Autophagy Regulates the NOTCH Signaling Pathway During 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

Research has indicated a crucial role for autophagy during skeletal muscle differentiation. More so, the inhibition of autophagy using 3MA (3-methyladenine), CQ (chloroquine), and shRNA against *Atg7* has been shown to impair myocyte fusion and differentiation. Thus far, research in skeletal muscle literature has primarily focused on the degradative system of autophagy, highlighting cell survival, and thereby overlooking the direct effects on numerous signaling processes, including the regulation of NOTCH signaling. The NOTCH signaling developmental pathway is implicated in a broad range of developmental processes, including cell fate, proliferation, and differentiation. Although, there is growing evidence of crosstalk between autophagy and NOTCH signaling in hemopoiesis, cardiogenesis, and neurogenesis, limited studies have investigated the role of autophagy in regulating NOTCH signaling during skeletal muscle cell differentiation. Therefore, in order to examine the role of NOTCH signaling, our laboratory has characterized NOTCH signaling during C2C12 myoblast differentiation. We have inhibited γ-secretase with DAPT treatment that decreased the production of NOTCH1 receptor and NOTCH1^{ICD} intracellular domain (ICD) levels to discern whether NOTCH signaling is required in myogenesis. Furthermore, autophagy was induced with rapamycin (RAPA), and inhibited with CQ to manipulate NOTCH signaling and assess whether autophagy is an important regulator of NOTCH signaling. Additionally, using a genetic approach, C2C12 cells were stably transfected with shRNA against *Atg7* (sh*Atg7*) to assess differences in NOTCH1^{ICD} levels during myogenic differentiation. We first confirmed that downregulation of NOTCH1 and NOTCH1^{ICD} occurs alongside increased autophagic flux during C2C12 myoblast differentiation. Furthermore, we found that induction of autophagy with RAPA was associated with decreased NOTCH1^{ICD} levels, while inhibition of autophagy with CQ was associated with increased NOTCH1^{ICD} levels in

proliferating myoblasts. Similarly, sh*Atg7* cells showed 0.6-fold increase in NOTCH1^{ICD} levels during differentiation. However, inhibition of γ-secretase with DAPT in sh*Atg7* cells decreased NOTCH1^{ICD} levels and was associated with rescued myogenic differentiation. Collectively, our results indicate that autophagy may be an important regulator of NOTCH signaling, thus playing a critical role in both skeletal muscle cell maintenance and myogenesis.

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List of Abbreviations

ADAM	a disintegrin and metallopeptidase
AMBRA1	autophagy and beclin 1 regulator 1
AMPK	AMP-activated protein kinase
ATG1	autophagy related protein 1
ATG10	autophagy related protein 10
ATG12	autophagy related protein 12
ATG13	autophagy related protein 13
ATG14L	autophagy related 14-like protein
ATG16L	autophagy related 16-like protein
ATG3	autophagy related 3 protein
ATG5	autophagy related 5 protein
ATG7	autophagy related 7 protein
BECN1	beclin-1
cdks	cyclin-dependent kinases
CKIs	cyclin-dependent kinase inhibitors
CQ	chloroquine
CSL	CBF1/suppressor of hairless/lag-1
DAPI	4′,6-diamidino-2-phenylindole
DAPT	N-[N-(3,5-Difluorophenacetyl-N-alanyl)]-S- phenylglycine t-Butyl Ester
DLL1	delta like canonical Notch ligand 1
DLL3	delta like canonical Notch ligand 3
DLL4	delta like canonical Notch ligand 4
DMD	Duchenne muscular dystrophy
DSL	Delta/Serrate/Lag2
EEA1	early endosomal antigen 1
EGF	epidermal growth factor
FGF	fibroblast growth factor
FIP200	focal adhesion kinase family interacting protein of 200 kDa
HES1	hairy and enhancer of split/hes family BHLH transcription factor 1
HEY1	hes related family BHLH transcription factor with YRPW motif 1
HEYL	hes related family BHLH transcription factor with YRPW motif like
HSC	hematopoietic stem cells
JAG1	jagged canonical Notch ligand 1
JAG2	jagged canonical Notch ligand 2
LAMPs	lysosome associate membrane proteins
MAML1	mastermind like transcriptional coactivator 1

MAP1LC3B	microtubule associated protein 1 light chain 3 beta
MAP1LC3B2	microtubule associated protein 1 light chain 3 beta 2
mATG9A	autophagy related 9A
MPC	myogenic progenitor cells
MRF6	myogenic factor 6
mTOR	mammalian target of rapamycin
MuSC	muscle stem cells
MYF5	myogenic factor 5
MYH	myosin heavy chain
MYOD1	myogenic differentiation 1
MYOG	myogenin
NOTCH-ECD	notch extracellular domain
NOTCH-ICD	notch intracellular domain
NOTCH1	notch receptor 1
NOTCH2	notch receptor 2
NOTCH3	notch receptor 3
NOTCH4	notch receptor 4
NUMB	NUMB endocytic adaptor protein
P13K	class-III phosphatidylinositol-3-kinases
P13P	phosphatidylinositol-3-phosphate
P150	chromatin assembly factor 1, subunit A
PAX3	paired box 3
PAX7	paired box 7
PCNA	proliferating cell nuclear antigen
PE	phosphatidyl ethanolamine
pEGFP	plasmid with Enhanced Green Fluorescent Protein
qRT-PCR	Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction
RBPJ	recombination signal binding protein for immunoglobulin kappa J region
RUBCN	rubicon autophagy regulator
SCs	satellite cells
SHH	sonic hedgehog signaling molecule
shRNA	short hairpin RNA
SQSTM1	sequestosome 1
T1D	Type I diabetes
ULK1	unc-51 like autophagy activating kinase 1
VPS34	vacuolar protein sorting 34
Wnt	Wnt signaling pathway
3-MA	3-Methyladenine

Chapter 1: Introduction and Literature Review

1.1 Background

Skeletal muscle accounts for 30-40% of total human body mass and is critical for locomotive activity (1, 2). The specialized arrangement of skeletal muscle is dependent on several intracellular signaling systems to regulate muscle development, remodeling and regeneration (2). The NOTCH signaling cascade is one of few pathways that is repeatedly used in embryonic, fetal, postnatal, and regenerative tissue (3). More specifically, NOTCH signaling is critical for developmental processes such as cell fate decisions, cell proliferation and differentiation to progress proper development and maintenance of skeletal muscle (4-6). However, during aging, skeletal muscles are constantly challenged with a multitude of mechanical, heat and oxidative stresses, which can result in cellular damage and death (3). Therefore, skeletal muscles require a system that not only facilitates the recycling of damaged organelles and proteins, but also degrades cytoplasmic material to meet the energy demands of the body (7). Macroautophagy, herein autophagy, is an intracellular degradation pathway that transports dysfunctional organelles and proteins to the lysosome for targeted degradation (7). Thus, the molecular mechanisms underlying NOTCH signaling and autophagy are critical for cell proliferation, cellular degradation and the remodelling of highly specialized skeletal muscle architecture (7).

1.2 NOTCH Signaling Pathway

The NOTCH signaling pathway is a highly conserved system that plays an important role in cell fate determination, proliferation, differentiation, regeneration, repair and cell death (21,22). Canonical NOTCH signaling employs a form of cell-to-cell communication that consist of transmembrane ligands and receptors, which are able to interact within the same cell (*cis*) and

across cell boundaries (*trans*) (1). Activation of NOTCH signaling requires the *trans* neighboring cell-to-cell interaction that involves sender cells producing paracrine signals to induce changes in nearby receiving cells (3, 9, 70). Sender cells receive extracellular signals that generate high of levels NOTCH ligands in comparison to receptors. Whereas receiver cells generate high levels of NOTCH receptors in comparison to ligands (70). In mammals, sending cells consist of multiple type 1 transmembrane ligands DSL (Delta/Serrate/Lag2) termed DLL1 (delta like canonical Notch ligand 1), DLL3 (delta like canonical Notch ligand 3), DLL4 (delta like canonical Notch ligand 4), and two homologues of serrate termed JAG1 (jagged canonical Notch ligand 1) and JAG2 (jagged canonical Notch ligand 1) (21–24). Whereas, receiving cells consist of NOTCH receptors NOTCH1 (notch receptor 1), NOTCH2 (notch receptor 2), NOTCH3 (notch receptor 3), NOTCH4 (notch receptor 4) (21–24).

Upon DSL ligand binding to NOTCH receptor, NOTCH signaling is initiated and activates the first ADAM (a disintegrin and metallopeptidase) proteolytic enzyme that functions to cleave the $NOTCH^{ECD}$ (NOTCH extracellular domain) from the NOTCH receptor (24,26). Subsequently, a second proteolytic enzyme termed γ-secretase cleaves the $NOTCH^{ICD}$ (NOTCH intracellular domain) from the NOTCH receptor (24). The $NOTCH^{ICD}$ is translocated to the nucleus and initiates the assembly of the CSL (CBF1/suppressor of hair- less/Lag1) transcriptional activation complex (23–25). This complex is made up of CSL (DNA binding protein), related co-activator MAML1 (mastermind like transcriptional coactivator 1), and co-repressor (Co-R). Specifically, $NOTCH^{ICD}$ binds to CSL protein and releases the Co-R from the activated complex to promote the transcription of NOTCH target genes (23–25). CSL transcriptional activation complex then transcribes NOTCH target genes *Hes1* (hes family BHLH transcription factor 1), *Hey1* (hes related

family BHLH transcription factor with YRPW motif 1), and *HeyL* (hes related family BHLH transcription factor with YRPW motif like) to promote cell growth and division (Figure 1) (6, 10).

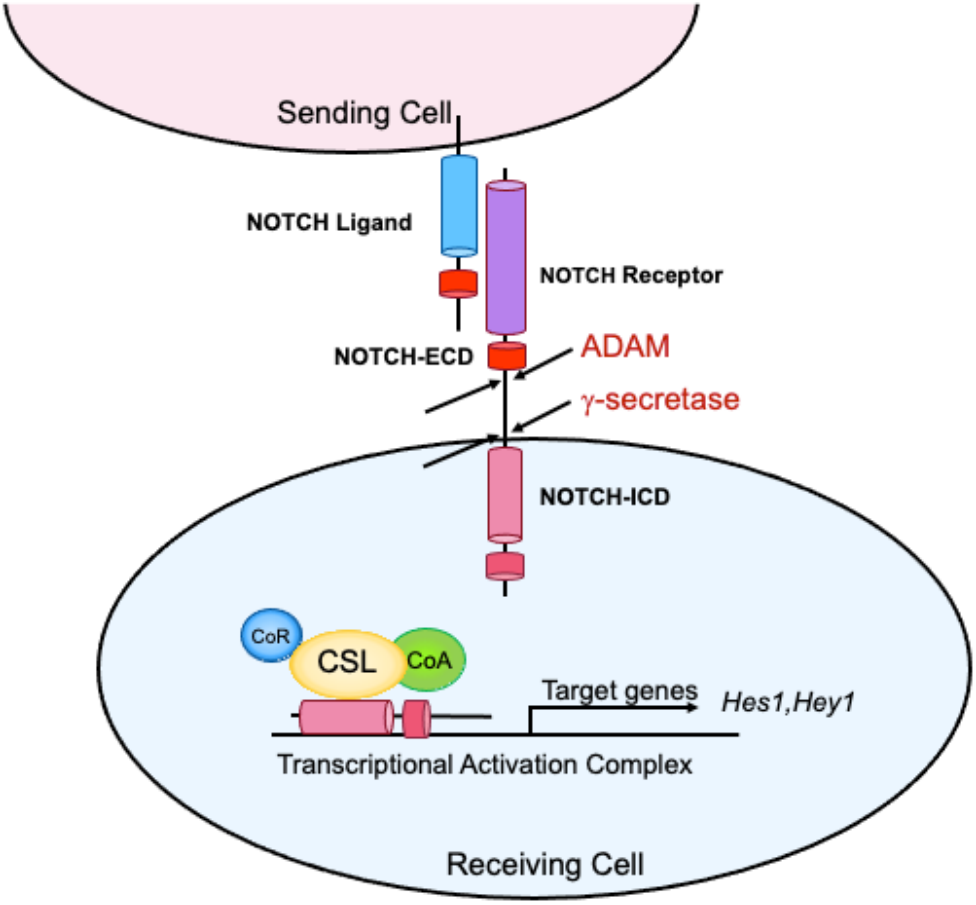


Figure 1. Basic overview of the Notch signaling pathway.

Initiation of NOTCH signaling requires NOTCH ligands (DLL1, DLL3 DLL4, JAG1, JAG2) expressed on sending cells to bind to NOTCH receptors (NOTCH1-4) found on receiving cells. Upon NOTCH ligand-receptor binding, the proteolytic enzyme ADAM first cleaves $NOTCH^{ECD}$ and subsequently γ-secretase cleaves $NOTCH^{ICD}$ from the NOTCH receptor. $NOTCH^{ICD}$ is translocated to the nucleus and activates the CSL transcriptional activation complex. Activated CSL transcriptional activation complex promotes the transcription of NOTCH target genes (*Hes1, Hey1)* that are involved in cell-to-cell communication, growth and division.

1.2.1 The role of NOTCH signaling in cell-cycle regulation

The cell-cycle is a series of cellular events involving cell-growth and division to promote two new daughter cells. In eukaryotes, the cell-cycle consists of four well-coordinated phases: G_0/G1 phase, S phase, G2 phase, and M phase (11). The G1 phase is characterized by the initiation of cell growth through the synthesis of proteins and the induction of transcriptional cell-cycle control genes (12). Multiple cell-cycle checkpoints are required to ensure proper cell function and growth before entering the subsequent S phase (13). During the S phase, DNA synthesis and replication occurs to allow cells to enter mitosis and divide into two new identical daughter cells (12). DNA repair mechanisms and multiple checkpoints are present to prevent possible errors in DNA duplication (12). In the G2 phase, cells continue to grow and prepare for mitosis (M-phase) (14). The M-phase is the last stage of the cell-cycle, whereby cells undergo mitosis in which DNA (4N) segregates into two new daughter cells (2N) (13). The daughter cells can withdraw from the cell-cycle and undergo cell-growth-arrest in order to terminally differentiate (13). A sub-population of cells can remain in a quiescent G_0 phase or re-enter the G1 phase to resume in the cell-cycle (13).

Cell checkpoint mechanisms are required to detect DNA errors and halt the cell-cycle in an attempt to repair DNA damage or promote apoptosis (14). Proper cell-division is dependent on the activation of CDKS (cyclin-dependent kinases) and cyclin proteins during mitosis (15). Specifically, CDK proteins are temporally and spatially activated in the cell-cycle to either promote or inhibit cell growth and division (14). Importantly, in response to DNA damage, cyclins/CDKs are rapidly inhibited and prevent mitosis (14, 15). CDK inhibitors (CKIs) negatively block cell-cycle activation through two well-known inhibitory families, INK4 and CIP/KIP proteins (14, 15). INK4 proteins (P16, P15, P18, P19) directly bind to CDKs to limit

association and activation by cyclins (13). In contrast, CIP/KIP proteins, P21, P27, and P57 function by binding to and inhibiting CDK-cyclin complexes (13). The cell-cycle requires the tight regulation of specific CDKs/cyclins and CKIs to prevent uncontrolled and unchecked cell division, typically characteristic of cancer (13-15).

Interestingly, the activation of NOTCH signaling promotes the transcription of NOTCH target genes *C-Myc*, *P21*, and *Cyclin-D1* that are involved in cell-cycle progression (4, 16). The NOTCH1 receptor has been shown to upregulate downstream *Hes1* and repress the transcription of *P21* to promote cell-cycle entry through the activation of CDK2 and CYCLIN-D1 proteins (19). P21 is a well-known negative regulator of the cell-cycle and has been shown to inhibit DNA synthesis by binding to the proliferating cell nuclear antigen (PCNA) (16, 17). Interestingly, white blood cells of the immune system called T-cells also require NOTCH signaling to upregulate G1 phase related proteins, CYCLIN-D, CDK4 and CDK6, progressing cell-cycle growth progression (10, 18). Thus, NOTCH signaling plays an important in regulating cell-cycle progression to promote cell growth and division (4, 10, 16, 18).

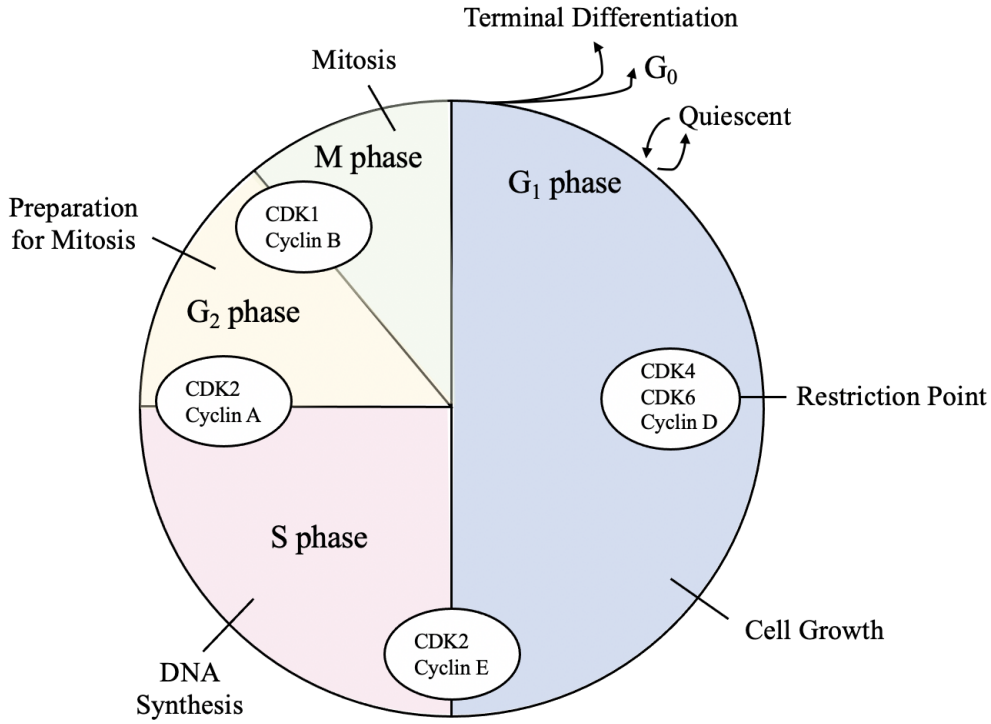


Figure 2. Schematic representation of the cell-cycle phases and check-point sites.

Cell growth and division undergo four distinct phases: G_0/G1 phase, S phase, G2 phase, and M phase. Cells are initiated for cell growth in G1 phase, which then enters the S-phase for DNA replication and synthesis. In the G2 phase, these cells continue to grow by synthesizing proteins required for the first stage of mitosis. During the M-phase, cells are divided into two new daughter cells and are able to withdraw from the cell-cycle to terminally differentiate. Cells can also re-enter the G1 phase resuming the cell-cycle or undergo cell-cycle growth-arrest and remain in a quiescent G_0 phase. Importantly, the spatial and temporal regulation of specific CDK/Cyclin complexes at each phase is critical to ensure that cell growth and division progresses without error.

1.2.2 Embryogenesis and regulation of asymmetric cell division by NOTCH signaling

During embryogenesis, the male sperm enters the female ovum forming a zygote (20). The single cell zygote undergoes mitosis, dividing into multiple primitive cells referred to as stem cells. Stem cells are defined as pluripotent, meaning that they the ability to become any type of cell in the body other than the placenta and umbilical cord (20). Stem cells can further specialize into embryonic stem cells and undergo multiple rounds of symmetrical and asymmetrical cell division to give rise to organs such as the heart, brain, muscle and kidney (20). Symmetrical cell division produces two identical cells, whereby one stem cell can either divide into two proliferative or differentiated cells that are identical (21). NOTCH signaling is involved in regulating asymmetric cell division through unequal distribution of cell fate determinants that results in two completely different daughter cells with distinct properties and cell fates (20). Specifically, asymmetrical cell division is primarily involved in cell fate determination and lineage specificity, promoting further cellular diversity (21). The NOTCH signaling asymmetric distribution was first discovered in the peripheral nervous system of *Drosophila melanogaster* (*D. melanogaster*). In detail, a sensory organ precursor cell is produced within ectodermal proneural cluster cells and undergoes multiple rounds of asymmetric cell division to form the different cell types of a sensory bristle (22). Sensory bristles are often referred to as short stiff hair typically found on the skin of an animal that is known to be involved in sensory processes like sight, smell, taste, touch and sound (23). Collectively, developmental processes like embryogenesis require NOTCH signaling to regulate cell fate decisions through controlled asymmetric cellular division, thus, promoting cellular diversity and tissue specificity (3, 5, 9).

1.3 Embryogenesis and development of skeletal muscle

NOTCH signaling is involved in a broad range of developmental processes like cell-to-cell communication, cell-cycle growth and proliferation, as well induce asymmetric cell division to promote cellular growth and diversity (3, 5, 9, 23, 24). Nonetheless, the activation of NOTCH signaling is critical for cell growth and division to progress the specialization of cells that will eventually form the skeletal muscle (3, 21). Skeletal muscle is one of the largest organs of the body and originates from embryonic and pluripotent stem cells that have the potential to differentiate into specialized skeletal muscle precursor cells (25). During late embryogenesis, stem cells form three distinctive layers called the endoderm, mesoderm and ectoderm that give rise to several organ systems (20). The mesodermal layer is then separated into the axial, paraxial, intermediate and lateral plate mesoderm that gives rise to the skeletal muscle tissue (26).

In the paraxial mesoderm, thick bands of mesodermal cells line the neural tubes that is an essential precursor for the central nervous system form blocks of cells called somites (27). Somites are transient epithelial spheres that divide dorsally and ventrally from the paraxial mesoderm (27). During embryonic muscle development, periodic NOTCH signaling defines the anterior boundary of somites and determines progenitor cell fate within somites (3, 5). Specifically, NOTCH signaling upregulates DELTA ligands to undergo multiple rounds of cell division to promote the proliferation of specialized myogenic progenitor cells (MPCs) (5). MPCs originate in the dorsal compartment of somites and are involved in forming the dermomyotome (5). Further specialization of MPCs result in transient expression of paired box transcription factors, PAX3 (paired box 3) and PAX7 (paired box 7) (28), which will migrate and delaminate to form the first combined muscle mass called the myotome (27).

Embryonic, fetal and postnatal myogenesis is controlled by NOTCH signaling to promote MPCs specification, proliferation, self-renewal, stem cell quiescence, and transiently inhibit terminal myogenic differentiation (3).

Myogenic differentiation is initiated through neighboring embryonic structures Wnt (Wnt signaling pathway) and SHH (sonic hedgehog signaling molecule) developmental signals that downregulate PAX3 and PAX7 (3, 27) and activate myogenic regulatory factors (MRFs) MYF5 (myogenic factor 5) and MYOD1 (myogenic differentiation 1) (3). The activation of MRFs localizes NOTCH inhibitor, NUMB endocytic adaptor protein (NUMB) to downregulate NOTCH signaling (6). Specifically, NUMB localization in MPCs initiates asymmetric cell division to generate two distinct daughter cells termed myogenic progenitor cells and myoblasts (3, 6, 23). Committed myoblasts express *Myf5* and *MyoD1* and exit from the cell-cycle, which is the hallmark of progression into the myogenic differentiation lineage (5). Specifically, cell cycle activators (cyclins, CDKs) are downregulated, while cell-cycle inhibitors (P21, P27, P57) are upregulated (25). The upregulation of MYOD1 in committed myoblasts activates downstream MRFs, MYOG (myogenin) and MRF6 (myogenic factor 6) that are mediators of terminal differentiation (3). Terminal differentiation is marked by the upregulation of MYH (myosin heavy chain) that promotes the formation of multinucleated myotubes (3, 29). Myotubes further differentiate into functional, contractile, and multinucleated myofibers (3, 29).

Unique to skeletal muscle, a sub-population of $PAX7^{+}$ cells are redistributed to a specific microenvironment between the sarcolemma of the myofiber and the surrounding basal lamina during the fetal stages of myogenesis (3, 29). The specialized cells are called satellite cells (SCs) that continually express *Pax7*, remaining in a quiescent, non-proliferative phase, and are characterized by reversible mitotic arrest (Figure 3) (3). Upon muscle injury, transient activation

of NOTCH signaling activates SCs to replenish the stem-cell pool (3, 29). Post activation of quiescent SCs, NOTCH signaling is then inhibited and prevents cell-cell entry. This allows for MPCs to undergo the process of myogenic differentiation through the upregulation of MYF5, MRF6, MYOD1^{+}, MYOG^{+}, MYH^{+} to form fully functional and contractile myotubes and myofibers (3, 29).

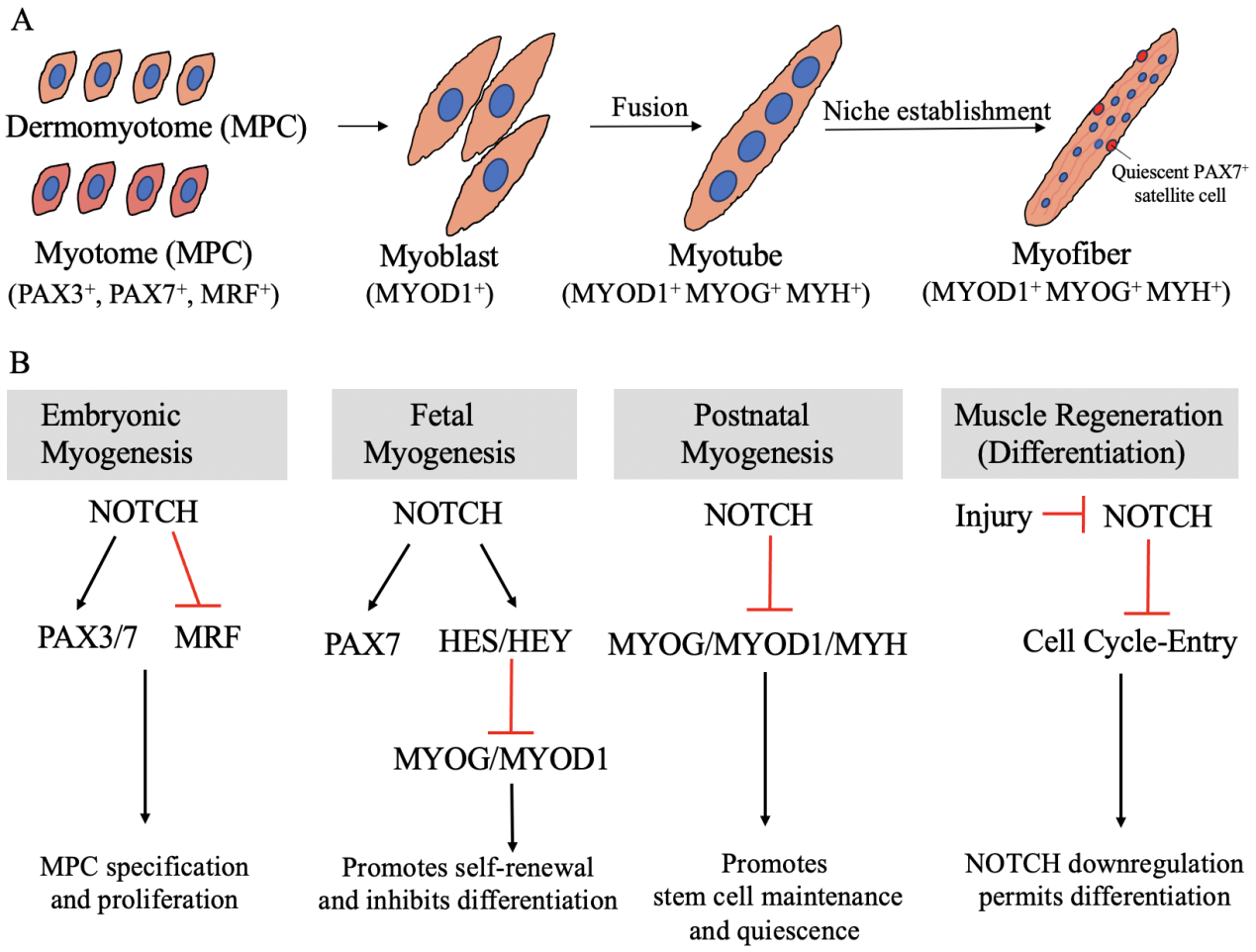


Figure 3. NOTCH signaling plays an important role in skeletal muscle development and stem cell maintenance.

(A) NOTCH signaling activates myogenic progenitor cells (MPCs) that are first found in the dermomyotome. Subsequently, MPCs transiently express *Pax3*$^{+}$ and *Pax7*$^{+}$, which migrate and delaminate to form the myotome. Further specialization of MPCs gives rise to undifferentiated

MYOD1$^+$, termed myoblasts. Myoblasts undergo fusion to form multinucleated myotubes that express *Myod1*$^+$, *Myog*$^+$, and *Myh*$^+$ and terminally differentiate into myofibers. Myofibers consist of quiescent, non-proliferative (G_0 phase) PAX7$^+$ SCs that are positioned between the sarcolemma of the myofiber and the surrounding basal lamina. However, in response to damage, SCs can self-renew, re-enter the cell-cycle to proliferate, differentiate, and regenerate muscle fibers. **(B)** NOTCH signaling is tightly regulated during embryonic, fetal, postnatal myogenesis and muscle regeneration. During embryonic myogenesis, NOTCH signaling promotes MPCs specialization (PAX3$^+$/PAX7$^+$), thereby inhibiting MRFs (MYF5, MRF4, MYOD1) involved in differentiation. In fetal myogenesis, MPCs delaminate from myotome, migrate to muscle fibers and localize between the basal lamina and sarcolemma of myofibers. In fetal and postnatal myogenesis, growth and development are maintained through the upregulation of NOTCH target genes, *Hes1* and *Hey1* that suppress myogenic regulatory factors, *Myod1* and *Myog.* Therefore, the activation of NOTCH signaling SCs promotes the self-renewal and replenishment of the stem cell pool and inhibits the process of terminal differentiation. However, upon muscle injury, quiescent cells are activated and NOTCH signaling is inhibited to prevent cell-cycle entry. During muscle regeneration, the downregulation of NOTCH signaling allows SCs to undergo the process of terminal differentiation to form multinucleated myotubes and myofibers.

1.4 NOTCH signaling acts as a suppressor of differentiation

In skeletal muscle, NOTCH signaling is shown to have multiple different roles during the proliferation and differentiation of SCs (29). However, the role of NOTCH signaling is multifaceted due to the fact that different combinations of ligand (DLL1-4, JAG1-2) and NOTCH receptor (NOTCH1-4) binding have very distinct outcomes during myogenesis (9). For example, elevated DDL1 mediated NOTCH signaling activates PAX7 and inhibits myogenic

differentiation at the medial border of the dermomyotome (31). Similarly, NOTCH signaling promotes the MPCs specification, proliferation, stem-cell renewal, maintenance, quiescence by transiently inhibiting myogenic differentiation (29). Another study highlighted the stem-cell renewal proliferative role of NOTCH signaling in a myofiber culture system that mimicked muscle-injury (32). The study found that NOTCH1 was rapidly activated in SCs and promoted the proliferation of myogenic precursor cells that transiently express PAX3 (4).

Several studies have shown that activation of NOTCH signaling prevents myoblast differentiation through repression of MYOD1 and promotes SCs self-renewal through upregulation of NOTCH target gene, *Hes1* (9, 31). Similarly, another study found that NOTCH ligand, DLL4, induced NOTCH signaling and blocked *Myog* expression in skeletal muscle. Specifically, this study cultured and differentiated C2C12 cells with fusion proteins, which are plasmids containing the extracellular domain of DLL4 (fc-DLL4) (34). Interestingly, DLL4-fc induced NOTCH signaling cells were unable to form multinucleated myotubes and failed to show *Myog* expression (34). Another recent study found that cultured muscle stem cells (MuSCs) with increased HEY1 and HEYL levels exerted anti-myogenic effects (33), suggesting that HEY1, HEYL and HES1 synergistically act to suppress myogenic differentiation (36). Through the use of conditional mutagenesis methods, the research suggests that HEY1 and HEYL are both required to maintain MuSCs pool and lacking HEY1 and HEYL results in the upregulation of differentiation markers MYOD1 and MYOG (37). Collectively, several studies have alluded to role of NOTCH signaling in SCs proliferation, maintenance and self-renewal, and suppression of myogenic differentiation (9, 31, 33, 36-37).

1.4.1 NOTCH signaling acts an inducer of myogenic differentiation

On the contrary, NOTCH inhibitor, NUMB localizes in MPCs to downregulate NOTCH signaling to promote terminal myogenic differentiation (6). Studies have also found that activation of JAG1 (NOTCH ligand) induces smooth muscle differentiation through the CSL transcriptional activation complex, also known as the RBPJ (recombination signal binding protein for immunoglobulin kappa J region) dependent pathway (33). Specifically, the study found that physiological stimulation of NOTCH signaling via JAG1 ligand, not DLL4, induced MYH expression through the RBPJ dependent pathway (33). Lastly, prior studies in C2C12 cell culture have used multiple cDNA constructs that express mutated forms of NOTCH1 to show impaired myogenic differentiation in the absence of NOTCH1(34). Researchers engineered two mutated forms of NOTCH1; 0CDN1, which lacks the majority of the cytoplasmic domain, and mutated CDN1 that lacks the signal peptide, the entire extracellular domain, the transmembrane region, cytoplasmic sequences containing the initial nuclear localization signal, and several CFB1-binding sites (34). Interestingly, this study found that the CDN1 mutation that lacks CBF1-interacting sequences blocked myoblast fusion and differentiation (34). In this study, researchers found that control C2C12 myoblasts showed little to no protein of MYOG at day 0, and increased MYOG and MYH levels at day 2 of differentiation (34). However, C2C12 myoblasts co-expressed with mutant NOTCH1-CDN1 showed dramatic reduction of myoblast fusion, and a significant decrease in MYOG and MYH levels at day 2 (34), further strengthening that NOTCH signaling is required to progress myogenic differentiation. Nonetheless, the activation of JAG1-2 ligands and DLL1-4 upregulation (34) and (37) induces and inhibits myogenic differentiation, respectively (34, 37). Therefore, differences in NOTCH ligand responses can be multifaceted and consequently have opposing outcomes during myogenesis (9).

1.4.2 Outcomes of hyperactivation and sustained NOTCH signaling

The tight regulation of NOTCH signaling is critical for cell fate decisions, embryonic and developmental processes, proliferation, and differentiation during myogenesis (3, 5, 9, 23, 24). Therefore, the dysregulation of NOTCH signaling has severe detrimental effects in SC renewal and myogenesis (38). For example, DMD (Duchenne muscular dystrophy) is characterized by muscle weakness and degeneration due to a mutation in the dystrophin gene, resulting in truncated protein or no protein (36, 39, 40). Furthermore, DMD dKO (dystrophin/utrophin knockout mouse) display repeated rounds of muscle degeneration and regeneration (35). Interestingly, dKO mice display over-activation of NOTCH signaling that result in negative physiological effects such as sustained inflammation, impaired muscle regeneration, and the rapid depletion and senescence of MPCs (i.e., PAX7+ cells) (35). However, dKO mice treated with γ-secretase inhibitor, DAPT (N-[N-(3,5-Difluorophenacetyl-N-alanyl)]-S-phenylglycine t-Butyl Ester) effectively suppressed muscle regeneration impairments that were associated with elevated NOTCH levels (35). DAPT is a γ-secretase chemical inhibitor that blocks proteolytic cleavage of $NOTCH^{ICD}$, decreasing $NOTCH^{ICD}$ levels and further repressing the activation of CSL transcription complex (36). Therefore, DAPT treated dKO mice effectively promoted stem-cell renewal and diminished increased senescence of MPCs (35). Another study found that transient activation of NOTCH signaling is critical for the upregulation of PAX3/PAX7 in myoblasts. However, excessive and sustained NOTCH signaling in myoblasts results in dramatic upregulation of *Pax7*, decreased *Myod1* expression, impairments in differentiation, SC depletion and senescence (37). The hyper-activation of NOTCH signaling not only is shown to impair myogenic differentiation, but can also promote brown adipogenesis at the expense of myogenic differentiation (37). Overexpression of $NOTCH1^{ICD}$ results in decreased expression of the

muscle-specific genes *Myf5, Myod,* and *Myog* and upregulates brown fat-specific genes (37). In this particular study, $Pax7^{CE/Fl}$: $Rosa^{Notch}$ mice were administered cardiotoxin to induce acute muscle damage, and interestingly SCs that overexpress NOTCH failed to effectively regenerate muscle, showed decreased muscle mass and as a consequence brown adipose tissue was formed within the regenerating muscle (37).

A more recent study researching Type I diabetes (T1D) in human subjects and insulin deficient $Ins2^{Akita}$ (Akita) mice found that persistent activation of NOTCH signaling impairs the functionality of SCs in skeletal muscle (38). The study suggested the dual and temporal functionality of NOTCH signaling during myogenesis, whereby early activation of NOTCH signaling promotes PAX7 and self-renewal of SCs (38). The second mode of NOTCH signaling requires reduced negligible levels of NOTCH to facilitate the progression of muscle differentiation (38). Researchers found that $Ins2^{Akita}$ myofibers show a 1.9-fold increase of $NOTCH1^{ICD+}/Pax7^{+}$ nuclei in activated $Ins2^{Akita}$ myofibers compared to wild-type (WT), which was indicative of elevated NOTCH signaling and reduced capacity of SCs to proliferate and progress differentiation (38). Further analysis of sustained NOTCH activity led to *in-vitro* experimental methods that treated isolated $Ins2^{Akita}$ myofibers with DAPT (24 hr) to reduce elevated NOTCH signaling (38). Supporting previous findings, DAPT treatment increased primarily PAX7 expression by 1.6-fold suggesting that the downregulation of NOTCH signaling progressed satellite cell activation and differentiation of $Ins2^{Akita}$ myofibers (38).

1.5 Autophagy Pathway

Autophagy is an intracellular degradation system that regulates key cellular processes, including cell-cycle division, DNA repair, apoptosis, cell growth, and differentiation (12). Several forms of autophagy exist, including chaperone-mediated autophagy, microautophagy,

and macroautophagy that have distinct cellular cytoplasmic transport mechanisms to the lysosome (13). Of interest in this thesis is macroautophagy (autophagy), whereby dysfunctional cytoplasmic contents are sequestered into double membraned structures called autophagosomes and delivered to lysosomes for degradation (15). A less-studied mechanism of autophagy is nutrient breakdown that involves the degradation of unwanted protein and nutrient stores to meet the energy needs of the body (7). For example, during starvation (nutrient poor conditions), autophagy is induced to breakdown macromolecules and organelles to supply nutrients and energy to the body (2,15). The autophagy pathway can be described in five main stages, which are initiation, elongation, maturation, fusion and degradation (7).

1.5.1 Initiation

The initiation stage requires two important phosphorylation platforms, ULK1 (unc-51 like autophagy activating kinase 1) complex and the BECN1 (Beclin-1) complex to initiate autophagosome formation (39, 40). Induction of autophagy activates the ULK1 complex containing ULK 1/2 (unc-51 like autophagy activating kinase 1) proteins, ATG13 (autophagy related 13), and FIP200 (focal adhesion kinase family interacting protein of 200 kDa) (39, 40). Under normal conditions, the ULK1 complex is hyperphosphorylated and inhibited, however during autophagic starvation ULK1/2 is hypo-phosphorylated and activated (39, 40). ULK1/2 activation promotes the autophosphorylation and phosphorylation of distinct residues on ATG13 and FIP200 (39, 40). Specifically, these phosphorylation events induce the translocation of the ULK1 complex to localize at pre-autophagosome production sites (39, 40).

The second phosphorylation platform BECN1 (Beclin-1) complex is activated and is responsible for the assembly and activation of autophagosomes (40). Furthermore, the BECN1 complex acts as a class-III phosphatidylinositol 3-kinases (PI3K) and induces the production of

phosphatidylinositol 3-phosphate (PI3P), required for assembly and activation of autophagosomes (41). In more detail, the BECN1 complex consists of core proteins, including PIK3c3/VPS34 (phosphatidylinositol 3-kinase catalytic subunit type 3), PIK3R4/VPS15 (phosphatidylinositol 3-kinase regulatory subunit 4), ATG14 (autophagy related 14), AMBRA1 (autophagy and beclin 1 regulator 1), and BECN1 (41). The entirety of the BECN1 complex synthesizes PI3P and regulates the nucleation of the double membrane (41) . During the final stage of initiation, BECN1 complex produces the PI3P end product that is involved in elongating the pre-autophagosomes and mediates the recruitment of other adaptor proteins (41).

1.5.2 Elongation and maturation

Pre-autophagosome (phagophore) elongation and maturation primarily involves two ubiquitin-like conjugation systems (42). The first system involves ATG7 (autophagy related 7) that function as E1 ubiquitin-conjugated enzyme and activates ATG12 (autophagy related 12) (42). ATG7-ATG12 is transferred to ATG10 (autophagy related 10) that functions as a E2-like ubiquitin-conjugated enzyme), which is covalently linked by its C-terminal domain to internal lysine residues of ATG5 (autophagy related 5) (42). ATG12-ATG5 heterodimer is then conjugated with ATG16L (autophagy related 16-like protein) and forms an 800-kDA complex (42). The ATG12-ATG5-ATG16L complex elongates and adds curvature to the autophagosome membrane (42). The second ubiquitin-like conjugation system, MAP1LC3B (microtubule associated protein 1 light chain 3 beta) is the synthesized precursor form and cleaved by ATG4B (autophagy related 4B) to produce the cytosolic isoform, LC3B-I (41-43). LC3B-I conjugates PE (phospholipid phosphatidylethanolamine) in a reaction mediated by ATG7 (E1 enzyme) and ATG3 (autophagy related 3), E2 enzyme. PE-LC3B-I conjugation and lipidation results in non-soluble form MAP1LC3B2 (microtubule associated protein 1 light chain 3 beta 2), hereafter

referred to as LC3B-II (42). LC3B-II is a standard marker for the presence of autophagosomes and found throughout the internal and external autophagosome membrane (15). At the end of the maturation stage, the phagophore expands and gains curvature to become a double membraned structure called the autophagosome (Figure 4) (15).

General autophagy was initially characterized as bulk degradation (7), however, it is now thought as selective degradation of sequestered damaged proteins and organelles (44). Autophagy mediated degradation involves the Ubiquitin-Proteasome-System (UPS) that tags misfolded and damaged protein with a small protein called ubiquitin (45). SQSTM1/P62 (sequestosome 1) is a ubiquitin-interacting domain receptor protein that functions to identify and sequester ubiquitinated proteins into larger aggregates for degradation (12,13,15). Interestingly, SQSTM1 interacts with LC3B-II through the LC3 interacting region (LIR) and facilitates the sequestering of ubiquitinated substrates to the autophagosome for degradation (16).

1.5.3 Fusion and degradation of autophagosome

The last step in the autophagy pathway is autophagosome fusion with lysosome to degrade damaged and unwanted cytoplasmic content (46). Specifically, the outer membrane of the autophagosome fuses to the lysosomal membrane through the regulation of several proteins, including RUBCN (Rubicon autophagy regulator), UVRAG (UV radiation resistance associated), RAB7A (RAB7A, member RAS oncogene family), SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors), and LAMPs (lysosome associated membrane proteins) (46). Post autophagosome-lysosome fusion, lysosomal hydrolases break down the targeted cytoplasmic content (46). Lastly, lysosomal permease transports the end products back to the cytosol and are used for energy production and protein synthesis (42).

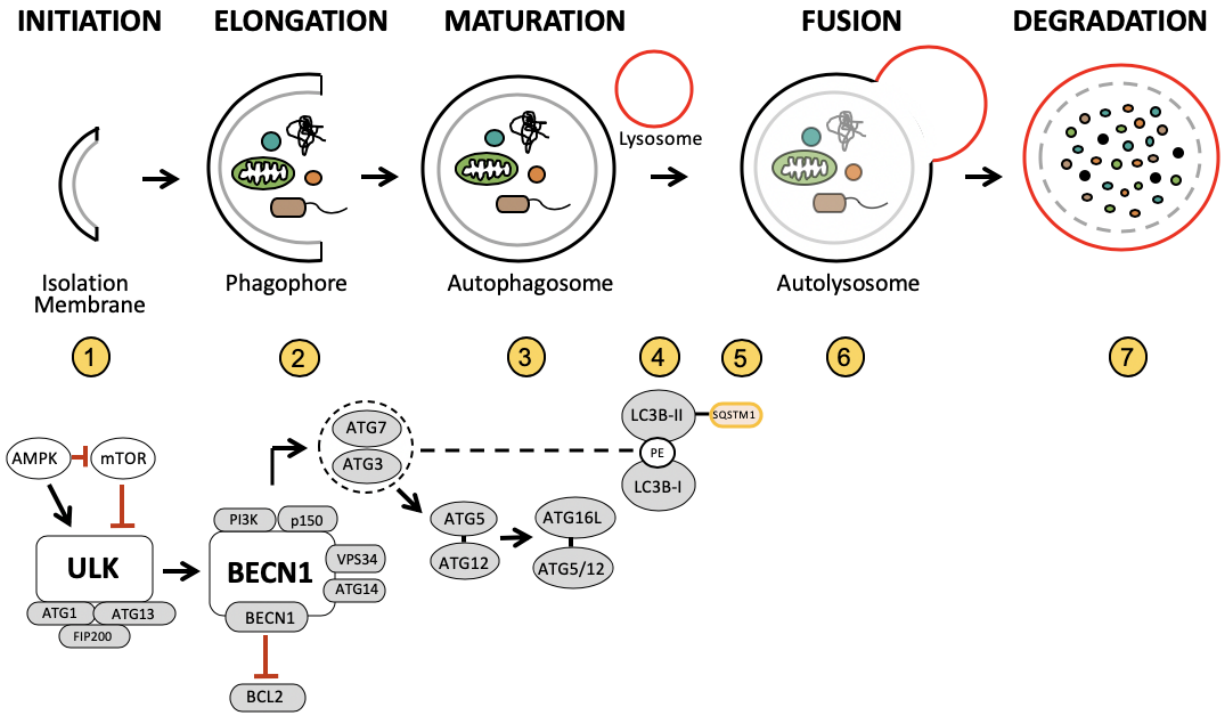


Figure 4. Stages of autophagy. 1. Activation of the ULK protein complex (ULK1/2 + ATG13 + FIP200) forms the isolation membrane (initiation). 2. ULK complex activates the BECN1 (BECN1 + P13K + ATG14 + P150 + AMBRA1) complex that initiates elongation of phagophore, furthermore BECN11 is disassociated from BCL2 to allow for nucleation of the phagophore. 3. Activation of ATG7 catalyzes the conjunction of ATG5, which then forms ATG5/12 that connects to ATG16 elongating the phagophore (elongation). 4. ATG7 and ATG3 conjugates PE to LC3B-I, whereby lipidation results in LC3B-II that inserts into the membrane to become a double membraned structure called the autophagosome (maturation). 5. SQSTM1 and LC3B-II recruits damaged organelles and proteins for degradation. 6. Autophagosome fuses with the lysosome to form the autolysosome (fusion). 7. Autolysosome degrades the targeted proteins and organelles (degradation).

1.5.4 Autophagy as a signaling mechanism in regulating apoptosis

The emerging concept that autophagy may function as fine-tuning signaling pathway and cross communicate with other signaling pathways was evidenced in a similar cell death process referred to as apoptosis (71). Apoptosis is a form of programmed cell death, whereby cells undergo morphological changes that include chromatin condensation, cell shrinkage and membrane blebbing (71). Cysteine aspartic acid proteases (caspases) are key mediators of apoptotic cell death that function to cleave substrates between cysteine and aspartic acid residues (72). Generally, caspases are grouped based on their primary apoptotic role: initiators (i.e., CASP8 (caspase8), CASP9 (capase9) or effectors (i.e., CASP3 (caspase3), CASP6 (caspase6). The induction of apoptosis activates certain caspases to cleave proteins (72), which results in cell death characteristics like cellular degradation, DNA fragmentation and blebbing (71).

Autophagy differs from apoptosis due to the fact that it accumulates autophagosomes within a dying cell (7) that is not associated with chromatin condensation and membrane blebbing (71). Autophagy has been shown to act as the first line of defence through elimination of cellular stresses (pro-cell survival), however, accumulation of cellular stresses beyond the beneficial threshold results in apoptotic cell death (48). In conditions where cellular stresses exceed the beneficial threshold, apoptosis is triggered and inactivates autophagy through caspase mediated cleavage of autophagy proteins (48). The interplay between autophagy and apoptosis has been studied extensively in skeletal muscle cell differentiation (48). Interestingly, the inhibition of autophagy with 3MA is associated with elevated CASP3 activity, DNA fragmentation and nuclear condensation (49). In addition, autophagy deficient cells indicate decreased autophagy signaling, elevated CASP3 activation and impaired myogenic differentiation (49). More so, autophagy inhibition has been shown to sensitize cells to apoptosis

in skeletal muscle (49), suggesting that autophagy plays an important regulatory role in apoptosis during skeletal muscle differentiation.

1.5.5 Autophagy as a fine-tuning regulator of NOTCH signaling

Several studies have examined the role of NOTCH signaling or autophagy during myogenesis. However, few investigations have been done in understanding the interaction of autophagy and NOTCH signaling during myogenic differentiation. These findings are limited to cardiac, neuronal and hemopoietic tissue, and have not been clearly defined in skeletal muscle. It is well-established that NOTCH signaling is required during early embryogenesis giving rise to muscle precursor cells that can proliferate and differentiate into skeletal muscle (3). Furthermore, several studies have provided thorough evidence that autophagy is essential to facilitate myogenic differentiation (7, 44, 47-50), and that inhibiting ATG7 drastically impairs the formation of mature multinucleated myotubes (44, 47). One of the first studies looking at the crosstalk between autophagy and NOTCH signaling found that impaired autophagy resulted in NOTCH hyperactivity (36). In this study, researchers conditionally knocked out the *Atg7* gene in the hematopoietic system by crossing Vav-iCre^{+} with *Atg7*$^{flox/flox}$ mice, referred to as Vav- *Atg7*$^{-/-}$ mice. Vav-*Atg7*$^{-/-}$ mice showed severe myeloid proliferation likely due to NOTCH hyperactivity and in some cases caused lethality to Vav-Atg7$^{-/-}$ mice (36). Interestingly, physiological autophagic signaling in mice was inversely correlated with NOTCH signaling (36). Experimental conditions that show a lack of autophagy are associated with upregulated and excessive NOTCH signaling in impaired hematopoietic stem cells (HSCs) (36). A more recent study has also postulated that autophagy selectively targets and degrades NOTCH1^{ICD} in order to downregulate NOTCH signaling to promote the differentiation of HSCs (36). The same study also restored hematopoietic differentiation potential in autophagy deficient HSCs cells by downregulating

NOTCH signaling through the use of γ-secretase inhibitor, DAPT and through genetic inhibition with the RNA interference of NOTCH effector RBPJ (CSL activation transcription complex) (36). Collectively, the study supported existing evidence of autophagy mediated degradation of NOTCH signaling is required to sustain hematopoietic differentiation (36).

The interplay between NOTCH signaling and autophagy has been thoroughly investigated during neurogenesis. Firstly, researchers suggested that NOTCH1 can be either degraded through endocytosis or the autophagic pathway (51). The endocytic pathway consists of early endosomes, late endosomes, and lysosomes that synthesizes vesicle membranes (52). Endocytosis functions similarly to autophagy such that endocytic membranes are recycled to remodel endocytic compartments and ensure the maintenance of organelle integrity (52). Interestingly, early and late endosomes can fuse with autophagosomes to become amphisomes (51) (Figure 5).

The NOTCH1 interaction in endocytic and autophagy pathways was further studied in human embryonic kidney (HEK) cultured cells (51). Specifically, a triple staining method was used to show that NOTCH1 is localized in early endosomes EEA1 (early endosomal antigen 1) positive vesicles via endocytosis, which were also positive for autophagy related protein, LC3 (51). This study first showed that an interaction between endocytosis and autophagy pathway exists with respect to NOTCH1 localization (51). In addition, NOTCH1 was also found in autophagosomes that were independent of the endocytosis pathway, indicated by LC3-positive and EEA1-negative autophagosomes (51). This finding in particular provides evidence that NOTCH1 is being targeted and sequestered to the autophagosome for degradation (51). Follow-up studies transfected HEK cells with plasmid coding Enhanced Green Fluorescent Protein (pEGFP)-LC3 and then treated with DMSO (control) or RAPA for 8 hrs to induce autophagy

(51). RAPA treated HEK cells demonstrated increased colocalization of NOTCH1 and LC3, and EEA1-negative autophagosomes, providing stronger evidence that autophagy is likely a key regulator of NOTCH1 degradation (51).

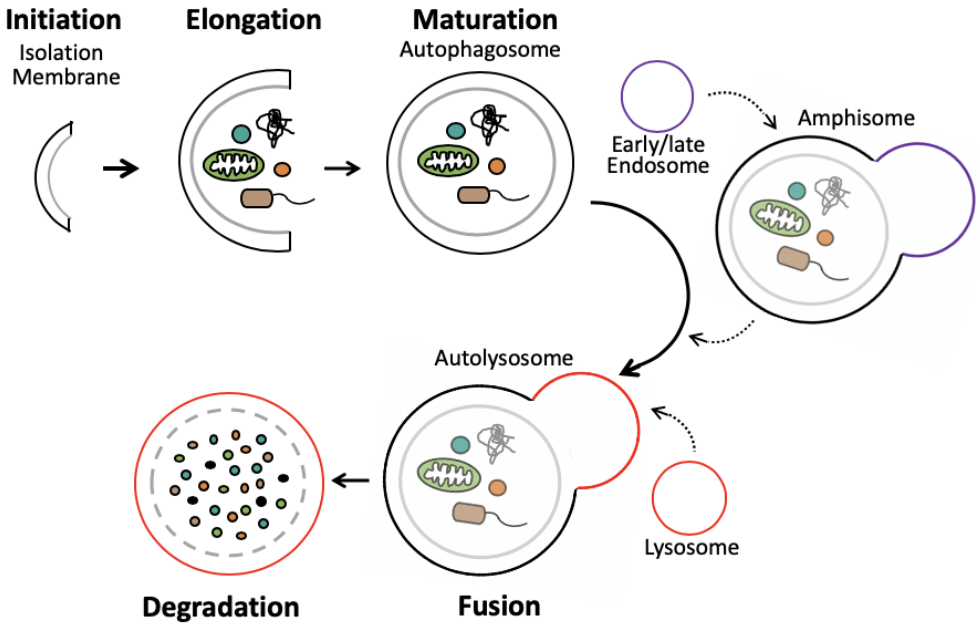


Figure 5. Autophagy and the endocytic pathway. Autophagosomes form at the pre-autophagosomes or phagophore assembly site and fuse with early endosomes, late endosomes, and lysosomes, becoming an amphisome or autophagosomes fuse with the lysosomes, becoming an autolysosome.

Considering that NOTCH1 co-localizes with LC3 in autophagosomes, researchers were interested to determine how early NOTCH1 receptor is present in the autophagic pathway (51). Therefore, another experiment was conducted to examine whether NOTCH1 is present in pre-autophagosomal structures that upregulate early autophagy markers like mATG9 (autophagy related 9A) and ATG16L1 (51). Interestingly, pre-autophagosomal structures contained NOTCH1 receptor, which suggests early targeting of NOTCH1 for degradation (51). The researchers also used a hypomorphic mutation that results in partial loss of the *Atg16l1* gene

function, not complete loss (53). Hypomorphic ATG16L1 mutated mice showed increased NOTCH1, $NOTCH^{ICD}$, HES1 levels, significantly higher proportion of stem cells, and developmental delays in neurogenesis, haematopoiesis, and in the gut villi (51). The *in-vivo* model strongly supported the hypothesis that impaired autophagy is linked to excessive NOTCH signaling, resulting in diminished degradation of NOTCH1 and differentiation during neurogenesis (51).

The model that proposes autophagy inversely downregulates NOTCH signaling was again shown in cardiac cells (43). Experiments conducted in this study also found that impaired autophagy is correlated with elevated NOTCH signaling (43). Specifically, ATG7 and ATG5 knock down cardiac cells that were treated with RAPA to induce autophagy resulted in the downregulation of NOTCH signaling levels and restored cardiac differentiation impairments (43). Furthermore, co-immunoprecipitation (co-IP) assays and immunofluorescence methods found that the autophagy pathway interacts with the NOTCH signaling pathway to form $NOTCH1^{ICD}$-LC3B-II and $NOTCH1^{ICD}$-SQSTM1 complexes during cardiac differentiation (43). The formation of $NOTCH1^{ICD}$-LC3B-II and $NOTCH1^{ICD}$-SQSTM1 complexes further strengthen previous findings that autophagy targets $NOTCH1^{ICD}$ for degradation in order to promote cardiac differentiation (43). Collectively, these findings suggest that autophagy sustains differentiation through the targeted degradation of $NOTCH1^{ICD}$, therefore, the same relationship should similarly exist in skeletal muscle differentiation (36, 43, 51).

Chapter 2 Purpose

Previous research has demonstrated a key role for autophagy during myoblast differentiation (44, 47-49). The induction of autophagy is essential to progress myogenic differentiation and inhibiting ATG7 drastically impairs the formation of mature multinucleated myotubes (47, 48). In contrast, NOTCH signaling has been implicated in developmental processes as a suppressor of myogenesis (6). However, studies examining the relationship between autophagy and NOTCH signaling in skeletal muscle are limited. We propose autophagy may function as fine-tuning signaling pathway that downregulates NOTCH signaling to sustain myogenic differentiation (36, 43).

The process of myogenesis is regulated by spatial and temporal activation of NOTCH signaling, specifically during embryonic, fetal and postnatal myogenesis to promote MPCs growth, proliferation and development (3). Thus, this thesis sought to test the hypothesis that autophagy downregulates NOTCH signaling through selective and targeted degradation of NOTCH proteins to allow for the remodeling of myoblasts into myotubes during C2C12 myogenic differentiation (36, 43). Our findings suggest that basal levels of NOTCH signaling are required to promote the development of myoblasts, however after the growth and proliferative period, we postulate that autophagy plays a critical role in downregulating NOTCH signaling to transition myoblasts into fully differentiated myotubes. Furthermore, we also postulate that NOTCH signaling is a novel target for rescuing myogenic differentiation potential in autophagy deficient conditions (36, 43).

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

1) Characterize NOTCH-related mRNA and protein levels during C2C12 myoblast differentiation (NOTCH1, NOTCH1^{ICD}, *Notch1*, *Hes1*, *Hey1*)

a. Determine the role of NOTCH signaling in response to γ-secretase inhibition in attempt to decrease the production of NOTCH1 and NOTCH1^{ICD} levels during C2C12 myoblast differentiation

2) Determine the role of autophagy in NOTCH signaling in response to:
    a. Pharmacological induction of autophagy using RAPA in proliferating myoblasts
    b. Pharmacological inhibition of autophagy using CQ in proliferating myoblasts
    c. Short hairpin RNA (shRNA) against *Atg7* during C2C12 myoblast differentiation
3) Investigate the interplay between autophagy and NOTCH signaling in response to DAPT treatment during autophagy deficient C2C12 myoblast differentiation.

Experiment 1: Downregulation of NOTCH1 during C2C12 myoblast differentiation

The murine skeletal muscle C2C12 cell line is commonly used as an *in vitro* model to study the interaction between NOTCH signaling and autophagy during differentiation. C2C12 myoblasts were initially cultured in growth media maintaining a proliferative and undifferentiated state. Subsequently, C2C12 myoblasts were substituted with differentiation media to induce the fusion and differentiation of myoblasts to form multinucleated myotubes. More specifically, C2C12 cells were collected at various states of growth and differentiation as follows, D0 (24 hr), D1 (48 hr), D2 (72 hr), D3 (96 hr), and D4 (120 hr). In the following experiment, to better elucidate the role and function of NOTCH1, γ-secretase inhibitor, DAPT was used to markedly decrease NOTCH1 and NOTCH1^{ICD} levels during C2C12 myoblast differentiation . Immunoblotting was used to assess autophagy related proteins (ATG7, SQSTM1, LC3B), myogenic markers (MYH, MYOG) and NOTCH signaling proteins (NOTCH1, NOTCH1^{ICD}) to assess autophagic signaling as well as NOTCH signaling during myogenesis. Additionally, qRT-PCR was used to establish the transcript levels of *Notch1 gene,* and downstream NOTCH target genes *Hes1 and Hey1*.

Experiment 2: Role of autophagy in regulating NOTCH signaling

In order to better determine the crosstalk between autophagic signaling and NOTCH signaling, autophagy was manipulated in experimental conditions that either increase or decrease autophagy in proliferating myoblasts. Firstly, autophagy was increased with RAPA treatment at 2 μM to assess the effect on $NOTCH1^{ICD}$ levels. In contrast, CQ treatment and sh*Atg7* cells were used as impaired autophagy models to determine whether the lack of autophagy results in increased NOTCH signaling. The effect of autophagy induction and inhibition was assessed by immunoblotting for various autophagy related proteins (ATG7, SQSTM1, LC3B), myogenic markers (MYH, MYOG) and NOTCH signaling proteins (NOTCH1, $NOTCH1^{ICD}$) in proliferating myoblasts at various time-points of growth (0 hr, 6 hr, 12 hr, 24 hr).

Experiment 3: Differentiation of autophagy deficient cells in the absence of NOTCH

To better elucidate if the effect of reduced ATG7 levels in C2C12s results in impaired myogenic differentiation, we studied the influence of NOTCH signaling pathway in a system lacking autophagy. Interestingly, the loss of autophagy leads to sustained and elevated levels of $NOTCH1^{ICD}$ in sh*Atg7* cells. We pharmacologically treated sh*Atg7* cells with DAPT to determine whether reduced $NOTCH1^{ICD}$ levels may improve the potential of myogenic differentiation in autophagy deficient cells. Immunoblotting was performed to assess differences in autophagy related proteins (ATG7, SQSTM1, LC3B), myogenic markers (MYH, MYOG) and NOTCH signaling proteins (NOTCH1, $NOTCH1^{ICD}$) during differentiation. Immunofluorescence methods were used to morphologically analyze differentiation and fusion indexes. In addition, the effect of DAPT treatment and decreased $NOTCH1^{ICD}$ levels in cell cycle regulation was assessed by flow cytometry, comparing differences between scr, sh*Atg7*+Veh, and sh*Atg7*+DAPT treatment groups.

2.1 Hypotheses

It was hypothesized that:

1) NOTCH signaling will decrease during differentiation, which simultaneously occurs alongside increased autophagic flux.
    a. Protein content of NOTCH1 and NOTCH1^{ICD} will transiently increase during early myogenesis and gradually decrease during myogenic differentiation
    b. Transcript levels of *Notch1*, *Hes1* and *Hey1* will similarly transiently increase during early myogenesis and gradually decrease during myogenic differentiation

1-2) Inhibition of γ-secretase will decrease NOTCH1 and NOTCH1^{ICD} levels and decrease myogenic differentiation markers

    a. Protein content of NOTCH1 and NOTCH1^{ICD} will decrease
    b. Myogenic markers MYH and MYOG will decrease
    c. There will be a slight increase in autophagic signaling

2) Induction of autophagy (RAPA) will decrease NOTCH1^{ICD} levels, while inhibition of autophagy with CQ or shRNA against ATG7 (sh*Atg7*) will increase NOTCH1^{ICD} levels
    a. NOTCH1^{ICD} levels will decrease in RAPA treated cells
    b. NOTCH1^{ICD} levels will increase in CQ treated cells and sh*Atg7* cells

3) Reduction of NOTCH1^{ICD} levels with DAPT treatment will improve the myogenic potential of autophagy deficient (sh*Atg7*) cells
    a. DAPT treatment will decrease NOTCH1 and NOTCH1^{ICD} levels
    b. DAPT treatment will increase MYH and MYOG levels
    c. DAPT treatment will increase S phase and G2/M phase indicative of cell-cycle exit

Chapter 3 Methods

3.1 Cell culture and transfections

Murine C2C12 skeletal myoblasts (ATCC, CRL-1772) were plated in polystyrene cell culture dishes (BD Biosciences) in growth media (GM) consisting of low-glucose Dulbecco's Modified Eagle's Medium (DMEM; ThermoFisher Scientific, SH30021) containing 5% fetal bovine serum (FBS; ThermoFisher Scientific, SH30396), 5% Serum Plus II (Serum Plus Medium Supplement-14008C), with 1% penicillin/streptomycin (ThermoFisher Scientific, SV30010) and incubated at 37ºC in 5% CO_2. Myoblast differentiation was induced by replacing GM with differentiation media (DM) consisting of DMEM supplemented with 2% horse serum (ThermoFisher Scientific, SH30074) and 1% penicillin/streptomycin. Cells were collected at 90% confluency prior to the addition of DM (D0) as well at 48 h (D1), 72 h (D2), 96 h (D3), 120 h (D4), after the addition of DM. In some experiments, CQ (10 μM); Sigma-Aldrich, C6628) was added for 24 hr to assess autophagic flux. To inhibit NOTCH activity 10-15 μM of DAPT GSI-IX (Selleck) was added to warm media and replaced every 24 hr throughout differentiation.

C2C12 cells stably expressing shRNA against *Atg7* (sh*Atg7*) or a scramble (SCR) control sequence were generated as previously described (49). Sh*Atg7* and SCR cell lines were generated previously in our lab (48). Briefly, C2C12s were grown in 12-well plates and transfected with vectors encoding an shRNA against ATG7 (Origene TG504956), a scramble control sequence (Origene TR30013), or a CRISPR control sequence (Origene). For each transfection, vector DNA and Lipofectamine 2000 were diluted in Opti-MEM and the mixture was added to cells and incubated for 6 hr. 24 hr after transfection, cells were transferred to 100 mm plates and grown in GM with puromycin (2 μg/mL) to allow for stable clone selection. Immunoblotting was then used to evaluate ATG7 protein levels in selected clones.

3.2 Immunoblotting

Briefly, cells were lysed in ice-cold lysis buffer (LB) [20mM Hepes, 10mM NaCl, 1.5mM MgCl, 1mM DTT, 20% glycerol and 0.1% Triton X-100 (pH7.4)] containing a protease (Roche Applied Sciences) inhibitor cocktail. Equal amounts of protein were loaded and separated on 7.5% or 12% SDS-PAGE gels, transferred onto PVDF membranes (Bio-Rad), and blocked in 5% milk in TBS-T for 1 hour at room temperature. Membranes were incubated overnight at 4℃ in primary antibodies against: ATG7 (Cell Signaling-D12B11), LC3B (Cell Signaling-D11), cleaved NOTCH1 (Cell Signaling-Val1744-D3B8), SQSTM1 (MBL-PM045), MYOG (Developmental Studies Hybridoma Bank-F5D), MYH (Developmental Studies Hybridoma Bank-MF-20), GAPDH (Cell Signaling-14C10), NOTCH1-EP1238Y (Abcam52627). Membranes were then washed in TBS-T and incubated for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). TBS-T washes were repeated, bands were visualized using the Clarity Western ECL substrate (Bio-Rad) and the ChemiDoc Imaging System (ImageLab).

3.3 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 hr, 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 mins, washed in PBS, kept in fresh PBS, and visualized using

Cytation5 (BioTek) imaging reader. The differentiation index was calculated at the percentage of MYH positive cells related to total nuclei. The fusion index was calculated as the percentage of nuclei present in multi-nucleated (two or more nuclei) cells relative to total nuclei.

3.4 Flow Cytometry Analysis of Cell Cycle

In order to assess the impact of differentiation and ATG7 knockdown with DAPT treatment on cell cycle properties, cells were collected at day 0 (D0; just prior to induction of differentiation). Cells were harvested via trypsinization and centrifuged at 100g for 5 mins. After removing the supernatant, the pellet was resuspended in 1mL of PBS and centrifuged a second time at 100 *g* for 5 mins. The supernatant was removed again, and the cells were resuspended in 100 µL 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 hr. To prepare the samples for flow cytometry, cells were centrifuged at 1000 *g* for 5 mins. After removing the supernatant, samples were washed twice with PBS. 100 µL of RNAse was added followed by 400 µL of propidium iodide (PI) solution (50 µg/mL in 0.1% Triton-X PBS). Samples were then incubated at room temperature in the dark for 30 mins at which point PI fluorescence was measured using flow cytometry (FACSCalibur, BD BioSciences). Resultant data was analyzed using Cell Quest Pro Software (BD Biosciences).

3.5 Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Briefly, cells were collected and homogenized in TRIzol Reagent (Sigma-Aldrich). RNA was isolated using chloroform phase separation and ethanol precipitation. RNA concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Maltham, MA, USA). Reverse transcription using the qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MA, USA) was used to synthesize cDNA. PCR primer sequences were described

previously and were constructed (Sigma-Aldrich) for the following: *Notch1, Hes1, Hey1* (38). PCR cDNA amplification was performed with the PerfeCTa SYBR Green SuperMix Low ROX Kit (Quanta Biosciences) and analyzed with the 7500 RT-PCR System (Applied Biosystems, Invitrogen Life Technologies, Carlsbad, CA, USA). Expression was normalized to levels of GAPDH mRNA.

3.6 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 within each group independently. To examine differences between groups at a certain time-point (within the same day) a student's *t*- test was employed in figure 8 and 11. A one-way ANOVA was used to assess time-matched group differences in multiple groups (≥ 3) along with post-hoc analysis as appropriate in figure 7, 9, 10 and 12. $p<0.05$ was considered statistically significant and $p\leq 0.10$ considered a statistical trend.

Chapter 4 Results

Differentiation of C2C12 myoblasts results in a downregulation of NOTCH levels, which simultaneously occurs along with increased autophagic flux.

Characterization of NOTCH1 receptor and NOTCH1^{ICD} protein content showed transiently increased levels during early differentiation that progressively decreased during late differentiation. More specifically, NOTCH1 levels steadily increased at D0, showing 24% increase and the highest peak at D1 (1.3-fold), followed with decreased levels during late differentiation (Figure 6a-b) ($^{*}p<0.05$). Similarly, NOTCH1^{ICD} levels were less abundant at D0, which dramatically increased by 57% at D1 (2.4-fold) and gradually declined during late differentiation (Figure 6c) ($^{*}p<0.05$). As previously demonstrated in the Quadrilatero lab, myogenic marker, MYOG rapidly increased at D0-D1, which increased by 86% (7.2-fold) from D0 to D2 timepoint ($^{*}p<0.05$) (Figure 6g). Similarly, terminal differentiation marker, MYH, indicated low levels at D0 timepoint and continually increased in parallel with MYOG (Figure 6g-h). However, MYOG gradually decreased after D2, while MYH continually increased with the highest levels at D4 of differentiation. (Figure 6h) ($^{*}p<0.05$). Autophagic signaling marker, ATG7 increased from D0 to D1 by 1.6-fold (40%), peaking at D2 ($^{*}p<0.05$), and markedly reduced protein content at D3 and D4 of differentiation (Figure 6d). LCB-I levels gradually increased from D0-D2 and showed decreased protein content at D3-D4 (Figure 6f). Whereas LC3B-II levels increased from D0 to D4 of differentiation ($^{*}p<0.05$) (Figure 6f). In opposition, SQSTM1 protein content showed highest levels at D0, and rapidly decreased during differentiation (Figure 6e) ($^{*}p<0.05$). Similarly, qRT-PCR was used to measure *Notch1* and downstream NOTCH target genes *Hes1* and *Hey1*. *Notch1* mRNA levels showed a similar progression towards decreased gene expression during C2C12 myoblast differentiation (Figure

6b and 6i). Although no significant differences exist, NOTCH signaling target genes *Hes1* and *Hey1* gene expression increased during early differentiation and decreased during late differentiation (Figure 6j-k).

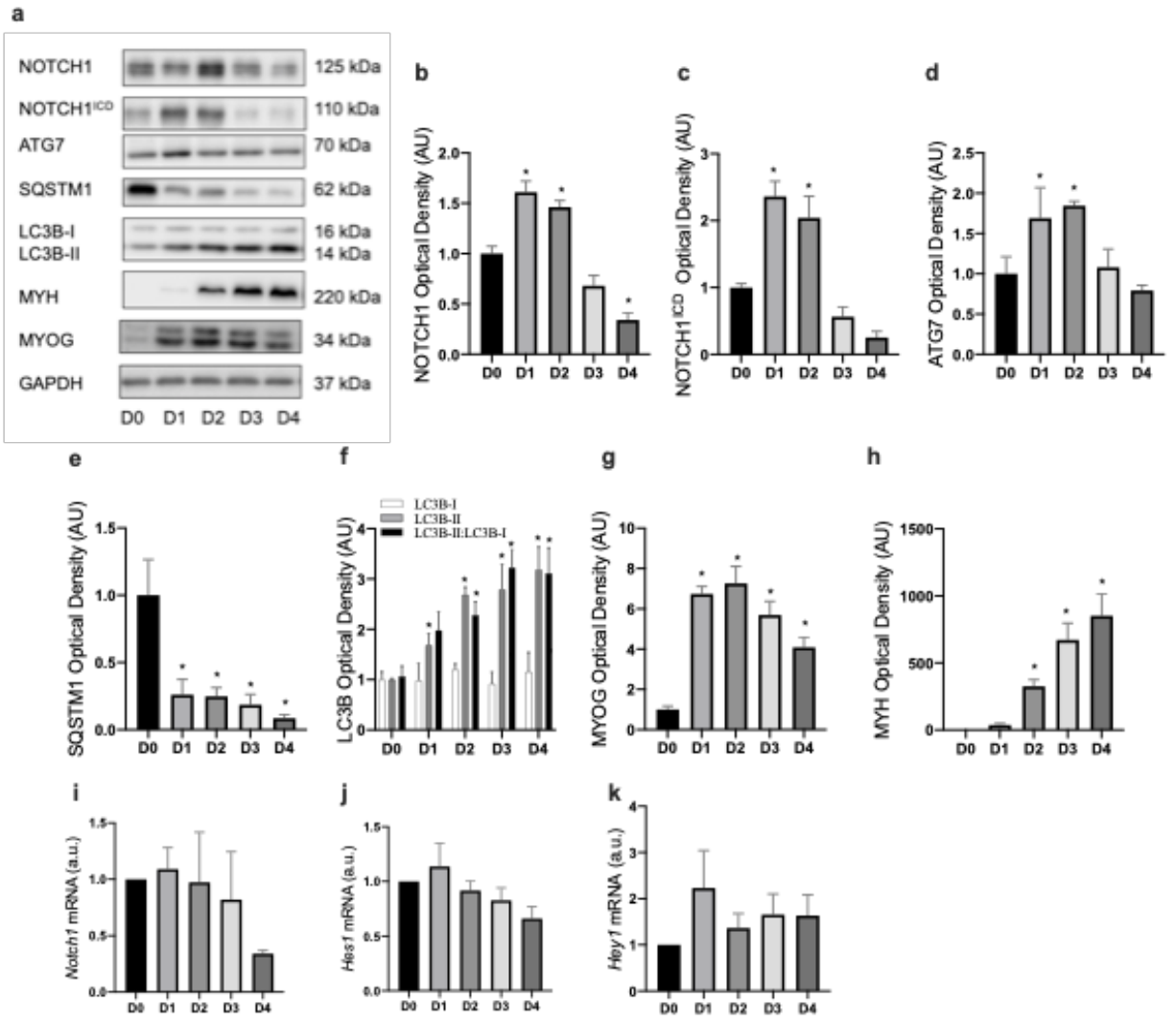


Figure 6. NOTCH signaling is downregulated and autophagy is upregulated during myoblast differentiation. Representative Immunoblots (a) and quantitative analysis of (b) NOTCH1, (c) NOTCH1^{ICD}, (d) ATG7, (e) SQSTM1, (f) LC3B, (g) MYOG, and (h) MYH during myoblast differentiation. Also shown here GAPDH loading control. *p<0.05 compared to D0 (within group). (n=3-5). Representative qRT-PCR analysis of (i) *Notch1*, (j) *Hey1* (k) *Hes1* gene expression during myoblast differentiation. Data are normalized to GAPDH house keeping gene. Data represent the mean value ± SEM (n=3). One-way ANOVA *p<0.05 compared to D0 (within group).

DAPT treatment decreased NOTCH1 and NOTCH1^{ICD} levels and was correlated with decreased myogenic differentiation

In experiment 1, we found transiently increased NOTCH1 and NOTCH^{ICD} levels at D0-D2 timepoints that steadily declined during differentiation. The pharmacological γ-secretase inhibitor, DAPT decreased NOTCH1 and NOTCH^{ICD} levels and we measured differences in NOTCH signaling, autophagy and myogenic related protein content. Comparisons were made between vehicle (Veh) cells and DAPT (10-15 μM) at each timepoint. In experimental conditions with DAPT (10 μM), NOTCH1 receptor levels were moderately reduced by 44% (1.8-fold) ($^{\#}p<0.05$) at D0, 43% (1.7-fold) ($^{\#}p<0.05$) at D1, and 47% D2 of differentiation (1.9-fold) ($^{\#}p<0.05$). Similarly, NOTCH1 receptor levels were decreased in the DAPT (15 μM) condition ($^{\dagger}p<0.05$) (Figure 7a-b). More importantly, DAPT treatment inhibited γ-secretase that led to the dramatic reduction of NOTCH1^{ICD} levels at both 10 μM and 15 μM concentrations (Figure 7b). In detail, DAPT treatment at 10 μM reduced NOTCH1^{ICD} levels (90.1-99.5%) from D0-D4, respectively. Whereas DAPT at 15 μM markedly decreased NOTCH1^{ICD} levels by 99.7% to 99.9% from D0-D4 (Figure 7c) ($^{\dagger}p<0.05$, $^{\dagger}p<0.05$, $^{\dagger}p<0.06$, $^{\dagger}p<0.05$), respectively. Our findings increased our confidence that NOTCH levels were significantly decreased with γ-secretase inhibitor, and we assessed myogenic markers to determine differences in the myogenic program. Interestingly, MYOG protein levels were significantly decreased in DAPT 10 μM at time point D0 ($^{\#}p<0.05$) and trended towards decreased levels at D1 ($^{\#}p<0.09$) (Figure 7e). However, DAPT treatment at 15 μM resulted in significantly decreased MYOG levels at D0 of differentiation (D0) ($^{\dagger}p<0.05$), and such trends continued throughout differentiation (D1-D4) (Figure 7e). Similarly, DAPT at 10 μM decreased MYH levels by 41% (1.71-fold) at D2 and decreased by 13% at D4 (1.15-fold) ($^{\#}p>0.10$), which was associated with reduced myogenic

differentiation levels. In addition, DAPT (15 μM) treated cells showed decreased MYH levels at D2 by 41% and 33% decreased levels at D4. However, autophagy markers SQSTM1, LC3B-I, LC3B-II and ATG7 levels were not significantly different between DAPT (10-15 μM) groups (Figure 7f-j) ($^{\#-\dagger}p>0.10$).

Autophagy induction or inhibition was associated with an inverse effect on NOTCH signaling

In order to investigate the crosstalk between autophagy and NOTCH signaling, we manipulated autophagy to identify differences in NOTCH1 protein content during early myogenesis. Autophagy induction with RAPA treatment at 2 μM was associated with decreased $NOTCH1^{ICD}$ levels at 12 hr of growth ($^{*}p>0.10$) and was correlated with significantly decreased at 24 hr of growth ($^{*}p<0.05$) (Figure 8a-b). $NOTCH1^{ICD}$ levels decreased by 2.8-fold (64%) after 24 hr of RAPA treatment ($^{*}p<0.05$) (Figure 8a-b). In contrast, inhibition of autophagy with CQ (10 μM) treatment was associated with significantly increased $NOTCH1^{ICD}$ levels in proliferating myoblasts ($^{*}p<0.05$) and associated with slightly increased NOTCH1 receptor levels ($^{*}p>0.10$) (Figure 8c-d). After 24 hr of CQ treatment, $NOTCH1^{ICD}$ levels increased by 49% (1.9-fold), and further elevated LC3B-I, and SQSTM1 levels ($^{*}p<0.05$) (Figure 8c-d). Similarly, sh*Atg7* cells showed increased $NOTCH1^{ICD}$ levels (34%, 0.6-fold) after 24 hr of growth (D0) compared to scr control cells ($^{*}p>0.10$) (Figure 9b). Collectively, the data showed that whether autophagy is increased or decreased, NOTCH signaling was associated with the inverse effect during the growth phase of proliferating myoblasts.

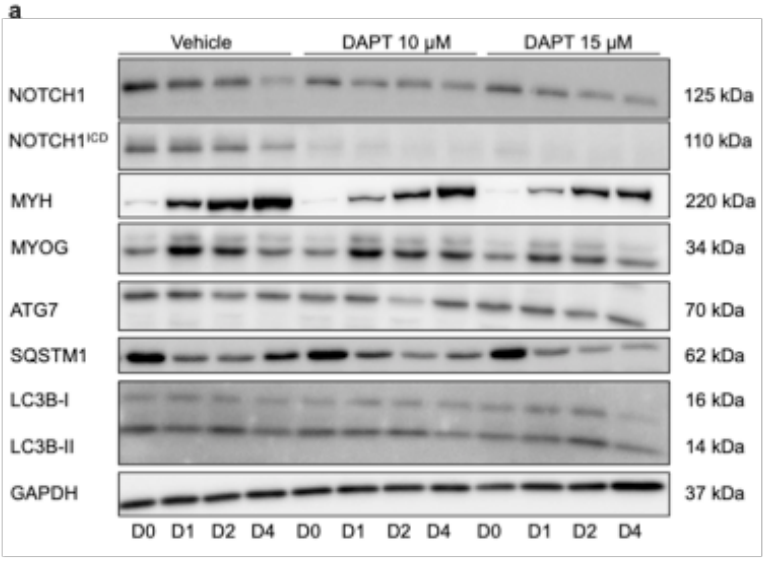
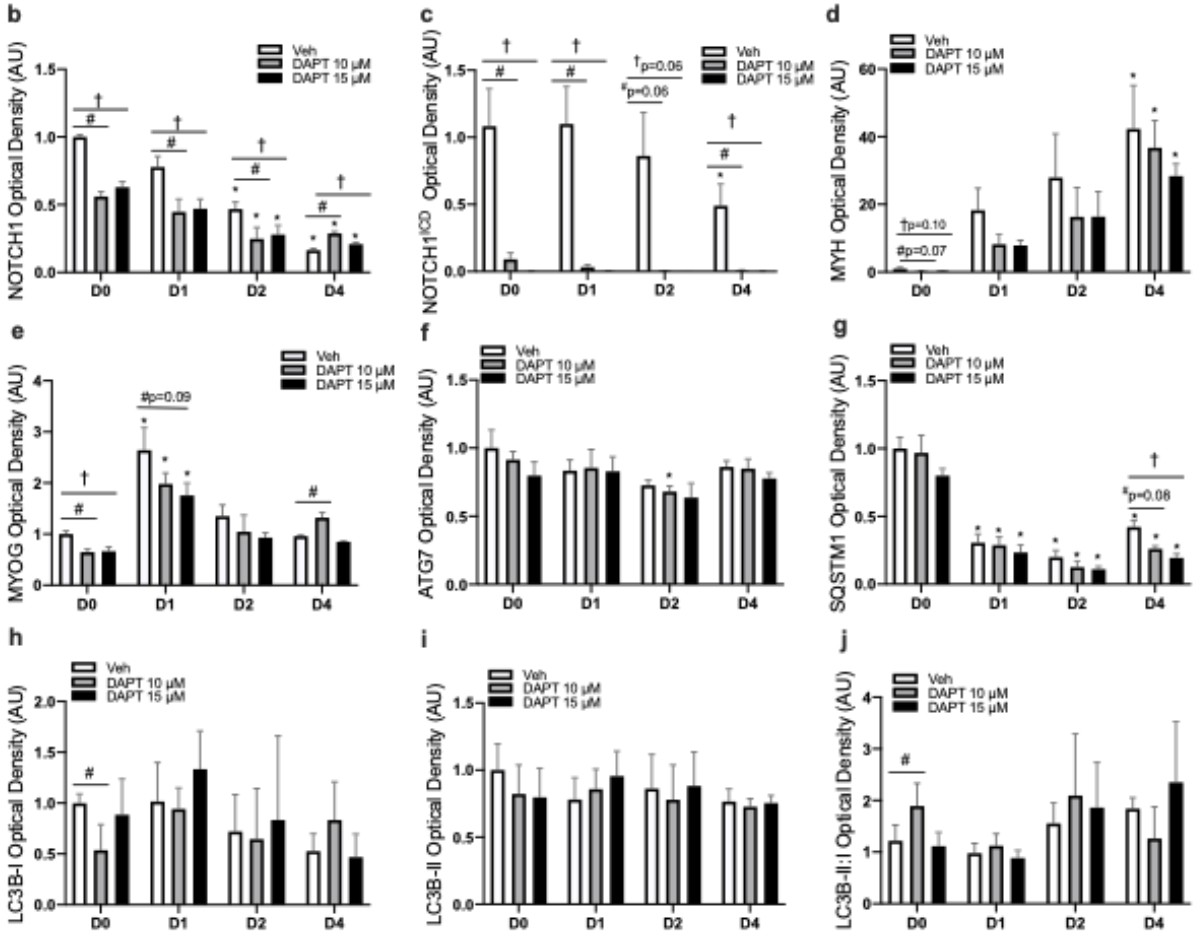


Figure 7. DAPT treatment led to decreased NOTCH1 and NOTCH1^{ICD} levels and was associated with trends towards decreased myogenic differentiation. Differentiating C2C12 myoblasts were treated with vehicle (Veh) or 10-15 μM DAPT. Representative Immunoblots (a) and quantitative analysis of (b) NOTCH1, (c) NOTCH1^{ICD}, (d) MYH, (e) MYOG, (f) ATG7, (g) SQSTM1, and (h) LC3B-I, (i) LC3B-II, (j) LCB-II: LC3B-I ratio. Also shown is a representative GAPDH loading control blot. *p< 0.05 compared to D0 (within group). #p< 0.05 between groups (Veh vs DAPT10) at the same time point. †p< 0.05 between groups (Veh vs DAPT15) at the same time point. (n=3)



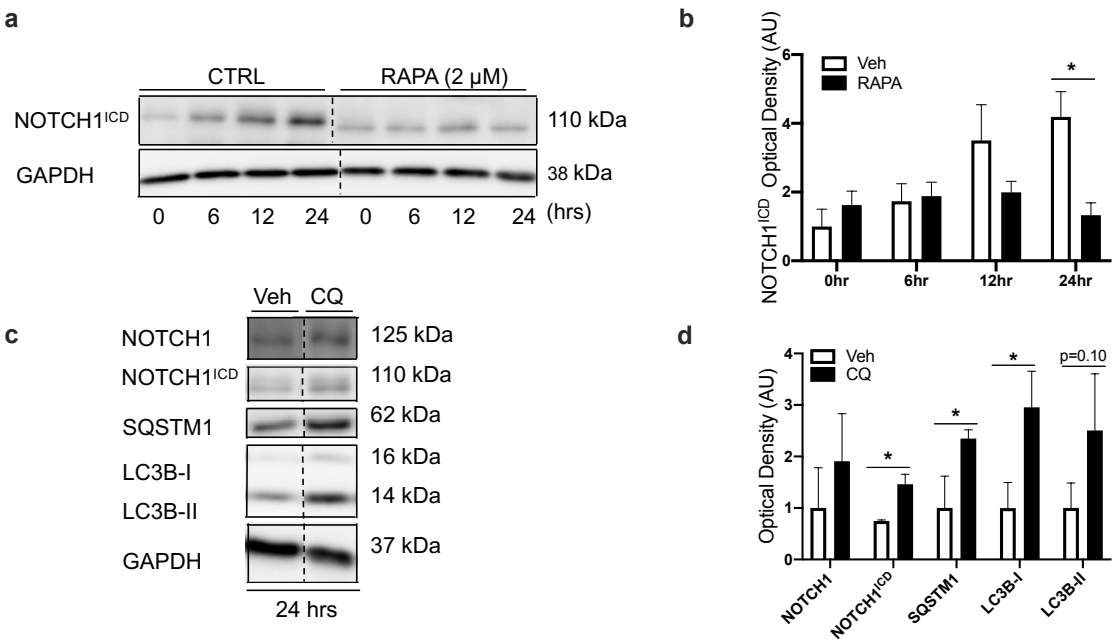


Figure 8. Autophagy inhibitor CQ was associated with increased NOTCH1^{ICD} levels, while the autophagy inducer rapamycin (RAPA) was associated with decreased NOTCH1^{ICD} levels in proliferating myoblasts. C2C12 myoblasts were treated with RAPA (2 μM) for 6, 12, 24hrs or CQ (10 μM) for 24hrs in proliferating myoblasts. Representative immunoblots of (a) RAPA (2 μM), (c) CQ (10 μM) and quantitative analysis of (b) RAPA and (c) CQ. Also shown is a representative GAPDH loading control blot. RAPA (n=4) CQ (n=3) *p<0.05 compared to CTRL vs treated (CQ or RAPA).

Inhibition of γ-secretase led to decreased *NOTCH1^{ICD} levels and was associated with rescued myogenic differentiation in autophagy deficient shAtg7 C2C12 cells*

In the previous experiment, we found that CQ treated cells (impaired autophagy) was associated with increased levels of NOTCH1^{ICD}. CQ treatment in C2C12 cells was preliminary evidence that impaired autophagy may result in the dysregulation of NOTCH1 and NOTCH1^{ICD} levels. Therefore, in experiment 3, we measured NOTCH signaling, autophagy and myogenic related protein content in sh*Atg7* cells during myogenic differentiation. As previously described by members of our lab, *Atg7* gene deficiency significantly impairs differentiation and myogenesis (44). ATG7 protein levels were significantly knocked down from D0-D4 in sh*Atg7* cells ($^{\#}p<0.05$) (Figure 9f). In addition, MYOG levels were significantly decreased compared to scr control cells at D1, D2, and D4 ($^{\#}p<0.05$) (Figure 9e). Furthermore, sh*Atg7* cells showed

dramatically decreased MYH levels by 92% (14-fold) at D2 and 98% (40-fold) at D4 of differentiation compared to scr control cells ($^{\#}p<0.05$) (Figure 9d), which indicated impaired differentiation. Immunofluorescent images showed that sh*Atg7* cells have impaired ability to undergo fusion indicated by decreased fusion index ($^{\#}p<0.05$) and reduced potential for myotube formation indicated by decreased differentiation index ($^{\#}p<0.05$) at D4 of differentiation (Figure 10a-c).

However, sh*Atg7* cells treated with γ-secretase inhibitor, DAPT decreased $NOTCH1^{ICD}$ levels and was associated with a trend towards improved myogenic differentiation. Sh*Atg7*+DAPT treated cells showed increased MYOG levels at D0 ($^{\dagger}p<0.07$). Although no significant differences exist, MYOG levels appeared to increase from D1-D4 of differentiation (Figure 9e). Importantly, MYH protein content increased in the sh*Atg7*+DAPT condition compared to sh*Atg7*+Veh cells by 94% and 97% at D2 and D4 timepoints, respectively, which may indicate a potential rescue of myogenic differentiation ($^{\dagger}p\geq0.10$). In addition, immunofluorescence methods showed that sh*Atg7* cells treated with DAPT was correlated with increased myogenic potential at D2 and D4 as evidenced by the differentiation index ($^{\dagger}p\geq0.10$) (Figure 10a-b). The fusion index also indicated a slightly increased number of nuclei (multinucleation) per myotube (Figure 10c) ($^{\dagger}p\geq0.10$). LC3B-I and LC3B-II levels trended towards decreased expression in sh*Atg7* cells compared to scr control during differentiation (Figure 9i). However, no significant differences were present in autophagy related makers LC3B-I, LC3B-II, and SQSTM1 in sh*Atg7*+DAPT treated cells (Figure 9g).

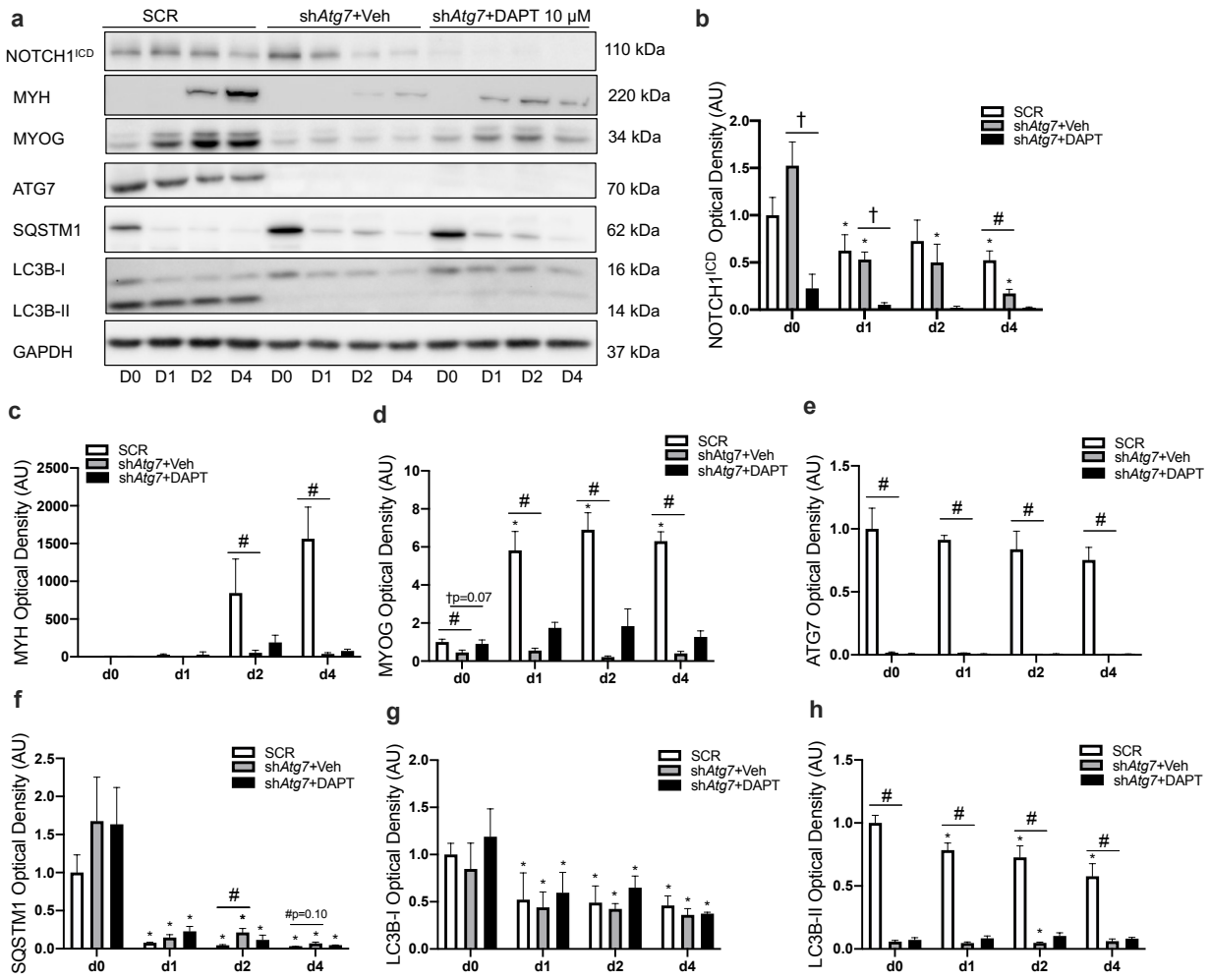


Figure 9. DAPT treatment in autophagy deficient sh*Atg7* cells was associated with trends towards rescued myogenic differentiation. Sh*Atg7* C2C12 cells were treated with DAPT at 10 μM every 24hrs. Representative Immunoblots (a) and quantitative analysis (b) of NOTCH1^{ICD}, (c) MYH, (d) MYOG, (e) ATG7, (f) SQSTM1, (g) LC3B-I and (h) LC3B-II during myoblast differentiation. *p< 0.05 compared to D0 (within group). $^{\#}$p< 0.05 between groups (SCR vs sh*Atg7*+Veh) at the same time point. †p< 0.05 between groups (sh*Atg7*+Veh vs sh*Atg7*+DAPT10) at the same time point. (n=3-4)

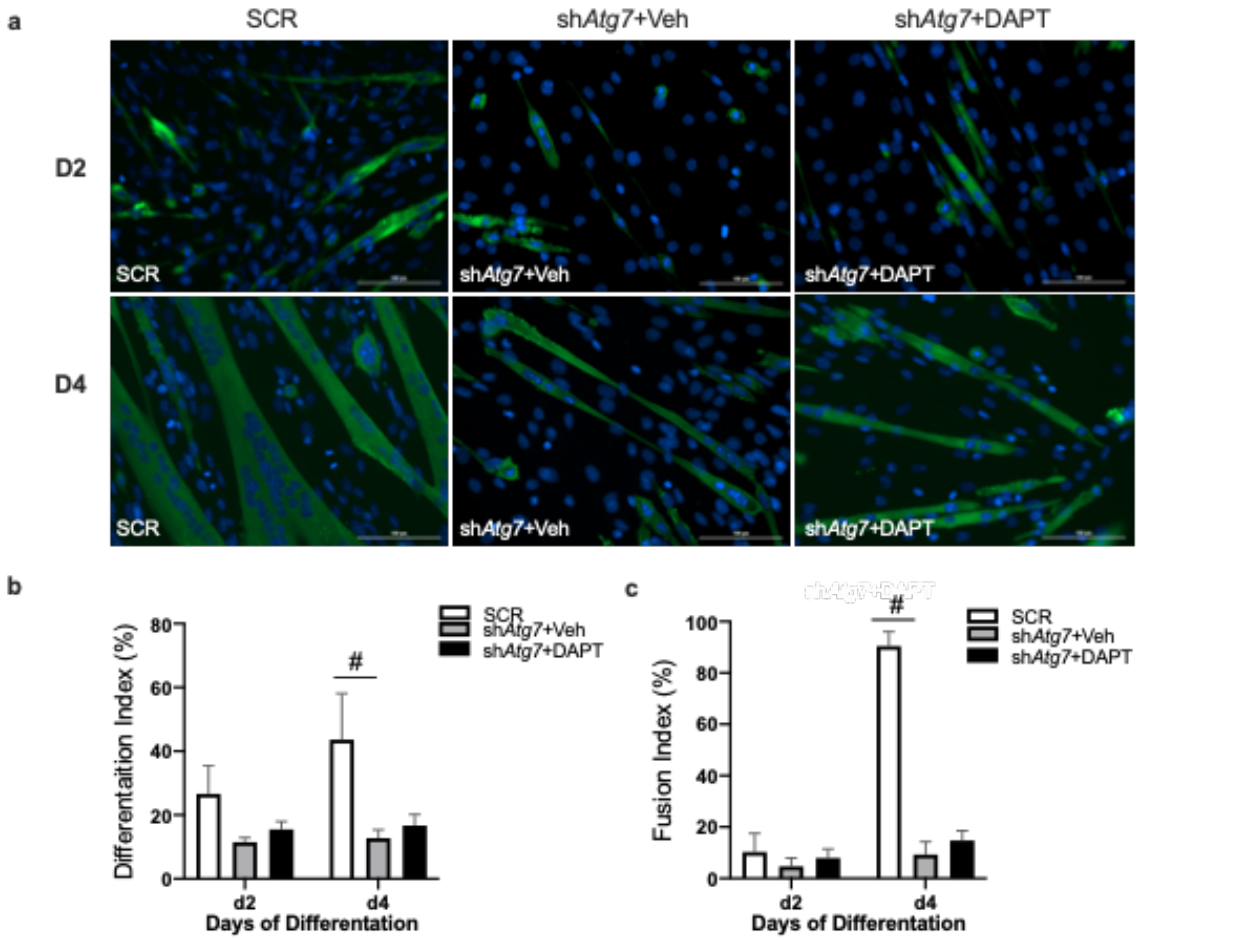


Figure 10. Sh*Atg7* cells treated with DAPT was associated with a trend towards slightly improved myotube fusion and formation. Representative immunofluorescence microscopy images (a) of myotube formation in sh*Atg7*+DAPT treated cells at D2 and D4 of differentiation. Cells were stained with DAPI (blue) and for MF20 (green) to observe nuclei and MYH. Scale bar 100 μM. Quanitative analysis of the differentiation index (b) and fusion index (c) $^{\#}p<0.05$ compared to SCR vs sh*Atg7*+Veh (between group). $^{\dagger}p<0.05$ compared to sh*Atg7*+Veh vs sh*Atg7*+DAPT (between group).

DAPT treatment in autophagy deficient shAtg7 cells was associated with improved myogenic differentiation

A one-way ANOVA test was used to compare differences between multiple groups; however, we were interested in the effect of the drug treatment of DAPT during myogenic differentiation. Therefore, a student's t-test was employed to compare differences between sh*Atg7*+Veh and sh*Atg7*+DAPT cells. Interestingly, sh*Atg7*+DAPT treated cells indicated a

significant increase in MYOG levels at D1 ($^{\dagger}p<0.05$) and trended towards increased levels at D4 of differentiation ($^{\dagger}p<0.06$), suggesting the potential recovery of myogenic differentiation (Figure 11d). Similarly, increased MYH levels at D2 and D4 of differentiation in sh*Atg7*+DAPT treated cells suggested reduced $NOTCH1^{ICD}$ levels was associated with rescued myogenic differentiation. Student's t-test analyses of representative immunofluorescent images showed increased differentiation index at D2 and D4 of differentiation ($^{\dagger}p<0.05$), (Figure 11i-j). Surprisingly at D2, LC3B-I levels were significantly increased in DAPT treated sh*Atg7* cells in the absence of the *Atg7* gene. Collectively, the pharmacological γ-secretase inhibitor, DAPT, decreased $NOTCH1^{ICD}$ levels in sh*Atg7* cells and was associated with improved myogenic differentiation.

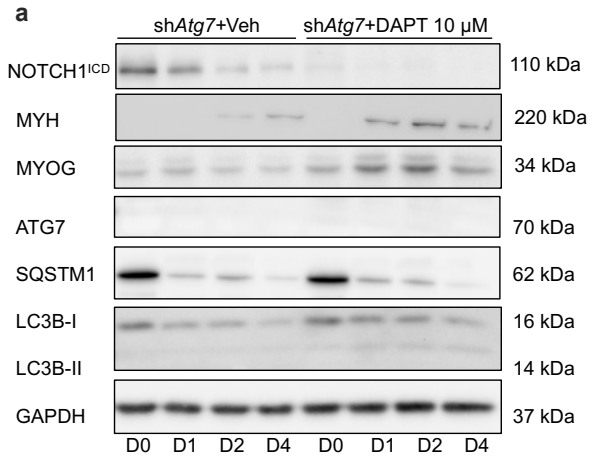
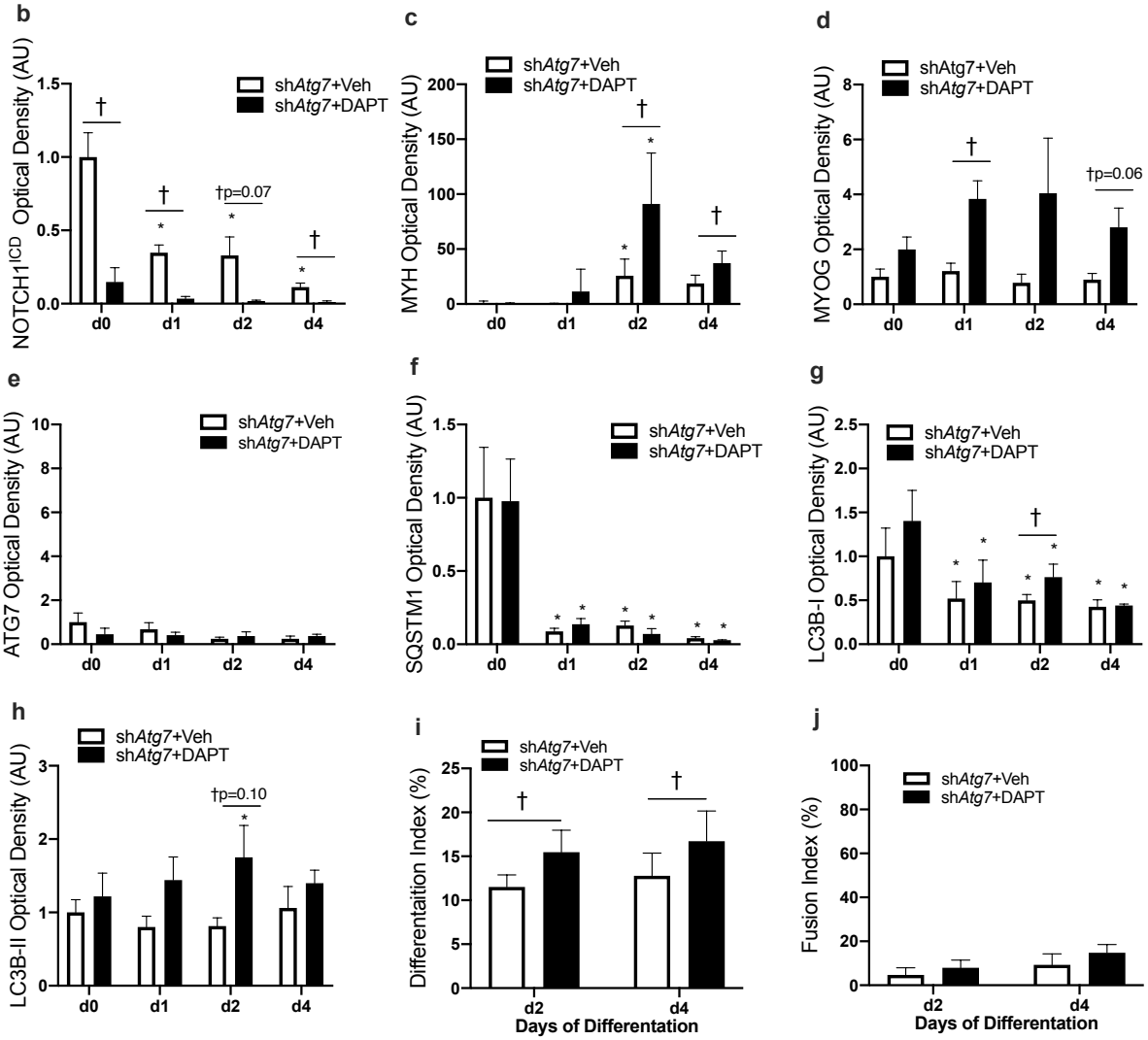


Figure 11. Pharmacological γ-secretase inhibitor, DAPT treatment was associated with rescued myogenic differentiation in autophagy deficient sh*Atg7* cells. Sh*Atg7* C2C12 cells were treated with DAPT at 10 μM every 24hrs. Representative Immunoblots (a) and quantitative analysis (b) of NOTCH1^{ICD}, (c) MYH, (d) MYOG, (e) ATG7, (f) SQSTM1, (g) LC3B-I and (h) LC3B-II during myoblast differentiation. *p< 0.05 compared to D0 (within group). $^{\#}$p< 0.05 between groups (SCR vs sh*Atg7*+Veh) at the same time point. †p<0.05 between groups (sh*Atg7*+Veh vs sh*Atg7*+DAPT 10 μM) at the same time point. Also, shown is a GAPDH loading control blot. Quanitative analysis of (i) the differentiation index and (j) fusion index †p< 0.05 compared to sh*Atg7*+Veh vs sh*Atg7*+DAPT 10 μM (between group).



DAPT treatment in sh*Atg7 cells showed no significant differences in cell cycle regulation*

As previously mentioned, NOTCH signaling has been found to play an important role in cell-cycle regulation. Therefore, flow cytometry was used to measure PI fluorescence to determine cell-cycle characteristics in scr, and sh*Atg7* cells in the presence or absence of DAPT treatment. Interestingly, we found sh*Atg7*+Veh had dramatically increased cells in the G_0/G1 phase compared to scr control groups that may indicate increased cell cycle growth arrest ($^{\#}p<0.05$) (Figure 12a-b). In comparison to scr control, sh*Atg7*+Veh cells indicated potentially decreased DNA synthesis and replication in the S phase ($^{\#}p<0.05$) (Figure 11a and 11c), along with decreased mitosis in G2/M phase ($^{\#}p<0.05$) (Figure 12a and 12d). Interestingly, after 24 hrs (D0) of DAPT (10 μM) treatment, no significant differences were found between sh*Atg7*+Veh and sh*Atg7*+DAPT treated cells in G_0/G1, S, and G2/M phases of cell cycle regulation (Figure 5a-d). Although further research is required, the data suggested that DAPT treatment in sh*Atg7* cells may not be associated with cell-cycle regulation at the D0 timepoint (Figure 12a-d).

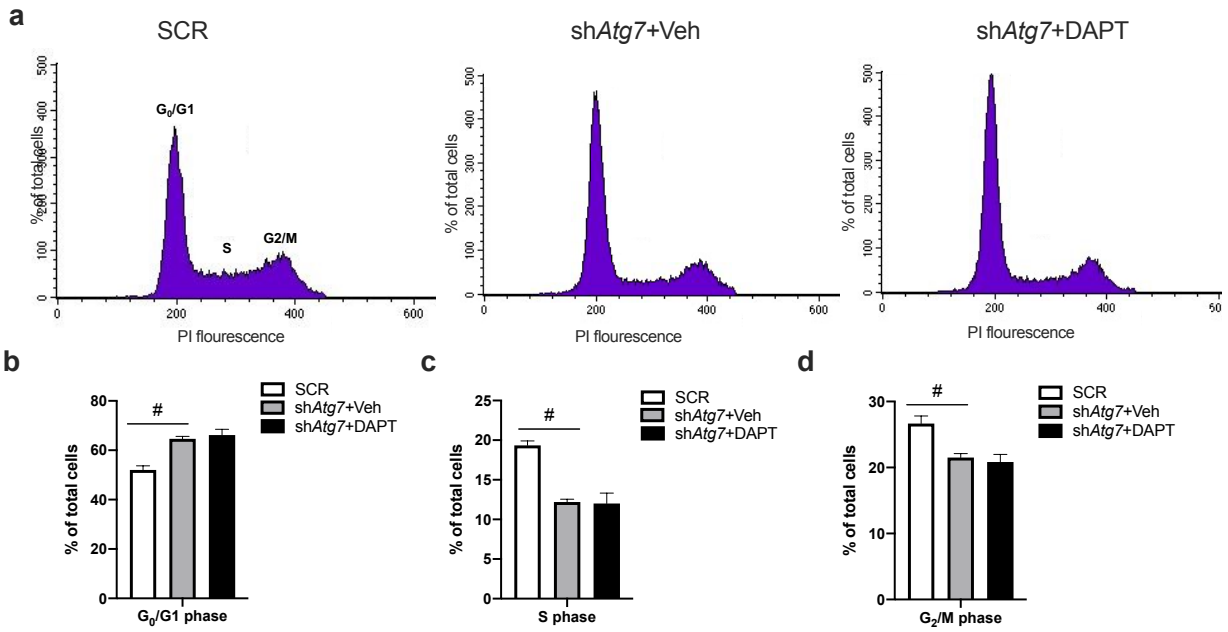


Figure 12. Sh*Atg7* vehicle cells exhibit increased growth arrest (G_0/G1 phase) compared to SCR cells and inhibition of γ-secretase with DAPT at 10 μM shows no significant differences in cell cycle regulation. Sh*Atg7* cells were treated with DAPT at 10 μM for 24hrs. Representative flow cytometry histograms (a) and quantitative analysis of (b) G_0/G1 phase, (c) M phase, and (d) G_2/M phase, at 24 hr of growth. $^{\#}p<0.05$ between groups (SCR vs sh*Atg7*) at the same time point. $^{\dagger}p< 0.05$ between groups (sh*Atg7*+Veh vs sh*Atg7*+DAPT10) at the same time point. (n=3)

Chapter 5 Discussion

The primary focus of this study was to investigate the crosstalk between NOTCH signaling and autophagy that is involved in cellular remodeling and reprogramming of myogenic differentiation. The role of autophagy in skeletal muscle differentiation has been extensively studied (44, 48, 49), however, we have overlooked the interaction between NOTCH signaling and autophagy during myogenesis, as the NOTCH signaling pathway is implicated in a broad range of developmental processes, including determining cell fate, proliferation and differentiation (3, 9, 23). Therefore, the present work aimed to study the fine-tuning signaling mechanism of autophagy in regulating NOTCH signaling to sustain myogenic differentiation (50). In order to test the hypothesis whether NOTCH signaling is required for myogenic differentiation, we first characterized NOTCH signaling related mRNA and protein levels (NOTCH1, NOTCH1^{ICD}, *Notch1*, *Hes1*, *Hey1*) that are downregulated during terminal myogenic differentiation. In follow-up experiments, we inhibited γ-secretase with DAPT to decrease the production of NOTCH1 and NOTCH1^{ICD} levels in C2C12 cells to assess the importance and function of NOTCH1 related proteins during myogenesis. Characterization of NOTCH signaling indicated that NOTCH1 and NOTCH1^{ICD} levels are downregulated, *Notch1*, *Hes1*, *and Hey1* gene expression is downregulated, while autophagy levels are upregulated during C2C12 myoblast differentiation (Figure 6). Subsequent findings indicated that basal levels of NOTCH signaling are likely required for the development of C2C12 myoblasts, as near-complete inhibition of γ-secretase led to decreased NOTCH1^{ICD} levels and was associated with impaired myogenic differentiation (Figure 7). Furthermore, the induction and inhibition of autophagy with RAPA and CQ, respectively, suggested that autophagy may play a role in regulating NOTCH signaling. Collectively, the findings increased our confidence that autophagy may have an

inverse effect on NOTCH signaling during early myogenesis. We found that increased autophagy with RAPA treatment was associated with the negative regulation of NOTCH1^{ICD} levels, while the inhibition of autophagy with CQ treatment was associated with increased NOTCH1^{ICD} levels. Lastly, we treated autophagic deficient sh*Atg7* cells with DAPT and found an association with improved myogenic differentiation. The results of experiment 1 likely demonstrates an additional, yet critical role of autophagy as a fine-tuner of NOTCH signaling to promote myogenic differentiation (50).

NOTCH1 and NOTCH1^{ICD} levels are downregulated during myogenic differentiation

NOTCH signaling has been extensively studied as a regulator of several fundamental cellular processes which include cell fate, cell-cycle regulation, proliferation and differentiation (3, 9, 23, 24). In order to determine the role of NOTCH signaling during myogenesis, NOTCH related mRNA and protein levels were characterized during C2C12 myoblast differentiation. The present study confirms prior findings by Bloemberg and Quadrilatero that autophagic flux occurs during early differentiation (49), however, we have included the novelty of transiently increased NOTCH1 and NOTCH1^{ICD} protein levels and *Notch1, Hes1, Hey1* mRNA levels during the induction of myogenesis, followed with a progressive decline during terminal differentiation that occurs alongside autophagic flux. Considering that NOTCH signaling regulates asymmetrical cell division, cell fate, and proliferation in embryonic and adult tissue (50), our results suggest NOTCH1 may be required to initiate myoblast proliferation during myogenesis. Skeletal muscle studies have found that the activation of NOTCH signaling prevents myoblast differentiation through the upregulation of HES1. HES1 upregulation represses MRF, MYOD1 and promotes the self-renewal of SCs (9, 31). The present work showed decreased gene expression of *Hes1*

and *Hey1* during terminal differentiation (D3-D4), which may suggest increased myogenic differentiation when *Hes1* and *Hey1* are downregulated. Another study further evidenced that HEY1, HEYL, and HES1 act synergistically to suppress myogenic differentiation (33, 36). Conditional mutagenesis methods were used to show that HEY1 and HEYL are both required to maintain skeletal muscle SCs pool and loss of HEY1 and HEYL function results in the upregulation of differentiation markers MYOD1 and MYOG (37).

NOTCH signaling is predominant during embryonic and fetal myogenesis to promote asymmetrical cell division of MPCs and maintain the self-renewal of SCs (3). Furthermore, the activation of NOTCH signaling promotes the specialization and proliferation of MPCs through the upregulation of PAX3/PAX7 (3). However, during postnatal myogenesis and muscle regeneration NOTCH signaling must be downregulated to allow for myoblasts to undergo terminal differentiation into myotubes and myofibers (3). Therefore, the present data increases our confidence that NOTCH signaling is likely important for the growth and proliferation of myoblasts, however, NOTCH signaling must return to negligible levels to allow for terminal differentiation (3, 5, 9).

Therefore, another intracellular mechanism is likely required to attenuate NOTCH signaling during postnatal myogenesis in order to progress terminal differentiation (50). Early onset of autophagy was speculated to be involved in downregulating NOTCH1 and NOTCH1^{ICD} levels in order to promote myogenic differentiation (36). Specifically, autophagy was postulated to play a role in the downregulation of NOTCH signaling and suppress the anti-myogenic effects of HES1 and HEY1, thus allowing for the upregulation of MYOG and MYH that are required for terminal differentiation (4). C2C12 cells also showed pronounced autophagic flux indicated by increased LC3B-II levels and decreased SQSTM1 levels during C2C12 differentiation,

Furthermore, myogenic markers MYOG gradually increased and declined, while MYH continually increased expression during terminal differentiation (D4). A large body of evidence suggests that autophagy regulates cellular quality control and energy balance (7). Numerous studies have found that the loss of autophagy is detrimental to development and differentiation of various tissues (36, 43, 51). For example, the loss of autophagy in adipose tissue has been found to impair $MYF5^{+}$ progenitor cells from undergoing cellular remodeling and differentiation, which resulted in defective brown adipose tissue differentiation at the expense of impaired myogenic differentiation in ATG7 KO mice (55).

Therefore, the downregulation of NOTCH signaling simultaneously occurring alongside increased autophagic flux during myogenesis was the first evidence suggesting that autophagy may be downregulating NOTCH signaling in order to promote myogenic differentiation. Experiment 1 findings suggest that the downregulation of NOTCH signaling, and upregulation of autophagy is likely required to promote proper myogenic differentiation. Collectively, autophagic signaling may play an important role in the selective removal of NOTCH proteins to maintain quality control (43), thereby, progressing the terminal differentiation of myoblasts during C2C12 myogenesis.

Increased Inhibition of γ-secretase decreased *NOTCH signaling and was associated with impaired myogenic differentiation*

We further investigated the role of NOTCH signaling through pharmacological inhibition of γ-secretase with DAPT that decreased the production of NOTCH1 and $NOTCH1^{ICD}$ during C2C12 myogenesis. Decreased NOTCH1 and $NOTCH1^{ICD}$ levels was postulated to decrease both the proliferative and differentiative potential of C2C12 myoblasts, and therefore impair

myogenesis. In support of our hypothesis, the results indicate that markedly reduced NOTCH1^{ICD} levels is likely associated with impaired myogenic differentiation, which was indicated by decreased MYOG levels ($^{\#\text{-}\dagger}p<0.05$), decreased MYH levels and significantly decreased NOTCH1^{ICD} in DAPT treated cells. In more detail, the findings increased our confidence that DAPT treatment significantly decreased NOTCH1^{ICD} levels and was correlated with reduced MYOG levels along with a trend towards decreased MYH levels in comparison to vehicle cells.

Literature in NOTCH signaling has suggested the importance of the activation of NOTCH signaling to promote SCs proliferation to undergo terminal myogenic differentiation (29). Therefore, the present work suggests that dramatic reduction of NOTCH1^{ICD} levels with DAPT treatment may have impaired the proliferation potential of myoblasts, which may be associated with downstream impairments in myogenic differentiation.

Important to note, NOTCH inhibitor DAPT, is a γ-secretase inhibitor (GS1-IX, compound 3) (56) that blocks proteolytic cleavage of NOTCH^{ICD} from the extracellular domain of NOTCH receptor (3). Specifically, the inhibitor utilizes a small molecule non-transition-state inhibitor that has a binding site different from the active site, which is speculated to be at the interface of the γ-secretase complex dimer (56). The binding of DAPT (GSI-IX) to the γ-secretase complex has been found to alter the protein conformation and block γ-secretase cleavage of NOTCH^{ICD}. Inhibition of γ-secretase prevents NOTCH1^{ICD} translocation to the nucleus (3, 56), formation of CSL transcriptional activation complex, transcription of NOTCH target genes, and ultimately suppresses the proliferation of myoblasts (3). Our findings indicate that DAPT treatment decreased NOTCH1 and NOTCH1^{ICD} levels and may be associated with impairments in differentiation, suggesting that early myoblasts may be impaired to undergo

proper remodeling to form mature myotubes. Collectively, the present work suggests that a certain level of γ-secretase is likely required to maintain NOTCH1 and NOTCH1^{ICD} levels in order to promote functional and proliferative myoblasts into the myogenic differentiation program (5).

Although the present work suggests that inhibition of γ-secretase largely decreased the production of NOTCH1^{ICD} levels and was associated with impaired myogenesis, inhibiting the γ-secretase enzyme also effects several other target proteins (73). The γ-secretase enzyme is primarily involved in proteolytic cleavage of numerous type 1 membrane proteins such as ß-amyloid precursor protein (APP), NOTCH, and E-CADHERIN (73). E-CADHERIN controls various cellular behaviours including cell-cell adhesion, differentiation and tissue development (74). Specifically, γ-secretase cleavage dissociates E-CADHERIN and releases ß-catenin and α-catenin to the cytosol (73). Free cytosolic ß-catenin is a key potent regulator of Wnt signaling pathway (73) that functions similarly to NOTCH signaling in regulating cell proliferation, fate specification and differentiation (75). Furthermore, Wnt signaling has been shown to interact with NOTCH signaling to activate NOTCH ligands during the development embryonic and adult tissues (75). Another study indicates that NOTCH signaling can modulate the active form of ß-catenin to reduce activity in transcriptional assays (73). Mutations in ß-catenin can result in constitutively activated ß-catenin signaling that prevent its degradation, leads to excessive stem cell renewal and proliferation that predisposes cells to tumorigenesis (73).

Therefore, it is likely that the γ-secretase inhibitor DAPT may exert secondary effects such as decreased ß-catenin that is attributed to decreased NOTCH1^{ICD} levels, which may function in conjunction to impair myogenic differentiation (73-75). Collectively, the present data suggests that inhibition of γ-secretase with DAPT likely alters the proliferative function of

myoblasts and disrupts the differentiation program, which is in agreement with our finding that DAPT treated C2C12 cells may be associated with impaired myogenic differentiation.

Contrary to our findings in Figure 7, research has suggested that NOTCH signaling is a well-known suppressor of myogenesis (34). For example, a study found that increased NOTCH ligand, DLL4, induced NOTCH signaling and blocked MYOG levels in skeletal muscle (6). Specifically, this study differentiated C2C12 cells with fusion proteins that consists of the extracellular domain of DLL4 (fc-DLL4) (6). Interestingly, C2C12 cells with DLL4-fc induced NOTCH signaling were unable to form multinucleated myotubes and failed to show *Myog* expression (6). Therefore, we would have expected that decreasing NOTCH levels with DAPT treatment may also suppress NOTCH associated DLL4 ligand and upregulate MYOG levels to increase C2C12 myogenic differentiation (Appendix Figure 1) (6).

Furthermore, considering that the upregulation of NOTCH1, NOTCH1^{ICD}, HES1, and HEY1 synergistically function to suppress differentiation, moderately decreasing NOTCH1 levels may repress downstream NOTCH target gene, *Hes1*. Reduced HES1 levels may partially activate the CSL complex, reducing the positive-feedback of the transcription of NOTCH target genes, *Hes1* and *Hey1* (4). In addition, suppression of HES1 would in turn promote the upregulation of myogenic regulatory factors, MYOD1 and MYOG to undergo terminal differentiation (4, 6). Therefore, we would have expected that the utilization of γ-secretase inhibitor DAPT to moderately reduce levels of NOTCH1, NOTCH1^{ICD} rather than a dramatic reduction may slightly decrease associated NOTCH proliferative proteins (HES1, NOTCH1, NOTCH1^{ICD}) (51) and result in increased C2C12 myogenic differentiation.

However, the present work primarily focused on the importance of the dramatic reduction of NOTCH1^{ICD} levels to study the importance of NOTCH signaling during

myogenesis. Therefore, we found that DAPT treatment dramatically reduced NOTCH1 and NOTCH1^{ICD} levels and was correlated with impaired myogenic differentiation evidenced by a trend towards decreased MYOG and MYH levels. The present data suggests that drastically decreasing NOTCH1^{ICD} levels may disrupt cell-division, proliferation and myoblast remodeling (3, 6, 50). Interestingly, DAPT treatment severely decreased NOTCH1^{ICD} levels and was associated with slightly increased LC3B levels during C2C12 differentiation in DAPT (15 μM) condition ($p>0.10$). Although there are no clear differences, we found a trend towards increased LC3B-I (19%) and LC3B-II (11.9%) at D1 of differentiation. Research in adipogenesis differentiation of human bone marrow mesenchymal stem cells (BM-MSCs) found that DAPT treatment increased autophagy related proteins LC3B and BECN1 levels (50). Therefore, increased levels of LC3B may suggest an increased clearance of excessive NOTCH and other damaged proteins that was associated with DAPT treatment to promote cellular quality control and sustain myogenic differentiation (7). Collectively, the present data suggests that NOTCH signaling is likely required for the development of myoblasts to properly progress into the differentiation program.

Induction of autophagy with RAPA treatment was associated with decreased NOTCH1^{ICD}

To further support our hypothesis that autophagy is a likely regulator of NOTCH signaling, we first experimented with autophagy activator RAPA to assess the effect on NOTCH1^{ICD} levels. The present data showed that treatment with RAPA increased autophagy and was associated with decreased NOTCH1^{ICD} levels in proliferating myoblasts. Interestingly, mammalian target of rapamycin (mTOR) pathway is a well-studied master regulator of cell growth and metabolism (57). RAPA was first discovered as an antifungal metabolite produced

by *Streptomyces hygroscopes* (57). Subsequent work demonstrated that RAPA also inhibits S6K1 (40S ribosomal S6 kinase 1) that is involved in proliferation and more importantly functions as an inducer of autophagy through the inhibition of mTOR pathway (57). In nutrient rich conditions (basal autophagy), mTOR dependent phosphorylation of ATG13 suppresses the ULK1 complex, preventing the activation of AMPK (AMP-activated protein kinase) (40). However, in nutrient poor conditions (induced autophagy), AMPK and DNA response-pathway inhibit mTOR and activate the ULK1 complex, initiating autophagosomal nucleation and elongation (40). Several studies have found that RAPA treatment effectively inhibited mTORC1 and increased levels of autophagy (57). For example, a study conducted in human neuroblastoma cells found that RAPA treatment elevated expression of autophagy related proteins BECN1 and LC3B-I/LC3B-II, while autophagy markers SQSTM1 and mTOR decreased (57).

A number of studies have shown that autophagy may function as a fine-tuning signaling pathway that plays a role in promoting differentiation (44, 48, 49). Considering that autophagy may be a negative regulator of NOTCH signaling, it was postulated that increased autophagy would further decrease $NOTCH1^{ICD}$ levels to promote terminal myogenic differentiation (6). Consistent with other research in myofibroblasts differentiation (58), autophagy induction using RAPA treatment was associated with decreased $NOTCH1^{ICD}$ levels in C2C12 proliferating myoblasts. Interestingly, RAPA inhibits S6K1 that is involved in proliferation (57), therefore, RAPA treatment related decreased proliferation may be correlated with decreased $NOTCH1^{ICD}$ levels. Another study has suggested that autophagy plays a role in the degradation of $NOTCH1^{ICD}$ in order to promote cardiac differentiation (43). In experiment 1, increased autophagic flux during C2C12 myoblast differentiation suggested possible targeted degradation of NOTCH1 proteins that was associated with the downregulation of $NOTCH1^{ICD}$ levels at D3

and D4 of differentiation. Similarly, immunofluorescence methods in cardiac cells have shown positive LC3 staining present in NOTCH1^{ICD} at D4 during differentiation, indicating the formation of the LC3-NOTCH1^{ICD} complex (43). Similarly, autophagy marker and adaptor protein SQSTM1 also formed a complex with NOTCH1^{ICD}, suggesting possible autophagy mediated recruitment of NOTCH1^{ICD} to the autophagosome for degradation (43). SQSTM1 is a selective adaptor protein that transports ubiquitinated proteins and large aggregates to the autophagosome for degradation (59). Jia et al. measured ubiquitination via western blot assay and found ubiquitinated smaller weight proteins at D0 and ubiquitinated larger molecular weight proteins like NOTCH1^{ICD} (110 kDa) were present at D4 of differentiation (43). Similarly, Co-IP assays showed increased ubiquitinated NOTCH1^{ICD} at D4 compared to D0 of differentiation. Therefore, it is likely that ubiquitinated SQSTM1-NOTCH1^{ICD} and LC3-NOTCH1^{ICD} complexes are transported to the autophagosome for targeted degradation (43). The preliminary finding that RAPA treatment was correlated with decreased NOTCH1^{ICD} suggests that autophagy may play a role in the selective clearance of NOTCH1^{ICD}. Therefore, autophagy signaling may negatively regulate NOTCH signaling to maintain basal NOTCH levels, thus, allowing for the progression of myogenic differentiation.

Inhibition of autophagy with CQ was associated with upregulated NOTCH signaling

In order to understand the crosstalk between autophagy and NOTCH signaling, we determined whether the inhibition of autophagy with CQ antagonistically affects NOTCH signaling. CQ is a commonly used autophagy inhibitor that effectively impairs the terminal stage of autophagosome fusion to the lysosome. Inhibition of autophagy with CQ treatment was associated with excessive and sustained NOTCH1^{ICD} levels, suggesting that impaired autophagy

may be inefficient at NOTCH related protein degradation and progressing myogenic differentiation (51). Interestingly, NOTCH signaling was first evidenced in *Drosophila*, and subsequent studies have suggested a link between autophagy and NOTCH signaling during Drosophila oogenesis (60). In this recent study, researchers found that loss of autophagy leads to precocious activation of the NOTCH signaling evidenced by elevated expression of downstream NOTCH effectors, *Cut* and *Hindsight* genes that are involved in egg chamber development (23, 60). Strengthening existing findings in our lab, CQ treatment disrupts autophagic flux indicated by increased SQSTM1, LC3B-I, and LC3B-II levels during differentiation (Figure 8). Interestingly, CQ inhibits lysosomal enzymes that alters the acidic environment of lysosomes and blocks the binding of autophagosomes to lysosomes. Increased levels of SQSTM1 and LC3B levels in CQ treated cells suggests the accumulation of SQSTM1 and LC3B proteins present on the inner membrane of autophagosome that are unable to undergo degradation.(61). In addition, CQ treatment may also be associated with the accumulation of NOTCH1and $NOTCH1^{ICD}$ proteins that would be normally degraded within autophagosomes in the presence of autophagy. Therefore, significantly increased $NOTCH1^{ICD}$ levels in CQ treated cells suggests that impairments in the autophagy pathway likely diminishes the downregulation of NOTCH signaling. In addition, C2C12 cells that were genetically and stably transfected with shRNA against *Atg7* showed impaired ability to form functional autophagosomes (62). Interestingly, autophagy deficient sh*Atg7* cells indicated dramatically elevated levels of $NOTCH1^{ICD}$ by 0.6-fold at the D0 (24 hr) timepoint.

Therefore, our hypothesis that autophagy likely regulates NOTCH signaling was demonstrated with two different chemical treatments, RAPA and CQ that was associated with decreased and increased $NOTCH1^{ICD}$ levels, respectively. In detail, RAPA treatment induced

autophagy and was associated with decreased NOTCH1^{ICD}, suggesting possibly increased autophagy mediated downregulation of NOTCH signaling that may further increase the clearance and degradation of NOTCH related proliferative proteins (NOTCH1, NOTCH1^{ICD}, HES1) (36, 43, 51). Secondly, impaired autophagy with CQ treatment was correlated with significantly elevated NOTCH1^{ICD} levels, suggesting autophagy related impairments in the degradation of NOTCH-related proteins (51). The present work suggests that impaired autophagy is likely accompanied with reduced ability to downregulate NOTCH signaling, which may result in undesirable accumulation and hyperactivity of NOTCH signaling (36, 43, 50, 60). Collectively, the data provides preliminary evidence that autophagy is likely an important regulator of NOTCH signaling.

Autophagy deficient (shAtg7) cells treated with DAPT may be associated with increased autophagy levels and improved the potential of myogenic differentiation

The present findings in this study suggest the importance of the temporal and coordinated activation of autophagy and NOTCH intracellular signaling systems to regulate proper development of C2C12 myoblast differentiation (51). As previously mentioned, CQ treatment impaired autophagy and was associated with the accumulation of NOTCH1^{CD} levels in proliferating myoblasts. Secondly, autophagy deficient sh*Atg7* cells showed a trend that suggests impaired autophagy results in excessive and sustained levels of NOTCH1^{ICD} signaling, which was indicated by 0.6-fold increase at D0 compared to scr control cells. High levels of activated NOTCH1^{ICD} appeared to be above the beneficial threshold, which may have resulted in undesirable outcomes such as over-proliferation of myoblasts and suppression of myogenic differentiation. Decreased autophagy levels and increased NOTCH1^{ICD} levels may suggest faulty crosstalk between autophagy and NOTCH signaling and is correlated with impaired myogenic

differentiation. Lack of autophagy may suggest that NOTCH related proteins are inadequately targeted to the autophagosome for degradation (51). More so, disrupted targeted degradation of NOTCH related proteins may negatively upregulate downstream NOTCH target genes (*Hes1, Hey1)* that synergistically suppress myogenic regulatory factors MYOD1 and MYOG (4, 51). Therefore, we speculate that impaired autophagy is likely associated with upregulated NOTCH signaling that sustains a proliferative state, impairing the ability of myoblasts to undergo terminal myogenic differentiation.

Several studies have found that sustained NOTCH signaling has detrimental effects in satellite cell renewal and myogenesis (38). For example, DMD KO mice show that over-activation of NOTCH signaling resulted in adverse physiological effects such as sustained inflammation, impaired muscle regeneration, and the rapid depletion and senescence of muscle progenitor cells (i.e., PAX7+ cells) (38). Hyperactivation of NOTCH signaling has been shown to transiently inhibit differentiation (38), therefore, decreasing elevated NOTCH levels in autophagy deficient cells appears to be a novel target in improving myogenic differentiation.

Therefore, in attempt to recover myogenic differentiation in sh*Atg7* cells, DAPT treatment was used to decrease $NOTCH1^{ICD}$ hyperactivity and increase myogenic differentiation. In support of our hypothesis, we found that inhibition of γ-secretase decreased $NOTCH1^{CD}$ levels and was associated with improved myogenic differentiation.

DAPT treatment reduced high levels of $NOTCH1^{ICD}$ present in sh*Atg7* vehicle cells by 85.2% at D0 of differentiation ($^{\dagger}p<0.05$) (Figure 9b). Furthermore, significantly decreased $NOTCH1^{ICD}$ levels in sh*Atg7* deficient cells may have potentially rescued myogenic differentiation indicated by increased MYH levels at D2 and D4 of differentiation. Immunofluorescent staining also showed increased differentiation index marked by the

formation of multinucleated myotubes in sh*Atg7*+DAPT treated cells. Although the fusion index indicated no significant differences, a slight increase in the number of nuclei per myotube was found in sh*Atg7*+DAPT treated cells.

Collectively, the reduction of elevated NOTCH1^{ICD} levels appears to be in agreement with improvements in myogenic differentiation, suggesting that decreasing hyperactive NOTCH1^{ICD} levels may potentially alleviate the repression of myogenic activity and promote the differentiation of myoblasts (4). It is well-established that autophagy upregulates myoblast differentiation (44), therefore, autophagic signaling may play a role in the recruitment of NOTCH1^{ICD} before translocation to the nucleus, thereby preventing the activation of the CSL complex (7). In addition, autophagy mediated inhibition of NOTCH1^{ICD} may partially diminish the expression NOTCH transcription genes, *Hes1* and *Hey1* (7). Partially diminished NOTCH target genes allows for the upregulation of MRFs, MYOD1 and MYF5, which commits myoblasts to the myogenic lineage for terminal differentiation (8). In the event that autophagy is impaired, it is likely that an absence of NOTCH recruitment and degradation exists (3). The lack of autophagy may be attributed to substantially increased NOTCH signaling, which may be associated with the suppression of myogenic differentiation (9).

Interestingly, LC3B-I levels were slightly increased in sh*Atg7*+DAPT treated cells at D2 of differentiation, compared to both scr control and sh*Atg7*+Veh cells (Figure 11). Considering the large body of evidence that has indicates LC3B is synthesized as a precursor that is cleaved by ATG4B to produce the cytosolic isoform, LC3B-I, which is responsible for autophagosome formation (41). The precursor form LC3B-I indicating slightly increased levels at D2 in sh*Atg7*+DAPT treated cells may suggest the possible activation of an alternative autophagy mechanism via member of RAS oncogene family (RAB9) in the absence of the *Atg7* gene. (63).

The RAB9 dependent pathway generates autophagosomes through the fusion of phagophores with vesicles that are derived from the *trans*-Golgi and late endosomes (63). Therefore, DAPT treatment that led to decreased NOTCH1^{ICD} levels may have alternative secondary effects that transiently increases autophagic signaling through an ATG7 independent pathway (i.e., RAB9), thereby progressing and improving myogenic differentiation. In another study, BM-MSC cells were treated with DAPT (5 μM) + CQ (20 μM) and indicated increased levels of autophagy related proteins (BECN1, LC3B) and adipose differentiation compared to vehicle control (DMSO) group (50). Although the results from this study are comparable to ours, differences in pharmacological treatments (CQ) versus the usage of a ATG7 deficient cell line should be accounted for. Collectively, autophagy deficient sh*Atg7* cells showed dramatically increased NOTCH1^{ICD} levels, which may be associated with impaired formation of myotubes (43). However, DAPT treatment reduced elevated NOTCH1^{ICD} levels in sh*Atg7* cells through the inhibition of γ-secretase, which was associated with partially restored myogenic differentiation and potentially increased autophagy signaling.

DAPT treatment in sh*Atg7 cells indicated no differences in cell cycle regulation*

Lastly, the present study was interested in further exploring cell-cycle characteristics related to NOTCH signaling. The downstream NOTCH signaling cascade involves NOTCH1^{ICD} translocation to the nucleus that activates the formation of the CSL transcriptional activation complex. Furthermore, the transcription of NOTCH target genes like *C-Myc, P21,* and *Cyclin D1* are upregulated during cell-cycle entry and progression (4, 16). Interestingly, a form of cancer known as acute lymphoblastic leukemia found in human T-cell (white blood cells) was treated with γ-secretase inhibitor and showed a dramatic reduction in medulloblastoma growth, induced G0/G1 cell-cycle arrest and apoptosis (15). Another study found that under normal conditions, T

cells show increased cell-cycle activators, CDK4 and CDK6 (10). However, the inhibition of NOTCH signaling abolished *Cdk4* and *Cdk6* expression suggesting NOTCH signaling plays an important role in promoting cell-cell cycle progression (10).

Therefore, the last experiment attempted to determine whether elevated NOTCH1^{ICD} levels in sh*Atg7* cells was associated with increased cell-cycle growth arrest (G_0) and decreased cell-cycle exit. Cellular differentiation requires coordinated cell-cycle growth arrest in G_0/G1 phase that results in irreversible cell-cycle exit and tissue-specific gene expression (11). In addition, decreased NOTCH1^{ICD} levels with DAPT treatment was postulated to increase cell-cycle exit that may explain increased myogenic differentiation found in sh*Atg7*+DAPT treated cells. Increased myogenic differentiation found in sh*Atg7*+DAPT treated cells was postulated to increase G_0/G1 phase to promote cell-cycle exit (differentiation), further decrease DNA synthesis in S phase and mitosis in the G2/M phase. Flow cytometry methods were used to measure PI fluorescence in scr control, sh*Atg7* vehicle, and sh*Atg7* +DAPT treated cells. Interestingly, sh*Atg7*+Veh cells showed markedly increased cell cycle growth arrest in G_0/G1 phase compared to scr control cells. In addition, sh*Atg7*+Veh cells also indicated less synthesis and duplication of DNA in the S phase followed with decreased mitosis in G2/M phase.

Surprisingly, there were no significant differences between sh*Atg7*+Veh and sh*Atg7*+DAPT treated cells in the G_0/G1, S, and G2/M phase of the cell cycle. However, a slight increase was present in the G_0/G1 phase in sh*Atg7*+DAPT cells, which may suggest a progression towards increased cell-cycle exit and terminal differentiation (10). Although further research is required, it is important to note that DAPT treated sh*Atg7* cells were collected after 24 hr of treatment (D0). In addition, the D0 timepoint is more indicative of 90% confluency and provides more evidence of cell-cycle exit. Therefore, an earlier time-point might capture a more

mitotic (rapidly dividing) environment and could better elucidate NOTCH related cell-cycle characteristics to measure the effect of the treatment (10, 16, 22). Collectively, cell-cycle analyses suggest that increased myogenic differentiation cannot be solely attributed to cell cycle regulation after cell-cycle exit, rather another more prominent signaling pathway like autophagy may be involved in the downregulation of NOTCH1^{ICD} levels in order to transition myoblasts into the differentiation program (51).

5.1 Summary and Conclusions

Thus far, research in skeletal muscle literature has typically focused on the degradative system of autophagy, highlighting cell survival, and thereby overlooking the direct effects on numerous signaling processes, including the regulation of NOTCH signaling (2,12,13,33). Evidence of autophagy mediated NOTCH signaling during differentiation has been studied in hemopoietic, cardiac, neuronal tissues (12, 32), however, it remains unclear in skeletal muscle. Therefore, the purpose of this study was to coherently investigate the role of autophagy in regulating NOTCH signaling during skeletal muscle cell differentiation. The results of this study highlights four main findings: 1) NOTCH signaling is downregulated and autophagy is upregulated during myogenic differentiation 2) increased autophagy with RAPA was associated with decreased NOTCH1^{ICD} levels, whereas inhibition of autophagy with CQ was associated with increased NOTCH1^{ICD} levels 3) near-complete inhibition of γ-secretase decreased NOTCH1 and NOTCH1^{ICD} levels and may be associated with decreased myogenic differentiation and 4) DAPT treated autophagy deficient cells (sh*Atg7*) potentiated autophagic signaling and had improved myogenic differentiation. Given the findings that NOTCH1 and NOTCH1^{ICD} levels are decreased during early myogenic differentiation, alongside autophagic flux, we propose an additional role of autophagy regulating NOTCH signaling to promote differentiation of C2C12 myoblasts. We

strengthened this hypothesis by showing that loss of autophagy dramatically increased and resulted in excessive NOTCH1^{ICD} levels in sh*Atg7* C2C12 cells. Furthermore, we found that sh*Atg7* cells treated with DAPT had improved the potential of autophagic signaling and myogenic differentiation. Collectively, the present data suggests that autophagy may be an important regulator of NOTCH signaling, thus playing an important role in both skeletal muscle cell maintenance and myogenesis (36, 51).

5.2 Limitations

NOTCH signaling is predominantly active in early stages of embryogenesis, regulating development and differentiation of a multitude of organ systems (angiogenesis, hematopoiesis, homeostasis, neurogenesis, myogenesis and somatogenesis) (3). A strong limitation of the present study was the use of an immortalized cell line, as C2C12 is not as effective for studying multifaceted functions of NOTCH signaling during embryonic and adult stem-cell renewal and stem cell quiescence (3, 6, 34, 64). The primary limitation of C2C12 cells is that the proliferative state occurs for a very short period of time and then rapidly matures into multinucleated myotubes in high serum conditions (64). C2C12 cells have been shown to have differences in morphology due to different adhesion factors in a petri-dish compared to in-vivo models (64, 65). Although, immortalized cell lines are generated from a well-known tissue type, certain mutations that make cells immortal may fluctuate the cell cycle machinery and senescent phase (64, 65). Research studying the differences between C2C12 gene expression and freshly harvested primary SCs (*ex-vivo*) found that 25% of regulatory genes present in C2C12s were absent in SCs (64). Therefore, to fully understand the entirety of the NOTCH signaling system, *in vivo* or *ex-vivo* experiments are highly recommended to understand the role of NOTCH signaling and autophagy during embryonic and postnatal muscle regeneration of SCs (3, 5, 37).

The present study primarily focused on NOTCH1 and NOTCH1^{ICD} levels during C2C12 myoblast differentiation. However, as previously mentioned there are four distinctive NOTCH (1-4) receptors and three unique NOTCH ligands (Delta/Delta-like and Serrate/Jagged ligand) (3). Interestingly, mammalians express three members of the Delta family (DDL1, DLL3, DLL4 and two members of the Jagged family (JAG1, JAG2), all of which have distinct structural and functional differences (3). Delta/Serrate/Lag-2 (DSL) domains have a series of epidermal growth factor (EGF)-like domains which all differ in number (9). In more detail, the JAGGED family has 16 EGF-like domains, DELTA family has 5-9, and Delta/Serrate ligand only has 1 (9). Paradoxically, this results in multiple different receptor-ligand combinations that generate very distinct cellular responses in proliferation and differentiation (9). For example, FRINGE is a well-known modulator of NOTCH signaling, specifically a glycotransferase that modifies O-fucose glycans in the NOTCH ECD EGF that enhances DELTA ligand binding NOTCH receptor and decreases SERRATE-NOTCH binding (9). More specifically, FRINGE proteins that are involved in glycosylation of NOTCH receptors strengthen the response of DLL1 and reduce the responsiveness of JAG, furthermore, exerting different effects on tissue organization (9). The present work in this thesis indicated elevated levels of NOTCH1^{ICD} was associated with impaired myogenesis (Figure 7). Although preliminary, C2C12 cells treated with JAG1 showed increased NOTCH1^{ICD} levels and was associated with increased MYH levels at D2 and D3 of differentiation, suggesting that JAG1 ligands may increase differentiation (Appendix Figure 2), while DELTA ligands may decrease myogenesis. Other studies have also suggested that activation of JAG1 ligand and binding to NOTCH1 receptor increases smooth muscle differentiation (33). However, these NOTCH receptor and ligand binding interactions are not clearly understood. Therefore, future studies should utilize plasmids with DLL4 and JAG1 to

transfect C2C12 cells and determine whether specific NOTCH ligands have opposite effects in myogenic differentiation.

Another limitation of the present study is examining markers of downstream autophagy (SQSTM1, LC3B-I, and LC3B-II) that interacts with NOTCH signaling without the consideration of upstream markers (i.e., PI3K, mTOR). It is well-established that NOTCH signaling is a developmental pathway that is activated during embryonic myogenesis (3), therefore, targeting early activators of autophagy like PI3K, mTOR may provide a more conclusive analysis on the crosstalk between the activation of autophagy and NOTCH signaling (9, 50). Although we measured the activity of late autophagy markers like LC3B, LC3B-II, conclusions regarding the levels of upstream autophagy activators cannot be fully understood. A recent study showed that upstream autophagy inhibitor, 3MA blocks the formation of autophagosomes by inhibiting PI3K and was associated with increased NOTCH signaling compared to CQ treated cells (50).

5.3 Future directions

Extensive research has shown that NOTCH signaling is a well-known suppressor of differentiation, thereby, inhibiting myoblast commitment and terminal differentiation. Initial experiments were aimed to understand the importance of NOTCH signaling during myogenesis. Characterization of NOTCH1 and NOTCH1^{ICD} protein levels suggested that NOTCH signaling is downregulated during differentiation. Furthermore, the near-complete inhibition of γ-secretase decreased NOTCH1 and NOTCH1^{ICD} and was associated with impaired myogenesis. The findings suggested that basal levels of NOTCH signalling are likely required to properly transition myoblasts from a proliferative to differentiative state (6). However, the present study overlooked the potential effects of slightly reduced NOTCH1 and NOTCH1^{ICD} levels. Therefore,

future studies should attempt to reduce the NOTCH related suppression of myogenesis by moderately decreasing NOTCH1^{ICD} levels in attempt to increase C2C12 myogenic differentiation (6, 34). Preliminary results suggest that DAPT treatment at very low concentration of 2.5 μM slightly decreases NOTCH1^{ICD} levels and may be sufficient to be associated with increased myogenic differentiation (Appendix Figure 1).

Furthermore, the present data suggested sh*Atg7* cells had high levels of NOTCH1^{ICD} signaling, therefore, DAPT treatment at 10 μM was used to considerably decrease NOTCH1^{ICD} levels. However, future experiments should moderately decrease NOTCH1^{ICD} levels in sh*Atg7* cells that is similar to baseline levels found in normal C2C12 differentiation to more accurately represent normal physiological conditions (Figure 6). More specifically, low concentrations of DAPT γ-secretase inhibitor at 2.5-5 μM may be sufficient to improve impaired myogenesis in sh*Atg7* cells and would reduce any potential side-effects of drug treatment.

Another avenue to explore in depth is the role of NOTCH signaling in regulating the cell-cycle. In this study, cell-cycle regulation was analyzed after 24 hr of DAPT treatment at the D0 timepoint that may be more representative of cells withdrawing from the cell-cycle. NOTCH signaling has been implicated in several developmental processes like cell-fate and cell-cycle regulation (4, 10, 16). Therefore, flow cytometry analysis at multiple timepoints like rapidly dividing cells (D-1), cell-cycle withdrawal (D0) and cell-cycle exit and terminal differentiation (D1) at a lower DAPT concentration of 5 μM may provide more evidence of NOTCH signaling activation and cell-cycle progression. Furthermore, flow cytometry analyses should be supplemented with immunoblotting for P21 protein to assess cell-cycle exit, which may indicate decreased P21 levels during C2C12 myoblast differentiation (16, 30).

The present study utilized various pharmacological activators and inhibitors to understand the crosstalk between autophagy and NOTCH signaling. Although chemical inhibitors are exceptional for clinical human-based drug trials, the addition of a genetic approach that utilizes an adenovirus expressing NOTCH inhibitory decoy is necessary to make any conclusions regarding the autophagy and NOTCH signaling relationship. Although preliminary, we have tested various multiplicity of infection (MOI) concentrations of the NOTCH1 decoy ($N1_{1\text{-}36}$). Adenovirus $N1_{1\text{-}36}$ encodes for 36-extracellular EGF-like repeats of human NOTCH1 and is fused to human IgGγ heavy chain that interferes with ligand-specific NOTCH activation (66). Furthermore, the NOTCH/CSL reporter assay was used to confirm that $N1_{1\text{-}36}$ decoy significantly blocks DLL4 and JAG1-induced NOTCH1 signaling (66). N1-decoys compete for binding site on the extracellular domains of DLL-class and JAG-class ligands that results in blocked ligand-specific NOTCH signaling (66). Preliminary data suggests partial inhibition of NOTCH levels at MOI of 100-250 in C2C12 cells and MOI of 300-770 in sh*Atg7* cells (Appendix Figure 3 and 4).

Lastly, *in vivo* experimental studies should be employed to further examine the interaction between NOTCH signaling and autophagy. Wu et al. found that hypomorphic *ATG16L1* mutated mice show increased levels of NOTCH1, $NOTCH1^{ICD}$, and HES1; significantly higher proportion of stem cells; and developmental delays in neurogenesis, haematopoiesis, and in the gut villi (51). Future experiments should consider using conditional *Atg7* KO mice, more specifically *Atg7* KO in SCs to measure NOTCH levels in 1-4-week-old male and female mice. Other representative *in-vivo* models presently studied include *Atg5* and *Becn1* KO mice (67). $Becn1^{F/F;Pcp2\text{-}Cre}$ KO mice indicated rapid degeneration of neuronal purkinje cells (PCs) found in the cerebral cortex (67). More importantly, the loss of PCs was evident

between 21 days and 1 month of birth and further progressed severe PCs neuronal loss at 2 months (67). However, downstream autophagy marker, *Atg5* conditional KO mice showed greater intact PCs at 1 month and decreased loss of PCs at 2 months compared to *Becn1*$^{F/F;Pcp2\text{-}Cre}$ KO mice. Therefore, research suggests that knocking-out upstream autophagy markers like *Becn1* may have greater detrimental effects on the PCs population (67). In conclusion, *in-vivo* conditional *Atg7* KO mice models may provide more evidence of the role of NOTCH signaling in SC quiescence and muscle regeneration (69) that is limited in the *in-vitro* C2C12 model.

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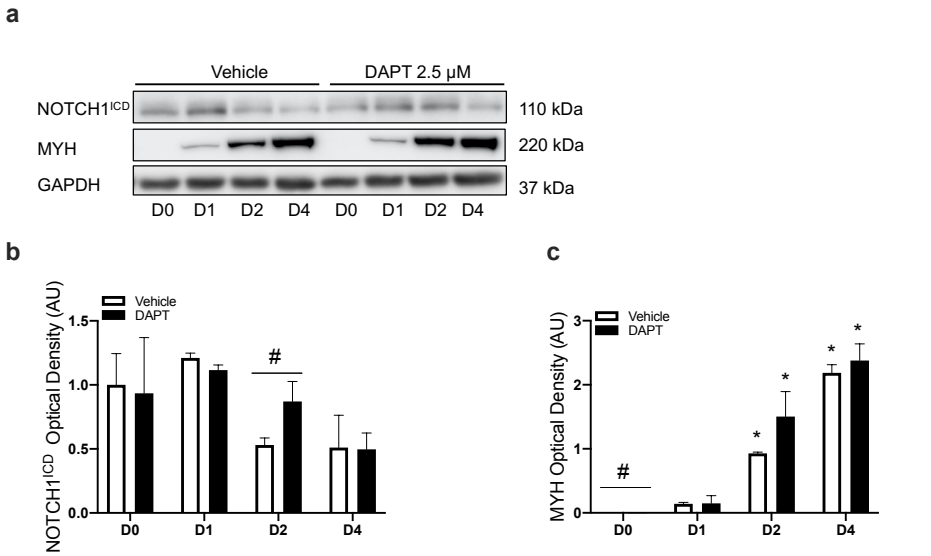
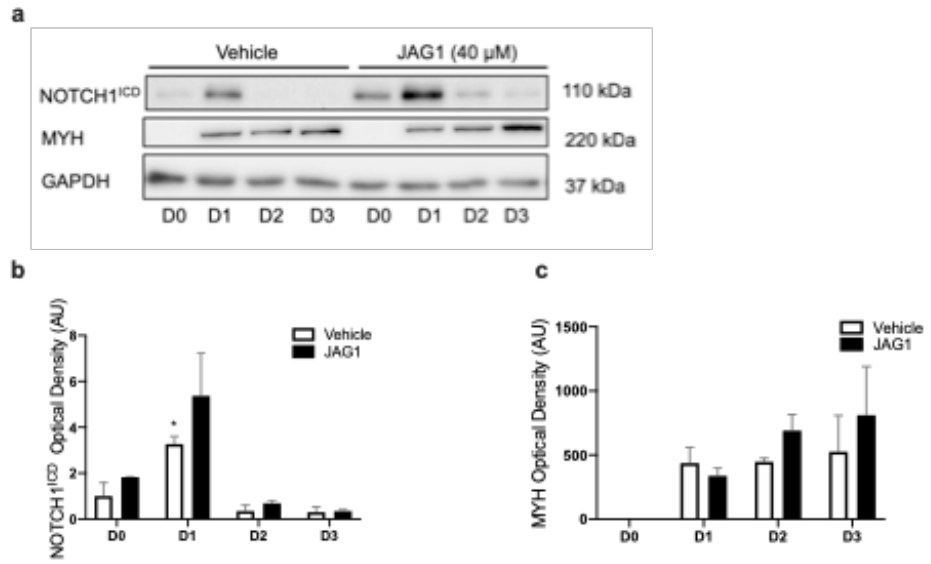
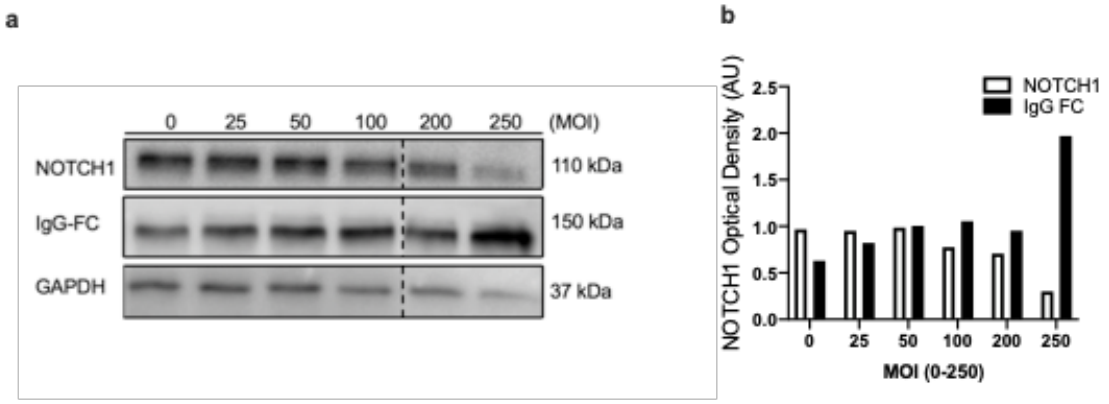
Appendices



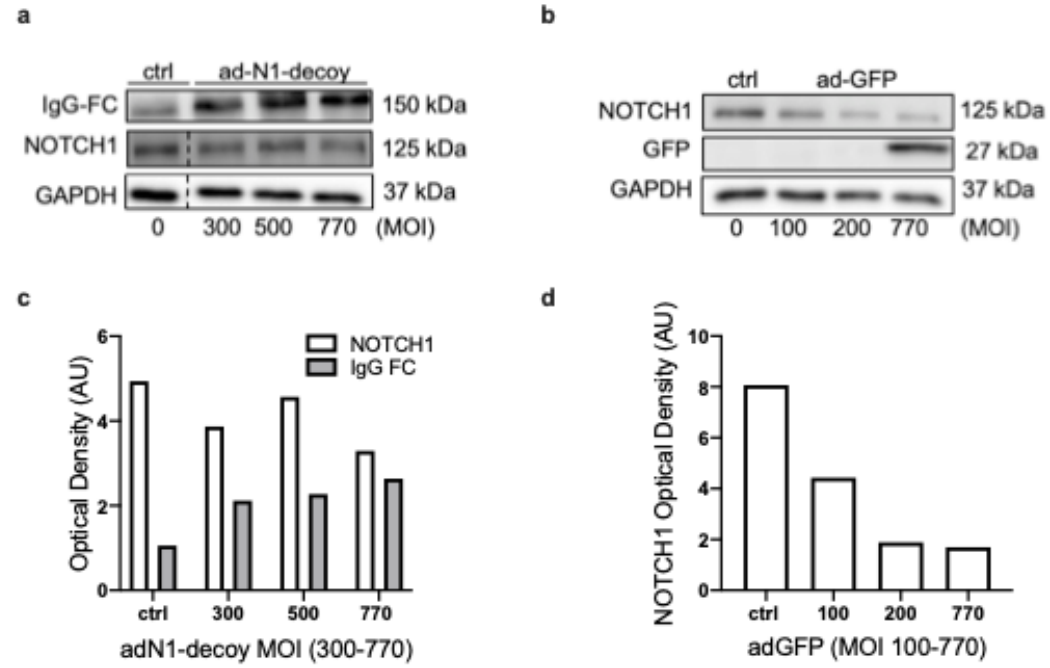
Appendix Figure 1. Acute inhibition of γ-secretase trended towards slightly decreased NOTCH1^{ICD} levels and was associated with increased MYH protein content during C2C12 myoblast differentiation. C2C12 myoblasts were treated with DAPT (2.5 μM) every 24 hrs of growth. Representative Immunoblots (a) and quantitative analysis (b) of NOTCH1^{ICD}, and (c) MYH during C2C12 myoblast differentiation. Also shown is a representative GAPDH loading control blot. $^{\#}$p<0.05 between groups DAPT (2.5 μM) at the same time point. One-way ANOVA *p<0.05 compared to D0 (within group). (n=2)



Appendix Figure 2. JAG1 treatment showed a trend towards increased NOTCH1^{ICD} levels and was associated with increased MYH levels during C2C12 myoblast differentiation. C2C12 myoblasts were treated with JAG1 (40 μM) for 48 hr prior to induction of differentiation. Representative immunoblot (a) and quantitative analysis (b) of NOTCH1^{ICD} and (c) MYH. Also shown is a representative GAPDH loading control blot. $^{\#}$p<0.05 compared to D0 (between group). One-way ANOVA *p<0.05 compared to D0 (within group). (n=2)



Appendix Figure 3. C2C12 cells transduced with NOTCH1-decoy resulted in slightly decreased levels of NOTCH1. Transduction of NOTCH1-decoy for 24 hrs was tested at various MOI concentrations (25, 50,100, 200, 250) Representative Immunoblots (a) and quantitative analysis (b) of NOTCH1 and IgG-FC. Also shown is a representative GAPDH loading control. (n=1)



Appendix Figure 4. Sh*Atg7* cells transduced with adenovirus expressing NOTCH1-decoy resulted in slightly decreased levels of NOTCH1. Transduction of NOTCH1-decoy for 24 hrs was tested at various MOI concentrations (300, 500, 770). Representative Immunoblots of (a) Ad-N1-decoy [MOI] test for NOTCH1 and IgG (0-770), (b) Ad-GFP [MOI] test (0-770) for NOTCH1 and GFP and quantitative analysis of (c) Ad-N1-decoy NOTCH1 and IgG, (d) Ad-GFP NOTCH1. Also shown is a representative GAPDH loading control. (n=1)