitor cocktails. Equal amounts of cell lysates were loaded and separated on 7.5-15% SDS-PAGE gels, transferred onto PVDF membranes (Bio-Rad Laboratories), and blocked for 1 hour at room temperature or overnight at 4°C with 5% milk-Tris-buffered saline (20

mM Tris, 137 mM NaCl, pH 7.5)-0.1% Tween 20 [BioShop, TWN508] (milk-TBST). Membranes were incubated either overnight at 4°C or for 1 hour at room temperature with primary antibodies against: BECN1, GAPDH, MAP1LC3B, CASP3 (Cell Signaling Technology, 3738, 2118, 2775, 9662, respectively), ATG12, ATG5, BCL2 (Santa Cruz Biotechnology, sc-271688, sc-133158, and sc-7382, respectively), cleaved CASP3 (Sigma C8487), MYH (Developmental Studies Hybridoma Bank, MF20), SQSTM1 (MBL, PM045), and AMBRA1 (Elabscience, E-AB-36142). Membranes were then washed with TBST, incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (BioRad, 170-6515, 170-6516) for 1 hour at room temperature, washed with TBST, and bands visualized using enhanced chemiluminescence western blotting detection reagents (BioVision, K820-500) and the BioRad ChemiDoc™ MP Imaging System. The approximate molecular weight for each protein was estimated using Precision Plus Protein WesternC Standards and Precision Protein Strep-Tactin HRP Conjugate (Bio-Rad Laboratories, 161-0385).

3.4 Microscopy and Immunofluorescence

Cells were washed with PBS, fixed with 4% formaldehyde/PBS at room temperature, and washed again with PBS. Cells were then permeabilized with 0.5% Triton X-100 and washed in PBS, blocked with 10% goat serum (Sigma–Aldrich; G9023), incubated with a primary antibody against myosin heavy chain (Developmental Studies Hybridoma Bank; MF20) diluted in blocking solution for 2 hours, and washed with PBS. An anti-mouse AlexaFluor488-conjugated secondary antibody (Invitrogen; A11001) was diluted in blocking solution and incubated with cells for 1 hour before the cells were washed in PBS,

counterstained with DAPI nuclear stain (Life Technologies; D3571) for 5 minutes, washed in PBS, kept in fresh PBS, and visualized using Cytation5 (BioTek) imaging reader. The fusion index was calculated as the percentage of nuclei present in multi-nucleated (two or more nuclei) cells relative to total nuclei.

3.5 Statistical Analyses:

A one-way repeated measure ANOVA was used to assess the effect of differentiation. If a main effect of differentiation was observed, Bonferroni's posthoc test was used to determine the differences from D0. Differences between treatments were assessed using a Students *t*-test. $P<0.05$ was considered statistically significant.

Chapter 4 Results

Treatment of C2C12 cells with ad-DN-ATG5 resulted in decreased levels of ATG12-ATG5 and ATG12 as well as increased levels of ATG5

C2C12 cells were transduced with adenovirus expressing DN-ATG5 at different MOIs (multiplicity of infection) and collected post ad-DN-ATG5 transduction. Increasing MOI of ad-DN-ATG5 resulted in decreased ATG12-ATG5 and ATG12 levels. The ATG12-ATG5 protein complex levels appeared in higher levels at MOI=0 while its levels decreased about 30% at MOI=60, 52% at MOI=100, 54% at MOI=200, and almost disappeared at MOI=300 (Figure 7 a, b). No ATG5 protein was detected at MOI=0 likely because ATG5 is mainly engaged in ATG12-ATG5 complex in the absence of its antagonist, DN-ATG5. However, ATG5 protein levels increased by 3-, 4-, 9-, and 18-fold as ad-DN-ATG5 MOI increased to 60,100,200, and 300, respectively (Figure 7 a, c). The increase in ATG5 levels is probably because i) cells were delivered with high amounts of DN-ATG5 (MOI=60-300) that possess similar molecular weight as ATG5 and ii) DN-ATG5 treatment prevented the formation of ATG12-5 complex resulting in the presence of more unbound ATG5. ad-DN-ATG5. The pattern of changes in ATG12 protein were similar to ATG12-ATG5; its levels decreased by 20%, 81%, 79%, and 100% at MOI=60, MOI=100, MOI=200, and MOI=300, respectively (Figure 7 a, d). We chose to continue the remaining experiments with the MOI=100 as this was the lowest MOI affecting ATG12-ATG5, ATG12, and ATG5 protein levels.

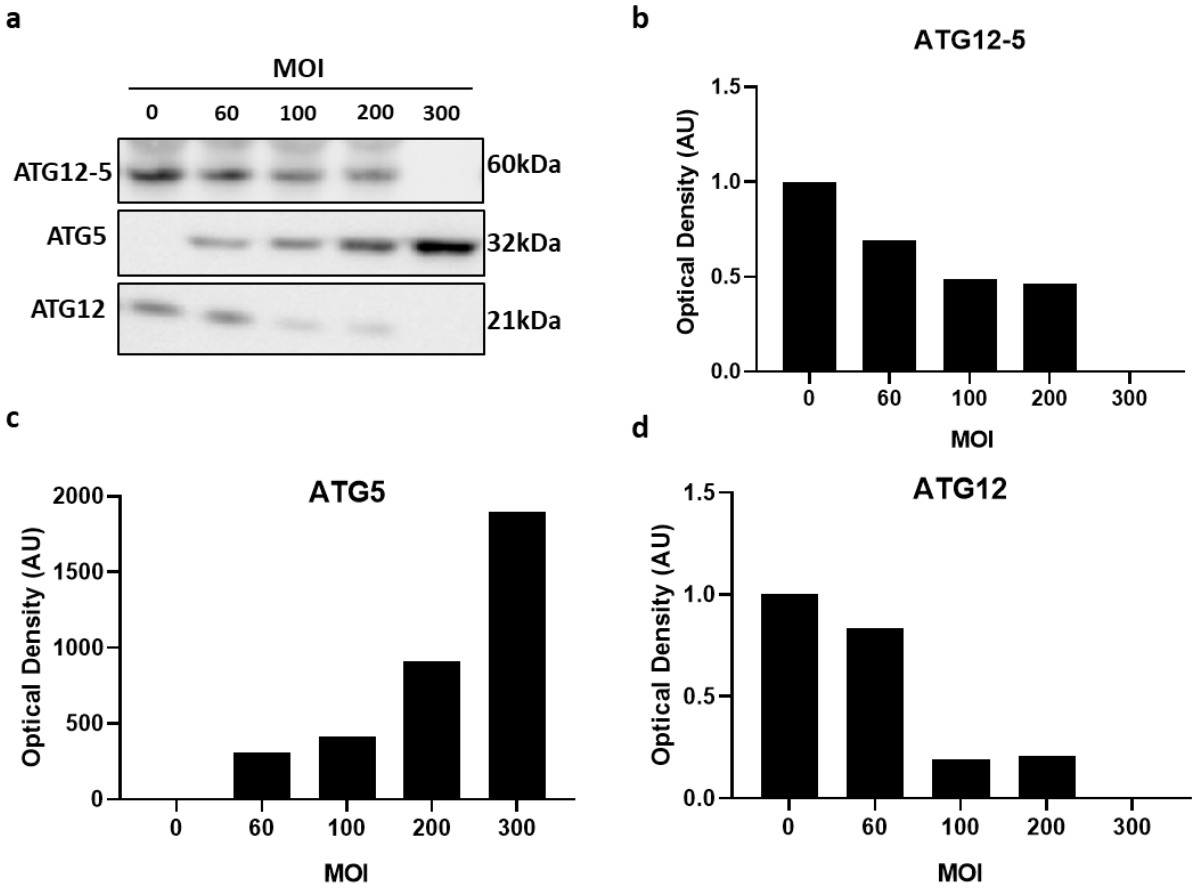

Figure 7 : ATG12-ATG5, ATG5, and ATG12 response in C2C12 cells with different MOIs of ad-DN-ATG5. Representative immunoblots (a) and quantitative analysis of ATG12-ATG5 (b), ATG5 (c), and ATG12 (d) protein in C2C12 cells treated with ad-DN-ATG5 MOI 0-MOI 300 (n=1).

Ad-DN-ATG5 treatment resulted in reduced levels of ATG12, ATG12-ATG5 and BECN1 in differentiating C2C12 cells

To look at the effect of ad-DN-ATG5 treatment on autophagy during skeletal muscle differentiation, we transduced C2C12 cells with either ad-GFP (CTRL) or ad-DN-ATG5 and collected them at different timepoints throughout differentiation. Compared to CTRL (ad-GFP), ATG5 levels were consistently higher in the ad-DN-ATG5 treated C2C12 cells during

differentiation (Figure 8 a, b). Conversely, the ATG12-ATG5 protein levels were significantly lower ($p<0.05$) at D1 to D5 of differentiation in ad-DN-ATG5 treated cells compared to ad-GFP treated ones (Figure 8 a, c). Similarly, ad-DN-ATG5 treated cells exhibited significantly lower ATG12 levels at D2, D3, and D5 of differentiation (Figure 8 a, d). The increased ATG5 and decreased ATG12-ATG5 levels confirm that DN-ATG5 has displaced the ATG5 from ATG12-ATG5 complex thus disrupted the assembly and function of the ATG12-ATG5 complex. LC3BII and SQSTM1 are used as indicators of autophagosome degradation as they are degraded via lysosomal enzymes after autophagosome-lysosome fusion. Surprisingly, the LC3BII:I and SQSTM1 levels remained nearly unchanged between ad-GFP and ad-DN-ATG5 groups throughout differentiation (Figure 8 a, e-g) indicating that the lower ATG12-ATG5 levels have not affected autophagosome degradation and turnover. However, ad-DN-ATG5 cells displayed lower BECN1 protein levels at D0 which further decreased at D1 ($p=0.05$), followed by significant decreases at D2 and D3 ($p<0.05$), and remained low at D5 of differentiation (Figure 8 a, h). Collectively, these data show that DN-ATG5 treatment with MOI=100 successfully reduced ATG12-ATG5, ATG12, and BECN1 levels while it did not affect autophagy markers SQSTM1 and LC3BII levels.

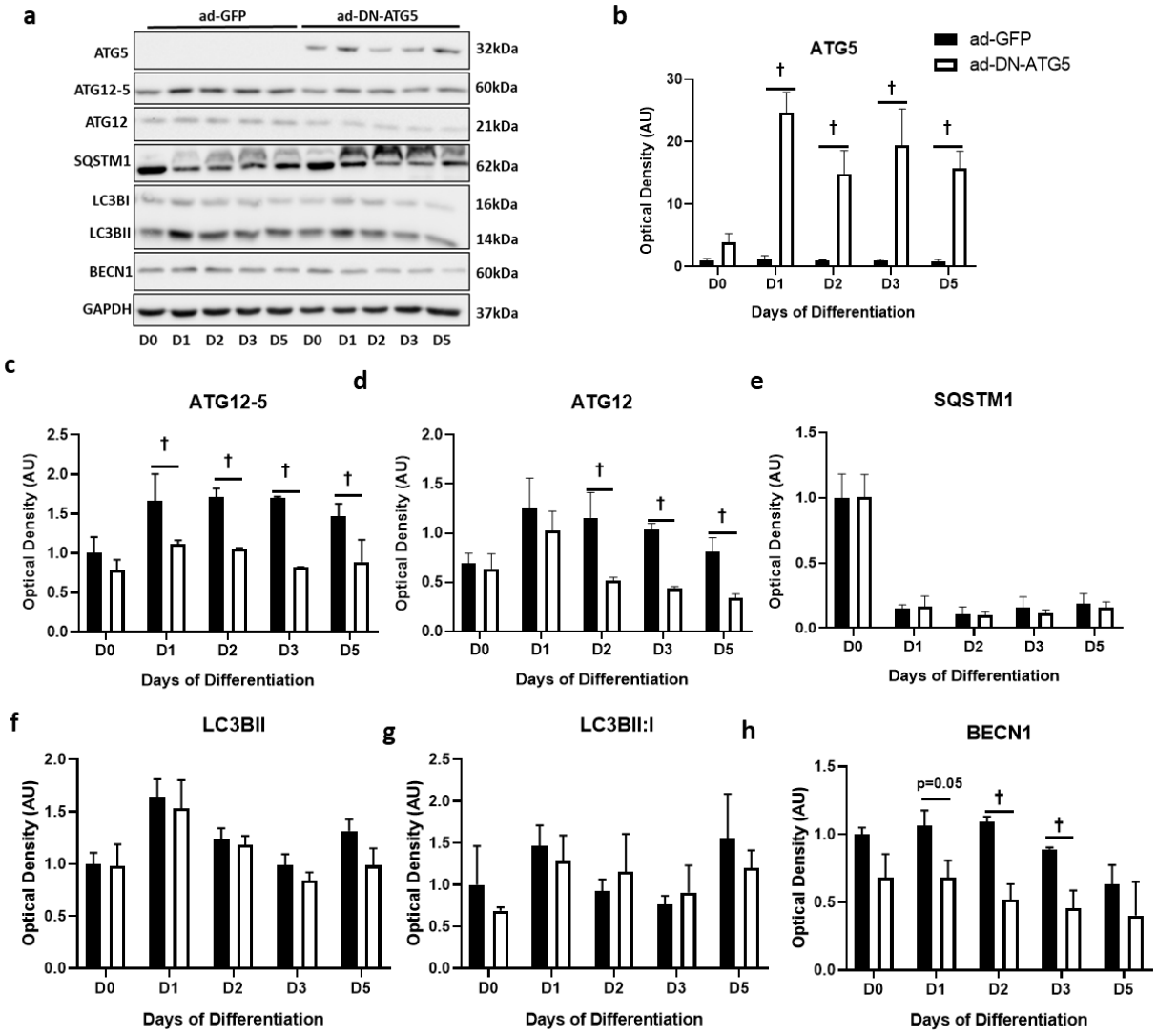

Figure 8 : Autophagy protein response in ad-GFP and ad-DN-ATG5 treated C2C12 cells during differentiation. Representative immunoblots (a) and Quantitative analysis of ATG5 (b), ATG512-5 (c), ATG12 (d), SQSTM1 (e), LC3BII (f), LC3BII:I (g), and BECN1 (h) levels in differentiating ad-GFP and ad-DN-ATG5 treated C2C12 cells. Also shown is a representative GAPDH blot. $^{\dagger}p<0.05$ compared to CTRL at the same timepoint (mean ± SEM, n=3 independent experiments).

ad-DN-ATG5 treatment augmented CASP3 activation

Previously we showed that CASP3 activity increases early during differentiation and is important for proper myogenesis [22]. However, further augmentation of CASP3 activity in

autophagy-deficient cells impairs differentiation [53]. Thus, we looked at cleaved CASP3 (the active form) in the differentiating ad-GFP and ad-DN-ATG5 administered C2C12 cells. Interestingly, we observed that cleaved CASP3 (the active form of CASP3) was significantly elevated in cells with DN-ATG5 at D1 of differentiation (Figure 9 a, b).

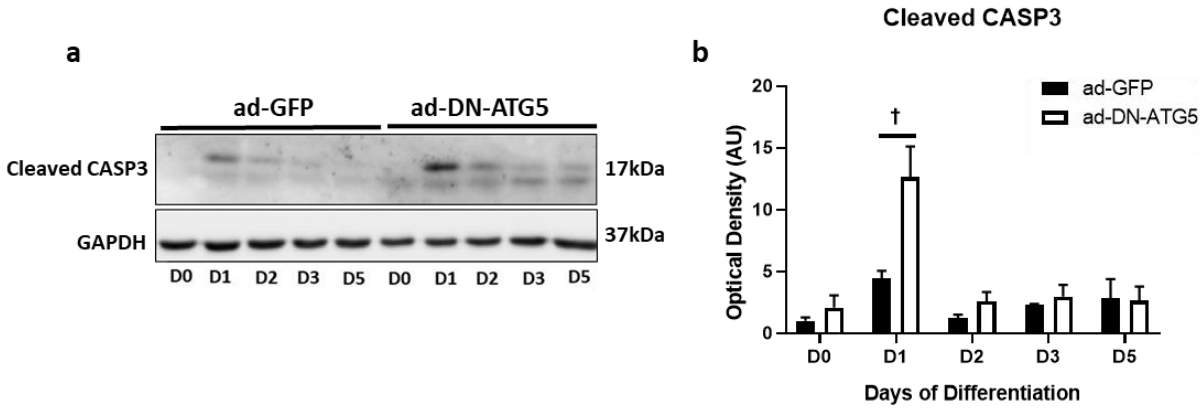

Figure 9 : CASP3 activation in response to ad-DN-ATG5 treatment. Representative immunoblots (a) and quantitative analysis (b) of cleaved CASP3 protein in ad-GFP and ad-DN-ATG5 treated C2C12 cells during differentiation. Also shown is a representative GAPDH immunoblot. $^{\dagger}p<0.05$ compared to CTRL at each timepoint (mean ± SEM, n=3 independent experiments).

Skeletal muscle differentiation was impaired in ad-DN-ATG5 treated cells

In a previous study we demonstrated that elevated CASP3 activation impairs skeletal muscle differentiation in autophagy- and mitophagy-deficient C2C12 cells [53]. Therefore, we examined skeletal muscle differentiation characteristics following ad-DN-ATG5 administration. The skeletal muscle differentiation marker, MYH, was lower by 10-, 5-, and 3-fold at D2, D3, and D5 of differentiation in ad-DN-ATG5 cells compared to ad-GFP cells (Figure 10 a, b). Microscopy images of D5 C2C12 cells also revealed a significantly reduced

fusion index ($p<0.05$) (Figure 10 c, d).

BECN1 protein levels decreased following CASP3 activation

In Figure 8 it was shown that BECN1 levels decreased during differentiation of ad-DN-ATG5 treated cells. Additionally, the reduced BECN1 levels was accompanied with elevated CASP3 activation. Interestingly, a previous study showed that CASP3 could cleave BECN1 at two sites thereby inactivating BECN1 autophagic activity [29]. Thus, we induced CASP3 activation by treating differentiating C2C12 cells for 8 and 3 hours with 125 nM and 2 μM of STS, respectively and assessed BECN1 levels. Compared to CTRL cells, 8 hours of 125 nM- and 3 hours of 2 μM STS treatment caused an average of 7- and 14-fold increases in cleaved CASP3 levels, respectively (Figure 11 a, c). This increase in cleaved CASP3 levels was accompanied by an average of 70% decrease in BECN1 levels (Figure 11 a, d). Consistent with data from Figure 8 these data indicate a decrease in BECN1 levels following increased CAP3 activation (Figure 11).

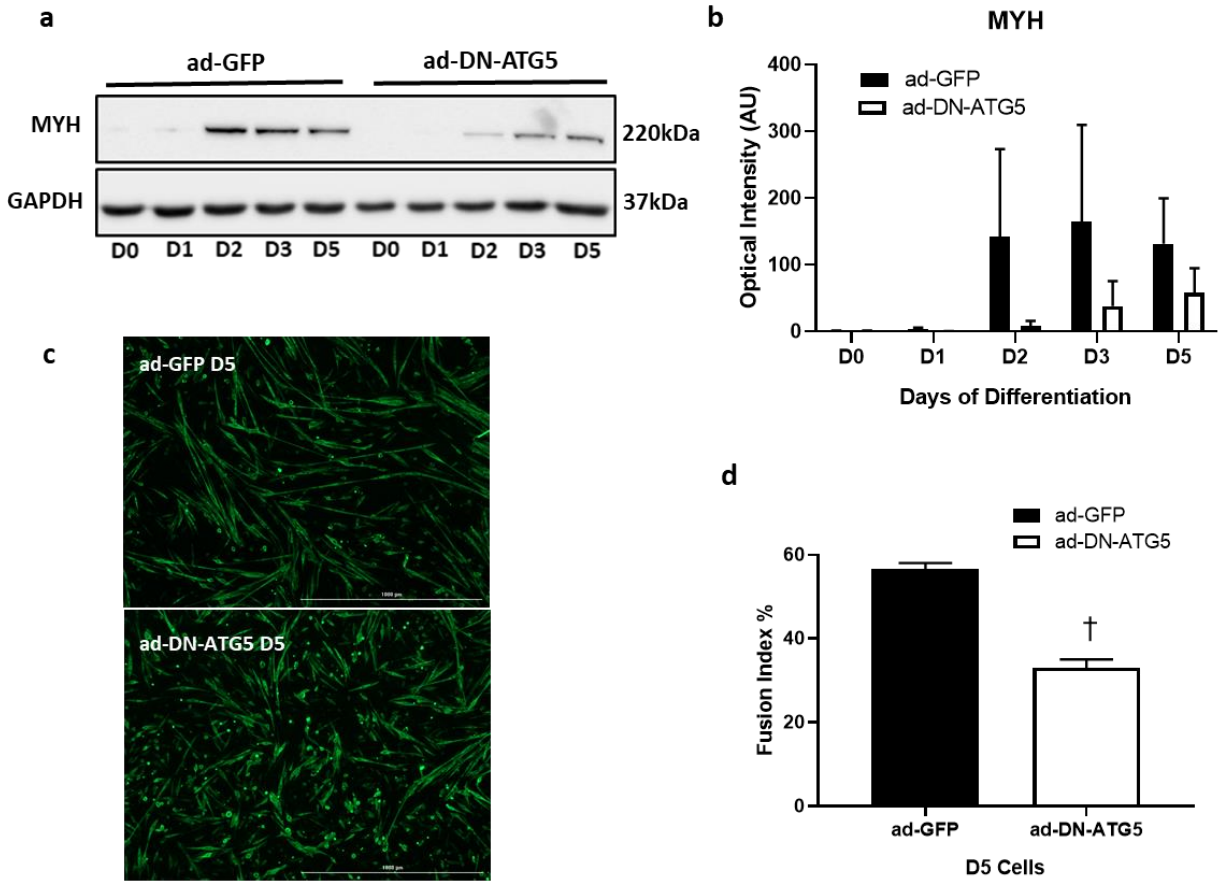

Figure 10 : Impaired skeletal muscle differentiation in ad-DN-ATG5 treated cells.

Representative immunoblots (a) and quantitative analysis (b) of myosin heavy chain (MYH) protein in ad-GFP and ad-DN-ATG5 treated C2C12 cells during differentiation (mean ± SEM, n=3 independent experiments). Also shown is a representative GAPDH blot of the membranes. Representative images of MYH (c) and quantitative analysis of fusion index (d) in D5 ad-GFP and D5 ad-DN-ATG5 cells. Scale bar is 1000 μm. $^{\dagger}p<0.05$ between groups at each timepoint (mean ± SEM, n=2 independent experiments).

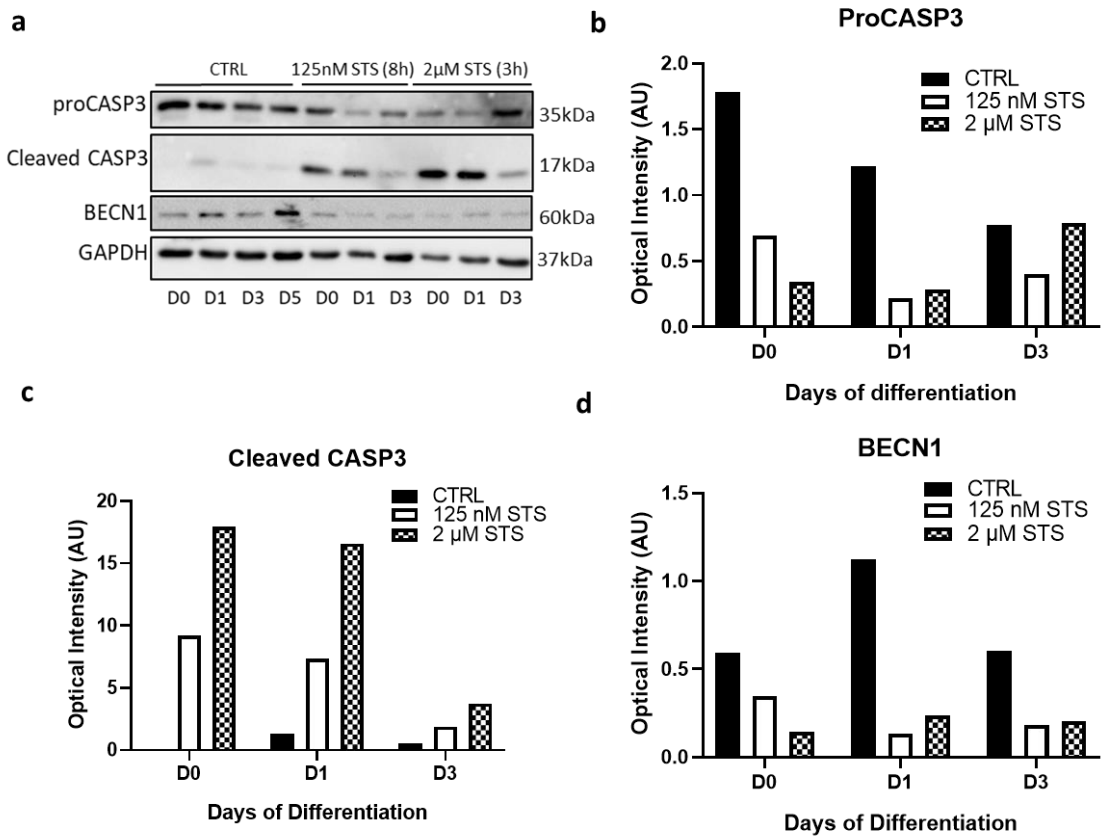

Figure 11: BECN1 protein levels decreased following CASP3 activation. Representative immunoblots (a) and quantitative analysis of ProCASP3 (b), Cleaved CASP3 (c), and BECN1 (d) protein in CTRL (vehicle), 12 nM (8h), and 2 μM (3h) STS treated differentiating C2C12 cells (n=1). Also shown is a representative GAPDH blot.

AMBRA1 protein levels increased early in differentiation, and decreased later during differentiation

AMBRA1 is a BECN1 associating partner that regulates its pro-autophagic action. AMBRA1 dissociates the BCL2 inhibition from BECN1 thus promoting BECN1 to initiate autophagosome formation [51]. AMBRA1 levels increased by 30% at D1 post differentiation which was followed by its significant decreases ($p<0.05$) later at D2, D3, and D5 of

differentiation (Figure 12 a, b).

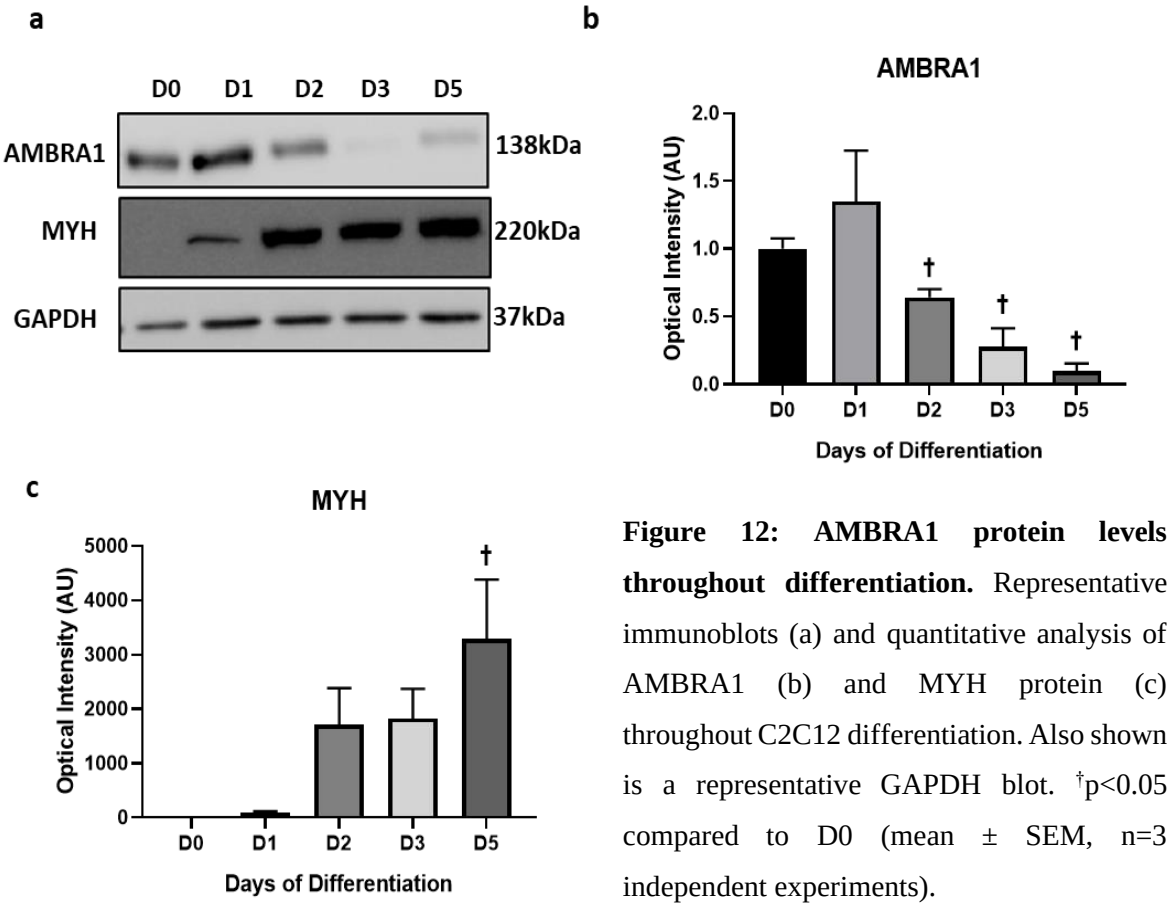

Figure 12: AMBRA1 protein levels throughout differentiation. Representative immunoblots (a) and quantitative analysis of AMBRA1 (b) and MYH protein (c) throughout C2C12 differentiation. Also shown is a representative GAPDH blot. $^{\dagger}p<0.05$ compared to D0 (mean ± SEM, n=3 independent experiments).

AMBRA1 siRNA treatment decreased AMBRA1 levels in myoblasts

To inhibit AMBRA1 expression and investigate its importance during skeletal muscle differentiation we transfected C2C12 cells with either CTRL siRNA or 50 nM, 80 nM, and 100 nM of AMBRA1 siRNA. AMBRA1 levels were decreased by 93%, 95%, and 99% in C2C12 cells transfected with 50, 80, and 100 nM of AMBRA1 siRNA, respectively (Figure 13). The 50 nM was the lowest concentration that caused efficient knockdown, so we performed the remaining experiments using 50 nM siRNA.

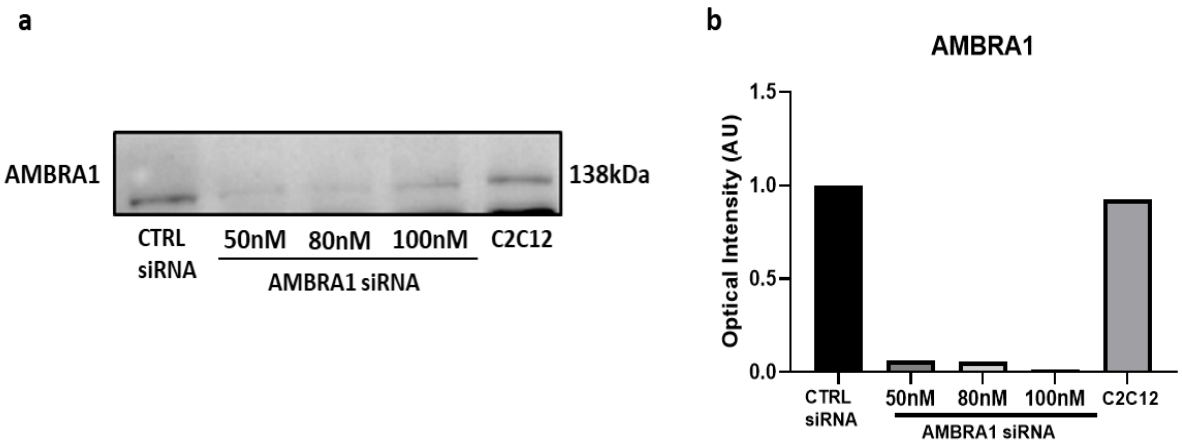

Figure 13: Knockdown of AMBRA1 in C2C12 cells using different concentrations of AMBRA1 siRNA. Representative immunoblots (a) and quantitative analysis (b) of AMBRA1 protein in C2C12 cells transfected with CTRL siRNA or 50 nM, 80 nM, and 100 nM AMBRA1 siRNA (n=1).

AMBRA1-deficient C2C12 cells displayed decreased BCL2 levels and impaired differentiation

When autophagy is inactive, BCL2 binds to BECN1 and inhibits its action. However, upon autophagy activation, AMBRA1 competes with BCL2 in binding to BECN1 thus removing the BCL2 inhibition from the BECN1 and promoting autophagy [51]. To look at changes in BCL2 levels and skeletal muscle differentiation C2C12 cells were transfected with AMBRA1 siRNA. AMBRA1 knockdown was confirmed in D0 C2C12 cells as noted by an 80% decrease in AMBRA1 protein in AMBRA1 siRNA transfected cells compared to CTRL siRNA transfected cells (Figure 14 a, b). Further, compared to CTRL cells, AMBRA1 siRNA treated cells displayed a 60% decrease in BCL2 levels (Figure 14 a, c). MYH expression was also reduced dramatically at D2 and D3 in AMBRA1 knockdown cells which became significantly lower at D5 ($p<0.05$) of differentiation (Figure 14 d, e). These data

suggest an important role for AMBRA1 in regulating BCL2 levels and skeletal muscle differentiation.

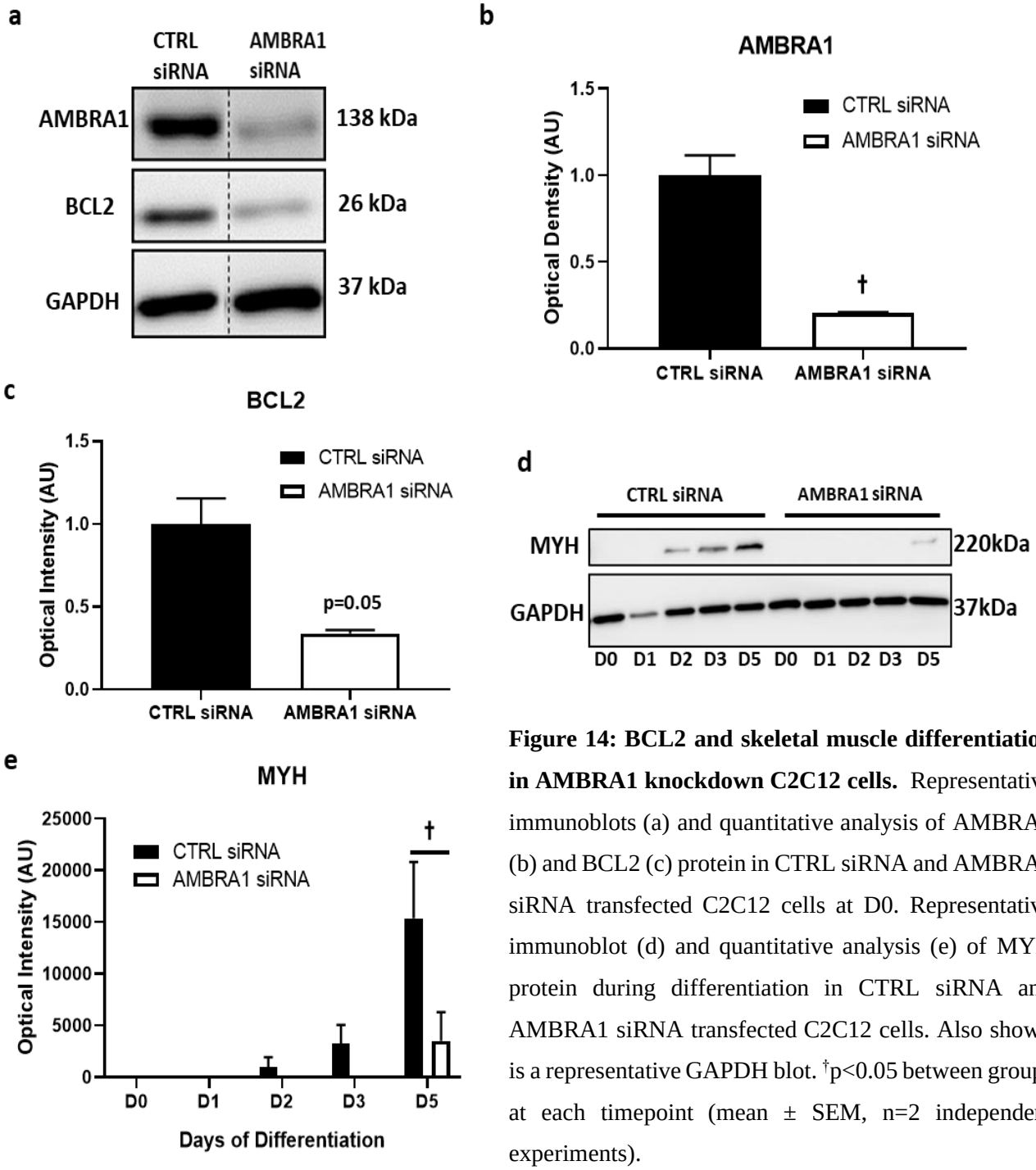

Figure 14: BCL2 and skeletal muscle differentiation in AMBRA1 knockdown C2C12 cells. Representative immunoblots (a) and quantitative analysis of AMBRA1 (b) and BCL2 (c) protein in CTRL siRNA and AMBRA1 siRNA transfected C2C12 cells at D0. Representative immunoblot (d) and quantitative analysis (e) of MYH protein during differentiation in CTRL siRNA and AMBRA1 siRNA transfected C2C12 cells. Also shown is a representative GAPDH blot. $^{\dagger}p<0.05$ between groups at each timepoint (mean ± SEM, n=2 independent experiments).

Chapter 5 Discussion

The focus of this study was to further characterize the effect of defective autophagy on apoptosis regulation and skeletal muscle differentiation as well as studying BECN1 and its regulator AMBRA1 in the context of skeletal muscle differentiation. Therefore, we first investigated the effect of ad-DN-ATG5 on CASP3 activation and C2C12 differentiation. Then, we examined the impact of CASP3 activation on BECN1 levels during C2C12 differentiation. Finally, we studied the pattern of AMBRA1 change during skeletal muscle differentiation, as well as its importance for the differentiation process. Previously, we demonstrated that autophagy regulates CASP3 activation and skeletal muscle differentiation [41]. BECN1 is an important regulator of autophagy during skeletal muscle differentiation as BECN1 expression increases during differentiation while dissociating from its inhibitor BCL2 early during differentiation [41]. BECN1 seems to be a target for CASP3 cleavage leading to autophagy inactivation in other cells and conditions [54-56]. However, BECN1 changes following elevated CASP3 activation during skeletal muscle differentiation remains to be elucidated. BECN1 complex consists of core proteins of BECN1, PIK3C3/VPS34, PIK3R4/VPS15, ATG14, and AMBRA1 [57]. Among these proteins, AMBRA1 is the first one that joins BECN1, activates its autophagic function, and initiates the recruitment of other proteins on BECN1 complex [32]. Nonetheless, the expression and function of AMBRA1 has not been studied during skeletal muscle differentiation.

Differentiating C2C12 cells with ad-DN-ATG5 showed elevated CASP3 activation and impaired skeletal muscle differentiation. Elevated CASP3 activation in both ad-DN-ATG5 and STS-treated C2C12 cells was accompanied by decreased BECN1 levels, which

might be due to its cleavage. Immunoblots of AMBRA1 in differentiating C2C12 cells showed that its levels increased early during differentiation, followed by its significant decrease later during differentiation. We also assessed the importance of AMBRA1 for skeletal muscle differentiation by transfecting C2C12 cells with siRNA against AMBRA1. MYH expression was significantly reduced in AMBRA1-deficient C2C12 cells.

Altogether, our results suggest that i) intact autophagy regulates apoptosis and is required for proper skeletal muscle differentiation, ii) CASP3 over-activation might be responsible for BECN1 decrease in ad-DN-ATG5 and STS treated cells, and iii) AMBRA1 is critical to skeletal muscle differentiation possibly through regulating BCL2 levels.

ad-DN-ATG5 Treatment Decreased ATG12, ATG12-ATG5, and BECN1 Levels while Increased CASP3 Activation and Impaired Skeletal Muscle Differentiation

Studies in our lab have demonstrated a critical role for autophagy in regulating skeletal muscle differentiation, and apoptosis [41, 50]. Previously in our lab, autophagy-deficiency in C2C12 cells was studied using 3MA-treatment and an ATG7 knockdown model [41]. In order to make a new autophagy-deficient C2C12 model and further investigate the downstream effects of autophagy deficiency on skeletal muscle differentiation and apoptosis, we treated C2C12 cells with adenovirus expressing the dominant-negative form of ATG5 (ad-DN-ATG5), previously shown to block autophagy [52]. To achieve deficiency using the dominant-negative (DN) approach, a mutated form of a protein that lacks specific functions of the wildtype (WT) is expressed along with the endogenous WT form. The mutated form then competes with the WT, hinders the assembly of the WT to its partnering proteins, and therefore, inhibits the WT function [58]. We used an adenovirus to deliver the DN-ATG5 into

C2C12 cells. Notably, viral infection can activate stress pathways in some cells and possibly induce autophagy; thus, we used an adenovirus that expresses GFP tag as the negative control in our experiments [59].

As mentioned earlier, the ATG5-ATG12-ATG16 complex is recruited on the expanding autophagosome double membrane and is required for autophagosome elongation [60]. ATG5-ATG12-ATG16 complex is assembled by ligation of ATG12 to an internal lysine (K130) residue of ATG5, thus forming an ATG12-ATG5 heterodimer which then binds to ATG16 [1]. To impede the formation of ATG12-ATG5 complex, we treated C2C12 cells with adenovirus expressing a mutant form of ATG5 (ad-DN-ATG5) whose ATG12-binding site is mutated (lysine130 is substituted with arginine (K130R)) [61]. The DN-ATG5 competes with the wildtype ATG5 in binding to ATG12, thereby disabling the formation of ATG5-ATG12-ATG16 complex. Normally, the endogenous ATG5 and ATG12 are mainly present as ATG12-ATG5 conjugates [62], which explains why little ATG5 is present in CTRL (ad-GFP treated) cells (Figure 8 a, b). However, when cells are treated with ad-DN-ATG5, the ATG5 protein appears as a 32kDa single band which might be the DN-ATG5, the unbound WT ATG5, or both. ad-DN-ATG5. Further, cells treated with higher MOI of ad-DNTG5 displayed lower ATG12-ATG5 levels (Figure 7 a, b), confirming that ad-DN-ATG5 treatment has blocked ATG12-ATG5 formation. Differentiating ad-DN-ATG5 treated C2C12 cells also displayed significantly lower ATG12-ATG5 levels compared to CTRL cells (Figure 8 a, c). Previous data obtained in differentiating C2C12 cells showed a significant increase in ATG12-ATG5 levels early during differentiation [41]. Similarly, our CTRL cells exhibited a significant increase in ATG12-ATG5 levels early during

differentiation; however, differentiating ad-DN-ATG5 treated cells did not show this effect (Figure 8 a, c).

BECN1 is a main regulator of autophagy; it is activated following autophagy induction and is involved in the initiation step of autophagosome formation [63]. The BECN1 complex has also been implicated in regulating autophagy during myogenesis. We have shown that, i) BECN1 protein increased during myoblast differentiation, ii) BCL2, which binds to BECN1 and inhibits BECN1-dependent autophagy in myoblasts, dissociates from BECN1 in early myogenesis, iii) inhibiting JNK (c-Jun N-terminal kinase); the protein kinase responsible for phosphorylating BCL2 and dissociating BCL2 from BECN, maintained the association between BECN1 and BCL2 resulting in impaired autophagy in early myogenesis, and iv) chemical inhibition of the BECN complex via 3MA treatment severely impaired myogenesis [41]. Importantly, our data showed that BECN1 levels were significantly lower in cells with ad-DN-ATG5, suggesting a decreased rate of autophagosome formation in these cells (Figure 8 a, h).

Another important autophagy regulator that mediates phagophore expansion is LC3BII [64]. LC3BII is created when the cytosolic LC3BI is conjugated to phosphatidylethanolamine (PE) in a reaction mediated by ATG7 and ATG3 [64, 65]. One study has shown that the ATG12-ATG5 complex acts as an E3-like ubiquitinating enzyme by transferring the PE from ATG3 to LC3BI and is necessary for LC3BI transition to LC3BII [66]. The LC3BII then localizes on developing double membrane, remains on autophagosome until autophagosome-lysosome fusion, and is degraded by lysosomal hydrolases [64]. Surprisingly, we did not observe any significant changes in LC3BII:I levels following ad-

DN-ATG5 treatment (Figure 8 a, g). The similar ratio of LC3BII:I in CTRL and ad-DN-ATG5 might, in fact, be a result of more LC3BII degradation in CTRL cells, which should be addressed by inhibiting lysosomal clearance of LC3BII and then measuring its content in future studies. Additionally, the decrease in ATG12-ATG5 levels might not have been enough to affect LC3BII lipidation and function.

SQSTM1 is another autophagy marker that links ubiquitinated cargos to LC3BII, thus delivering the cargos to autophagosomes. SQSTM1 becomes incorporated into autophagosome and is degraded via lysosomal enzymes upon autophagosome-lysosome fusion, thereby serving as a readout of autophagic flux [67]. Decreased SQSTM1 levels are associated with autophagy activation, while its accumulation correlates with autophagy inhibition [67]. Even though the decreased level of ATG12-ATG5 and BECN1 implied impaired autophagy in ad-DN-ATG5 treated cells, our data did not show SQSTM1 accumulation in ad-DN-ATG5 treated cells during C2C12 differentiation (Figure 8 a, e, f). Importantly, it has been shown that SQSTM1 changes due to autophagy alterations can be cell-type and context-specific [68]. During C2C12 differentiation, autophagy upregulation is followed by a significant decrease in SQSTM1 levels, indicating that SQSTM1 responds to autophagy activation in differentiating C2C12 cells. However, in the context of ad-DN-ATG5 treatment the reduced levels of SQSTM1 might not reflect increased autophagy since the SQSTM1 level might also decrease due to; i) its degradation through an autophagy-independent pathway called ubiquitin-proteasome pathway [69], ii) its lower transcription rate as a result of autophagy downregulation [70], and iii) its cleavage by the apoptotic proteases CASP6 (caspase 6), CASP8 and calpain [71]. Altogether, data obtained from

differentiating ad-DN-ATG5 treated C2C12 cells suggest that autophagy has been decreased at the autophagosome nucleation and elongation steps (shown as lower BECN1 and ATG12-ATG5 levels) in ad-DN-ATG5 treated cells, while the autophagosome degradation step might have remained unchanged (indicated by no significant changes in LC3BII and SQSTM1 levels).

To discover the effect of ad-DN-ATG5 treatment and decreased BECN1 and ATG12-ATG5 levels on apoptosis, we assessed CASP3 activation by immunoblotting for cleaved CASP3, the activated form of CASP3. Consistent with previous work in our lab [38], these data indicated a spike for cleaved CASP3 at D1 of differentiation in CTRL cells (Figure 9 a, b). However, the activation of CASP3 was significantly higher in D1 of ad-DN-ATG5 treated cells compared to CTRL ones (Figure 9 a, b). The crosstalk between autophagy and apoptosis has been highlighted by numerous studies showing that apoptosis is elevated when autophagy is inhibited [72-74]. For instance, ATG5-dependent autophagy is shown to protect dopaminergic cells against apoptotic cell death [74]. Differentiating skeletal muscle cells also use autophagy to offset elevated apoptosis [41, 50]. Autophagy deficiency by either 3MA administration or ATG7 knockdown was accompanied by dramatic increases in CASP3 and CASP9 activation during skeletal muscle differentiation [41, 50]. Moreover, differentiating C2C12 cells that lacked selective mitochondrial autophagy displayed CYCS release, increased levels of cytosolic AIFM1, CASP9 and CASP3 activation, hallmarks of mitochondria-induced apoptosis [50]. In our previous study, autophagy inhibition in the 3MA treated and ATG7-deficient C2C12 cells were accompanied by dramatic reductions in LCBII levels [41]. Interestingly, in the present study, even though ATG12-ATG5 depletion did not

lead to decreased LC3BII levels following ad-DN-ATG5 treatment, CASP3 activation was augmented (Figure 9). This finding suggests that even partial alterations in the autophagy pathway could sensitize differentiating C2C12 cells to apoptotic cell death.

The augmented CASP3 activation in ad-DN-ATG5 treated C2C12 cells was accompanied by impaired C2C12 differentiation, as noted by reduced MYH levels and significantly decreased differentiation and fusion index (Figure 10). This observation is in agreement with Baechler et al. that demonstrated an impaired C2C12 differentiation (marked as lower MYOG and MYH expression in addition to decreased differentiation and fusion index) following augmented CASP3 and CASP9 activation in autophagy-deficient cells [50] . In the same work, it was shown that CASP3 and CASP9 inhibition could partially restore the MYOG and MYH expression as well as C2C12's differentiation and fusion index [50]. Studies have found that elevated apoptosis and CASP activation in satellite cells of aged animals and DMD (Duchenne muscular dystrophy) patients associates with depleted numbers of satellite cells and impaired skeletal muscle [75, 76]. Overall, these data underline the detrimental effects that elevated CASP3 activation could impose on skeletal muscle regeneration.

BECN1 Levels were Reduced Following STS-induced CASP3 Activation

In the ad-DN-ATG5 treated cells, elevated CASP3 activity was accompanied by decreased BECN1 levels. To identify whether CASP3 activation in other settings also accompanies reduced BECN1 levels, we treated differentiating C2C12 cells with Staurosporine (STS).

STS functions as a non-selective kinase inhibitor that blocks the ATP binding sites of protein kinases [77]. STS is often used to trigger apoptosis; however, it also could induce autophagy depending on its concentration and incubation period [54, 78, 79]. For instance, autophagosome formation is elevated after 3 hours of incubating HeLa cells with 0.1μM STS and drops to basal levels 24 hours post-treatment while apoptosis reaches its highest level at the 24 hours post-treatment timepoint [54]. Earlier research by Bloemberg et al. identified a significant increase in apoptosis in proliferating C2C12 cells that were treated with 125 nM and 2 μM of STS [80]. Likewise, in the present work, we observed elevated CASP3 activation in STS-treated differentiating C2C12 cells as determined by an increased conversion of proCASP3 (inactive form) to cleaved CASP3 (active form) in these cells (Figure11). Our data showed that higher CASP3 activity is associated with lower BECN1 levels (Figure11), similar to what we observed in ad-DN-ATG5 treated cells (Figure 8, 9).

A growing body of evidence suggests that activated CASP3 can cleave BECN1 as a mechanism to inactivate autophagy [30, 54, 81]. For instance, STS- and interleukin-3 withdrawal-induced CASP3 activation in HeLa, neuron, and Ba/F3 cells have been shown to cleave a full-length BECN1 to a C-terminal and an N-terminal fragment; an effect that could be rescued using a CASP3 inhibitor [30, 54, 81]. The BECN1 C-terminal fragment then localizes on mitochondria, promotes mitochondrial membrane permeability, further elevating CASP3 activation, and sensitizing cells to apoptotic cell death [54]. Hence, the reduced BECN1 levels after STS and ad-DN-ATG5 treatment of differentiating C2C12 cells might also be a result of CASP3-associated cleavage. However, in order to clarify this hypothesis,

future studies should examine BECN1 cleavage before and after applying the CASP3 inhibitor and investigate its C-terminal localization on mitochondria.

AMBRA1 Levels Increased Early During C2C12 Differentiation, and its Deficiency Impaired C2C12 Differentiation

BECN1 is a principal regulator in autophagosome formation during autophagy process during skeletal muscle differentiation [41, 82]. AMBRA1 has been found as a positive regulator of BECN1-dependent autophagy in HEK293 (human embryonic kidney) cells, SH-SY5Y (human neuroblastoma) cells, and during the development of the nervous system in mouse embryos [32, 57, 83]. As mentioned previously, the BECN1 complex, with the aid of its lipid kinase PIK3C3, provides the pool of PI3P (phosphatidylinositol 3-phosphate), which is required for the formation of the autophagosome double membrane. The proposed scenario for the mechanism through which AMBRA1 regulates BECN1-dependent autophagy is that AMBRA1 directly binds to BECN1 and stabilizes the BECN1-PIK3C3 interaction, thereby promoting the initiation of autophagosome formation [82]. Furthermore, AMBRA1 can interact with a region of BECN1 adjacent to its BH3 domain, thus competes with the binding of the inhibitory factor BCL2 to BECN1 [51].

Given the importance of autophagy for myogenesis and the proposed role of AMBRA1 in regulating autophagy, we investigated AMBRA1 expression during C2C12 differentiation. AMBRA1 levels increased at D1 of differentiation; however, at D2, D3, and D5 of differentiation, its content significantly decreased to below its initial levels at D0 (Figure 12). Typically, other autophagy regulating proteins like BECN1, ATG12-ATG5, LC3BII, and ATG7 levels significantly increase at D1 of C2C12 differentiation and remain

relatively elevated up to D5 of differentiation [41]. Therefore, the changes in AMBRA1 levels during C2C12 differentiation doesn't seem to follow a similar pattern as other autophagy protein. However, the elevation in AMBRA1 levels during differentiation corresponds with BECN1-BCL2 dissociation, and its reduction corresponds with BECN1-BCL2 re-association. A previous study from our lab has presented that the interaction between BECN1-BCL2 significantly drops at D1 and D2 of C2C12 differentiation, which returns to near D0-levels at D3 and further increases at D5 [41]. The same study also introduced pJNK (phosphorylated c-Jun N-terminal kinase) as the kinase that phosphorylates BCL2 and mediates the BECN1-BCL2 dissociation [41]. Interestingly, similar to AMBRA1, pJNK levels upregulate early during differentiation (D0.5 and D1), followed by its gradual decrease at D2, D3, and D5 of differentiation [41]. Collectively, it is possible that AMBRA1 facilitate the BECN1-BCL2 dissociation upon autophagy induction during C2C12 differentiation; however, future studies could further clarify this.

The BECN1-BCL2 dissociation is central for autophagy induction and has been found as the target for different proteins to induce or inhibit autophagy. For instance, BNIP3 disrupts the BECN1-BCL2 complex to trigger autophagy initiation in hypoxia conditions [26]. This function of BNIP3 is mediated by its BH3 domain, as the BH3 mutant BNIP3 did not activate autophagy [26]. During starvation also JNK phosphorylates BCL2 at multiple sites, thereby dissociating BCL2 from BECN1 and stimulating autophagy in mammalian cells [84]. Similarly, glucose withdrawal in mammalian cells activates autophagy by phosphorylating BECN1 at its Thr388 location, thus separating it from the BCL2 inhibitor [85]. Further, pro-apoptotic protein MST1 is found to phosphorylate BECN1 at its Thr108

residue hence enhancing its association with BCL2 leading to autophagy suppression [86]. Collectively, these findings highlight the importance of BECN1-BCL2 complex for the decision to progress with autophagy or cell death.

The observed increase in AMBRA1 levels at D1 of C2C12 differentiation and its subsequent decrease at D2, D3, and D5 might also be important with respect to AMBRA1 role in cell cycle regulation [87]. Proliferating C2C12 cells should exit from the cell cycle and terminate their proliferation to properly begin the differentiation program as it is indicated by a significant decrease in the rate of proliferating cells following differentiation induction [38]. AMBRA1 could mediate the cell cycle withdrawal and cease cell growth by mediating the degradation of a proto-oncogene protein c-MYC (MYC proto-oncogene, bHLH transcription factor), whose accumulation has been found to enhance cell proliferation [87, 88]. Interestingly, silencing AMBRA1 expression in mouse has resulted in a noticeable increase in cell density in its skeletal muscle tissue, which was accompanied by reduced number of myofibers, suggesting a cell cycle regulatory role for AMBRA1 in skeletal muscles [89]. Altogether, it is hypothesized that AMBRA1 levels increase at D1 to facilitate the transition from C2C12 proliferation to C2C12 differentiation. However, since at D2, D3, and D5 C2C12 cells have started the differentiation program, there is no need for AMBRA1 levels to remain elevated. Nevertheless, future studies that focus on the impact of AMBRA1 deficiency on cell cycle regulation would be beneficial.

In order to suppress AMBRA1 expression in C2C12 cells, we transfected myoblasts with a siRNA against AMBRA1 mRNA. siRNAs (small interfering RNAs) are small double-stranded RNA molecules that silence gene expression by binding to their complementary

mRNAs, inducing the formation of an RNA-induced silencing complex (RISC) leading to cleavage of the targeted mRNAs. Immunoblot analysis revealed a significant decrease for AMBRA1 levels in AMBRA1 siRNA treated C2C12 cells compared to CTRL siRNA treated cells (Figure 14 a, b). The decrease in AMBRA1 levels in AMBRA1 depleted myoblasts was accompanied by a 7-fold decrease in BCL2 levels (Figure 14 a, c). As mentioned earlier, BCL2 possess an anti-apoptotic function [21]. Typically, a decrease in BCL2 level is associated with an increase in apoptotic cell death events as fewer BCL2 would exist to inhibit pro-apoptotic proteins like BAX and BAK [20, 90, 91]. However, lower BCL2 levels might also be due to its higher engagement in complexes with its interacting partners such as BAK and BAX. Thus, BCL2 content should be analyzed along with other pro-apoptotic proteins to diagnose apoptosis [92].

Even though autophagy is significantly upregulated upon differentiation induction, proliferating myoblasts also undergo a basal autophagy level. The reduced BCL2 levels in AMBRA1-deficient myoblasts might indicate that in the absence of AMBRA1, BCL2 is more engaged in the BECN1-BCL2 complex thereby inducing a higher level of inhibition on BECN1-dependent autophagy [51]. It has been shown that basal autophagy is required for skeletal muscle stem cells to maintain their stemness [93]. Autophagy failure in satellite cells of young mouse causes loss of proteostasis as well as increased mitochondrial dysfunction and oxidative stress all leading to satellite cells entry into senescence and their inability to regenerate skeletal muscles [93]. Myoblasts also should preserve the basal autophagy levels. In AMBRA1 depleted C2C12 cells BCL2 might bind to BECN1 at a higher rate thus inhibiting BECN1 and preveningt basal autophagy.

To identify the importance of AMBRA1 for C2C12 differentiation, we inhibited its expression using AMBRA1 siRNA and assessed their ability to differentiate by analyzing their MYH levels. Differentiating CTRL C2C12 cells showed MYH at D2 of differentiation, whose levels increased at D3 and D5 (Figure 14 d, e). Conversely, MYH was not expressed at D2 and D3 of AMBRA1-deficient cells, and its levels at D5 were significantly lower than the CTRL cells (Figure 14 d, e). This finding is in line with the studies performed in developing embryos of zebrafish and mouse, showing that AMBRA1 knockdown is accompanied by severe myopathy, along with structural and functional defects of skeletal muscles [89]. These defects are likely attributed to the absence of AMBRA1-associated autophagy since the reduced number of autophagosomes and accumulation of dysfunctional organelles was also observed in muscle fibers of the AMBRA1-deficient mouse model [89, 94]. Further, a study in zebrafish showed that AMBRA1 depletion altered the gene expression program responsible for correct muscle development, including *Myod1* gene expression, suggesting that AMBRA1 might also contribute to regulating the myogenic gene expression [89]. Therefore, future studies are required to elucidate the exact mechanism through which AMBRA1 regulates skeletal muscle differentiation.

In this work we demonstrated that autophagy impairment via DN-ATG5 and AMBRA1 siRNA treatment results in impaired skeletal muscle differentiation. The impaired skeletal muscle differentiation in ad-DN-ATG5 treated cells was accompanied by significant increases in apoptotic signaling. Previously in our lab, we suppressed autophagy during skeletal muscle differentiation by treating C2C12 cells with 3MA, and sh*Atg7,* as well as knocking out *Bnip3* gene via CRISPR-Cas9 system, all three leading to increased apoptosis

and impaired C2C12 differentiation. Therefore, an intact autophagy process is crucial for proper skeletal muscle differentiation [41, 53]. Interestingly, studies have shown that sarcopenia, age associated loss of muscle, is highly associated with a decline in autophagy [93, 95]. For instance, satellite cells of geriatric mice loose their regenerative ability, which is attributed to their incompetence to perform autophagy as re-establishment of autophagy restores the regenerative functions in geriatric satellite cells [93]. Further, muscle samples from patients affected by DMD (Duchenne muscular dystrophy) and *mdx* mice (a model of the disease) displayed severely impaired autophagy while autophagy stimulation improved muscular dystrophy in the *mdx* mice [96, 97]. Collectively, similar to the literature, the results of this study indicate an important role for autophagy during skeletal muscle differentiation. Our results also suggest that autophagy protects the differentiating cells from apoptotic cell death thereby maintaining the ability of skeletal muscles to regenerate.

5.1 Summary and Conclusions

This thesis serves as an examination of the interplay between autophagy and apoptosis during skeletal muscle differentiation. Using the new approach of treating C2C12 cells with ad-DN-ATG5, we reinforced the finding that autophagy downregulation sensitizes differentiating myoblasts to apoptotic cell death leading to impaired skeletal muscle differentiation. We also proposed that BECN1 is cleaved by elevated CASP3 activation since augmented CASP3 activation was accompanied by reduced levels of BECN1. Further, we demonstrated that AMBRA1-deficiency in myoblasts is accompanied by a dramatic reduction in BCL2 levels; an event that could be interpreted as increased apoptosis.

Additionally, this study shows AMBRA1 (the activating molecule in BECN1 regulated autophagy protein 1) contribution to skeletal muscle differentiation. Our data indicated that AMBRA1 levels increase early during differentiation while its content significantly drops at D2, D3, and D5 of differentiation. Importantly, the changes in AMBRA1 levels during C2C12 differentiation inversely correspond to the levels of BECN1-BCL2 complex, suggesting that AMBRA1 might mediate the BECN1-BCL2 dissociation. Further, in response to AMBRA1 depletion, myoblasts did not differentiate properly to myotubes, as indicated by significantly reduced MYH expression in those cells.

Collectively, an interplay exists between autophagy and apoptosis during skeletal muscle differentiation in which autophagy activation downregulates apoptosis and apoptosis tends to inhibit autophagy partly through cleaving its regulators.

5.2 Limitation

One of the makers that we used here to analyze autophagy in ad-DN-ATG5 treated cells was LC3BII, and the LC3BII:I ratio. LC3BII is often an excellent marker for autophagic structure and degradation; however, measuring LC3BII levels on their own do not show autophagic flux [96]. LC3BII could be degraded by lysosomal enzymes; therefore, it is essential to determine whether an increase in LCBII levels represents increased flux, or a block in fusion or degradation by using a lysosomal enzyme inhibitor such as chloroquine (CQ). Consequently, higher LC3BII levels with treatment plus CQ compared to CQ alone may indicate that the treatment increases the synthesis of autophagy-related membranes [68]. If the treatment by itself increases LC3BII levels, but the treatment plus CQ does not increase LC3BII levels compared to CQ alone, this may indicate that the treatment induced a partial

block in autophagic flux [68, 92]. Thus, a treatment condition increasing LC3BII on its own that has no difference in LC3BII in the presence of CQ compared to treatment alone may suggest a complete block in autophagy at the terminal stages [92].

In this work, an examination of skeletal muscle differentiation was performed based on analyzing MYH. MYH levels were analyzed during differentiation of C2C12 cells while fusion index was also measured based on MYH and DAPI staining. MYH is a muscle-specific protein, and its increase indicates a higher rate of myogenesis in different settings [36, 98]. However, since it is expressed later during differentiation, it is not informative about the early myogenesis. Myogenin (MYOG) on the other hand, is a muscle-specific transcription factor that is involved in the early stages of skeletal muscle differentiation [36, 98]. Therefore, to precisely track C2C12 differentiation, MYOG expression should also be analyzed.

The data that reported an inverse relationship between STS-induced CASP3 activation and BECN1 content in Figure 11 has not been replicated (n=1). Therefore, it is not clear whether the same observation is reproducible in another independent experiment of differentiating C2C12 cells [99]. Taking measurements from different independent biological replicates shows how reproducible a set of data is against the intrinsic biological variation that exists in samples [99]. Further, the experimental manipulation or interventions could impact the results at different levels [99]. Thus, to draw a strong conclusion from this set of experiments, more biological replicates need to be carried out.

5.3 Future Directions

The results of this study suggest impaired autophagy in differentiating ad-DN-ATG5 treated C2C12 cells as indicated by reduced levels of autophagy regulating proteins ATG12-ATG5, BECN1, and ATG12. However, since SQSTM1 and LC3BII did not exhibit noticeable alterations, further experiments are required to confirm that autophagy has been completely inhibited. Therefore, delivering C2C12 cells with an LC3B protein that is tagged to a GFP reporter would aid in visual monitoring of autophagic punctate formation and degradation thus further elucidate autophagic profile in these cells.

Although data obtained from the second experiment indicated that CASP3 overactivation is accompanied by reduced BECN1 level, additional evidence is required to elucidate how CASP3 affects BECN1 levels. One scenario is that activated CASP3 cleaves BECN1, which could be examined by inhibiting CASP3 activation and checking whether BECN1 levels are restored [29]. The cleavage product of BECN1 has been shown to localize on mitochondria and promote the release of pro-apoptotic factors from mitochondria in other cells and context [30]. Therefore, it is also of interest to test the BECN1 translocation to mitochondria by performing a BECN1 and mitochondria colocalization assay following elevated CASP3 activation in STS- and ad-DN-ATG5-treated cells.

The results of this study provide evidence for the importance of AMBRA1 protein for C2C12 differentiation. Future studies should attempt to investigate the mechanism through which AMBRA1 exerts its regulatory function during skeletal muscle differentiation. Our data indicate a decrease in the anti-apoptotic protein BCL2 following AMBRA1 deficiency; however, to confidently interpret an increase in apoptosis following AMBRA1 depletion

other apoptosis markers like BAX, activated CASP3, and mitochondrial CYCS should also be analyzed. Further, one of the means through which AMBRA1 regulates autophagy is to bind to BECN1, thereby displacing BCL2 inhibition from it, leading to the activation of BECN1-associated autophagy [83]. This could be elucidated by investigating the levels of BECN1-BCL2 complex in differentiating AMBRA1-deficient C2C12 compared to differentiating AMBRA1-competent C2C12 cells.

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