Examining the role of autophagy and mitophagy in regulating muscle differentiation

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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

Statement of Contributions

Chapter 1 was written by Brittany Baechler, and was partially written as part of my Comprehensive Examination in April 2016 and edited by Dr. Joe Quadrilatero.

Chapter 2 and Chapter 3 experiments were designed by Brittany Baechler and Dr. Joe Quadrilatero. Brittany Baechler performed experiments, analyzed data, and made figures. Dr. Darin Bloemberg generated the sh*Atg7*, SCR, Scram, and *bnip3*$^{-/-}$ cell lines used for these experiments. These chapters were written by Brittany Baechler and edited by Dr. Joe Quadrilatero. Some results from Chapter 2 and Chapter 3 of this thesis are published in the following paper:

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Chapter 4 was designed by Brittany Baechler and Dr. Joe Quadrilatero. Brittany Baechler performed experiments, analyzed data, and made figures. sh*Atg7*, SCR, Scram, and *bnip3*$^{-/-}$ cell lines were previously made by Dr. Darin Bloemberg. Fatemeh Keyvani assisted with flow cytometry experiments. Alessia Roma from the lab of Dr. Paul Spagnuolo assisted with respirometry experiments and analysis. Chapter 4 was written by Brittany Baechler and edited by Dr. Joe Quadrilatero.

Chapter 5 was written by Brittany Baechler and edited by Dr. Joe Quadrilatero.

Abstract

Autophagy is a degradative process that is used to eliminate intracellular organelles and protein aggregates. Further, a selective form of autophagy, termed mitophagy, is used to specifically degrade mitochondria. Autophagy/mitophagy is important for eliminating damaged/dysfunctional mitochondria to limit ROS levels and apoptosis, and is also required during erythrocyte and myoblast differentiation. Moreover, recent studies have demonstrated that mitophagy is required to initiate mitochondrial biogenesis during myogenic differentiation. Previous work in our lab has demonstrated that autophagy-deficient myoblasts fail to differentiate, have increased mitochondrial dysfunction, and have elevated levels of apoptotic signaling. Therefore, the purpose of this thesis was to determine the role of autophagy- and mitophagy-related proteins during myogenic differentiation. Chapter 2 demonstrated that canonical mitophagy is disrupted in ATG7-deficient cells, but that mitochondria can still be degraded using an alternative mitophagy pathway. However, we also determined that mitochondrial damage was increased in ATG7-deficient cells, suggesting that targeted degradation of damaged mitochondria specifically is impaired in ATG7-deficient cells. Moreover, we found that increasing the expression of the mitophagy receptor protein BNIP3 was able to partially recover myogenesis in ATG7-deficient cells. Chapter 3 then explored the requirement for the mitophagy-related proteins BNIP3L/NIX and BNIP3 during myogenic differentiation, and found that a deficiency in either of these proteins was disruptive to myogenesis. Further, we demonstrated that *bnip3*$^{-/-}$ cells showed elevated levels of mitochondria-mediated apoptotic signaling, suggesting impairment in the elimination of dysfunctional mitochondria. Moreover, *bnip3*$^{-/-}$ cells had increased autophagy-related protein

expression. Interestingly, we found that overexpression of ATG7 or treatment with the autophagy inducer rapamycin can disrupt myogenic differentiation in C2C12 myoblasts, suggesting that elevated autophagy might inhibit myogenesis. Additionally, Chapter 2 and Chapter 3 demonstrated that mitochondrial signaling and mitochondrial protein expression is reduced in both sh*Atg7* and *bnip3*$^{-/-}$ cells, suggesting impairment in mitochondrial remodelling during differentiation. Therefore, Chapter 4 examined whether upregulating mitochondrial biogenesis can compensate for a potential reduction in autophagy/mitophagy during differentiation. Interestingly, we found that treating ATG7- and BNIP3-deficient cells with SNP, a mitochondrial biogenesis inducer, caused increased mitochondrial biogenesis- and mitochondria-related protein expression, as well as an increase in differentiation and myotube formation. Overall, this thesis demonstrated that autophagy and mitophagy are important during myogenic differentiation, and that these processes must be tightly regulated in order to ensure that cell death is limited and differentiation can progress properly.

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Dedication

I dedicate this work to my Dad, Dale Baechler. He was both my toughest critic and my #1 fan – I couldn't have done this without him.

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List of Abbreviations

AICAR: 5-Aminoimidazole-4-carboxamide-ribonucleoside
AIFM1: apoptosis-inducing factor, mitochondrion-associated 1
ATG5: autophagy related 5
ATG7: autophagy related 7
BCL2: B cell leukemia/lymphoma 2
BECN1: beclin 1
BNIP3: BCL2/adenovirus E1B interacting protein 3
BNIP3L/NIX: BCL2/adenovirus E1B interacting protein 3-like
BFA: brefeldin A
CI: complex I
CII: complex II
CASP: caspase
CASP3: caspase 3
CASP9: caspase 9
$CoCl_2$: cobalt chloride
CQ: chloroquine
CYCS: cytochrome c
DNM1L: dynamin 1-like
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
GFP: green fluorescent protein
LC3: microtubule-associated protein 1 light chain 3 beta
MTOR: mechanistic target of rapamycin kinase
MYH: myosin
MYOG: myogenin
BNIP3L/NIX: BCL2/adenovirus E1B interacting protein 3-like
OPA1: mitochondrial dynamin like GTPase
OXPHOS: oxidative phosphorylation
PPARGC1A: peroxisome proliferative activated receptor, gamma, coactivator 1 alpha
p-H2AFX: phosphorylated H2A histone family, member X
PINK1: PTEN induced putative kinase 1
PRKN: parkin RBR E3 ubiquitin protein ligase
RAB9: RAB9, member RAS oncogene family
RFP: red fluorescent protein
ROS: reactive oxygen species
SCR: scramble shRNA
sh*Atg7*: short hairpin RNA against ATG7
SIRT1: sirtuin 1
SLC25A4: solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator)
SNP: sodium nitroprusside
SOD2: manganese superoxide dismutase
TFAM: transcription factor A, mitochondrial
ULK1/ATG1: unc-51 like autophagy activating kinase 1
VDAC1: voltage-dependent anion channel 1

Chapter 1: Introduction & Literature Review

Skeletal muscles allow the body to move, and because of this important function, they possess a highly organized structure (Sandri, 2010) and possess complex and tightly regulated intracellular signaling mechanisms (Yin et al., 2013). Mature skeletal muscle is made up of multinucleated myofibers, which contain the contractile proteins necessary for muscle contraction (Sandri, 2010). Muscle also contains a sophisticated network of mitochondria to meet its demanding metabolic requirements (Sin et al., 2016). In addition to these features, muscle is also more resistant to stress and is highly responsive and adaptable to intracellular changes (Quadrilatero et al., 2011; Ceafalan et al., 2014). Although it is considered to be a post-mitotic tissue (Wang & Walsh, 1996), skeletal muscle has the ability to regenerate (Ceafalan et al., 2014) due to a specialized population of satellite cells (SCs) (Dumont et al., 2015). SCs are undifferentiated muscle stem cells that can support muscle regeneration throughout adulthood and in response to damage (Dumont et al., 2015; Bentzinger et al., 2012; Shi & Garry, 2006).

Skeletal muscle development during embryogenesis

Skeletal muscle development begins during embryogenesis, following the separation of the mesoderm germ layer to form the paraxial mesoderm, which subsequently forms the somite (Bentzinger et al., 2012). The somite is then divided into dorsal and ventral portions, with the dorsal portion forming the dermomyotome, a structure from which most skeletal muscle is derived (Shi & Garry, 2006). Cells of the dermomyotome express the transcription factors paired box 3 (PAX3), paired box 7 (PAX7), and myogenic factor 5 (MYF5), and a portion of these cells will migrate and delaminate to form an additional structure known as the myotome. Cells of the myotome show strong expression of the transcription factor MYOD and will terminally differentiate to form

skeletal muscle. However, a subset of the PAX3/PAX7-expressing cells of the dermomyotome, migrate to the myotome but remain in an undifferentiated state. It is believed that this undifferentiated population of cells gives rise to adult muscle satellite cells (SCs) (Bentzinger et al., 2012; Shi & Garry, 2006).

SCs and the surrounding niche

SCs represent less than ten percent of the myonuclei found in adult skeletal muscle, and are positioned between the sarcolemma of the myofiber and the surrounding basal lamina (Dumont et al., 2015). SCs are generally maintained in a quiescent, non-proliferative G_o phase (Cheung & Rando, 2013). However, SCs can re-enter the cell cycle to proliferate and subsequently differentiate and fuse to regenerate muscle fibers in response to damage (Dumont et al., 2015), as well as self-renew to maintain the stem cell population (Yin et al., 2013). The instructions that govern whether SCs are in a quiescent or activated state come from the surrounding microenvironment known as the SC niche. The SC niche consists of the surrounding cells and extracellular structures, which sense cellular changes and send signals to the SCs to promote quiescence or activation (Thomas et al., 2015), depending on the circumstances.

As described above, adult SCs are specified during embryonic myogenesis, and maintain high levels of PAX7 expression (Dumont et al., 2015). Over a decade ago, Seale et al (2000) showed that *pax7* is expressed in the undifferentiated SC-derived myoblasts, and that *pax7* expression decreases as cells differentiate (Seale et al., 2000), suggesting that PAX7 is a reliable marker of the SC/myoblast population (Yin et al.,

2013). Moreover, studies using PAX7-deficient (*pax7*$^{-/-}$) mice demonstrated that PAX7 is required for the establishment of SCs in adult skeletal muscle (Seale et al., 2000). More recent studies have sought to determine if the role of PAX7 in SC regulation is limited to the initial specification of SCs, or if PAX7 is necessary for SC function during adulthood. One particular study showed that muscle regeneration was not impaired in a tamoxifen-inducible, SC-specific, PAX7 knockdown mouse model (Lepper et al., 2009). However, another study challenged these findings, and demonstrated that PAX7 is necessary for SC maintenance and muscle regeneration (von Maltzahn et al., 2013). Additionally, although PAX7 expression is needed in undifferentiated SCs, muscle formation and regeneration is dependent on the commitment of SCs to the myogenic lineage and their subsequent differentiation, processes that are dependent on the expression and functions of a group of myogenic regulatory factors (Yin et al., 2013).

Myogenic regulatory factors

Muscle differentiation during embryonic muscle development and from adult SCs (Braun & Gautel, 2011) is orchestrated by a group of myogenic regulatory factors (MRFs), which includes MYOD, MYF5, MYOG, and MRF4. These basic helix-loop-helix (bHLH) proteins heterodimerize with E proteins and specifically bind E-box DNA sequences to regulate target gene expression (Sabourin & Rudnicki, 2000). Interestingly, these MRFs are recognized as being sufficient to induce myogenic differentiation even in non-muscle progenitors (Bentzinger et al., 2012).

MYOD and MYF5 are considered to be the MRFs responsible for the commitment of cells to the myogenic lineage. Although these proteins mediate unique transcriptional requirements, they share some overlapping functions, allowing them to sometimes compensate for one another (Sabourin & Rudnicki, 2000). Early work had shown that MYOD-deficient mice were able to form skeletal muscle, and that loss of MYOD was associated with a compensatory increase in *myf5* expression (Rudnicki et al., 1992). Additionally, skeletal muscle formation was not disrupted in MYF5-deficient mice (Braun et al., 1992); however, mice lacking both MYF5 and MYOD failed to generate skeletal muscle, suggesting that although these transcription factors show some redundancy, each one plays a role in supporting proper myogenic determination and differentiation (Rudnicki et al., 1993; Sabourin & Rudnicki, 2000). MYF5 and MYOD are both expressed in committed myoblasts; however, MYF5 is detected earlier in SCs (Sincennes et al., 2016), with its expression being transcriptionally activated by PAX7 (McKinnell et al., 2008). When SCs undergo asymmetric cell divisions to generate an undifferentiated SC as well as a committed myoblast, MYF5 expression is induced in the committed myoblast but not in the uncommitted stem cell (Sincennes et al., 2016). Subsequently, MYOD is expressed in the committed myoblast, and forces the cell to undergo further myogenic differentiation. This is accomplished through the MYOD-dependent transcriptional regulation of numerous muscle-specific genes, including *myogenin (Myog)* (Fujimaki et al., 2013).

Although myoblast commitment is dependent on MYF5/MYOD, MYOG and MRF4 are the important downstream mediators of terminal differentiation in skeletal muscle (Sabourin & Rudnicki, 2000), with MRF4 also having a potential role in

myogenic determination (Braun & Gautel, 2011). Early work had shown that the specification of myoblasts does occur in MYOG-deficient mice; however, the formation of muscle fibers is severely impaired. This work suggested that MYOG is a necessary downstream mediator of myogenesis, and that its role is distinct from those of MYF5 and MYOD (Rawls et al., 1995). Additionally, more recent studies have shown that MYOG is not essential for muscle differentiation in the adult mouse or *in vitro*, suggesting a possible compensatory action of the other MRFs. However, although MRFs have overlapping functions and can compensate for one another, each factor is likely to regulate some unique skeletal muscle-specific genes (Meadows et al., 2008).

The C2C12 myoblast cell line

In order to study myogenic differentiation *in vitro*, studies will often use the C2C12 myoblast cell line (Tannu et al., 2004). C2C12 myoblasts were isolated from the thigh muscles of CH3 mice after the muscles had sustained a crush injury. These myoblasts have a 24-hour generation time and fuse upon reaching confluency to form multinucleated myofibers (Yaffe & Saxel, 1977). Additionally, there is a coordinated upregulation of myogenic proteins such as MYOG and MYOSIN (MYH) observed during differentiation in C2C12 cells (Andres & Walsh, 1996), which demonstrates that C2C12 myoblasts can serve as a powerful *in vitro* system for studying skeletal muscle differentiation.

The role of apoptotic proteins in muscle differentiation

Apoptosis is a form of programmed cell death that allows for the efficient removal of unwanted cells and plays an important role in normal development (Elmore, 2007). Moreover, apoptosis can also be induced to eliminate cells that become damaged or stressed, which can result in unplanned or undesirable cell death/loss (Saikumar et al., 1999; Kroemer et al., 2007). Apoptotic cell death can be induced extrinsically through the binding of ligands with cell membrane-associated death receptors, and can also be induced intrinsically as a result of DNA damage or mitochondrial outer membrane permeabilization (MOMP) (Marino et al., 2014). MOMP, which is largely dependent on the activity of BH3-only proteins, induces apoptosis by allowing pro-apoptotic factors such as cytochrome *c* (CYCS), endonuclease G (ENDOG), and apoptosis-inducing factor (AIFM1) to be released from the mitochondria. The release of these pro-apoptotic factors into the cytosol can then initiate apoptotic cell death through caspase-dependent or caspase-independent mechanisms (Marino et al., 2014; Quadrilatero et al., 2011). When CYCS is released into the cytosol, it can form a complex with apoptotic peptidase activating factor 1 (APAF1), a protein that has a caspase-recruitment domain (CARD). After this complex forms, pro-CASPASE 9 is recruited, becomes activated, and can then cleave and activate other caspases such as CASPASE 3 (CASP3) (Zou et al., 1999; Wang, 2001). Interestingly, CASP3 activation is known to occur during myogenic differentiation (Fernando et al., 2002); however, CASPASE 9 (CASP9) is not normally activated or needed to activate CASP3 during myogenesis (Bloemberg & Quadrilatero, 2014). Moreover, previous work in our lab revealed that CASPASE 2 (CASP2) is required for myogenic differentiation. Interestingly, CASP3 activity is reduced CASP2-

deficient cells, suggesting that CASP2 likely promotes CASP3 activation during differentiation (Boonstra et al., 2018).

Interestingly, cellular features associated with apoptosis are also observed in cells undergoing differentiation, suggesting that proteins involved in the former process might also mediate the latter (Fernando & Megeney, 2007). Chromatin modifications, such as DNA strand breaks, are induced by apoptotic proteins and have been shown to occur during skeletal muscle differentiation (Larsen et al., 2010). Additionally, chromatin organization and remodelling is important in promoting the expression of muscle-specific genes such as *Myog* during differentiation, and can also limit the expression of genes that may inhibit this process, such as *Pax7* (de la Serna et al., 2005; Palacios et al., 2010; Sincennes et al., 2016).

CASP3 is a proteolytic enzyme most often recognized for its role in promoting apoptotic cell death (Fernando et al., 2002; Fernando & Megeney, 2007). However, CASP3 is also required to induce cellular differentiation, a function that is independent of its apoptotic role (Fernando et al., 2002). Using C2C12 myoblasts, Fernando et al (2002) showed that inhibiting CASP3 activity causes a delay in cell cycle exit and cell differentiation. Moreover, overexpression of activated CASP3 is sufficient to promote premature myoblast differentiation, even under growth-promoting conditions *in vitro* (Fernando et al., 2002). Interestingly, CASP3 activity is required to initiate DNA strand breaks during myoblast differentiation (Larsen et al., 2010), and it accomplishes this by indirectly activating caspase-activated DNase (CAD). Further, CASP3/CAD-mediated DNA strand breaks were shown to occur in the promoter region of *p21,* and are necessary

for promoting *p21* expression to induce cell cycle arrest and cell differentiation (Larsen et al., 2010).

PAX7 expression is characteristic of undifferentiated SCs (Seale et al., 2000), and its expression must be reduced to allow for proper SC differentiation. Interestingly, studies have demonstrated that CASP3 plays an important role in mediating PAX7 protein degradation by cleaving PAX7 to produce non-functional protein fragments. Additionally, treatment with a CASP3 activator results in increased SC differentiation and a concurrent decrease in the number of PAX7-expressing cells in isolated myofibers (Dick et al., 2015). The temporal regulation of CASP3 activity is therefore critical for proper muscle differentiation.

Autophagy in skeletal muscle maintenance and differentiation

Autophagy is a process that allows for the degradation of cellular organelles and proteins (Kondo et al., 2005). The autophagic process can be initiated by numerous factors (Kondo et al., 2005) and begins with the formation of an isolation membrane known as a phagophore (Marino et al., 2014). Once the phagophore forms, it extends to form a double-membraned autophagosome surrounding elements of cytoplasm, such as organelles, that are to be degraded (Mizushima, 2007). Autophagosome formation and expansion is reliant on two ubiquitin-like conjugation systems (Tsujimoto & Shimizu, 2005), which both involve the E1-like activating enzyme ATG7. In the first reaction, ATG7 activates ATG12 so that it can be covalently linked to ATG5. Subsequently, the ATG12-ATG5 complex, along with ATG16L, associates with the expanding membrane.

In the second reaction, which also utilizes ATG7, the microtubule-associated protein 1 light chain 3 (MAP1LC3/LC3) protein is conjugated to phosphatidylethanolamine (PE) to form LC3B-II, and is then incorporated into the developing autophagosome membranes (Glick et al., 2010). Interestingly, LC3 plays an important role in determining which cytoplasmic components will be sequestered within the autophagosome. For example, LC3 has been shown to interact directly with the mitochondrial protein BNIP3 to mediate the degradation of old or damaged mitochondria (mitophagy) (Hamacher-Brady & Brady, 2016). Additionally, LC3 interacts with the scaffold protein SQSTM1 to degrade ubiquitinated protein aggregates (Scherz-Shouval & Elazar, 2011; Glick et al., 2010). After the autophagosome forms, it fuses with a lysosome so that the cytoplasmic components within the autophagosome can be degraded by lysosomal enzymes (Glick et al., 2010).

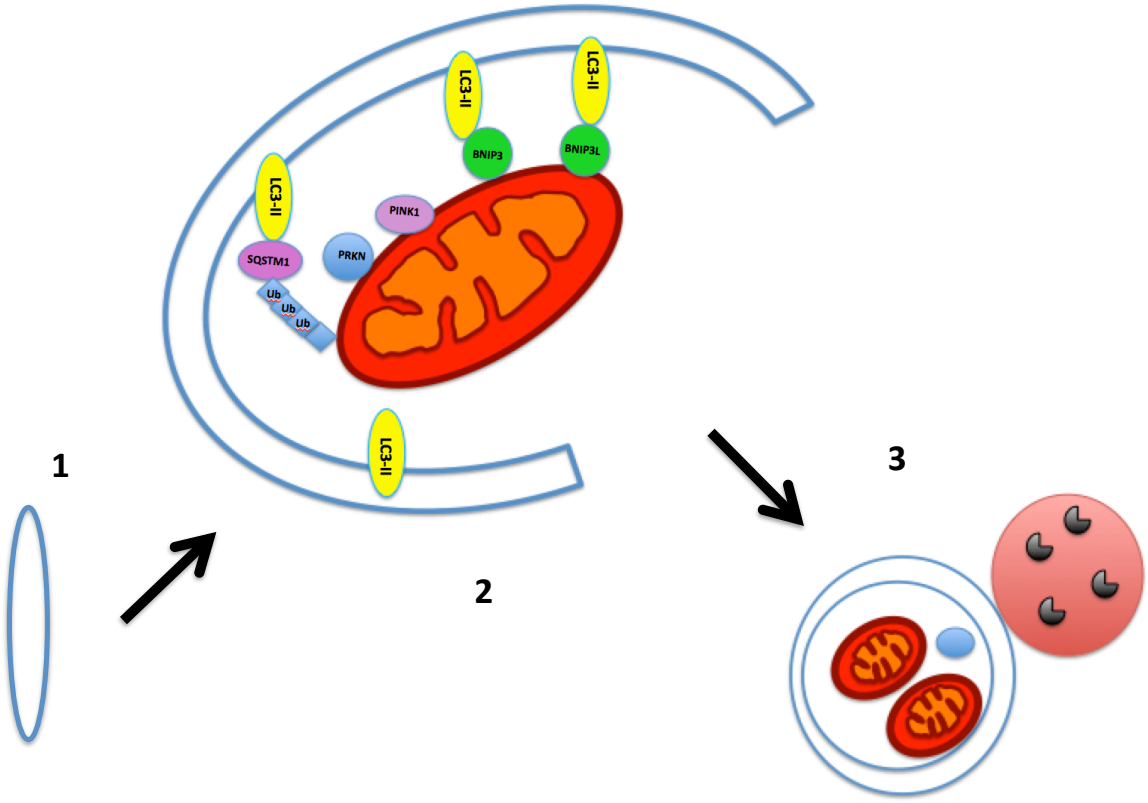

Figure 1. Overview of autophagy/mitophagy. 1) Formation of isolation membrane (phagophore). 2) The phagophore extends to form an autophagosome surrounding organelles or protein aggregates that are to be degraded. In this figure, a mitochondrion (red) is being targeted for degradation (mitophagy). 3) The autophagosome fuses with a lysosome to allow its cargo to be degraded by lysosomal enzymes. (Marino et al., 2014; Triolo & Hood, 2019).

Although this is the most commonly described or accepted mechanism of autophagy-mediated degradation, an alternative or non-canonical autophagy/mitophagy pathway has also been described (Grose & Klionsky, 2016). Approximately ten years ago, Nishida et al (2009) discovered that autophagosome formation was not disrupted in ATG7- or ATG5-deficient mouse cells (Nishida et al., 2009). Interestingly, LC3 lipidation does not occur and is not required for alternative autophagy; however, proteins that play an important role in canonical autophagy, such as ULK1 and BECN1, are also necessary for alternative autophagy (Nishida et al., 2009; Hirota et al., 2015; Shimizu et al., 2014; Honda et al., 2014). During alternative autophagy, it is hypothesized that

autophagosomes are generated through the fusion of isolation membranes with *trans*-Golgi-derived vesicles (Nishida et al., 2009). Moreover, although it is not needed for canonical autophagy, RAB9, a GTPase that mediates intracellular trafficking (Kucera et al., 2016), is required to execute alternative autophagy (Nishida et al., 2009; Wang et al., 2016;). Interestingly, studies using mouse embryonic fibroblasts (MEFs) and HeLa cells have suggested that RAB9-dependent/alternative autophagy, rather than canonical autophagy, is the main pathway used to degrade mitochondria in response to treatment with cellular stressors (Hirota et al., 2015). Further, alternative autophagy/mitophagy can be used to degrade mitochondria during erythrocyte differentiation (Nishida et al., 2009; Honda et al., 2014; Shimizu et al., 2014), and has recently been shown to limit ROS levels and apoptosis in erythroleukemia cells (Wang et al., 2016).

Autophagy allows cells to effectively remove damaged organelles, which could otherwise be detrimental to cell survival. Moreover, it can also be used as a method for recycling cellular materials to provide cells with energy during starvation (Fan et al., 2015; Kondo et al., 2005). To further complicate matters, although autophagy can promote cell survival by inhibiting apoptosis, autophagy can also induce cell death (Marino et al., 2014; Liu & Levine, 2015). Additionally, autophagy has also been shown to play an important role in stem cell maintenance and differentiation in numerous contexts (Phadwal et al., 2013).

Several years ago, Garcia-Prat et al (2016) demonstrated that autophagic signaling is highly active in quiescent SCs, with a suspected function being to remove proteins and organelles to allow SCs to remain in a quiescent state, while also preventing senescence (Garcia-Prat et al., 2016). The maintenance of stem cell quiescence and the prevention of

cellular senescence, are necessary requirements for preserving the regenerative capacity of adult skeletal muscle (Garcia-Prat et al., 2016). The decline in muscle regenerative capacity associated with aging is thought to occur because of a reduction in autophagic signaling (Sousa-Victor et al., 2014; Garcia-Prat et al., 2016). In support of this hypothesis, autophagosome formation and clearance have been shown to be disrupted in aged SCs; however, protein and organelle clearance can be effectively restored with autophagy-inducing rapamycin treatment (Garcia-Prat et al., 2016). Interestingly, an autophagy-deficient mouse model (*Atg7*$^{-/-}$), which uses PAX7-driven Cre recombinase expression to abolish *Atg7* expression specifically in SCs, was shown to phenocopy the aged wild-type model. More specifically, the number of SCs was significantly reduced in *Atg7*$^{-/-}$ mice, and transplanting *Atg7*$^{-/-}$ SCs to damaged wild-type muscle confirmed that these cells were less effective at regenerating muscle (Garcia-Prat et al., 2016).

Due to the observed accumulation of mitochondria in ATG7-deficient SCs and aged SCs, the authors speculated that reduced mitophagy and a resultant increase in reactive oxygen species (ROS) might account for the above-mentioned regeneration defects associated with aged SCs. Therefore, they used Trolox to inhibit ROS, which prevented the ROS-induced expression of the senescence-promoting gene *p16/INK4a*. In addition, they found that Trolox treatment improved the functionality of aged SCs, and prevented *Atg7*$^{-/-}$ SCs from becoming senescent (Garcia-Prat et al., 2016). These findings highlight autophagy's role in limiting ROS to support cellular homeostasis in SCs (Scherz-Shouval & Elazar, 2011).

In addition to its role in SC maintenance (Garcia-Prat et al., 2016), autophagy is needed to provide sufficient energy for SC activation (Tang & Rando, 2014), and is also

needed to promote myoblast differentiation (McMillan & Quadrilatero, 2014). C2C12 myoblasts show increased autophagy when induced to differentiate, and differentiation is impaired in ATG7 knockdown cells or cells treated with the autophagy inhibitor, 3MA (McMillan & Quadrilatero, 2014). Moreover, autophagy has been shown to assist with the necessary cellular remodelling associated with cell differentiation in other tissues (Phadwal et al., 2013), so it is not surprising that C2C12 myoblasts are dependent on autophagy to execute proper myogenic differentiation and myotube generation (McMillan & Quadrilatero, 2014; Sin et al., 2016).

As mentioned previously, skeletal muscle is a long-lived tissue that is quite resistant to cellular stressors (McMillan & Quadrilatero, 2011). With its multi-nucleated structure, it is uncommon for an entire muscle cell to die, and muscle longevity is highly reliant on apoptosis inhibitors (Quadrilatero et al., 2011). Therefore, it is not surprising that the maintenance of this tissue is largely dependent on autophagic signaling (Masiero et al., 2009), which can suppress apoptosis in some contexts (Marino et al., 2014). Interestingly, previous work in our lab has demonstrated that CASP3 activation and apoptotic signaling are elevated in ATG7-deficient C2C12 myoblasts and in cells treated with 3MA (McMillan & Quadrilatero, 2014; McMillan, 2015). Moreover, numerous animal studies have also shown that autophagy-deficiency is associated with increased apoptotic signaling in skeletal muscle (Pare et al., 2017; Masiero et al., 2009; Chrisam et al., 2015; Grumati et al., 2011). Therefore, autophagy is a necessary process for supporting cellular homeostasis and differentiation.

Mitophagy

Mitophagy is a selective form of autophagy that specifically eliminates mitochondria (Melser et al., 2013). Interestingly, there are numerous proteins that have been shown to regulate and/or induce mitophagy through unique mechanisms (Hamacher-Brady & Brady, 2016). Some examples of these mitophagy-related proteins include PINK1, PRKN, BNIP3, and BNIP3L/NIX, among others (Hamacher-Brady & Brady, 2016). PINK1/PRKN-mediated mitophagy allows for the efficient removal of depolarized mitochondria (Hamacher-Brady & Brady, 2016). When mitochondria are healthy and polarized, the PINK1 kinase is imported into the mitochondria, cleaved, and subsequently degraded (Jin et al., 2010). However, when mitochondria are depolarized, PINK1 is not imported into the mitochondria and accumulates at the outer mitochondrial membrane (OMM), which causes the E3 ubiquitin ligase PRKN to be activated and recruited to the mitochondria (Matsuda et al., 2010; Kondapalli et al., 2012). PRKN then ubiquitylates proteins on the OMM, and recruits autophagy receptors such as SQSTM1 to the mitochondria (Narendra et al., 2010). Although some reports suggest that SQSTM1 is required for PRKN-mediated mitophagy (Geisler et al., 2010), others suggest that SQSTM1 mediates the sequestration of mitochondria but is not needed for mitophagy (Narendra et al., 2010).

Mitophagy can also occur in a PINK1/PRKN-independent manner using other mitophagy receptor proteins, such as BNIP3 and BNIP3L (Hamacher-Brady & Brady, 2016). BNIP3 and BNIP3L are functionally related transmembrane proteins, which can localize to the mitochondria. At the mitochondria, BNIP3 and BNIP3L can induce both cell death and autophagy (Ney, 2015). Moreover, BNIP3 and BNIP3L possess an LC3-

interacting region (LIR), which allows them to interact directly with LC3 to facilitate autophagic degradation of mitochondria (Hanna et al., 2012; Rogov et al., 2017). Further, LC3 has been shown to interact with additional proteins and lipids such as FUNDC1 and Cardiolipin, respectively, in order to promote mitophagy in some contexts (Wu et al., 2014; Chu et al., 2013).

Mitophagy protects against unwanted cell death

A healthy and functional mitochondrial population is crucial for providing cells with energy and also for limiting unwanted cell death (Kubli & Gustafsson, 2012; Gomes & Scorrano, 2013). Damaged mitochondria can release pro-apoptotic factors to initiate cell death processes (Marino et al., 2014; Quadrilatero et al., 2011), and this is why it is important for cells to eliminate damaged mitochondria (Ding & Yin, 2012). Therefore, in order to limit cell death, damaged mitochondria are degraded by mitophagy (Kubli & Gustafsson, 2012). In support of this, PINK1/PRKN-mediated mitophagy has been shown to prevent apoptosis induced by mitochondrial dysfunction (Wu et al., 2015), and BNIP3 can eliminate damaged mitochondria to reduce CYCS release capacity (Zhu et al., 2013). Therefore, mitophagy is necessary for ensuring that cells survive and remain functional.

Mitophagy is important for myogenic differentiation

Mitophagy is an important process during cell differentiation. For example, mitophagy is needed to eliminate the mitochondrial population found in reticulocytes so that they can

differentiate to form erythrocytes (Novak et al., 2010; Sandoval et al., 2008). Interestingly, BNIP3L has been identified as a critical mediator of mitochondrial clearance during erythrocyte differentiation (Sandoval et al., 2008) and can also effectively restore mitophagy in PRKN-deficient models (Koentjoro et al., 2017). Moreover, a recent study has suggested that mitophagy is needed to initiate mitochondrial remodelling during myotube formation (Sin et al., 2016). The myoblast population is more glycolytic and requires fewer mitochondria, while the larger population of mitochondria found in differentiated myotubes is better suited for oxidative phosphorylation (OXPHOS), and can better support the energy requirements of this metabolically active tissue (Sin et al., 2016; Wagatsuma & Sakuma, 2013). Interestingly, BNIP3L, along with the small GTPase RHEB, has been shown to promote mitophagy during increased OXPHOS activity in order to renew the mitochondrial population to maintain a high level of energy production (Melser et al., 2013).

As mentioned previously, autophagy is required for proper differentiation of C2C12 myoblasts (McMillan & Quadrilatero, 2014), and work conducted by Sin et al (2016) has expanded on this finding to demonstrate the importance of mitophagy for regulating the cellular remodelling and mitochondrial changes necessary to support the transition from myoblast to myotube (Sin et al., 2016). In C2C12 myoblasts induced to differentiate, mitochondrial fragmentation is observed at early time-points and is accompanied by an increase in levels of DNM1L/DRP1, which is a mitochondrial fission protein, and there is also increased autophagosome formation (Sin et al., 2016). As differentiation progresses, there is an increase in mitochondrial biogenesis as well as increased expression of the fusion protein OPA1 (Sin et al., 2016). The transition from

increased fission to increased biogenesis and fusion is reliant on autophagy, and myotube formation and mitochondrial network remodelling fails to occur if autophagy is disrupted (Sin et al., 2016). Interestingly, it has also been suggested that peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PPARGC1A/PGC-1α), a critical regulator of mitochondrial biogenesis (Palikaras et al., 2015), limits mitophagy/mitochondrial degradation during myogenic differentiation to instead promote mitochondrial biogenesis (Baldelli et al., 2014). Thus, it is likely that the opposing processes of mitochondrial degradation (mitophagy) and biogenesis must be tightly regulated during myogenic differentiation.

Interestingly, the importance of PINK1 and PRKN in muscle development and function has been demonstrated in studies using *Drosophila* as a model organism (Greene et al., 2003; Clark et al., 2006). Notably, *Drosophila PRKN* mutants demonstrate muscle abnormalities and functional impairments, which are suspected to result from mitochondrial dysfunction and increased apoptotic signaling (Greene et al., 2003). Moreover, a similar phenotype is observed in *PINK1* mutants (Clark et al., 2006), and can be effectively rescued by overexpression of PRKN (Park et al., 2006). Interestingly; however, overexpression of PINK1 cannot rescue the impairments associated with PRKN-deficiency (Park et al., 2006). Similarly, overexpression of BNIP3 can ameliorate the muscle abnormalities characteristic of *PINK1 Drosophila* mutants (Zhang et al., 2016).

Mitochondrial Biogenesis

Although it is important for cells to eliminate damaged or unnecessary mitochondria to maintain cellular homeostasis and during differentiation (Drake et al., 2017; Naik et al., 2018), it is equally important for cells to rebuild and maintain a functional mitochondrial network to support metabolic needs (Xu et al., 2013; Wanet et al., 2015). Mitochondrial biogenesis is a process by which new and/or more extensive mitochondrial networks are generated and involves the division, fusion, and growth of pre-existing mitochondria (Jornayvaz & Shulman, 2010; Ploumi et al., 2017). Mitochondrial function and integrity is dependent on the coordination of numerous regulatory proteins to ensure that proteins encoded by both the nuclear and mitochondrial genome are correctly synthesized and incorporated into the mitochondria (Jornayvaz & Shulman, 2010; Friedman & Nunnari, 2014). PPARGC1A is a transcriptional co-activator, and is considered to be a master regulator of mitochondrial biogenesis and function (Palikaras et al., 2015). Therefore, in order to ensure that mitochondrial homeostasis is maintained, PPARGC1A coordinates the expression and activity of numerous biogenesis-promoting proteins. First, PPARGC1A promotes the expression of genes encoding nuclear respiratory factor-1 (NRF-1) and NRF- 2 (Wu et al., 1999), which are transcription factors required for the expression of numerous nuclear and mitochondria-encoded proteins required for mitochondrial function (Wagatsuma & Sakuma, 2013). Additionally, PPARGC1A binds to NRF-1 to enhance expression of the gene encoding mitochondrial transcription factor A (TFAM), a protein that regulates the replication and transcription of mitochondrial DNA (Wu et al., 1999). Interestingly, work done by Wu et al (1999) demonstrated that overexpression of PPARGC1A in C2C12 myoblasts caused increases in mitochondrial

protein expression and mitochondrial number without impacting myogenic differentiation (Wu et al., 1999).

Mitochondrial Biogenesis during cell differentiation

Stem cells or undifferentiated cells are generally more reliant on glycolysis to fulfill their energy requirements, while increased OXPHOS activity is more characteristic of differentiated cells (Wanet et al., 2015; Naik et al., 2018). This aligns with studies demonstrating that myoblasts are more glycolytic, but display a shift toward OXPHOS-based metabolism as they differentiate to form myotubes (Sin et al., 2016; Wagatsuma & Sakuma, 2013). In support of this, differentiated myotubes also contain more mitochondria than undifferentiated myoblasts, and also show increased expression of OXPHOS complex proteins, which demonstrates that the mitochondrial population associated with myotubes differs from the mitochondrial population found in myoblasts. Moreover, myogenesis and muscle regeneration is associated with an increase in the expression of the major mediators of mitochondrial biogenesis such as PPARGC1A, NRF-1, and TFAM (Remels et al., 2010; Wagatsuma et al., 2011; Wagatsuma & Sakuma, 2013).

Thus, given the mitochondrial changes that must occur when cells differentiate, it is important to consider that mitochondrial biogenesis and remodelling might influence the likelihood or the ability of a cell to differentiate (Wanet et al., 2015; Xu et al., 2013). In agreement with this, previous studies have shown that inhibiting mitochondrial function in undifferentiated proliferating cells prevents the cells from differentiating (Mandal et al., 2011). Moreover, treating cells with the mitochondrial biogenesis inducer

S-NitrosoAcetylPenicillamine (SNAP) has been shown to enhance hepatocyte and cardiomyocyte differentiation (Sharma et al., 2009; Kanno et al., 2004), while overexpression of PPARGC1A can promote adipocyte differentiation (Huang et al., 2011). Further, previous work in our lab demonstrated that C2C12 myoblasts treated with mitochondrial biogenesis inducers are more resistant to cell death (Dam et al., 2013). Therefore, it is possible that mitochondrial biogenesis might also protect against and/or limit apoptotic signaling during myogenic differentiation.

Drosophila as a model organism to study skeletal muscle

Drosophila melanogaster, or the fruit fly, is a useful model organism because it is easy to maintain and has a short life cycle. Additionally, there are many genetic tools and fly lines readily available that allow for gene knockdown, overexpression experiments, and fluorescent labelling by simply performing genetic crosses (Orfanos, 2008).

Adult muscle progenitors (AMPs) are undifferentiated muscle progenitor cells, which differentiate to form adult muscle in *Drosophila* (Dobi et al., 2015). AMPs are specified during embryogenesis, when three types of mesoderm-derived myoblasts are specified: muscle founder cells (FCs), fusion-competent myoblasts (FCMs), in addition to the above-mentioned AMPs. The FCs and FCMs differentiate first to form both embryonic and larval muscles, while the AMPs remain in an undifferentiated state throughout larval development and differentiate following metamorphosis to generate the adult muscles (Dobi et al., 2015). Although the assumption was that all AMPs differentiate during adult muscle formation, without reserving a population of

undifferentiated satellite cells (Piccirillo et al., 2014), a recent study has suggested that adult *Drosophila* muscle does contain a population of satellite-like cells capable of proliferating and regenerating damaged muscle (Chaturvedi et al., 2017). Therefore, muscle differentiation can be studied at numerous stages of development, and in response to damage or disease.

Although it is not the most commonly used model organism for *in vivo* skeletal muscle research, *Drosophila* muscle is both functionally and structurally similar to mammalian muscle (Piccirillo et al., 2014). Moreover, the signaling pathways that govern cellular behaviour in mammals are highly conserved in *Drosophila*, suggesting that *Drosophila* can serve as an ideal *in vivo* system for studying skeletal muscle (Piccirillo et al., 2014; Gunage et al., 2017). Moreover, numerous aging-associated changes in mammalian skeletal muscle are also observed in *Drosophila*, such as structural changes and decreased functional capacity, as well as changes in autophagic signaling (Demontis et al., 2013). Additionally, work done in *Drosophila* muscle has provided an *in vivo* system to complement *in vitro* studies highlighting the importance of mitophagy-related proteins in maintaining both mitochondrial and whole muscle integrity (Zhang et al., 2016). Moreover, the recent development of a *Drosophila* line carrying the mt-Keima fluorescent mitophagy reporter has provided a powerful *in vivo* system to study mitophagy mechanisms in muscle (Lee et al., 2018; Cornelissen et al., 2018). Thus, the fruit fly can be an excellent model organism for studying skeletal muscle maintenance and differentiation.

Overall Purpose & Objectives

Autophagy and mitophagy promote myogenic differentiation and support the rebuilding of the mitochondrial network during differentiation (McMillan & Quadrilatero, 2014; Sin et al., 2016). However, studies thus far have utilized models deficient in autophagy-specific proteins, such as ATG5 and ATG7 (McMillan & Quadrilatero, 2014; Sin et al., 2016), and have not addressed the requirement for any specific mitophagy-related proteins during differentiation. Moreover, previous work in our lab has demonstrated that autophagy-deficient myoblasts contain dysfunctional mitochondria and have elevated apoptotic signaling (McMillan, 2015; McMillan & Quadrilatero, 2014). The retention of dysfunctional mitochondria in autophagy-deficient myoblasts would suggest that they are also mitophagy-deficient; however, the requirement for mitophagy specifically during differentiation has not been thoroughly investigated. Therefore, the purpose of this thesis was to investigate the roles of autophagy and mitophagy proteins during myogenic differentiation. Further, we wanted to determine if mitophagy is blocked in ATG7-deficient cells, and if this might account for the differentiation impairments observed in ATG7-deficient cells. Moreover, although there have been numerous mitophagy-related proteins and mechanisms identified (Hamacher-Brady & Brady, 2016), the necessity for these proteins/pathways has not been investigated during myogenic differentiation.

Therefore, the first study (Chapter 2) examined if mitophagy is disrupted in autophagy-deficient myoblasts to determine if proteins involved in regulating autophagy are also necessary for mitophagy. Additionally, we overexpressed mitophagy-related proteins in ATG7-deficient cells in an attempt to enhance mitophagy and improve differentiation. The second study (Chapter 3) investigated the requirement for specific

mitophagy receptors during myogenic differentiation and mitochondrial remodelling. Overall, the goal of these studies was to evaluate if mitophagy and mitochondrial degradation occur in cells deficient in ATG7, or the mitophagy receptor proteins BNIP3 and BNIP3L/NIX, and determine if these mitophagy-related proteins are required for proper myogenesis. Finally, the third study (Chapter 4) investigated whether myogenic differentiation can be recovered in myoblasts deficient in autophagy/mitophagy-related proteins by enhancing mitochondrial biogenesis. Mitochondrial biogenesis is known to accompany myogenesis and is thought to play an important role in regulating cell differentiation (Duguez et al., 2002; Wagatsuma & Sakuma, 2013). Moreover, it has been suggested that mitophagy is a pre-requisite for mitochondrial biogenesis during myogenic differentiation (Sin et al., 2016). Thus, by enhancing mitochondrial biogenesis, our goal was to ameliorate some of the downstream effects of insufficient mitophagy levels in order to enhance myotube formation, reduce cell death and promote the rebuilding of the mitochondrial network.

Therefore, the experiments comprising this thesis attempted to enhance our understanding of how autophagy and mitophagy mediate myogenic differentiation. In summary, the overall objectives of this thesis were:

1) To determine if mitophagy is impaired in ATG7-deficient cells and if enhancing mitophagy can improve myogenic differentiation in ATG7-deficient cells.

2) To determine if the mitophagy-related proteins BNIP3 and BNIP3L are required for myogenesis and mitochondrial network generation.

3) To determine if enhancing mitochondrial biogenesis can compensate for autophagy- or mitophagy-deficiency with respect to myogenic differentiation.

Chapter 2: Examination of mitophagy and mitophagy-related protein expression in ATG7-deficient cells during myogenic differentiation

Project Rationale and Objectives

Autophagy is required for myogenic differentiation, and previous work in our lab has demonstrated that autophagy-deficient myoblasts fail to differentiate properly and show increased apoptotic signaling (McMillan & Quadrilatero, 2014). Sin et al (2016) suggested that it is a deficiency in mitophagy specifically that disrupts myogenic differentiation in autophagy-deficient cells by preventing the clearance of old mitochondria to initiate mitochondrial biogenesis and remodelling during myotube formation (Sin et al., 2016). Further, we have demonstrated that ATG7-deficient myoblasts show increased oxidative stress and mitochondrial dysfunction (McMillan 2015; Baechler et al., 2019). Moreover, studies examining ATG7-deficient muscles have shown that they accumulate dysfunctional and abnormal mitochondria (Masiero et al., 2009; Garcia-Prat et al., 2016). However, it is unclear if mitophagy is completely blocked in autophagy-deficient cells and if they show an increase in the total number of mitochondria relative to controls. Therefore the focus of our initial experiments was to determine if mitophagy increases when C2C12 myoblasts are induced to differentiate, and if there is a complete absence of mitophagy in ATG7-deficient cells. We next focused on measuring proteins associated with mitochondria and mitochondrial biogenesis to determine if mitochondrial remodelling is impaired during myogenic differentiation in sh*Atg7* cells.

Interestingly, although ATG7-deficient erythrocytes show some disruption in mitochondrial degradation (Zhang et al., 2009), this effect is much more severe in BNIP3L-deficient cells (Ney, 2015), suggesting that mitophagy receptor proteins may be more important than general autophagy-related proteins for mitochondrial degradation

during development. Previous reports have suggested that inhibition of autophagy disrupts mitophagy in muscle cells (Sin et al., 2016), so we wondered if overexpressing a mitophagy-related protein in ATG7-deficient cells could augment mitophagy levels, reduce cell death, and rescue myogenic differentiation.

Therefore, the main objectives for Chapter 2 were:

1) To determine if mitochondrial degradation is completely blocked in ATG7-deficient myoblasts/myotubes.

2) To determine if overexpression of a mitophagy-related protein is sufficient to reduce cell death and improve differentiation in ATG7-deficient cells.

We hypothesized that mitophagy would be reduced but not completely blocked in ATG7-deficient cells. Moreover, we speculated that overexpression of mitophagy-related proteins would enhance mitophagy in sh*Atg7* cells and reduce cell death by eliminating dysfunctional mitochondria. Further, we hypothesized that enhancing mitophagy would not rescue myogenic differentiation in sh*Atg7* cells because insufficient mitochondrial degradation is likely not the sole cause of myogenic impairment.

Abbreviations

AIFM1: apoptosis-inducing factor, mitochondrion-associated 1; ATG5: autophagy related 5; ATG7: autophagy related 7; BCL2: B cell leukemia/lymphoma 2; BECN1: beclin 1; BNIP3: BCL2/adenovirus E1B interacting protein 3; BFA: brefeldin A; CASP: caspase; CASP3: caspase 3; CASP9: caspase 9; CQ: chloroquine; CYCS: cytochrome c; DNM1L: dynamin 1-like; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFP:

green fluorescent protein; LC3B: microtubule-associated protein 1 light chain 3 beta; MYH: myosin; MYOG: myogenin; BNIP3L/NIX: BCL2/adenovirus E1B interacting protein 3-like; OPA1: mitochondrial dynamin like GTPase; PPARGC1A: peroxisome proliferative activated receptor, gamma, coactivator 1 alpha; p-H2AFX: phosphorylated H2A histone family, member X; PINK1: PTEN induced putative kinase 1; RAB9: RAB9, member RAS oncogene family; RFP: red fluorescent protein; ROS: reactive oxygen species; SCR: scramble shRNA ; sh*Atg7*: short hairpin RNA against ATG7; SLC25A4: solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator); SOD2: manganese superoxide dismutase; VDAC1: voltage-dependent anion channel 1

Introduction

Cell differentiation is associated with numerous intracellular signaling changes and remodelling events, and it has been suggested that a cell must eliminate its "old" parts to make room for "new" parts to better support its changing structure and metabolic requirements (Mizushima & Komatsu, 2011). Autophagy is a degradative process used to eliminate protein aggregates as well as whole organelles, and requires the coordination of numerous autophagy-related (ATG) proteins, including ATG7 (Kondo et al., 2005; Glick et al., 2010). When autophagy is initiated, a double-membraned autophagosome forms surrounding the organelles/proteins that are to be degraded. Subsequently, the autophagosome delivers its contents to a lysosome for degradation (Kondo et al., 2005; Mizushima & Komatsu, 2011). Autophagic degradation can be quite selective and one form of autophagy, called mitophagy, is used to specifically degrade mitochondria

(Melser et al., 2013). Numerous mitophagy-related proteins have been identified and their necessity is often context dependent, meaning that certain proteins act to eliminate depolarized or dysfunctional mitochondria, while others might eliminate mitochondria for developmental purposes (Hamacher-Brady & Brady, 2016). For example, the mitophagy-related proteins PINK1 and PRKN support the degradation of damaged/depolarized mitochondria (Durcan & Fon, 2015), while the mitophagy receptor BNIP3L serves to remove unnecessary mitochondria during cell differentiation (Sandoval et al., 2008; Schweers et al., 2007).

Autophagy and mitophagy have been shown to play important roles during myogenic differentiation (McMillan & Quadrilatero, 2014; Sin et al., 2016). Previous work in our lab has demonstrated that autophagy-deficient myoblasts fail to differentiate properly (McMillan & Quadrilatero, 2014). Moreover, Sin et al (2016) suggested that blocking autophagy by treating C2C12 myoblasts with bafilomycin A_1 (BAF) disrupts the degradation of old mitochondria during myogenic differentiation, which prevents cells from rebuilding the new mitochondrial network required for myotube formation (Sin et al., 2016).

Further, autophagy/mitophagy is also required to limit unwanted cell death by removing damaged mitochondria that could otherwise release pro-apoptotic factors into the cytosol to trigger cell death processes (Kubli & Gustafsson, 2012; Marino et al., 2014; Quadrilatero et al., 2011). Interestingly, knocking down ATG7 in C2C12 myoblasts causes an increase in mitochondrial dysfunction and apoptotic signaling (McMillan, 2015; Baechler et al., 2019; McMillan & Quadrilatero, 2014). However, studies have shown that mitophagy is more disrupted during erythrocyte differentiation in

BNIP3L-deficient cells than in ATG7-deficient cells (Zhang et al., 2009; Ney, 2015), suggesting that mitophagy receptor proteins might play a more crucial role than general autophagy proteins in executing mitophagy during cell differentiation. Further, studies have also suggested that "alternative mitophagy," which occurs in an ATG7-independent manner and requires the RAB9 GTPase, can degrade mitochondria during differentiation and limit apoptosis (Nishida et al., 2009; Honda et al., 2014; Shimizu et al., 2014; Wang et al., 2016; Hirota et al., 2015). Moreover, additional mechanisms of autophagy-independent mitochondrial degradation have also been described (Hammerling et al., 2017; Oliveira et al., 2015).

Overexpression of mitophagy-related proteins is sufficient to induce mitophagy (Quinsay et al., 2010), limit mitochondrial CYCS release capacity and apoptosis (Zhu et al., 2013) and can improve mitochondrial abnormalities in muscle *in vivo* (Zhang et al., 2016). Therefore, the purpose of this study was to determine if mitophagy occurs in ATG7-deficient (sh*Atg7*) myoblasts during differentiation, and if overexpression of mitophagy-related proteins can enhance mitophagy to reduce cell death and restore myogenic differentiation in ATG7-deficient cells.

Results

Autophagy/mitophagy increases during myogenic differentiation

Autophagy is known to play an important role during cell differentiation in numerous contexts (Phadwal et al., 2013), and previous work in our lab has shown that autophagy increases when C2C12 myoblasts are induced to differentiate (McMillan & Quadrilatero, 2014). In agreement with this, we found that levels of SQSTM1 decrease when

myoblasts are induced to differentiate (Figure 1A, B). Moreover, treating cells with chloroquine (CQ) to inhibit autophagosome-lysosome fusion (Phadwal et al., 2013), confirmed that the decrease in SQSTM1 results from increased autophagy-mediated degradation. Similarly, we found that LC3B-II levels, indicative of autophagosome formation (Mizushima, 2007), showed a mild increase during differentiation that was preserved with CQ (Figure 1A, C).

Next, we wanted to specifically examine if mitophagy increases during differentiation. Therefore, C2C12 myoblasts were transfected with a tandem p-mito-mRFP-EGFP fluorescent mitophagy reporter (Kim et al., 2013). This reporter shows overlapping expression of RFP and GFP when targeted to mitochondria, but when mitochondria are targeted for degradation by autophagosomes/lysosomes, the GFP is degraded resulting in the appearance of RFP-only mitochondria (Kim et al., 2013; Hamacher-Brady & Brady, 2016). As shown in Figure 1D and Figure 1F, proliferating (D0) cells are positive for both RFP and GFP (yellow), suggesting that mitophagy is low or not occurring. Interestingly, when cells are induced to differentiate (D1), the number of cells displaying RFP-only mitochondria increases significantly (Figure 1E, F), suggesting that mitophagy increases during myogenic differentiation.

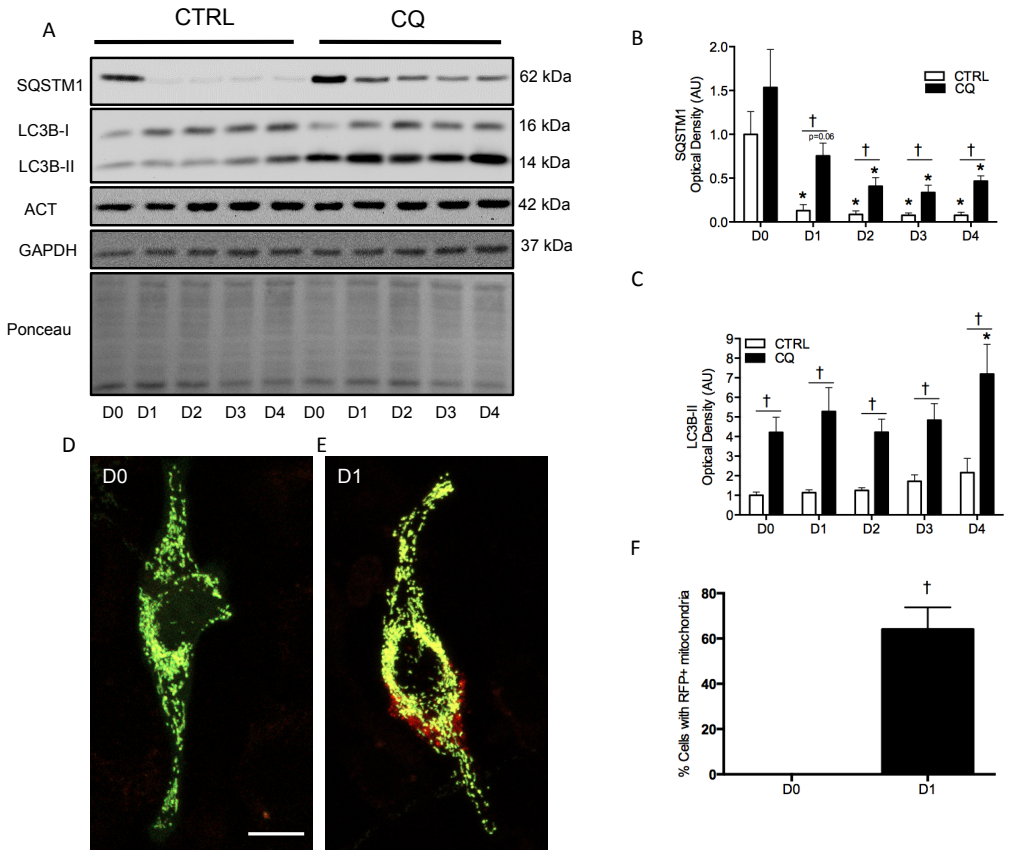

Figure 1. Autophagy and mitophagy increase during myogenic differentiation. Representative immunoblots (A) and quantitative analysis (B-C) of SQSTM1 and LC3B-II in CTRL (Vehicle) and chloroquine (CQ) treated myoblasts during differentiation. Also shown are representative ACT, GAPDH, and ponceau stained loading control blots/membranes. Representative images of proliferating (D) and differentiating (E) myoblasts transfected with p-mito-RFP-GFP. Scale bar=10 µm. Quantitative analysis (F) of the percentage of cells displaying RFP-only mitochondria (indicative of mitophagy). *$p<0.05$ compared to D0 (within group). †$p<0.05$ between groups.

Mitochondrial remodelling is impaired in autophagy-deficient cells

Work conducted by Sin et al (2016) has shown that inhibiting autophagy in C2C12 myoblasts prevents the cells from differentiating and they speculated that differentiation was impaired due to the requirement for mitophagy to allow mitochondrial remodelling to occur (Sin et al., 2016). Therefore, in order to further validate this interpretation, we differentiated ATG7-deficient (sh*Atg7*) and control (SCR) C2C12 myoblasts and measured the expression of mitochondria-related proteins (Figure 2A-H). Interestingly, expression of the mitochondrial biogenesis marker PPARGC1A (Palikaras et al., 2015)

(Figure 2A, B) was lower in sh*Atg7* relative to SCR cells. Moreover, Sin et al (2016) demonstrated that the fission and fusion proteins DNM1L and OPA1 increase during differentiation. In agreement with these findings, we showed that DNM1L (Figure 2A, C) and OPA1 (Figure 2A, D) expression increased in SCR cells during differentiation, but that the expression level of these proteins was lower in sh*Atg7* cells. Additionally, at early time-points (D1, D2) we found that sh*Atg7* cells had elevated expression of SOD2 relative to SCR cells (Figure 2A, E), which could indicate that mitochondria are not being degraded in the autophagy-deficient cell and that higher SOD2 levels result from the retention of mitochondria. However, given that SOD2 is a mitochondrial antioxidant (Bresciani et al., 2015; Candas & Li, 2014), the higher level of expression could suggest an increase in mitochondrial oxidative stress in sh*Atg7* cells. Other mitochondrial markers such as VDAC1 (Figure 2A, F), SLC25A4 (Figure 2A, G), and CYCS (Figure 2A, H), were lower in sh*Atg7* cells relative to SCR cells. This supports that mitochondrial biogenesis and/or remodelling is reduced in ATG7-deficient cells.

Given that others have speculated that mitophagy is needed to trigger mitochondrial remodelling during differentiation (Sin et al., 2016), we measured autophagy/mitophagy-related protein expression in both SCR and sh*Atg7* cells during differentiation (Figure 2I, 2J-L). Interestingly, we found that BECN1 (Figure 2I, J), BNIP3 (Figure 2I, K), and BNIP3L/NIX (Figure 2I, L) expression was generally lower in sh*Atg7* cells relative to SCR cells, which could suggest that autophagy/mitophagy induction is reduced in sh*Atg7* cells.

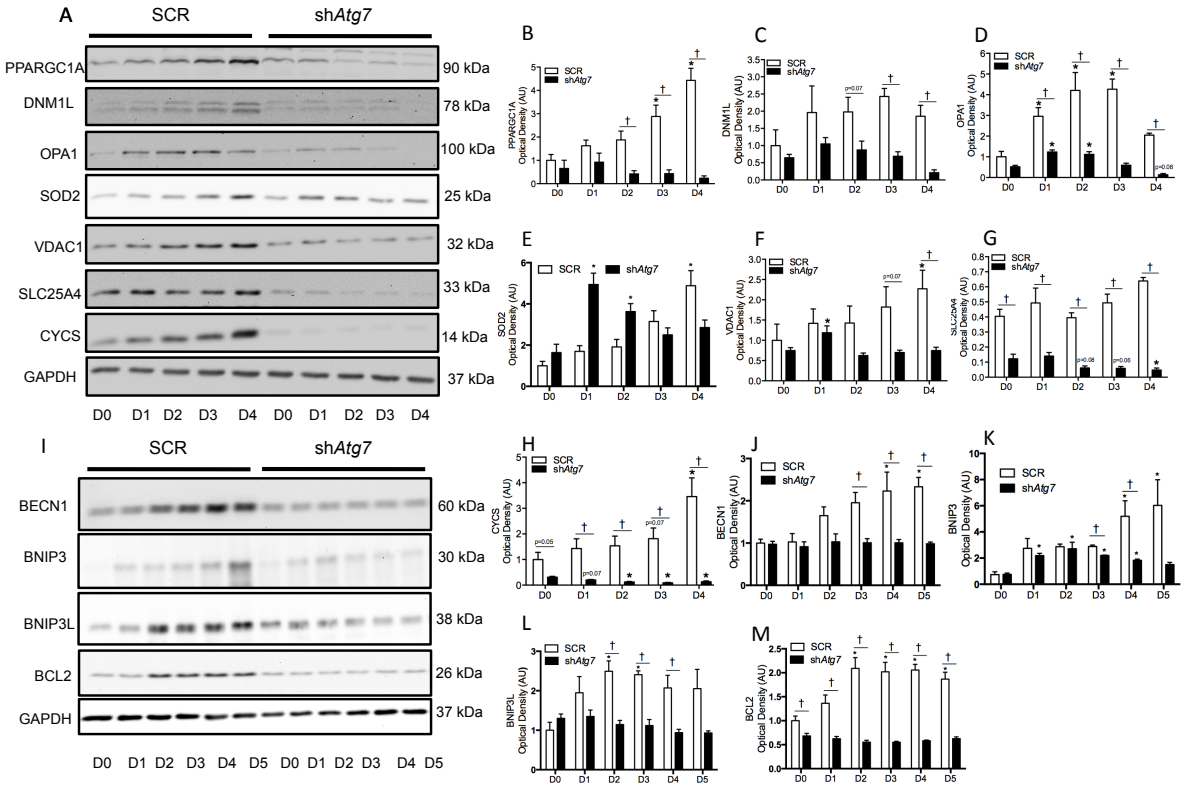

Figure 2. Mitochondrial and autophagy-related protein expression is reduced in sh*Atg7* cells . Representative immunoblots (A) and quantitative analysis (B-H) of PPARGC1A, DNM1L, OPA1, SOD2, VDAC1, SLC25A4, and CYCS in sh*Atg7* and SCR cells during differentiation. Also shown is a representative GAPDH loading control blot. Representative immunoblots (I) and quantitative analysis (J-M) of BECN1, BNIP3, BNIP3L, and BCL2 in sh*Atg7* and SCR cells during differentiation. Also shown is a representative GAPDH loading control blot. *$p<0.05$ compared to D0 (within group). †$p<0.05$ between groups at the same time point.

Mitochondrial damage is associated with increased oxidative stress and apoptotic signalling (Kubli & Gustafsson, 2012), and our previous work has shown that sh*Atg7* cells display more mitochondrial dysfunction which likely contributes to the increase in apoptotic signaling observed in these cells (McMillan, 2015; Baechler et al., 2019; McMillan & Quadrilatero, 2014). Moreover, subcellular fractionation revealed that CYCS and AIFM1 levels were higher in cytosolic-enriched fractions in *shAtg7* cells relatives to SCRs cells (Figure 3A-B). This suggests that mitochondrial integrity is compromised resulting in the release of CYCS and AIFM1 into the cytosol.

Additionally, we found that CASP3 activity was significantly elevated in sh*Atg7* cells at D3 of differentiation (Figure 3C), which could result from the mitochondrial release of CYCS (Wang, 2001). Moreover, we were also able to partially restore myogenic differentiation in sh*Atg7* cells by treating the cells with an adenovirus expressing dominant negative CASP9 (ad-DNCASP9). We observed increased MYOG and MYH expression in ad-DNCASP9-treated cells relative to ad-GFP-treated cells (Figure 3D-E), which supports previous interpretations that excessive CASP9 activity limits myogenic differentiation in sh*Atg7* cells (McMillan, 2015).

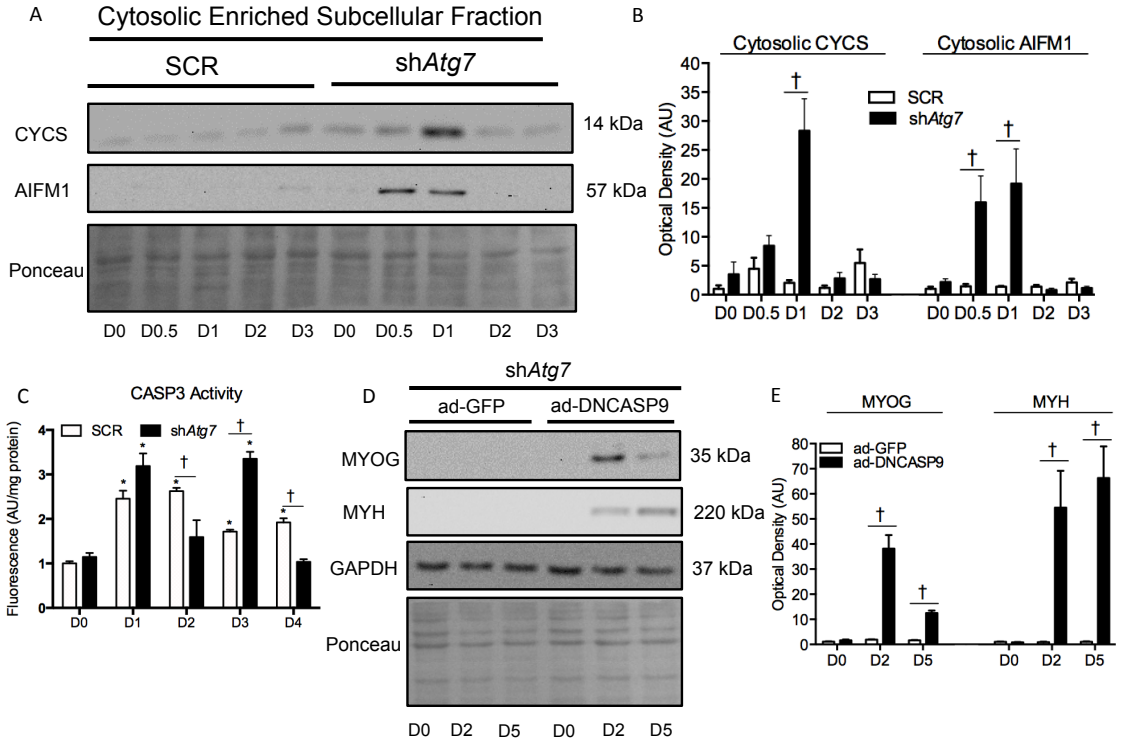

Figure 3. Mitochondrial release of pro-apoptotic factors and CASP3 activation in sh*Atg7* and SCR cells during differentiation. Representative immunoblots (A) and quantitative analysis (B) of cytosolic CYCS and AIFM1 in SCR and sh*Atg7* cells during differentiation. Also shown is a representative ponceau stained membrane. Quantitative analysis (C) of CASP3 activity in sh*Atg7* and SCR cells during differentiation. Representative immunoblots (D) and quantitative analysis (E) of MYOG and MYH in sh*Atg7* cells treated with ad-GFP (control) or an adenovirus expressing dominant-negative CASP9 (ad-DNCASP9) during differentiation. Also shown are representative GAPDH and ponceau control blots/membranes.*$p<0.05$ compared to D0 (within group). $^{\dagger}p<0.05$ between groups at the same time point.

Overexpression of some autophagy/mitophagy-related proteins fails to restore myogenic differentiation in ATG7-deficient cells

Given that the differentiation impairment observed in autophagy-deficient cells is thought to result from a disruption in mitophagy (Sin et al., 2016), we hypothesized that overexpressing an autophagy/mitophagy-related protein might increase mitophagy levels and therefore improve myogenic differentiation in sh*Atg7* cells. Initial experiments involved co-transfecting C2C12 myoblasts with p-mito-mRFP-EGFP and adenoviruses expressing autophagy/mitophagy-related proteins (Ad-PRKN, Ad-BECN1, Ad-BNIP3), and proliferating cells were observed using microscopy (Figure 4A-D). As shown in Figure 4A-C, RFP-only mitochondria were less readily observed in proliferating C2C12 myoblasts (Figure 4A, E) or cells expressing Ad-PRKN (Figure 4B, E) and Ad-BECN1 (Figure 4C, E). RFP-only mitochondria were; however, observed in C2C12 cells expressing Ad-BNIP3 (Figure 4D, E), suggesting that overexpression of BNIP3 might induce mitophagy in C2C12 myoblasts. Next, we treated sh*Atg7* cells with Ad-GFP (control), Ad-PRKN, Ad-BECN1, or Ad-BNIP3, and measured MYOG and p-H2AFX levels to determine if there was any recovery of myogenic differentiation and/or decrease in DNA fragmentation in response to mitophagy-related protein expression. Interestingly, there was no significant change in MYOG or p-H2AFX in cells treated with Ad-PRKN (Figure 4F, I-J) or Ad-BECN1 (Figure 4G, K-L) relative to controls; however, cells treated with Ad-BNIP3 (Figure 4H, M-N) showed a significant increase in MYOG relative to controls (Figure 4M), and reduced p-H2AFX at D2 (Figure 4N). Further, immunostaining for MYH (Figure 5A-F) revealed an increase in the appearance of MYH+ myotubes at D5 (Figure 5C, F) as well as a significantly higher fusion index (Figure 5G) in sh*Atg7* cells overexpressing BNIP3 relative to sh*Atg7* cells expressing

GFP (controls). Moreover, quantitative analysis revealed that although the number of cells decreased in both groups by D5, there was significantly less decline in the groups treated with Ad-BNIP3 (Figure 5H). Although this might indicate that the overexpression of BNIP3 prevents or delays cell death, there was no significant difference in CASP3 activity levels in Ad-BNIP3-treated cells relative to the controls (Figure 5I), suggesting that some cell death occurs in a CASP-independent manner.

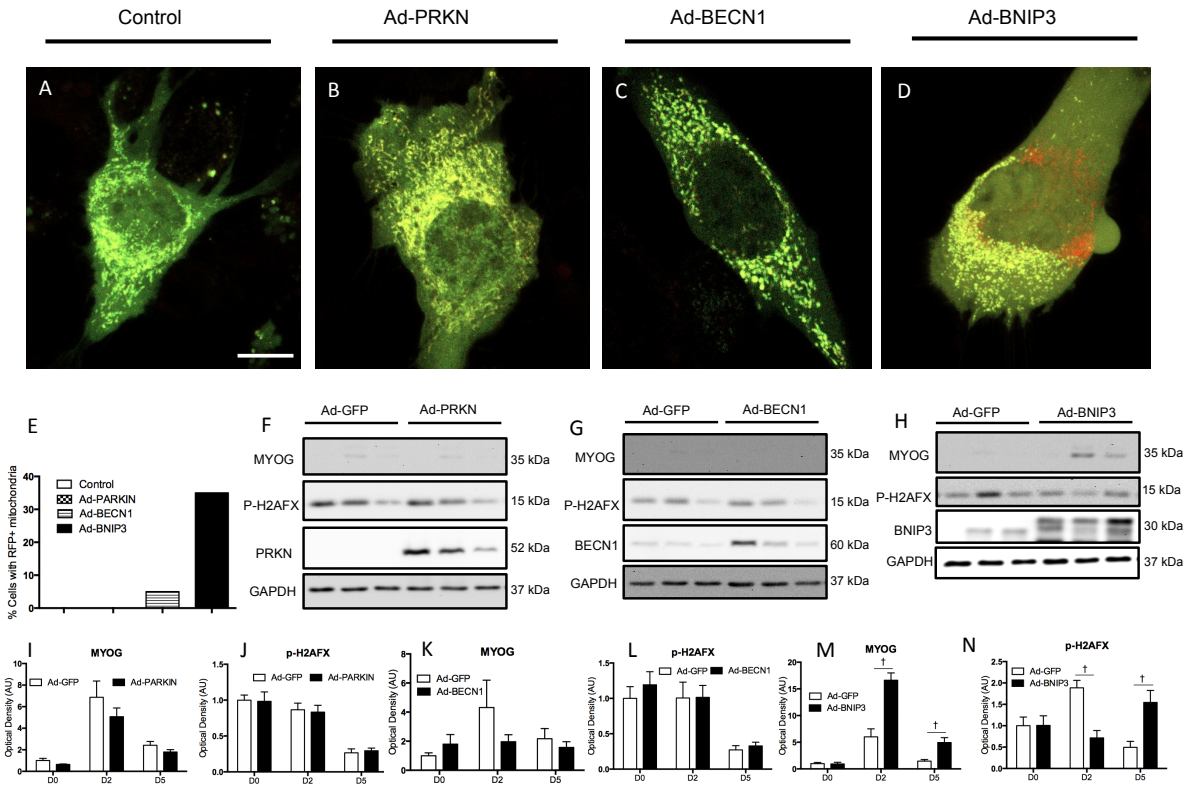

Figure 4. Overexpression of autophagy/mitophagy-related proteins in C2C12 myoblasts and sh*Atg7* cells. Representative images of proliferating C2C12 cells (A) and C2C12 myoblasts expressing Ad-PRKN (B), Ad-BECN1 (C), and Ad-BNIP3 (D). Cells were transfected with p-mito-RFP-GFP, and the appearance of RFP-only mitochondria is indicative of mitophagy. Scale bar=10 µm. Quantification of the percentage of cells containing RFP-only mitochondria (E). Representative immunoblots (F) and quantitative analysis (I-J) of MYOG and p-H2AFX in sh*Atg7* cells treated with Ad-GFP (controls) or Ad-PRKN. Representative immunoblots (G) and quantitative analysis (K-L) of MYOG and p-H2AFX in sh*Atg7* cells treated with Ad-GFP or Ad-BECN1. Representative immunoblots (H) and quantitative analysis (M-N) of MYOG and p-H2AFX in sh*Atg7* cells treated with Ad-GFP or Ad-BNIP3. $^{\dagger}p<0.05$ between groups at the same time point.

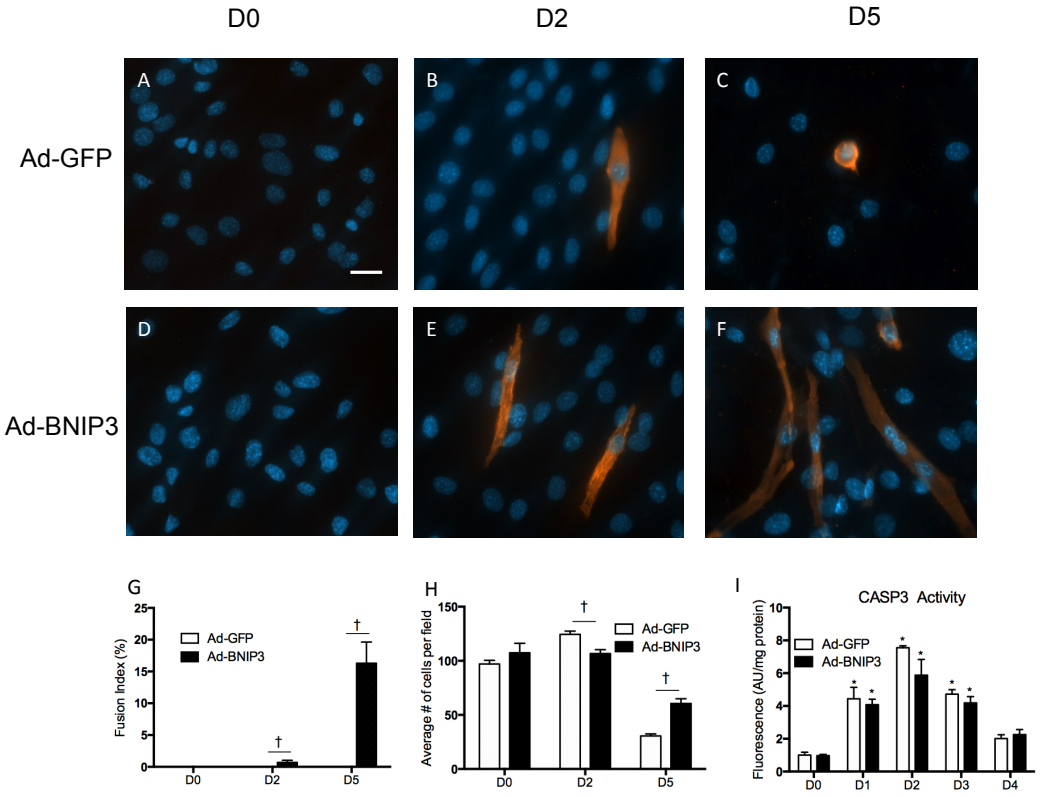

Figure 5. Overexpression of BNIP3 can partially rescue myogenic differentiation in sh*Atg7* cells by reducing cell loss. Representative images of sh*Atg7* cells treated with Ad-GFP (A-C) or Ad-BNIP3 (D-F) throughout differentiation. Anti-MYH (red) was used to visualize myotube formation, and nuclei are labelled with DAPI. Scale bar=10 µm. Quantitative analysis of fusion index (G), cell number (H), and CASP3 activity (I) in Ad-GFP- and Ad-BNIP3-treated sh*Atg7* cells during differentiation. $^{\dagger}p<0.05$ between groups at the same time point. *$p<0.05$ compared to D0 (within group).

ATG7-deficient cells degrade mitochondria in an LC3-independent manner

The next series of experiments aimed to determine if sh*Atg7* cells are in fact unable to degrade mitochondria. First we wanted to quantify the co-localization of mitochondria and autophagosomes as a measure of mitophagy during differentiation. sh*Atg7* and SCR cells were co-transfected with DsRed-Mito to fluorescently label mitochondria and Ad-GFP-LC3 to visualize autophagosome formation. In agreement with reports that mitophagy increases during differentiation (Sin et al., 2016), we saw increased co-localization of mitochondria and LC3-puncta (indicative of autophagosome formation)

early during differentiation in SCR cells (Figure 6A-D, 6I-L), but not in sh*Atg7* cells (Figure 6E-H, L). sh*Atg7* cells also maintained strong, cytosolic GFP expression, demonstrating that LC3 lipidation and autophagosome formation is impaired in sh*Atg7* cells (Figure 6E-H). Interestingly, however, RFP-only mitochondria were observed in both SCR (Figure 7A-C) and sh*Atg7* cells (Figure 7D-F) transfected with p-mito-mRFP-EGFP, suggesting that mitochondria are delivered to lysosomes for degradation in sh*Atg7* cells.

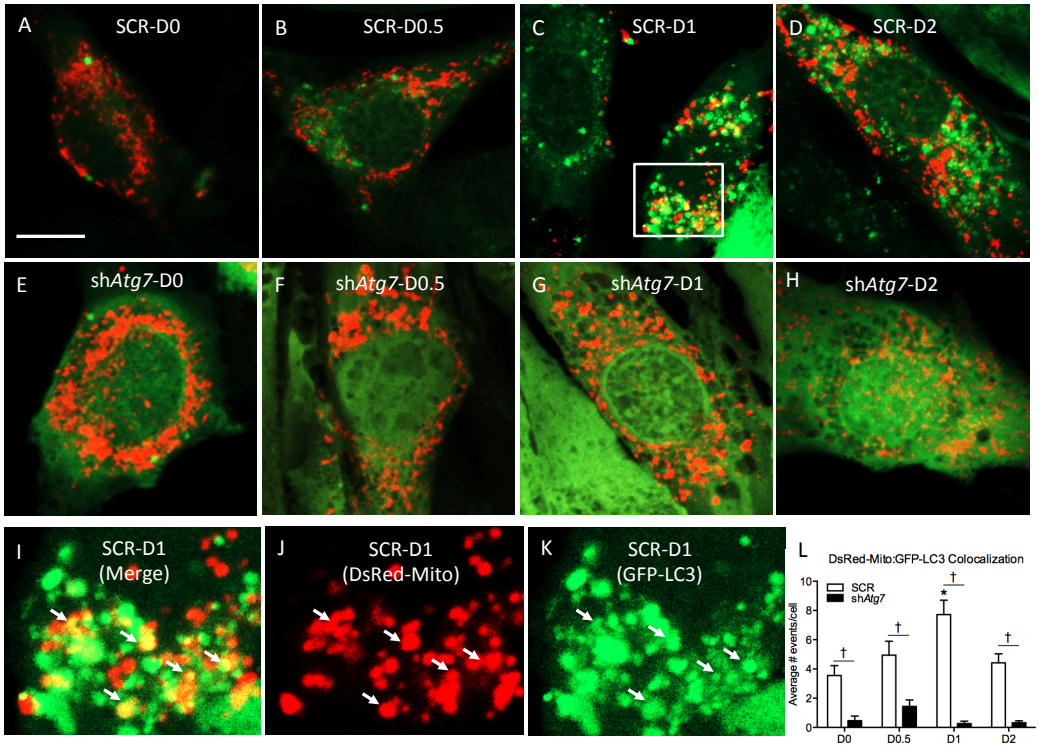

Figure 6. LC3-mediated mitophagy does not occur in differentiating sh*Atg7* cells. Representative images (A-K) and quantitative analysis (L) of co-localization (yellow) of LC3 (green) and mitochondria (red) in Ad-GFP-LC3 and DsRed-Mito co-transfected SCR and sh*Atg7* cells during differentiation. Zoomed in and split channel images of SCR cells at D1 (I-K) demonstrate co-localized mitochondria and LC3 puncta (yellow, arrows). Scale bar=10 μm. $^{\dagger}p<0.05$ between groups at the same time point. *$p<0.05$ compared to D0 (within group).

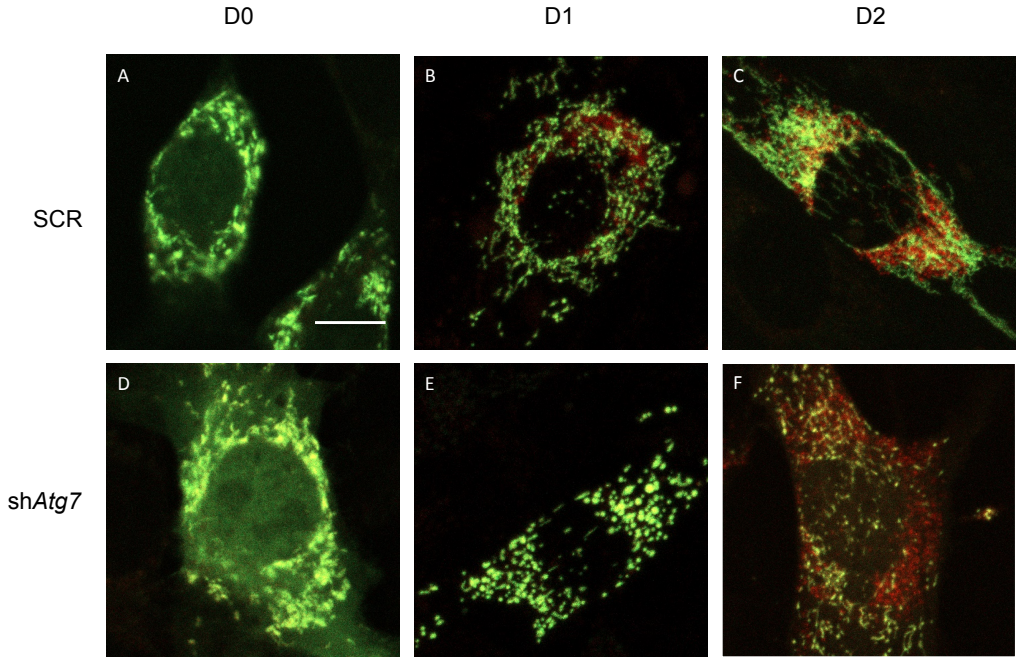

Figure 7. Mitochondrial degradation occurs in both SCR and sh*Atg7* cells during differentiation. Representative images of SCR (A-C) and sh*Atg7* cells (D-F) transfected with p-mito-RFP-GFP. RFP-only mitochondria are observed during differentiation in both groups. Scale bar=10 µm.

Although these results were initially surprising, others have reported that LC3-independent mitophagy does occur in autophagy-deficient cells and is reliant on RAB9 and BECN1 (Nishida et al., 2009; Hirota et al., 2015; Wang et al., 2016). Therefore, sh*Atg7* cells were treated with brefeldin A (BFA), an inhibitor of this alternative autophagy pathway (Nishida et al., 2009; Wang et al., 2016), to determine if sh*Atg7* cells might utilize alternative autophagy to degrade mitochondria. Cells were transfected with p-mito-mRFP-EGFP prior to being treated with BFA or vehicle, and differentiated for one day. Interestingly, RFP-only mitochondria (indicative of mitophagy) were frequently observed in vehicle-treated sh*Atg7* cells at D1 (Figure 8A, C), but rarely observed in BFA-treated cells (Figure 8B-C). Moreover, treating sh*Atg7* cells with BFA caused a more significant decrease in cell number during differentiation than vehicle treatment (Figure 8D-F); however, BFA treatment did not increase CASP3 activity (Figure 8G).

Interestingly, BFA treatment did not prevent C2C12 myoblasts from differentiating and forming numerous MYH-positive myotubes (Figure 8I), suggesting that alternative autophagy is not required for myogenic differentiation in healthy C2C12 cells, but that it might be a compensatory mechanism induced to promote survival in sh*Atg7* cells.

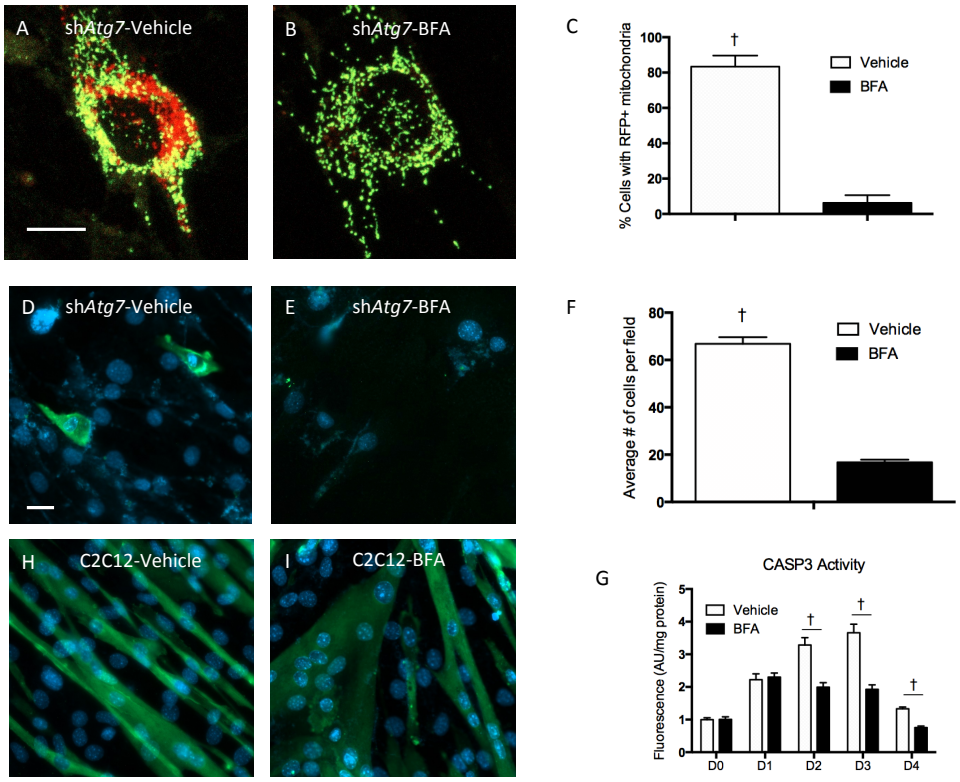

Figure 8. sh*Atg7* cells might utilize alternative mitophagy to degrade mitochondria and limit cell death. Representative images (A-B) and quantitative analysis (C) of the percentage of cells containing RFP-only mitochondria for vehicle- and BFA-treated sh*Atg7* cells transfected with p-mito-RFP-GFP. Cells are shown at D1 of differentiation. Scale bar=10 µm. Representative images (D-E) of myotubes (MYH+) in vehicle- and BFA-treated sh*Atg7* cells. Scale bar=20 µm. Quantitative analysis (F) of the average number of cells per field and CASP3 activity (G) in vehicle- and BFA-treated sh*Atg7* cells. $^{\dagger}p<0.05$ between groups at the same time point. Representative images (H-I) of myotube formation in both vehicle- and BFA-treated C2C12 myoblasts. Scale bar=20 µm.

Discussion

The purpose of this study was to determine if mitophagy occurs in ATG7-deficient myoblasts, and if enhancing mitophagy would reduce cell death and improve myogenic differentiation. It has been suggested that mitophagy increases during myogenic differentiation (Sin et al., 2016), and in this study we used a tandem RFP-GFP mitophagy reporter to support this interpretation (Figure 1). This reporter allows us to confirm that mitochondria are delivered to lysosomes for degradation (Kim et al., 2013; Hamacher-Brady & Brady, 2016) when C2C12 myoblasts are induced to differentiate.

Sin et al (2016) suggested that mitophagy occurs during myogenic differentiation to remove the mitochondria associated with undifferentiated myoblasts to allow mitochondrial biogenesis and remodelling to occur to build the mitochondrial network needed to support a differentiated myotube (Sin et al., 2016). Moreover, they demonstrated that mitochondrial remodelling is impaired in autophagy-deficient cells (Sin et al., 2016). Consistent with these observations, we found that sh*Atg7* cells had reduced levels of PPARGC1A, DNM1L, and OPA1, proteins required for mitochondrial biogenesis, fission, and fusion (Sin et al., 2016), relative to SCR cells (Figure 2A-D). Moreover, expression of the mitochondrial proteins VDAC1, SLC25A4, and CYCS was also lower in sh*Atg7* cells, which suggests that mitochondrial content is reduced in differentiated sh*Atg7* cells (Figure 2A, F-H). Taken together, these results indicate that the rebuilding of the mitochondrial network is impaired in sh*Atg7* cells, likely due to a failure to induce mitochondrial biogenesis.

Given that mitophagy is considered a pre-requisite for mitochondrial biogenesis during differentiation (Sin et al., 2016), and based on our results indicating that

mitochondrial biogenesis is impaired in sh*Atg7* cells, we next measured the expression of some autophagy- and mitophagy-related proteins. Interestingly, we found that BECN1, BNIP3, and BNIP3L/NIX levels were generally lower in sh*Atg7* cells (Figure 2I, J-L), which could suggest that autophagy/mitophagy is reduced. BECN1 is an important regulator of autophagy and also plays a role in canonical and alternative mitophagy, while BNIP3 and BNIP3L have been shown to mediate mitophagy in numerous contexts (Choubey et al., 2014; Hirota et al., 2015; Hamacher-Brady & Brady, 2016); therefore, insufficient levels of these proteins could prevent mitochondria from being effectively targeted and degraded. Moreover, SOD2 levels were higher in sh*Atg7* cells at early time-points during differentiation (Figure 2A, E), which could suggest an increased level of oxidative stress and be indicative of impairment in mitochondrial degradation (Bresciani et al., 2015; Sebori et al., 2018).

Additionally, expression of the anti-apoptotic protein BCL2 (Levine et al., 2008) was significantly lower in sh*Atg7* cells at all time-points throughout differentiation (Figure 2I, 2M), and previous work done in our lab has demonstrated that apoptotic signaling is elevated in sh*Atg7* cells (McMillan & Quadrilatero, 2014; McMillan, 2015). Increased apoptotic signaling can result from mitochondrial damage, and in support of this, subcellular fractionation revealed that cytosolic levels of CYCS and AIFM1 were elevated in sh*Atg7* cells relative to SCR cells (Figure 3A-B). CYCS and AIFM1 are mitochondrial proteins that can be released into the cytosol if mitochondrial integrity is disrupted, and can initiate CASP9 activation and CASP-dependent cell death as well as CASP-independent forms of cell death (Wang, 2001). Previous work in our lab has demonstrated that CASP9 activity is elevated in sh*Atg7* cells and that myogenesis is

partially restored when cells are treated with a CASP9 inhibitor (McMillan, 2015). In support of this, we found that treating sh*Atg7* cells with ad-DNCASP9 improved myogenic differentiation (Figure 3D-E), which suggests that mitochondria-mediated apoptotic signaling contributes to the differentiation impairments observed in sh*Atg7* cells. Interestingly, we saw differences in CASP3 activity in sh*Atg7* cells relative to SCR cells (Figure 3C); however, levels were not higher in sh*Atg7* cells at all time-points during differentiation. Therefore, we suspect that sh*Atg7* cells are eliminated using both CASP-dependent and CASP-independent cell death mechanisms, which could result from an accumulation of leaky/dysfunctional mitochondria (Kubli & Gustafsson, 2012). Thus, further investigation of both CASP-dependent and CASP-independent cell death in sh*Atg7* cells is warranted.

Mitophagy eliminates damaged/dysfunctional mitochondria that might otherwise induce intracellular cell death signaling; therefore, it is not surprising that the expression of mitophagy-related proteins can protect against apoptosis by inducing mitophagy (Zhu et al., 2013). Thus, we attempted to induce mitophagy in C2C12 myoblasts using adenoviruses expressing the autophagy/mitophagy-related proteins PRKN, BECN1, and BNIP3. Mitophagy was then assessed using the p-mito-RFP-GFP mitophagy reporter. This reporter labels mitochondria with overlapping RFP and GFP expression; however, if mitochondria are targeted to lysosomes, the GFP expression is lost first and the mitochondria will be labeled with RFP only (Kim et al., 2013; Hamacher-Brady & Brady, 2016) (Figure 4A-D). Using this reporter, RFP-only mitochondria (indicative of mitophagy) were rarely observed in proliferating myoblasts, but could easily be detected in C2C12 cells overexpressing BNIP3 (Figure 4A-E). Previous reports have shown that

BNIP3 can induce both autophagy and mitophagy (Bellot et al., 2009; Hanna et al., 2012; Zhang et al., 2016), as well as restore mitophagy in BNIP3L-deficient erythrocytes (Zhang et al., 2012), thus it is not surprising that we saw mitophagy occurring in BNIP3-overexpressing cells. However, given that the p-mito-RFP-GFP reporter provides a more qualitative measure of mitophagy, it is difficult to be certain that mitochondrial degradation occurred more frequently in BNIP3-overexpressing cells than in BECN1- or PRKN-overexpressing cells. Interestingly, although we have tried several antibodies, we have never detected endogenous PRKN expression in C2C12 myoblasts in our lab, although we can detect it in other cell types and in cells treated with Ad-PRKN (see Figure 4F; Bloemberg, 2017), which conflicts with previous reports (Peker et al., 2018; Baldelli et al., 2014). This could indicate that the antibodies are not appropriate for mouse cells, or it could mean that PRKN is not expressed in C2C12 cells and would not serve a mitophagy-related purpose. Other studies have shown that PRKN is expressed at very low levels or not at all in some cell types, and have often employed systems to overexpress PRKN to study mitophagy (Hirota et al., 2015; Narendra et al., 2008). Further, most studies have used mitochondrial stressors/depolarizing agents to study PINK1/PRKN-mediated mitophagy (Narendra et al., 2008; Hirota et al., 2015); however, endogenous PRKN has not been detected in our lab in response to treating C2C12 myoblasts with mitochondrial stressors (Bloemberg, 2017). However, in order to determine if overexpression of PRKN (using Ad-PRKN) can promote mitophagy in C2C12 myoblasts, it might be necessary to treat cells with a mitochondrial stressor or depolarizing agent. Additionally, we also failed to rescue myogenic differentiation by overexpressing BECN1 in sh*Atg7* cells. Thus, although BECN1 plays an important role

in mediating autophagy and mitophagy (Liang et al., 1999; Choubey et al., 2014; Hirota et al., 2015), its overexpression might cause a more broad effect and be less mitophagy-specific. Further, BECN1 overexpression has been shown to have negative consequences with respect to cell survival in some contexts (Zhu et al., 2018).

Interestingly, the assumption has been that BNIP3-mediated mitophagy requires the direct interaction between mitochondria-targeted BNIP3 and LC3 on the autophagosome (Hanna et al., 2012; Zhu et al., 2013; Shi et al., 2014). Therefore, given that we did not observe the formation of LC3 puncta in sh*Atg7* cells during differentiation, suggesting that LC3+ autophagosomes do not form in the absence of ATG7 (Figure 6), it is unlikely that overexpression of BNIP3 in sh*Atg7* cells is enhancing mitophagy through a direct interaction with LC3. However, if there is some LC3 lipidation/autophagosome formation occurring in sh*Atg7* cells, then one could speculate that having more BNIP3 available to interact with that small number of autophagosomes could cause a slight increase in mitochondrial degradation. Further, studies investigating mitophagy during erythrocyte differentiation have determined that the functionally similar protein BNIP3L contains a short sequence called a minimal essential region (MER), which does not interact with LC3 but is required for mitochondrial clearance (Zhang et al., 2012). The same authors also demonstrated that BNIP3 contains the same MER sequence as BNIP3L, and can compensate for BNIP3L to degrade mitochondria during erythrocyte differentiation (Zhang et al., 2012). Thus, they and others have suggested that this developmentally induced mitophagy might use an alternative autophagy pathway that is independent of LC3 (Zhang et al., 2012; Nishida et al., 2009). Given that BNIP3 is functionally similar to BNIP3L and also contains this MER

sequence (Zhang et al., 2012), this would suggest that BNIP3 may be able to promote LC3-independent mitophagy in sh*Atg7* cells to prevent the accumulation of unnecessary or damaged mitochondria that might otherwise disrupt differentiation and trigger cell death (Zhang et al., 2012; Zhu et al., 2013). Moreover, recent work has suggested that BNIP3 can promote LC3-independent, endosome-mediated mitochondrial degradation in autophagy-deficient cells (Hammerling et al., 2017). Therefore, the partial recovery of myogenic differentiation observed in sh*Atg7* cells overexpressing BNIP3 (Figure 4H, 4M, Figure 5A-G), but not in those overexpressing BECN1 (Figure 4G, K) or PRKN (Figure 4F, I) could result from BNIP3-induced mitochondrial clearance, which is necessary for myogenic differentiation (Sin et al., 2016). Interestingly, muscle-specific overexpression of BNIP3 can also effectively rescue the myogenic abnormalities observed in *Drosophila PINK1* mutants (Zhang et al., 2016).

In addition to its established role in limiting cell death by eliminating dysfunctional mitochondria (Zhu et al., 2013), BNIP3 can also inhibit cell death in a mitophagy-independent manner (Burton et al., 2009; Burton et al., 2013). Studies have shown that BNIP3 can transcriptionally repress the expression of the genes encoding apoptosis inducing factor (AIFM1) and death receptor-5 (DR5) to inhibit apoptosis (Burton et al., 2009; Burton et al., 2013), and potentially limit other forms of cell death (Artus et al., 2010; Baritaud et al., 2012). As shown in Figure 5I, we did not detect a significant change in CASP3 activity in Ad-BNIP3-treated cells relative to Ad-GFP-transfected cells (controls), suggesting that overexpression of BNIP3 did not cause a reduction in CASP-mediated apoptotic signaling. However, we did detect a significant reduction in p-H2AFX in BNIP3-overexpressing cells relative to controls at D2, although

p-H2AFX levels increased in BNIP3-overexpressing cells by D5 (Figure 4H, 4N). Cell loss/death generally increases after D3 of differentiation in the sh*Atg7* cells, and elevated p-H2AFX levels are associated with apoptotic cell death (Rogakou et al., 2000) as well as alternate forms of cell death like necroptosis/necrosis (Artus et al., 2010; Baritaud et al., 2012). This would suggest that overexpression of BNIP3 might delay cell death in sh*Atg7* cells, but that it does not provide long-term protection against cell death. Therefore, it would be interesting to determine if BNIP3 suppresses apoptotic and/or non-apoptotic cell death in sh*Atg7* cells by transcriptionally inhibiting the expression of genes such as *AIFM1*.

Although the co-localization of LC3+ puncta (autophagosomes) and mitochondria was rarely observed in sh*Atg7* cells during differentiation (Figure 6), transfecting sh*Atg7* cells and SCR cells with the p-mito-RFP-GFP mitophagy reporter revealed the presence of RFP-only mitochondria in both groups, which suggests that mitochondrial degradation does occur in sh*Atg7* cells during differentiation (Figure 7). This result was surprising; however, consultation with the literature revealed that others have observed LC3-independent autophagy/mitophagy in ATG7-deficient cells (Nishida et al., 2009; Hirota et al., 2015; Wang et al., 2016). Nishida et al (2009) had discovered that autophagosomes can form in the absence of ATG5 and ATG7, and that they are likely derived from the Golgi apparatus (Nishida et al., 2009). They also demonstrated that this alternative/non-canonical autophagy pathway does not involve LC3 lipidation, and requires the intracellular trafficking protein RAB9 (Nishida et al., 2009). Moreover, this group also suggested that mitophagy occurring during erythrocyte differentiation might actually utilize this alternative autophagy pathway, an interpretation that has gained

support through additional studies (Nishida et al., 2009; Honda et al., 2014). Additionally, others have shown that mitophagy induced by starvation or hypoxia requires RAB9, but is not affected by knocking down ATG7 or LC3 (Hirota et al., 2015). Thus these results, in agreement with previous reports (Nishida et al., 2009; Hirota et al., 2015), demonstrate that LC3 lipidation might not be the best or most accurate measure of autophagy, and that additional methods such as electron microscopy, should be considered to detect both canonical and alternative autophagy/mitophagy. Further, a more recent study has demonstrated that RAB5/RAB7-positive endosomes can deliver mitochondria directly to lysosomes for degradation (Hammerling et al., 2017).

In order to determine if the mitochondrial degradation observed in sh*Atg7* cells (Figure 7) is dependent on the alternative mitophagy pathway, we treated sh*Atg7* cells with the alternative autophagy inhibitor brefeldin A (BFA) (Nishida et al., 2009; Wang et al., 2016), and found that mitochondrial degradation was effectively blocked by BFA treatment (Figure 8A-C). Additionally, we found that myogenic differentiation and cell loss was more severe in sh*Atg7* cells treated with BFA relative to vehicle-treated cells (Figure 8D-F), which is consistent with reports that alternative mitophagy is needed to suppress cell death in autophagy-deficient cells (Wang et al., 2016). Moreover, CASP3 activation was actually lower in BFA-treated cells relative to controls (Figure 8G), which suggests that the increased cell loss observed in BFA-treated sh*Atg7* cells may result from CASP-independent cell death (Gudipaty et al., 2018; Tait et al., 2014). However, it is also possible that CASP3 levels were lower because the least healthy cells have already been eliminated and CASP3 activity is being measured in the surviving/healthiest cells. Interestingly, we saw no impairment in myogenic differentiation in C2C12 myoblasts

treated with BFA (Figure 8H-I), which suggests that alternative autophagy/mitophagy might limit cell loss in sh*Atg7* cells, but might not be required for proper myogenesis in healthy C2C12 myoblasts. Interestingly, Wang et al (2016) found that although ATG7-deficient leukemia cells fail to differentiate properly, they are also resistant to apoptosis because alternative mitophagy can effectively degrade mitochondria and regulate intracellular ROS levels (Wang et al., 2016). This supports previous work in our lab demonstrating that sh*Atg7* cells fail to differentiate (McMillan & Quadrilatero, 2014). Moreover, we also demonstrated that mitochondrial degradation does occur in sh*Atg7* cells and could be providing some protection against cell death (Figure 8). However, although we suspect that alternative mitophagy can help to limit apoptosis in sh*Atg7* cells, our previous work has shown that these cells retain dysfunctional mitochondria and show elevated ROS levels and apoptotic signaling (McMillan, 2015; Baechler et al., 2019; McMillan & Quadrilatero, 2014). Moreover, the cytosolic release of CYCS and AIFM1 (Figure 3A-B) suggests that leaky/damaged mitochondria are not being efficiently eliminated in sh*Atg7* cells. Therefore, alternative mechanisms of mitochondrial degradation might not be sufficient to compensate for impairment in canonical/ATG7-dependent autophagy/mitophagy during myogenic differentiation. Further, blocking the alternative mitophagy pathway in healthy C2C12 myoblasts during differentiation did not inhibit myogenesis (Figure 8H-I), suggesting that alternative autophagy/mitophagy is likely not as important as canonical/ATG7-dependent mitophagy during myogenic differentiation.

Given our evidence that the elimination of dysfunctional mitochondria is compromised in sh*Atg7* cells, it is important to consider that the numerous mechanisms in

place to degrade mitochondria might differ in how they identify and target mitochondria for degradation. In other words, autophagy/mitophagy-mediated mitochondrial degradation might be more important for eliminating damaged mitochondria, while endosomal-mediated mitochondrial degradation, which can occur more rapidly (Hammerling et al., 2017) might be less likely to distinguish between healthy and dysfunctional mitochondria. Moreover, the p-mito-RFP-GFP mitophagy reporter used for this study relied on a tandem RFP-GFP construct that localizes to the mitochondria (Kim et al., 2013), thus it is possible that the RFP and GFP proteins could have been degraded in an autophagy-independent manner.

Conclusion

Overall, these experiments have demonstrated that autophagy and mitophagy are important for myogenic differentiation. We also found that mitochondrial remodelling and biogenesis were impaired in sh*Atg7* cells, which could result from insufficient mitophagy. Interestingly, we found that LC3-dependent autophagy/mitophagy does not occur in sh*Atg7* cells, but that mitochondrial degradation can occur in an LC3-independent manner. However, we also suspect that the specific targeting of damaged or dysfunctional mitochondria is impaired in sh*Atg7* cells, given that sh*Atg7* cells have increased mitochondrial damage and dysfunction relative to SCR cells. Therefore, we speculate that canonical autophagy/mitophagy is critical to limit apoptotic signaling and cell death during myogenic differentiation, although additional studies are required to determine how mitochondrial targeting and degradation is regulated during myogenesis.

Materials and Methods

Cell culture, chemical treatments

Culturing conditions have been described previously (McMillan & Quadrilatero, 2014). Briefly, low pass C2C12 myoblasts (A.T.C.C.) were plated in polystyrene cell culture dishes in growth media (GM) composed of low- glucose Dulbecco's modified Eagle's medium (DMEM), 10% FBS, and 1% penicillin/streptomycin (P/S), and were maintained at 37 °C in 5% CO2. GM was changed every 24-48 hours. When cells were ready to differentiate (80-90% confluent), D0 cells were collected and GM was exchanged for differentiation media (DM) consisting of low-glucose DMEM, 2% horse serum, and 1%P/S in the remaining wells. DM was replaced each day until cells were collected at the appropriate time-points (D1, D2, etc.). For collections, cells were trypsinized (0.25% trypsin with 0.2g/l EDTA), centrifuged (1000g for 5 min), and stored at -80°C.

ATG7 knockdown and SCR cell lines were generated previously in our lab (Bloemberg, 2017) by growing C2C12s in 12-well plates and transfecting them with vectors encoding an shRNA against ATG7 (Origene TG504956), or a SCRble control sequence (Origene TR30013). For transfections, vector DNA and Lipofectamine 2000 was diluted in Opti-MEM and the mixture was added to cells for a 6 hour incubation. 24 hours post-transfection, cells were transferred to 100 mm plates and grown in GM containing puromycin (2 μg/mL) and stable clones were selected. Immunoblotting was then used to evaluate ATG7 levels in selected clones.

Brefeldin A (BFA) (B677240; Toronto Research Chemicals) treatments were performed as described previously to inhibit alternative mitophagy (0.1 μg/mL; Wang et al., 2016). BFA was diluted in DM and replaced each day during differentiation.

Fluorescent mitophagy reporters/microscopy

Cells were grown on glass coverslips coated with Cultrex BME (Trevigen, 3432-010-01) in 12-well plates and transfected with pDsRed2-Mito vector (generously provided by Dr. Douglas Green, St Jude's Children's Research Hospital, Memphis, TN) or p-mito-RFP-GFP vector (kindly provided by Dr. Andreas Till; Kim et al., 2013) upon reaching 60-70% confluence. jetPRIME Transfection Reagent (Polyplus-transfection, 114-07) was used for all transfections according to the manufacturer's instructions. For co-localization analysis, GFP-LC3 adenovirus (ad-GFP-LC3; kindly provided by Dr. Gökhan S. Hotamisligil, Harvard School of Public Health, Boston, MA) was added to wells containing cells transfected with pDsRed2-Mito for overnight incubation. The next day, cells were washed and imaged (D0) or induced to differentiate. For imaging, coverslips were mounted on glass slides using Prolong Gold Antifade Reagent (ThermoFisher Scientific, P36930) and imaged on a Zeiss LSM 800 (Carl Zeiss). To assess mitophagy, we counted the number of co-localization events (overlapping GFP-LC3 punta and pDsRed2-Mito-labelled mitochondria). Chloroquine (CQ;10 μm) was added to each well one day prior to imaging to assist with counting co-localization events. For p-mito-RFP-GFP qualitative experiments, we observed the change from GFP+RFP+ mitochondria to RFP+-only mitochondria as being indicative of mitophagy.

Adenoviruses, amplification, and tittering

The following adenoviruses were used for this study: Ad-PRKN, Ad-BNIP3, Ad-BECN1 (kindly provided by Dr. Abhinav Diwan), Ad-GFP, Ad-LC3-GFP (Yang et al., 2010; kindly provided by Dr. Gökhan S. Hotamisligil), and ad-DNCASP9 (generously provided by Dr. Paul Dent). Adenoviruses were amplified using the ViraPower Adenoviral

Expression System protocol (Life Technologies). Briefly, HEK293 cells were plated in growth media and viral lysate was added to each plate of confluent cells. The cells were then placed at 37°C (CO_2 incubator) until 80-90% of the cells had rounded up and were starting to detach from the plate. At this point, the cells and media were collected using a serological pipette and placed in a 15 mL falcon tube. The tube was then placed at -80°C for 30 minutes, then thawed in a 37°C waterbath for 15 minutes. This freeze/thaw cycle was repeated for a total of 3 times. The cell lysate was then centrifuged at 3000 rpm for 15 minutes at room temperature. Subsequently, the pelleted debris was discarded and the supernatant (crude virus) was transferred to cryotubes and stored at -80°C.

Viral titers were determined using the Adeno-X Rapid Titer Procedure (Clontech Laboratories, Inc.). HEK293 cells were grown on coverslips coated with Cultrex BME (Trevigen) in 12-well plates. Using growth media as a diluent, we prepared 10-fold serial dilutions of adenovirus stocks and added them to the appropriate wells. Cells were incubated at 37°C (5% CO_2) for 48 hours. Following incubation, cells were rinsed and fixed in 1 mL cold 100% methanol at -20 °C for 10 minutes. After fixation, cells were rinsed with PBS containing 1% BSA, then incubated with mouse anti-Hexon antibody (Santa Cruz Biotechnology) for 1 hour at 37°C on a shaker. Cells were then rinsed and incubated for 1 hour with goat anti-mouse Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific). Cells were subsequently washed in PBS and mounted on glass slides using Prolong Gold Antifade Reagent (ThermoFisher Scientific, P36930) and imaged on a Zeiss LSM 800 (Carl Zeiss). Six to ten fields containing 5-50 green (Hexon+) cells were counted, and used to calculate infectious units/ml. For all experiments, C2C12 myoblasts were treated with adenoviruses (overnight incubation) at

a multiplicity of infection (MOI) of 100 (for Ad-GFP, Ad-PRKN, Ad-BECN1, and Ad-BNIP3) or 60 (for ad-DNCASP9 and Ad-GFP experiments), and viruses were removed before differentiation was induced.

Immunoblotting

Western blotting was performed as described previously (McMillan & Quadrilatero, 2011; McMillan & Quadrilatero, 2014). Cells were sonicated 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 protease inhibitor cocktail. Protein concentrations were determined using the BCA method and equal protein amounts were loaded and separated on 12% SDS- PAGE gels. Proteins were then 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°C in primary antibodies against: SQSTM1 (PM045; MBL), BNIP3, ACT (B7931, A2066; Sigma-Aldrich), SOD2 (ADI-SOD-110; Enzo Life Sciences), GAPDH, BNIP3L, DNM1L, LC3B, ATG7, BECN1 (2118, 12396, 8570, 2775, 8558, 3738; Cell Signaling), MYOG, MYH, (F5D, MF20; Developmental Studies Hybridoma Bank), AIFM1, BCL2, SLC25A4, CYCS, PPARGC1A, VDAC1, p-H2AFX, OPA1, PRKN (sc-13116, sc-7382, sc-9299, sc-13156, sc-13067, sc-390996, sc-101696, sc-393296, sc-32282; Santa Cruz Biotechnology). Primary antibodies were removed and membranes were washed in TBS-T then incubated for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). TBS-T washes were then repeated and bands were visualized using the Clarity Western ECL substrate (Bio-Rad) and the ChemiGenius 2 Bio-Imaging System (Syngene).

Immunofluorescence and microscopy

Immunofluorescent staining of cells was performed as described previously (McMillan & Quadrilatero, 2014). Cells were grown and differentiated on glass coverslips coated with Cultrex BME (Trevigen). Cells were then fixed with ice-cold methanol, and washed with PBS. Cells were permeablized with 0.5% Triton X-100 for 10 minutes and washed in PBS. Cells were then blocked in 10% goat serum (Sigma–Aldrich) in PBS for 30 minutes, followed by incubation with anti-MYH primary antibody (MF20; DSHB) in fresh blocking solution for 1 hour. PBS washes were then repeated. Next, coverslips were incubated for 1 hour in the appropriate fluorochrome-conjugated secondary antibody diluted in blocking solution. Subsequently, PBS washes were repeated, cells were stained with DAPI nuclear stain (Life Technologies; D3571) for 5 minutes, and then washed with PBS. Coverslips were placed on slides and Prolong Gold Antifade Reagent was used for mounting. Slides were imaged the following day using an Axio Observer Z1 microscope equipped with an AxioCam HRm camera and AxioVision software (Carl Zeiss). Fusion index was calculated as the percentage of nuclei present in multinucleated (two or more nuclei) cells relative to total nuclei, and cell number was determined by counting the number of nuclei per field.

CASP assays

CASP3 and CASP9 assays were performed as described previously (McMillan et al., 2015; Baechler et al., 2019). Cells were sonicated in lysis buffer without protease inhibitors, and CASP3 and CASP9 activity was determined using the fluorogenic substrates Ac-DEVD-AFC and Ac-LEHD-AFC (AAT Bioquest, 13401; Tocris Bioscience, 1575), respectively. Fluorescence was measured using a Synergy H1

microplate reader (BioTek) in duplicate. CASP activity was normalized to total protein content determined using the BCA protein concentration assay.

Statistics

Statistical analysis was performed using GraphPad PRISM. A one-way ANOVA was used to assess the effect of differentiation within groups, with Bonferroni's multiple comparison test to compare differences from D0. Differences between time-matched SCR and sh*Atg7* cells, and treatment versus control groups were assessed using a Students T-test. For all experiments $p<0.05$ was considered statistically significant. For all immunoblotting and CASP activity assays, n = 3 or more. For fluorescent reporter and microscopy experiments, n = 15-30 cells.

Chapter 3: Examining the importance of autophagy/mitophagy-related protein expression during myogenic differentiation

Project Rationale and Objectives

Autophagy and mitophagy are considered to be necessary processes during myogenic differentiation (McMillan & Quadrilatero, 2014; Sin et al., 2016). In support of this, deficiencies in the autophagy-related proteins ATG7 and ATG5 and the mitophagy-related protein PRKN, have been shown to disrupt myogenesis and muscle quality (McMillan & Quadrilatero, 2014; Sin et al., 2016; Peker et al., 2018). Interestingly; however, our lab has not been able to detect PRKN expression in C2C12 cells (Bloemberg, 2017; Chapter 2- Figure 4). Therefore, we wondered if the expression of other mitophagy-related proteins might be more critical for degrading mitochondria during differentiation in C2C12 myocytes. Interestingly, previous work in our lab demonstrated that expression of the mitophagy receptor protein BNIP3 increases during myogenic differentiation (McMillan & Quadrilatero, 2014), which prompted us to explore if BNIP3 plays a role in regulating mitophagy during myogenesis. Moreover, in Chapter 2 we found that BNIP3 was able to partially rescue myogenic differentiation in ATG7-deficient cells. Further, the closely-related mitophagy receptor BNIP3L/NIX is known to play an important role in regulating mitophagy during erythrocyte differentiation (Sandoval et al., 2008; Schweers et al., 2007), thus, we also wanted to evaluate if it is required to support myogenic differentiation.

Therefore, the major objectives for Chapter 3 were:

1) To determine if the mitophagy-related proteins BNIP3 and BNIP3L are required for myogenic differentiation.

2) To determine if autophagy and mitophagy are altered in BNIP3-deficient cells.

We hypothesized that myogenic differentiation would be disrupted in myoblasts deficient in BNIP3L or BNIP3 because we suspected that one or both of these proteins would be involved in mediating mitophagy during differentiation. Further, we predicted that mitophagy would be impaired in BNIP3-deficient cells, resulting in the accumulation of mitochondria, and that this would cause an increase in autophagic signaling in an attempt to degrade the accumulated mitochondria.

Abbreviations

ATG5: autophagy related 5; ATG7: autophagy related 7; BCL2: B cell leukemia/lymphoma 2; BECN1: beclin 1; BNIP3: BCL2/adenovirus E1B interacting protein 3; BNIP3L/NIX: BCL2/adenovirus E1B interacting protein 3-like; CASP: caspase; CASP3: caspase 3; CASP9: caspase 9; CYCS: cytochrome c; DNM1L: dynamin 1-like; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFP: green fluorescent protein; LC3B: microtubule-associated protein 1 light chain 3 beta; SOD2: manganese superoxide dismutase; MTOR: mechanistic target of rapamycin kinase; MYH: MYH; MYOG: MYOG; OPA1: mitochondrial dynamin like GTPase; PPARGC1A: peroxisome proliferative activated receptor, gamma, coactivator 1 alpha; p-H2AFX: phosphorylated H2A histone family, member X; PINK1: PTEN induced putative kinase 1; RFP: red fluorescent protein; ROS: reactive oxygen species; SLC25A4: solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator); ULK1/ATG1: unc-51 like kinase 1; VDAC1: voltage-dependent anion channel 1

Introduction

Skeletal muscle differentiation and remodelling have been shown to rely heavily on a degradative process known as autophagy (McMillan & Quadrilatero, 2014; Fortini et al., 2016). When autophagy is initiated, cytoplasmic elements such as organelles are taken up by a double-membraned structure called an autophagosome. The autophagosome then delivers these cytoplasmic elements to a lysosome so that they can be degraded (Kondo et al., 2005). Autophagy can be quite a selective process, and one type of autophagy called mitophagy involves the specific targeting and degradation of mitochondria (Naik et al., 2018). Previous work has demonstrated that mitophagy is necessary during myogenic differentiation, and it is suggested to protect against mitochondria-mediated apoptosis (Sin et al., 2016; Baechler et al., 2019). Moreover, mitophagy degrades the myoblast mitochondrial population so that new mitochondria can be generated to fulfil the metabolic requirements associated with myotube formation and maintenance (Sin et al., 2016). Numerous mitophagy-related proteins and mitophagy pathways have been identified, and their induction and requirement are often context dependent (Hamacher-Brady & Brady, 2016). For example, PINK1 and PRKN are thought to be required for eliminating depolarized mitochondria (Hamacher-Brady & Brady, 2016), while the mitophagy receptor BNIP3L/NIX is essential for removing unnecessary but otherwise healthy mitochondria as part of a developmental differentiation program (Hamacher-Brady & Brady, 2016; Ney, 2015).

We among others have previously shown that autophagy-deficient myoblasts fail to differentiate properly (McMillan & Quadrilatero, 2014; Sin et al., 2016). Moreover, autophagy-deficient myoblasts contain more dysfunctional/damaged mitochondria and

elevated levels of ROS, and fail to sufficiently induce mitochondrial biogenesis during differentiation (McMillan, 2015; Baechler et al., 2019; Sin et al., 2016; Chapter 2). Further, the accumulation of dysfunctional/damaged mitochondria can disrupt differentiation by triggering cell death signaling events and apoptosis (Kubli & Gustafsson, 2012), and also contributes to cell senescence (Garcia-Prat et al., 2016). Thus, mitophagy plays a critical role in both myoblast/myotube survival and myogenic differentiation.

Although it is well established that autophagy-related proteins are required for myogenic differentiation, the role of mitophagy-related proteins in regulating myogenesis remains elusive. Although some studies have suggested that the PINK1/PRKN pathway is important in muscle (Peker et al., 2018; Yang et al., 2006), we have not been able to detect endogenous PRKN in C2C12 myoblasts (Bloemberg, 2017; Chapter 2 – Figure 4). Moreover, recent *in vivo* studies have suggested that PINK1 and PRKN are not needed for basal mitophagy (McWilliams et al., 2018; Lee et al., 2018), while others suggest a need for PINK1/PRKN-mediated mitophagy during aging (Cornelissen et al., 2018). Interestingly, previous work in our lab has demonstrated that expression of the mitophagy-related protein BNIP3 increases during myogenic differentiation (McMillan & Quadrilatero, 2014), and some have suggested that BNIP3 might be involved in mediating mitophagy during myogenesis (Baldelli et al., 2014).

BNIP3 and BNIP3L are structurally and functionally similar single-pass transmembrane proteins that are primarily found at the outer mitochondrial membrane (Ney, 2015). Both proteins contain a BCL2-homology 3 (BH3) domain, and a transmembrane domain that is crucial for inducing cell death (Ney, 2015). Similar to

other BH3-only proteins, BNIP3 and BNIP3L can induce apoptosis by interacting with BCL2 family proteins to encourage pro-apoptotic activity while also inhibiting those with anti-apoptotic functions (Marino et al., 2014). Further, by binding to BCL2 proteins, BNIP3 and BNIP3L can liberate BECN1, which would otherwise be inhibited by BCL2, to promote autophagy (Marino et al., 2014; Hamacher-Brady & Brady, 2016). However, although these proteins can promote autophagy, they are not necessarily required for proper induction and execution of autophagy. Interestingly, BNIP3-deficient tumour cells are more reliant on autophagy for survival than wild-type cells (Chourasia et al., 2015).

In addition to their potential to induce autophagy, BNIP3 and BNIP3L have been identified as important regulators of mitophagy (Hamacher-Brady & Brady, 2016). BNIP3L is required for eliminating mitochondria during erythrocyte differentiation (Sandoval et al., 2008; Zhang et al., 2012), and mitochondrial localization of BNIP3 increases during myogenic differentiation, suggesting that BNIP3 might be involved in differentiation-associated mitophagy (Baldelli et al., 2014). Additionally, BNIP3-deficient mammary tumours and lymphocytes show signs of impaired mitophagy, such as elevated ROS levels and the accumulation of dysfunctional mitochondria (Chourasia et al., 2015; O'Sullivan et al., 2015). Thus these proteins can regulate mitophagy as part of a developmental program, to support mitochondrial homeostasis and also to protect against mitochondria-related cellular stress (Hamacher-Brady & Brady, 2016; Sandoval et al., 2008; Kubli & Gustafsson, 2012). Further, BNIP3L and BNIP3 are thought to regulate mitochondrial turnover, and can mediate the removal of polarized and healthy mitochondria in addition to supporting the degradation of damaged mitochondria (Zhu et

al., 2013; Hamacher-Brady & Brady, 2016). Interestingly, both proteins contain an LC3-interacting region (LIR), which allows them to interact with autophagosome-associated LC3 directly to promote mitophagy (Novak et al., 2010; Hanna et al., 2012).

Thus, given that BNIP3 and BNIP3L have been described as important regulators of mitophagy in numerous contexts, it is possible that these proteins might play a mitophagy-related role during myogenic differentiation. Therefore, the purpose of this study was to determine if BNIP3 and/or BNIP3L are required for proper myogenic differentiation, and if a deficiency in one of these proteins results in autophagic/mitophagic impairments.

Results

BNIP3L/NIX and BNIP3 are required for proper myogenic differentiation

Based on previous reports supporting the importance of mitophagy during myogenic differentiation (Sin et al., 2016; Chapter 2), it is reasonable to speculate that mitophagy-related proteins are required to support myogenesis. Therefore, for this study we reduced the expression of the mitophagy receptors BNIP3L and BNIP3 (Ney, 2015) to determine if myogenic differentiation is disrupted. First, C2C12 myoblasts were transfected with siRNA targeting transcripts encoding BNIP3L or a non-targeting (Scr) siRNA, and differentiated for five days. BNIP3L expression was significantly reduced in the BNIP3L siRNA cells analyzed at D1 and D3 of differentiation relative to Scr siRNA cells; however, BNIP3L expression was almost restored by D5 (Figure 1A, B). Interestingly, BNIP3L siRNA cells displayed significantly higher levels of BNIP3 expression at time-

points when BNIP3L expression was reduced (Figure 1A, C). Moreover, MYH expression was reduced in BNIP3L siRNA cells compared to Scr cells (Figure 1A, D), suggesting impairment in myogenic differentiation. Further, p-H2AFX levels were lower in BNIP3L siRNA cells at D1 (Figure 1A, E), which could suggest that BNIP3L-deficient cells are more resistant to DNA fragmentation, but could also account for some alterations/impairments in differentiation, given that DNA strand breaks occur during normal myogenic differentiation (Larsen et al., 2010).

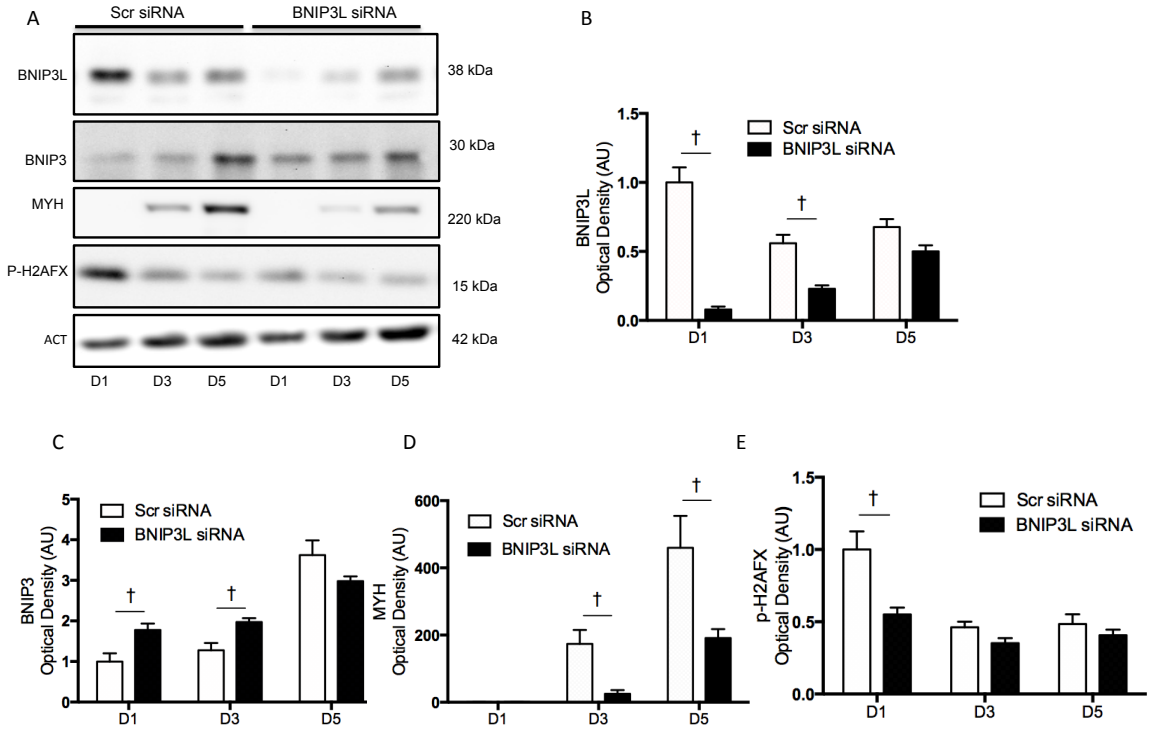

Figure 1. siRNA-mediated knockdown of BNIP3L impairs myogenic differentiation. Representative immunoblots (A) and quantitative analysis (B-E) of BNIP3L, BNIP3, MYH, and p-H2AFX in BNIP3L siRNA and Scr siRNA myoblasts during differentiation. Also shown is a representative ACT loading control blot. †$p<0.05$ between groups at the same time point.

Given the transient nature of the siRNA-mediated knockdown approach, a stable BNIP3-deficient (*bnip3*$^{-/-}$) C2C12 myoblast cell line, which was previously generated in our lab (Bloemberg, 2017), was used for subsequent experiments. As shown in Figure 2,

immunoblotting revealed no visible BNIP3 expression in *bnip3*$^{-/-}$ cells throughout differentiation (Figure 2A), although some background signal was detected at D0 (Figure 2B). Interestingly, we found that MYH (Figure 2A, C) and MYOG (Figure 2A, D) expression was significantly lower in *bnip3*$^{-/-}$ cells relative to Scram cells, demonstrating impaired myogenesis in cells lacking BNIP3. Further, CASP9 (Figure 2E) and CASP3 (Figure 2F) activity was elevated at D1, which was followed by higher p-H2AFX levels at D2 in *bnip3*$^{-/-}$ cells (Figure 2A, G). This suggests that cell death signaling is elevated during early differentiation time-points in BNIP3-deficient cells.

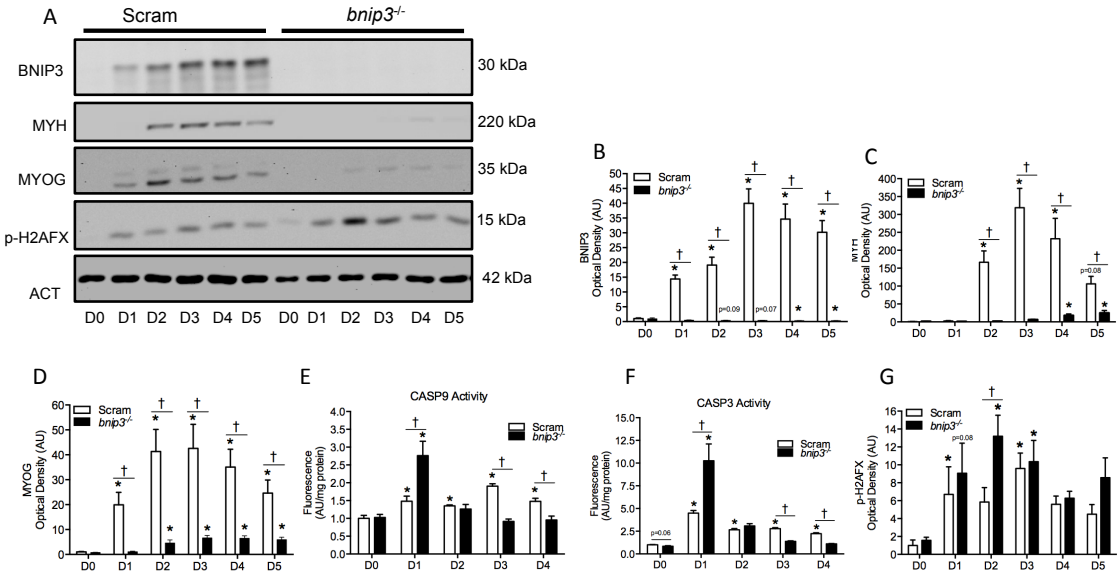

Figure 2. BNIP3 is required for myogenic differentiation. Representative immunoblots (A) and quantitative analysis (B-D, G) of BNIP3, MYH, MYOG, and p-H2AFX in *bnip3*$^{-/-}$ and Scram myoblasts during differentiation. Also shown is a representative ACT loading control blot. Quantitative analysis of (E) CASP9 and (F) CASP3 activity in *bnip3*$^{-/-}$ and Scram cells during differentiation. *$p<0.05$ compared to D0 (within group). $^{\dagger}p<0.05$ between groups.

Mitochondrial protein expression is altered in bnip3$^{-/-}$ *cells*

Mitophagy is said to be required for allowing mitochondrial remodelling to occur during differentiation (Sin et al., 2016), and BNIP3 has been shown to promote mitophagy (Zhang et al., 2016; Shi et al., 2014), thus we speculated that *bnip3*$^{-/-}$ cells might fail to

generate the mitochondrial network associated with myotubes. Interestingly we found that SOD2 expression was significantly elevated in *bnip3*$^{-/-}$ cells at early time-points during differentiation (Figure 3A, H), which could suggest that mitochondria are accumulating or that there is an increase in oxidative stress levels. Moreover, and similar to what we observed in sh*Atg7* cells (Chapter 2), the level of mitochondrial signaling- and mitochondria-related protein expression was generally lower in *bnip3*$^{-/-}$ relative to Scram cells (Figure 3A-G); however, the effect was not as severe as in sh*Atg7* cells (Chapter 2). This suggests that mitochondrial content and mitochondria-related signaling events are altered in *bnip3*$^{-/-}$ cells.

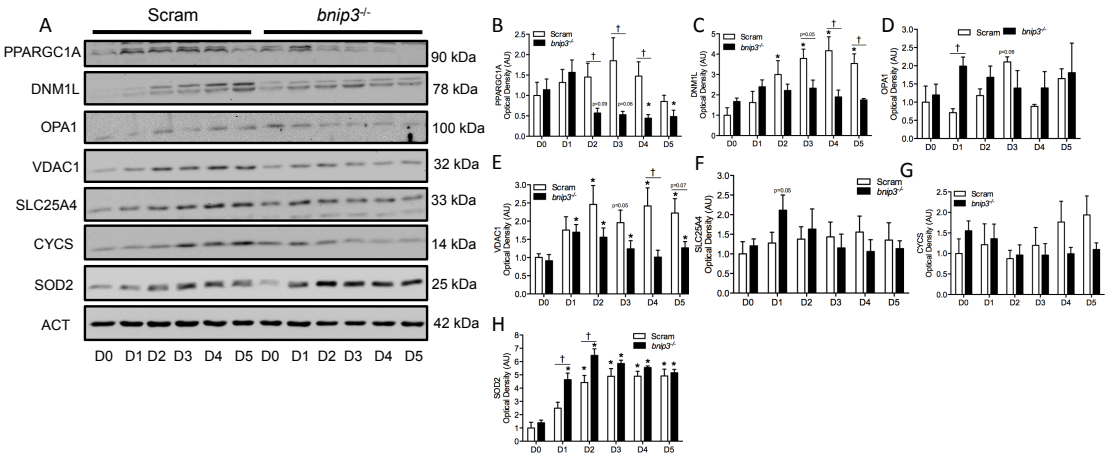

Figure 3. Mitochondrial network-related signaling and content is altered in *bnip3*$^{-/-}$ cells. Representative immunoblots (A) and quantitative analysis (B-H) of PPARGC1A, DNM1L, OPA1, VDAC1, SLC25A4, CYCS, and SOD2 in *bnip3*$^{-/-}$ and Scram myoblasts during differentiation. Also shown is a representative ACT loading control blot. *$p<0.05$ compared to D0 (within group). $^{\dagger}p<0.05$ between groups.

Mitochondrial degradation occurs in bnip3$^{-/-}$ *cells*

Next, we wanted to determine if mitophagy occurs in Scram and *bnip3*$^{-/-}$ cells during differentiation. Cells were transfected with DsRed-Mito and Ad-GFP-LC3 to visualize the co-localization of mitochondria (DsRed-labelled) and autophagosomes (GFP-LC3 puncta) during differentiation. As shown in Figure 4A-C, co-localization events occurred

in both Scram and *bnip3*$^{-/-}$ cells, and surprisingly, there were significantly more co-localization events at D0.5 in *bnip3*$^{-/-}$ cells relative to Scram cells (Figure 4C). Similarly, when cells were transfected with p-mito-mRFP-EGFP, RFP-only mitochondria were detected in both groups (Figure 4D-E), which indicates that mitochondria have been targeted to lysosomes. Thus, these findings suggest that mitochondrial degradation can and does occur in *bnip3*$^{-/-}$ myoblasts; however, given that there was elevated SOD2 levels (Figure 3H) and CASP9 activity (Figure 2E), it is likely that the targeting of specific, damaged/dysfunctional mitochondria is impaired in *bnip3*$^{-/-}$ cells.

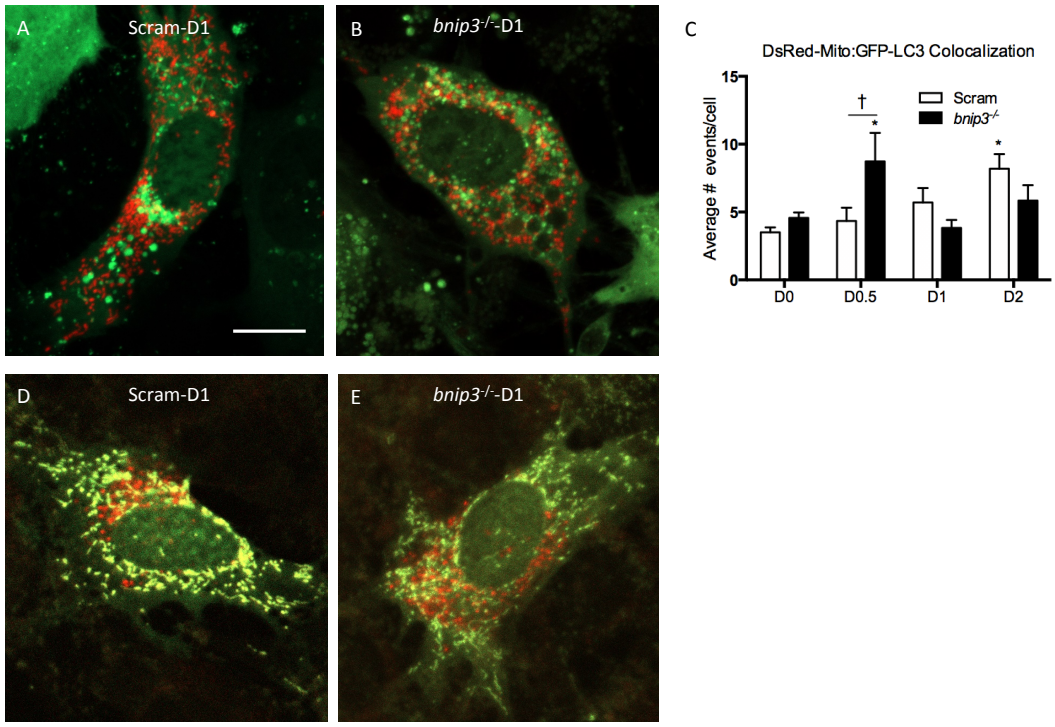

Figure 4. Mitochondrial degradation occurs in *bnip3*$^{-/-}$ cells during differentiation. Representative images (A-B) and quantitative analysis (C) of co-localization (yellow) of LC3 (green) and mitochondria (red) in Ad-GFP-LC3 and DsRed-Mito co-transfected Scram and *bnip3*$^{-/-}$ cells at D1 of differentiation. Representative images (D-E) of Scram and *bnip3*$^{-/-}$ cells transfected with p-mito-RFP-GFP. RFP-only mitochondria represent mitochondria that have been delivered to a lysosome. Scale bar=10 µm. *$p<0.05$ compared to D0 (within group). $^{\dagger}p<0.05$ between groups.

Increased expression of autophagy-related proteins can disrupt myogenesis

Based on our findings that mitochondrial degradation does occur in *bnip3*$^{-/-}$ cells (Figure 4), we speculated that other mitophagy-related proteins might compensate for the absence of BNIP3. In support of this hypothesis, *bnip3*$^{-/-}$ cells had higher levels of BNIP3L expression relative to Scram cells during differentiation (Figure 5A, B). This is consistent with other studies that have shown that BNIP3L expression increases in BNIP3-deficient cells (Shi et al., 2014; Chourasia et al., 2015).

BNIP3-deficient tumours and neurons have been reported to be more reliant on autophagy and show increased autophagy levels (Chourasia et al., 2015; Shi et al., 2014). Therefore, we measured autophagy-related protein expression in Scram and *bnip3*$^{-/-}$ cells during differentiation. Interestingly, we found that ATG7 (Figure 5A, C) and BECN1 (Figure 5A, D) expression was generally higher in *bnip3*$^{-/-}$ cells in comparison to the Scram cells. Moreover, the ratio of LC3B-II/LC3B-I was significantly elevated in *bnip3*$^{-/-}$ cells at most time-points (Figure 5A, E), suggesting an increased level of autophagosome formation and/or accumulation in *bnip3*$^{-/-}$ cells. This demonstrates that BNIP3-deficient cells show elevated expression of autophagy proteins and activation.

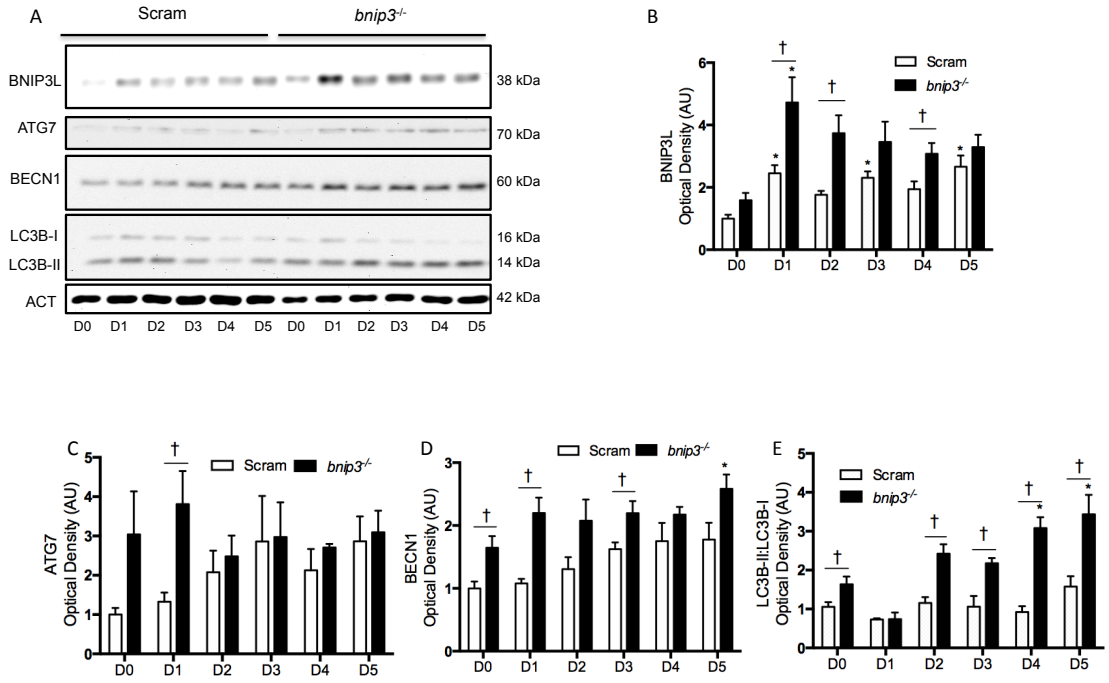

Figure 5. Autophagy/mitophagy-related protein expression is elevated in BNIP3-deficient cells. Representative immunoblots (A) and quantitative analysis (B-E) of BNIP3L, ATG7, BECN1, and the LC3B-II:LC3B-I ratio in *bnip3*$^{-/-}$ and Scram myoblasts during differentiation. Also shown is a representative ACT loading control blot. *$p<0.05$ compared to D0 (within group). $^{\dagger}p<0.05$ between groups.

Given that autophagy-related protein expression was higher in *bnip3*$^{-/-}$ cells, we wondered if increased autophagy might be preventing the cells from differentiating properly. Therefore, we decided to look more specifically at whether augmented expression of autophagy-related proteins impacts myogenic differentiation. In order to examine this, C2C12 myoblasts were treated with adenoviruses expressing the autophagy-mediating proteins BECN1 or ATG7 or treated with rapamycin, a known inducer of autophagy (Fan et al., 2016), and collected throughout differentiation. Interestingly, overexpression of BECN1 did not disrupt myogenic differentiation (Figure 6A-C) and BECN1-overexpressing cells actually had significantly higher MYH levels than control (GFP-expressing) cells (Figure 6B). In contrast, overexpression of ATG7 caused significant reductions in both MYH (Figure 6D, E) and MYOG expression

(Figure 6D, F). Similarly, MYH (Figure 6G, H) and MYOG (Figure 6G, I) levels were lower in rapamycin-treated cells, suggesting that differentiation can be impaired in response to augmented autophagic signaling.

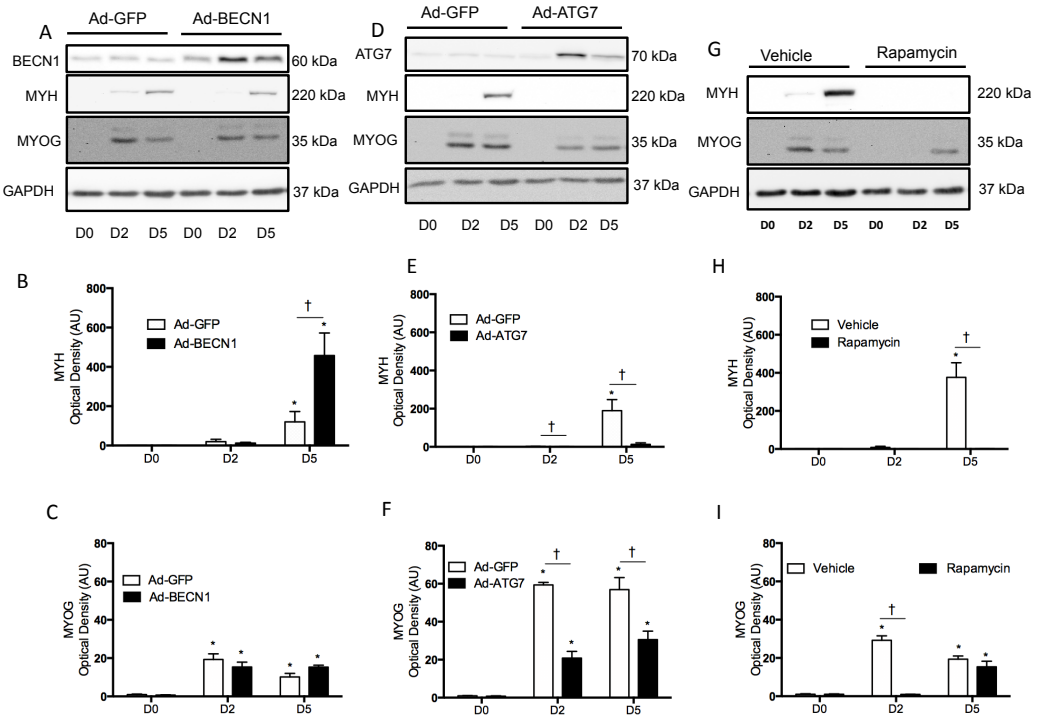

Figure 6. Autophagy-related protein expression can impact myogenic differentiation in C2C12 myoblasts. Representative immunoblots (A) and quantitative analysis (B-C) of MYH and MYOG in Ad-BECN1- and Ad-GFP-treated cells during differentiation. Representative immunoblots (D) and quantitative analysis (E-F) of MYH and MYOG in Ad-ATG7 and Ad-GFP-treated cells during myogenesis. Representative immunoblots (G) and quantitative analysis (H-I) of MYH and MYOG in rapamycin- and vehicle-treated myoblasts during differentiation. Also shown are representative GAPDH loading control blots. *$p<0.05$ compared to D0 (within group). †$p<0.05$ between groups.

Based on our previous findings that ATG7-deficiency is associated with increased cell death (McMillan & Quadrilatero, 2014; McMillan, 2015; Baechler et al., 2019), we wanted to determine if increased autophagy would reduce cell death signaling. First, we determined that p-H2AFX levels were similar in both Ad-ATG7-treated cells and Ad-GFP-treated cells (controls) at early time-points during differentiation, but that p-H2AFX expression was significantly elevated in the Ad-ATG7 groups at D5 (Figure 7A, B).

Interestingly, we found that CASP3 activity was elevated in Ad-ATG7 cells at D1, although not significant, but was actually lower than in control cells by D2 (Figure 7C). CASP3 activity was similar among groups at later time-points (Figure 7C), and there were no major differences in CASP9 activation throughout differentiation (Figure 7D).

Next we examined cells overexpressing BECN1 and found that p-H2AFX levels were lower than in control cells during differentiation (Figure 7E, F). Additionally, CASP3 activity was significantly lower in Ad-BECN1-treated cells at D1 (Figure 7G); however, CASP3 activity was similar between groups at later time-points and there were no significant differences in CASP9 activity levels (Figure 7H).

Interestingly, and similar to Ad-ATG7-treated cells, p-H2AFX was higher in rapamycin-treated cells at D5 (Figure 7I, J). However, CASP3 (Figure 7K) and CASP9 activity (Figure 7L) was significantly lower in cells treated with rapamycin at days 2-4 of differentiation, suggesting that rapamycin treatment might suppress CASP activation.

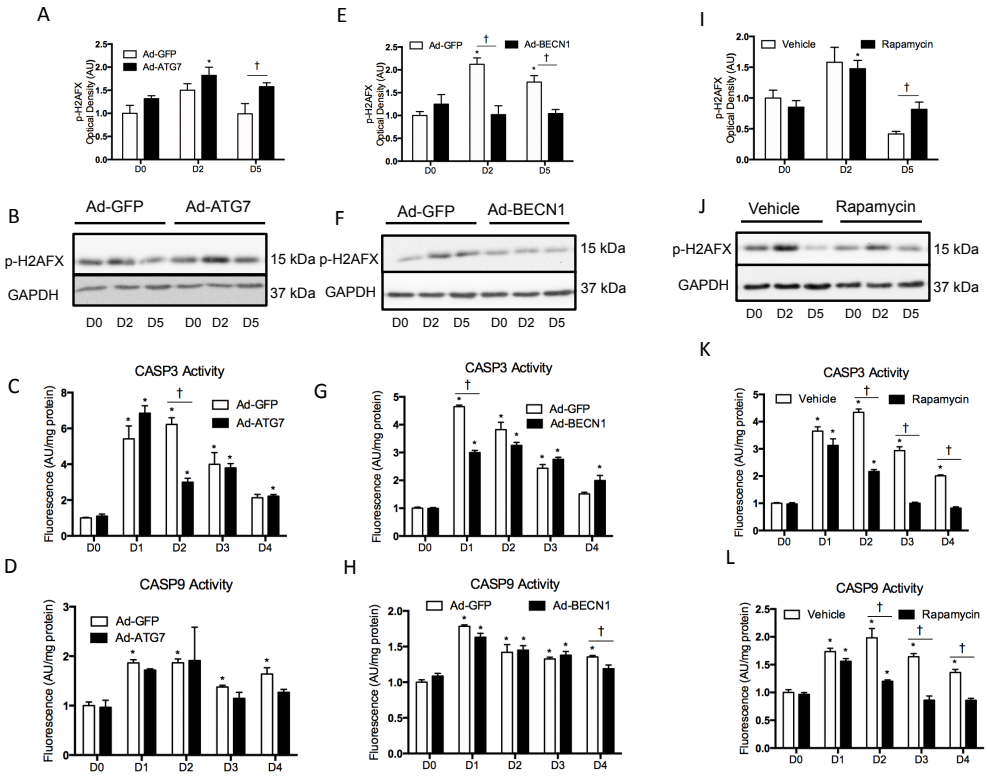

Figure 7. Enhanced expression of autophagy-related proteins influences CASP activation during differentiation. Quantitative analysis (A) and representative immunoblot (B) of p-H2AFX in Ad-ATG7- and Ad-GFP-treated myoblasts during myogenic differentiation. Quantitative analysis of (C) CASP3 and (D) CASP9 activity in Ad-ATG7- and Ad-GFP-treated cells during myogenesis. Quantitative analysis (E) and representative immunoblot (F) of p-H2AFX in myoblasts treated with Ad-BECN1 and Ad-GFP. Quantitative analysis of (G) CASP3 and (H) CASP9 activity in Ad-BECN1- and Ad-GFP-treated cells during myogenesis. Quantitative analysis (I) and representative immunoblot (J) of p-H2AFX in rapamycin- and vehicle-treated myoblasts during differentiation. Quantitative analysis of (K) CASP3 and (L) CASP9 activity in differentiating rapamycin- and vehicle-treated cells. Also shown are representative GAPDH loading control blots. *$p<0.05$ compared to D0 (within group). $^{\dagger}p<0.05$ between groups.

To further validate the importance of controlling autophagy levels during myogenic differentiation, we used *Drosophila* as a model system. Using the GAL4/UAS system of inducible gene expression, we were able to overexpress the autophagy-related protein ATG1/ULK1 specifically in muscle cells during development by using a muscle-specific Mef2-GAL4 driver. Additionally, the strength of the Mef2-GAL4 driver is regulated by temperature. Thus, at 18°C the GAL4 driver is not active and the UAS-ATG1 construct is not expressed; however the strength of the GAL4 driver increases at

temperatures above 25°C and provides maximum overexpression levels at 29°C. For all experiments, flies were grown at 18 °C until pupal stages, and then shifted to the indicated temperatures, which allowed us to examine the effect of augmenting autophagy during adult muscle development (Figure 8). Flies expressing UAS-GFP (controls) and those expressing UAS-ATG1 hatched out of their pupal cases properly (eclosed) when shifted to 25°C. However, when pupae were shifted to 27°C, some flies expressing UAS-ATG1 failed to eclose properly and died. Interestingly, flies that were shifted to 27°C during larval stages of development all failed to emerge from their pupal cases. Moreover, when pupae were shifted to 29°C, which would have allowed for maximal overexpression of ATG1, all pupae expressing UAS-ATG1 failed to eclose and died (Figure 8A). Further, flies overexpressing ATG1 that were grown at 25°C displayed wing posture changes (Figure 8B-F) that differed from flies expressing GFP (controls). More specifically, ATG1-overexpressing flies had either a “wings up” or “wings out” phenotype, which was not or rarely detected in GFP-expressing flies. Similar phenotypes have been reported in flies deficient in PINK1, which are known to have muscle abnormalities (Clark et al., 2006; Park et al., 2006; Zhang et al., 2016; Shiba-Fukushima et al., 2014). Taken together, these results suggest that expression levels of autophagy-related proteins must be tightly controlled to allow for proper development and cell survival.

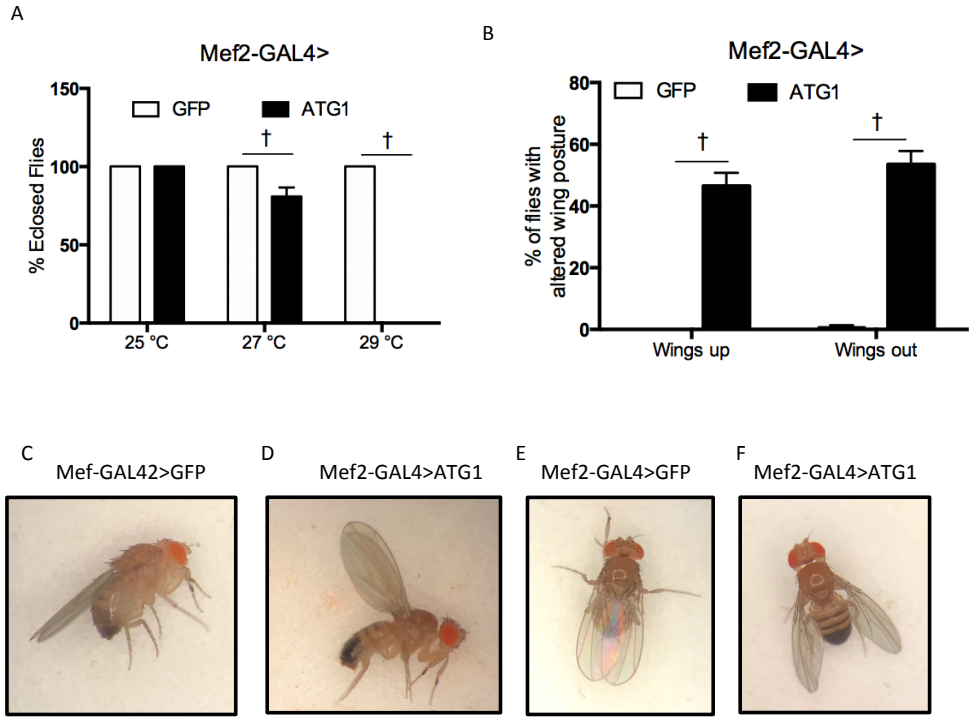

Figure 8. Overexpression of the autophagy-related protein ATG1/ULK1 in *Drosophila* muscle progenitors disrupts adult fly development. Quantitative analysis (A) of the percentage of adult flies that eclose properly (emerge from their pupal case) when UAS-GFP (control) or UAS-ATG1 is overexpressed specifically in muscle (Mef2-GAL4>). Flies were grown at 18°C until they reached pupal stages (UAS-constructs suppressed) and were then shifted to 25°C, 27°C (mild overexpression), or 29°C (maximum overexpression level) until adulthood. Quantitative analysis (B) of the percentage of flies overexpressing GFP or ATG1 in muscle with abnormal "wings up" or "wings out" wing postures. Representative images (C, E) of wing postures observed in control (Mef2-GAL4>GFP) flies, and the "wings up" (D) and "wings out" (F) postures observed in flies overexpressing ATG1. $^{\dagger}p<0.05$ between groups at the same time point.

Discussion

During myogenic differentiation, mitophagy is necessary to promote mitochondrial biogenesis and myotube formation, and also to limit unwanted cell death (Sin et al., 2016; Fortini et al., 2016; Baechler et al., 2019). Studies have suggested that the protein PRKN protects against muscle atrophy and is essential for mitophagy and mitochondrial turnover (Peker et al., 2018); however, we have been unable to detect endogenous PRKN

expression in C2C12 myoblasts in our lab (Bloemberg, 2017; Chapter 2 – Figure 4). Therefore, we turned our attention to the mitophagy receptor proteins BNIP3L and BNIP3, which have been shown to mediate the removal of damaged as well as healthy mitochondria, and could therefore play an important role in regulating mitophagy during differentiation (Hamacher-Brady & Brady, 2016; Zhu et al., 2013; Ney, 2015). Moreover, previous work has suggested that BNIP3 might regulate mitophagy during myogenic differentiation (Baldelli et al., 2014). Using siRNA- or CRISPR/Cas9-mediated approaches, we generated cells deficient in BNIP3L and BNIP3. Interestingly, we found that myogenic differentiation was disrupted in both BNIP3L- and BNIP3-deficient cells (Figure 1 and Figure 2); however, we found that mitochondria were degraded in *bnip3*$^{-/-}$ cells induced to differentiate (Figure 4), suggesting that the differentiation impairment does not result from a complete deficiency in mitochondrial degradation. Interestingly, several studies have demonstrated that mitochondria can be degraded in an autophagy- and LC3-independent manner (Hammerling et al., 2017; Honda et al., 2014; Rakovic et al., 2018; Oliveira et al., 2015). Moreover, studies have demonstrated that BNIP3 can promote both LC3-dependent mitophagy, as well as endosome-mediated mitochondrial degradation (Hanna et al., 2012; Hammerling et al., 2017). Thus, given that mitophagy as well as endosome-mediated mitochondrial degradation might normally be involved in degrading mitochondria during myogenesis, a deficiency in BNIP3 could compromise one or both of these pathways. In support of this, we found that SOD2 levels and mitochondria-related apoptotic signaling were elevated in *bnip3*$^{-/-}$ cells (Figure 3A, H; Figure 2E), suggesting a possible impairment in the elimination of damaged/dysfunctional mitochondria in *bnip3*$^{-/-}$ cells. Therefore, although gross

mitochondrial degradation occurs in *bnip3*$^{-/-}$ cells, it is possible that BNIP3 might be needed to identify and eliminate specific mitochondria that are damaged/dysfunctional. Further, we measured mitochondria-related proteins in *bnip3*$^{-/-}$ cells and found that mitochondrial biogenesis-related and mitochondrial protein levels were reduced in *bnip3*$^{-/-}$ cells, suggesting impairment in mitochondrial biogenesis (Figure 3). Interestingly, BNIP3-deficient tumour cells have been shown to be more reliant on glycolysis to support energetic demands (Chourasia et al., 2015), similar to undifferentiated myoblasts (Sin et al., 2016), and forced expression of BNIP3 in BNIP3-deficient tumours promotes a switch to oxidative metabolism (Chourasia et al., 2015). Thus, it seems reasonable to speculate that BNIP3 might be required to support mitochondrial remodelling and the transition from myoblast to myotube. Moreover, BNIP3 might also mediate the selective degradation of dysfunctional mitochondria to limit stress and cell death signaling during myogenic differentiation.

Although *bnip3*$^{-/-}$ cells showed signs of apoptotic signaling during early stages of differentiation, such as elevated CASP activity (Figure 2E, F) and increased DNA fragmentation (Figure 2A, G), the cells did not die. Additionally, p-H2AFX levels have been shown to increase at D1 as a result of the CASP3-mediated DNA strand breaks that must occur during myogenic differentiation (Larsen et al., 2010); however, significantly higher p-H2AFX levels were detected at D2 in *bnip3*$^{-/-}$ cells, a point at which levels should have decreased during differentiation (Larsen et al., 2010). Thus, it is likely that *bnip3*$^{-/-}$ cells have more DNA fragmentation than Scram cells, which could indicate an increase in cell death signaling. Interestingly, p-H2AFX levels were actually much lower in BNIP3L-deficient cells than in Scr cells at D1, a time-point at which BNIP3 was

significantly higher in BNIP3L-deficient cells (Figure 1A, C, E). BNIP3L can induce both apoptotic and non-apoptotic events which could result in increased p-H2AFX levels (Baines, 2010; Zhang & Ney, 2009; Ney, 2015; Diwan et al., 2009), thus it seems reasonable to speculate that BNIP3L might be indirectly required for initiating DNA strand breaks during myogenic differentiation, and could provide one possible explanation for why differentiation is impaired in BNIP3L-deficient myoblasts (Figure 1A, 1D). Moreover, the elevated and persistent p-H2AFX expression in *bnip3*$^{-/-}$ cells could result from inappropriately high levels of BNIP3L expression (Figure 5A-B), which could promote cell death signaling and disrupt myogenesis. Further, previous work has demonstrated that reduced BNIP3 expression is associated with a compensatory upregulation of BNIP3L expression; however, although BNIP3 and BNIP3L have similar structures and functions, they cannot fully compensate for one another (Chourasia et al., 2015; Shi et al., 2014), suggesting that BNIP3L and BNIP3 likely have some independent and unique intracellular roles. Moreover, studies have suggested that BNIP3 and BNIP3L might antagonize/limit the expression of one another (Bellot et al., 2009). Therefore, it is not surprising that we saw increased BNIP3L in *bnip3*$^{-/-}$ cells and elevated BNIP3 in BNIP3L-deficient cells. Further, given that BNIP3 and BNIP3L may have independent functions in myoblasts/myotubes, inappropriate or altered levels of either of these proteins could impact cell death signaling and differentiation.

Interestingly, studies have shown that cells can recover from the stress-induced induction of apoptosis and have named this process “anastasis” which means “rising to life” (Tang et al., 2012). Thus, CASP3 activation and DNA fragmentation do not necessarily result in apoptotic cell death; however, the cells that arrest apoptosis and

survive acquire permanent damage and genetic alterations (Tang et al., 2012). Therefore, *bnip3*$^{-/-}$ myoblasts might recover and survive following high levels of CASP activation, but the resulting cellular changes might limit their ability to differentiate. Moreover, a more recent study suggested that anastasis is not associated with changes in autophagic signaling (Sun et al., 2017). Interestingly, we found that autophagy-related protein expression was elevated in *bnip3*$^{-/-}$ cells relative to controls (Figure 5). This is not that surprising given that previous studies have shown that BNIP3-deficient cells upregulate autophagy as a survival mechanism (Chourasia et al., 2015; Shi et al., 2014). Moreover, we speculate that the increased induction of autophagy might occur in *bnip3*$^{-/-}$ cells to compensate for a deficiency in the removal of dysfunctional mitochondria. Increased expression of autophagy-related proteins as well as increased formation of autophagosomes, could enhance mitophagy and cell survival but it could also contribute to cell death (Liu & Levine, 2015; Gudipaty et al., 2018; Marino et al., 2014). Interestingly, autophagic cell death has been shown to occur during the development and remodelling of some tissues (Denton et al., 2009) but its role in myogenic differentiation has yet to be determined. Previous work in our lab has demonstrated that autophagy is needed to limit CASP activation during myogenesis (McMillan & Quadrilatero, 2014); however, inappropriately high levels of autophagy could also trigger cell death (Liu & Levine, 2015; Scott et al., 2007). One form of autophagy-mediated cell death termed autosis can be induced by starvation or treatment with a BECN1 peptide derivative (Liu et al., 2013; Liu & Levine, 2015). Interestingly, autotic cell death does not involve CASP activation and is associated with increased cell-substrate adherence (Liu et al., 2013; Liu & Levine, 2015). This is intriguing because although *bnip3*$^{-/-}$ cells had elevated CASP

activity at D1, levels were generally lower at later time-points during differentiation and the cells had elevated autophagy-related protein expression and did not die (Figure 2 and Figure 5). Further, when we overexpressed ATG7 and BECN1, or treated C2C12 myoblasts with rapamycin, CASP activity was generally reduced, cells remained adherent, and myogenic differentiation was impaired in ATG7- and rapamycin-treated cells (Figure 6 and Figure 7). These results could indicate that autophagy is elevated in *bnip3*$^{-/-}$ cells and that this level of autophagy, which could be similar to our overexpression models, is triggering autotic cell death. Therefore, although the cells remained undifferentiated but adhered to plates, they might be incapable of differentiating because autophagy levels are too high. Interestingly, overexpression of BECN1 in C2C12 myoblasts did not impair myogenic differentiation, and actually seemed to enhance it. We speculate that the reduction in CASP3 activity at D1 in BECN1-treated cells (Figure 7G) might have limited apoptotic cell death allowing there to be more cells available to fuse to form myotubes. Moreover, our work using *Drosophila* demonstrated that overexpression of the autophagy-related protein ATG1/ULK1 in muscle is lethal and prevents flies from eclosing properly or emerging from their pupal cases (Figure 8A). Similar phenotypes have been reported in flies with reduced MTOR signaling and is attributed to muscle weakness (Hatfield et al., 2015) thus it is possible that myogenic differentiation is impaired due to a failure in the necessary upregulation of MTOR signaling. Moreover, overactivation of autophagy can promote CASP activation and cell death (Mohseni et al., 2009; Cormier et al., 2012; Scott et al., 2007); therefore, it is possible that overexpression of ATG1/ULK1 is inducing some form of cell death which prevents muscle from forming/differentiating properly. Further, we

found that muscle-specific overexpression of ATG1 resulted in wing posture abnormalities (Figure 8B-F), similar to those which have been reported for flies deficient in mitophagy-related proteins (Zhang et al., 2016; Shiba-Fukushima et al., 2014), demonstrating that the controlled regulation of autophagy/mitophagy-related protein expression in muscle is critical for normal development.

Conclusion

Overall this work demonstrates that mitophagy-related proteins are required during myogenic differentiation. Based on our results, we speculate that BNIP3 and BNIP3L might play multiple mitochondria-related as well as mitochondria-independent roles during myogenesis, and that BNIP3 and BNIP3L levels must be regulated appropriately. Interestingly, we determined that a deficiency in BNIP3 results in inappropriately high levels of other autophagy/mitophagy-related proteins, which might be a compensatory mechanism used to degrade dysfunctional/damaged mitochondria that would otherwise accumulate in *bnip3*$^{-/-}$ cells. Moreover, we suggest that overactivation of autophagy can impact cell death signaling in addition to disrupting myogenesis. Therefore, this work highlights the importance of maintaining a proper balance of mitophagy/autophagy-related protein expression to maintain cellular homeostasis during development and myogenic differentiation.

Materials and Methods

Cell culture and transfections

Culturing conditions have been described previously (McMillan & Quadrilatero, 2014). Low pass C2C12 myoblasts (A.T.C.C.) were plated in polystyrene cell culture dishes or on Cultrex BME-coated coverslips in growth media (GM) composed of low-glucose Dulbecco's modified Eagle's medium (DMEM), 10% FBS, and 1% penicillin/streptomycin, and incubated at 37°C in 5% CO_2. Media was replaced every 24-48 hours. Cells were induced to differentiate when they reached 80-90% confluency, by replacing GM with differentiation media (DM) comprised of low-glucose DMEM, 2% horse serum, and 1%P/S. Cells were collected at day 0 (D0) prior to switching GM to DM, and collected at the appropriate time-points (D1, D2, etc.). Cells were trypsinized (0.25% trypsin with 0.2g/l EDTA), centrifuged (1000g for 5 min), and stored at -80°C. For rapamycin treatments, rapamycin (1 μM) or vehicle was added to DM and replaced each day during differentiation.

Stable BNIP3 knockout and Scram cell lines were previously generated in our lab (Bloemberg, 2017). Briefly, C2C12 myoblasts were grown in 12-well plates and transfected with vectors encoding a BNIP3 CRISPR sequence, 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 hours. 24 hours 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 BNIP3 levels in selected clones.

Transient knockdown of BNIP3L was achieved using the jetPRIME siRNA transfection reagent protocol (Polyplus-transfection). Briefly, C2C12 myoblasts were transfected with BNIP3L siRNA (sc-37454) or Control siRNA-A (sc-37007) (Santa Cruz Biotechnology) diluted in jetPRIME buffer along with jetPRIME Transfection Reagent (Polyplus-transfection, 114-07), at 60% confluence. Cells were incubated in media containing siRNA overnight and until they were 80-90% confluent. GM was then replaced with DM and cells were collected at the appropriate time-points. Immunoblotting was used to validate BNIP3L knockdown.

Adenoviruses and amplification

The following adenoviruses were used for this study: Ad-BECN1 (kindly provided by Dr. Abhinav Diwan), Ad-ATG7, Ad-GFP, and Ad-LC3-GFP (Yang et al., 2010). Adenoviruses were amplified in HEK293 cells using the ViraPower Adenoviral Expression System protocol (Life technologies). Cells were grown in 100 mm plates and viral lysate was added to the growth media once the cells had reached confluence. Cells were harvested once 80-90% of the cells had rounded up and were starting to detach from the plate. Cells and media were collected using a serological pipette and placed in a 15 mL falcon tube. The tube was placed at -80°C for 30 minutes, then thawed in a 37°C waterbath for 15 minutes. This freeze/thaw cycle was performed a total of 3 times. The cell lysate was then centrifuged at 3000 rpm for 15 minutes at room temperature. The supernatant (crude virus) was transferred to cryotubes and stored at -80°C. Viral titers were determined using the Adeno-X Rapid Titer Procedure (Clontech Laboratories, Inc.). Briefly, HEK293 cells were seeded in 12-well plates on coverslips coated with Cultrex

BME (Trevigen, 3432-010-01), 10-fold serial dilutions of adenovirus stocks were prepared and the appropriate viral dilutions were added to each well. Cells were incubated at 37°C (5% CO2) for 48 hours. Following incubation, media was aspirated and cells were fixed in 1 mL cold 100% methanol at -20°C for 10 minutes. After fixation, cells were rinsed with PBS + 1% BSA. Cells were then incubated with mouse anti-Hexon antibody (Santa Cruz Biotechnology) for 1 hour at 37°C on a shaker. Cells were then rinsed and Alexa Fluor 488-conjugated secondary antibody (Thermo Fisher Scientific) was added for a 1 hour incubation. Cells were then washed in PBS and mounted on glass slides using Prolong Gold Antifade Reagent (Thermo Fisher Scientific, P36930) and imaged on a Zeiss LSM 800 (Carl Zeiss). Six to ten fields containing 5-50 were counted for green (Hexon+) cells, and used to calculate infectious units/ml. For all overexpression experiments, C2C12 cells were treated with adenoviruses (overnight incubation) at a multiplicity of infection (MOI) of 100.

Fluorescent mitophagy reporters/microscopy

To investigate mitophagy during differentiation, cells were grown on glass coverslips coated with Cultrex BME (Trevigen). Cells were transfected with pDsRed2-Mito vector (generously provided by Dr. Douglas Green, St Jude's Children's Research Hospital, Memphis, TN) or p-mito-RFP-GFP vector (provided by Dr. Andreas Till; Kim et al., 2013) upon reaching 60-70% confluence. Transfections were performed using jetPRIME Transfection Reagent (Polyplus-transfection) according to the manufacturer's instructions. Additionally, GFP-LC3 adenovirus (ad-GFP-LC3; kindly provided by Dr. Gökhan S. Hotamisligil, Harvard School of Public Health, Boston, MA) was added to

each well containing cells transfected with pDsRed2-Mito for overnight incubation. The next day, cells were washed and imaged (D0) or induced to differentiate. For imaging at the appropriate time-points (D0, D1, etc.), coverslips were removed from plates, washed and mounted on glass slides using Prolong Gold Antifade Reagent (ThermoFisher Scientific) and imaged on a Zeiss LSM 800 (Carl Zeiss). To assess mitophagy, we counted the number of times we saw co-localization of GFP-LC3 punta with pDsRed2-Mito (co-localization events). To assist with counting co-localization events, 10 μm chloroquine (CQ) was added to each well one day prior to imaging. For p-mito-RFP-GFP experiments, the appearance of RFP-only mitochondria indicated that mitophagy was happening in transfected cells.

Immunoblotting

Western blotting was performed as described previously (McMillan & Quadrilatero, 2011; McMillan & Quadrilatero, 2014). 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 inhibitor cocktail (Roche Applied Sciences). Equal amounts of protein were loaded and separated on 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: BNIP3, ACTIN (B7931, A2066; Sigma-Aldrich), SOD2 (ADI-SOD-110; Enzo Life Sciences), GAPDH, BNIP3L, DNM1L, LC3B, ATG7, BECN1 (2118, 12396, 8570, 2775, 8558, 3738; Cell Signaling), MYOG, MYH (F5D, MF20; Developmental Studies Hybridoma Bank), CYCS, PPARGC1A, SLC25A4, VDAC1, p-H2AFX, OPA1 (sc-13156, sc-13067, sc-9299, sc-390996, sc-101696, sc-393296; Santa

Cruz Biotechnology). 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 and bands were visualized using the Clarity Western ECL substrate (Bio-Rad) and the ChemiGenius 2 Bio-Imaging System (Syngene).

CASP activity assays

CASP3 and CASP9 assays were performed as described previously (McMillan et al., 2015; Baechler et al., 2019). Briefly, cells were sonicated in lysis buffer (LB) without added protease inhibitors, and CASP3 and CASP9 activity was determined using the fluorogenic substrates Ac-DEVD-AFC and Ac-LEHD-AFC (AAT Bioquest, 13401; Tocris Bioscience, 1575), respectively. Fluorescence was measured at the appropriate wavelength using a Synergy H1 microplate reader (BioTek) in duplicate. CASP activity measures were normalized to total protein content determined using the BCA protein concentration assay.

Fly stocks

All stocks were maintained on standard Drosophila medium at 25°C under a 12 h light/dark cycle, unless otherwise indicated. The following stocks were used for experiments: *tubGal80ts;; Mef2-Gal4+UAS-GFP* (Dr. Frank Schnorrer), *UAS-Atg1^{6A}; UAS-Atg1^{6B} (Dr. Bruce H. Reed)*. For all experiments, crosses were set up at 18°C and shifted to the appropriate temperatures (25°C-29°C) at larval/pupal stages.

GAL4/UAS System

The GAL4/UAS system is a method that allows for inducible expression of a sequence of interest in a tissue and temporal specific manner. Briefly, the sequence encoding the GAL4 protein is placed under the control of a tissue-specific promoter, and when expressed, it will bind to an *Upstream Activating Sequence (UAS*) to induce gene expression. A sequence of interest encoding a protein of interest or an RNAi sequence can be placed under the control of a *UAS* sequence, allowing for protein expression/knockdown wherever the GAL4 protein is produced. The $Gal80^{ts}$ system can also be combined with this to allow for temperature-dependent regulation of the GAL4 protein. Therefore, when flies are grown at 18°C, Gal80 prevents GAL4 from binding to the *UAS* sequence and gene expression is suppressed. Conversely, if flies are grown at 29°C, Gal80 is inactivated, allowing GAL4 to bind to its *UAS* sequence to induce target gene expression (Elliott & Brand, 2008). For all experiments, a muscle-specific GAL4 driver (*tubGal80ts;;Mef2-Gal4+UAS-GFP)* was used to allow for temperature regulated expression.

Statistics

Differences between time-matched groups were assessed using a Students T-test. For all experiments $p<0.05$ was considered statistically significant. A two-way ANOVA was used to assess temperature-matched group differences for *Drosophila* experiments, with Bonferroni's multiple comparisons test. A one-way ANOVA was used to assess the effect of differentiation within groups, with Bonferroni's multiple comparison test to compare differences from D0. For all immunoblotting and CASP activity assays, n = 3

or more. For fluorescent reporter analysis, n = 15-30 cells. For *Drosophila* development and wing posture analysis, at least 50 flies were counted for each group.

Chapter 4: Induced mitochondrial biogenesis to improve myogenic differentiation in autophagy/mitophagy-deficient cells

Project Rationale and Objectives

In order for cells to differentiate and survive, mechanisms must be in place to ensure that they are equipped with the necessary metabolic machinery. During myogenic differentiation, the undifferentiated myoblast, which primarily uses glycolysis, generates a new mitochondrial network better suited to support a shift to using oxidative phosphorylation (OXPHOS) to generate energy (Sin et al., 2016). The generation of the mitochondrial network and maintenance of mitochondrial homeostasis requires the coordination of autophagy/mitophagy-mediated mitochondrial degradation as well as mitochondrial biogenesis to generate new mitochondria (Palikaras et al., 2015). Studies have suggested that autophagy/mitophagy is required to initiate mitochondrial biogenesis during myogenic differentiation, and that an impairment in mitophagy prevents mitochondrial biogenesis and differentiation from occurring (Sin et al., 2016; Baechler et al., 2019). Further, mitochondrial function and biogenesis have been shown to play an important role in regulating differentiation in numerous cell types (Rochard et al., 2000; Seyer et al., 2006; Sharma et al., 2009; Kanno et al., 2004). In Chapter 2 and Chapter 3 we demonstrated that ATG7- and BNIP3-deficient cells show impairments in the up-regulation of mitochondrial and mitochondrial biogenesis-related proteins during differentiation. Interestingly, previous work in our lab has shown that mitochondrial respiration is also reduced in sh*Atg7* and *bnip3*$^{-/-}$ cells relative to SCR/Scram cells (Bloemberg, 2017). Therefore, it is likely that sh*Atg7* and *bnip3*$^{-/-}$ cells may not differentiate, at least in part, because they cannot fulfill the energetic requirements to form myotubes, and that this may be a direct or indirect consequence of alterations in autophagy/mitophagy. Moreover, we speculate that the increased cell death associated

with ATG7-deficiency (McMillan & Quadrilatero, 2014; McMillan, 2015; Baechler et al., 2019) might be partially attributed to failure to re-establish a mitochondrial network. Additionally, we considered that if sh*Atg7* or *bnip3*$^{-/-}$ cells are less efficient at eliminating damaged or dysfunctional mitochondria, then it might be possible to improve cellular function and homeostasis by simply increasing the number of healthy mitochondria (through mitochondrial biogenesis) in order to outnumber or compensate for dysfunctional mitochondria. Therefore, the major objectives of this project were:

1) To identify chemical treatments that will induce mitochondrial biogenesis in sh*Atg7* cells.

2) To determine if increased mitochondrial biogenesis can effectively restore myogenic differentiation in sh*Atg7* and *bnip3*$^{-/-}$ cells.

3) To determine if mitochondrial function improves in sh*Atg7* and *bnip3*$^{-/-}$ cells following treatment with an inducer of mitochondrial biogenesis.

We hypothesized that treating ATG7- and BNIP3-deficient cells with mitochondrial biogenesis inducers would enhance mitochondrial biogenesis. Moreover, we speculated that increased mitochondrial biogenesis would reduce cell death signaling in sh*Atg7* cells, allowing more cells to differentiate and fuse. We also hypothesized that mitochondrial health and function would improve in the sh*Atg7* and *bnip3*$^{-/-}$ cells that were treated with a mitochondrial biogenesis inducer.

Abbreviations

AICAR: 5-Aminoimidazole-4-carboxamide-ribonucleoside; ATG7: autophagy related 7; BNIP3: BCL2/adenovirus E1B interacting protein 3; CI: complex I; CII: complex II; CASP: caspase; CASP3: caspase 3; $CoCl_2$: cobalt chloride; CYCS/cyto c: cytochrome c; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFP: green fluorescent protein; MYH: myosin; MYOG: myogenin; OXPHOS: oxidative phosphorylation; PPARGC1A: peroxisome proliferative activated receptor, gamma, coactivator 1 alpha; RFP: red fluorescent protein; SIRT1: sirtuin 1; SNP: sodium nitroprusside; TFAM: transcription factor A, mitochondrial; VDAC1: voltage-dependent anion channel 1

Introduction

Mitochondria are important cellular organelles that are often described as being the "powerhouse" of the cell because of their important role in generating cellular energy (Ploumi et al., 2017). Moreover, in addition to their metabolic role, mitochondria also support cellular homeostasis and function by influencing apoptotic signaling events (Wagatsuma & Sakuma, 2013), and act as important regulators of cell differentiation (Barbieri et al., 2011).

Numerous morphological, intracellular signaling, gene/protein expression, and metabolic changes occur when stem or progenitor cells differentiate (Wanet et al., 2015; Barbieri et al., 2011; Mizushima & Komatsu, 2011). Some cell types, such as undifferentiated myoblasts, are heavily reliant on glycolysis to generate energy, but show increased reliance on oxidative phosphorylation (OXPHOS) to fulfill their energy

requirements during/following differentiation (Wanet et al., 2015; Sin et al., 2016; Wagatsuma & Sakuma, 2013). Thus, for proper differentiation to occur, cells must be able to effectively generate an extensive mitochondrial network using a process known as mitochondrial biogenesis (Sin et al., 2016). Mitochondrial biogenesis involves the division, fusion, and growth of pre-existing mitochondria (Jornayvaz & Shulman, 2010; Ploumi et al., 2017), and is dependent on the peroxisome proliferator-activated receptor gamma co-activator 1 (PPARGC1A) family of proteins, nuclear respiratory factor (NRF) proteins, as well as mitochondrial transcription factor A (TFAM) (Wu et al., 1999).

Skeletal muscle is a metabolically active tissue and serves as an excellent model for studying mitochondrial biogenesis (Sin et al., 2016; Duguez et al., 2002). During skeletal muscle regeneration and in response to exercise, markers of mitochondrial biogenesis increase significantly, suggesting that biogenesis plays an important role in the muscle recovery process (Duguez et al., 2002; Wagatsuma et al., 2011; Ju et al., 2016). Further, studies using C2C12 myoblasts have determined that undifferentiated myoblasts have a less extensive mitochondrial population consisting of single, distinct mitochondria and are more glycolytic, while differentiated myotubes have a more complex mitochondrial network suited to OXPHOS-based metabolism (Barbieri et al., 2011; Sin et al., 2016; Wagatsuma & Sakuma, 2013). Sin et al (2016) hypothesized that mitochondrial remodelling is necessary during myogenic differentiation and that myoblasts must eliminate their old mitochondria so that the new mitochondrial network can be built (Sin et al., 2016). Cells employ a process called autophagy to degrade protein aggregates and organelles, and this process involves sequestering cytoplasmic elements within a double-membrane structure known as an autophagosome, which then

fuses with a lysosome to allow the contents to be degraded and/or re-used (Kondo et al., 2005). Autophagy is crucial for myogenic differentiation (McMillan & Quadrilatero, 2014), and a specific type of autophagy, which is used to degrade mitochondria (mitophagy), is necessary to regulate apoptotic signaling and to eliminate mitochondria so that remodelling can occur during differentiation (Baechler et al., 2019; Sin et al., 2016). Moreover, failure to effectively degrade old mitochondria is thought to account for the differentiation impairments observed in autophagy-deficient myoblasts (Sin et al., 2016).

It is believed that mitophagy is necessary to trigger the induction of mitochondrial biogenesis and to fulfill the increased energy demands during differentiation (Sin et al., 2016; Wagatsuma & Sakuma, 2013). Thus, insufficient autophagy/mitophagy levels could cause energy-deficiency and prevent cells from generating the mitochondrial network necessary to support differentiation. Further, studies have shown that inhibiting mitochondrial function can block myogenic differentiation by preventing the necessary up-regulation of MYOG and downregulation of MYC (Rochard et al., 2000; Seyer et al., 2006), while treatment with mitochondrial biogenesis inducers has been shown to promote differentiation in some cell types (Sharma et al., 2009; Kanno et al., 2004). Therefore, it is evident that mitochondrial homeostasis is critical during differentiation.

We have previously demonstrated that cell lines deficient in the autophagy/mitophagy-related proteins ATG7 and BNIP3 fail to undergo proper myogenic differentiation and have reduced levels of mitochondrial and mitochondrial biogenesis-related protein expression (See Chapter 2 and Chapter 3). Therefore, the purpose of this

study was to determine if treating sh*Atg7* and *bnip3*$^{-/-}$ cells with mitochondrial biogenesis-inducers could effectively rescue myogenic differentiation.

Results

SNP treatment can increase mitochondrial biogenesis

In order to determine if mitochondrial biogenesis can be used to rescue myogenic differentiation in autophagy-deficient cells, we first performed a chemical screen to identify compounds that might increase mitochondrial biogenesis (Figure 1 and Figure 2). Differentiating sh*Atg7* cells were treated with leucine (Figure 1A-E), caffeine (Figure 1F-J), AICAR (Figure 2A-E), or SNP (Figure 2F-J), all of which have previously been shown to increase PPARGC1A levels and/or mitochondrial biogenesis (Liang et al., 2014; Schnuck et al., 2018; Wang et al., 2015). Following treatments, immunoblot analysis was performed to measure mitochondrial proteins (CYCS), markers of mitochondrial biogenesis (PPARGC1A, TFAM), as well as MYOG to determine if there were improvements in myogenic differentiation. There were no significant changes observed in cells treated with leucine (Figure 1A-E) or caffeine (Figure 1F-J), and MYOG and CYCS expression decreased in cells treated with AICAR (Figure 2A-E), suggesting that myogenic differentiation is further reduced in response to AICAR treatment in sh*Atg7* cells. Interestingly; however, sh*Atg7* cells treated with SNP showed dramatic improvements in differentiation- and mitochondria-related protein expression, indicated by increased levels of MYOG, TFAM, PPARGC1A, and CYCS (Figure 2F-J).

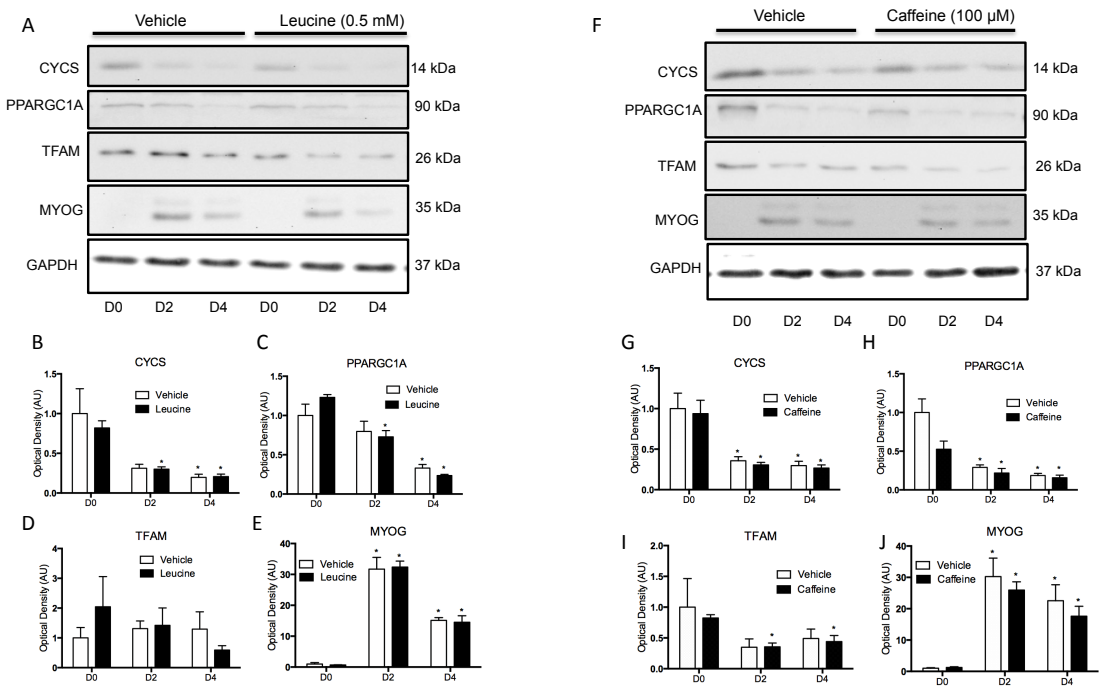

Figure 1. Leucine and caffeine treatment does not enhance mitochondrial biogenesis or myogenesis in sh*Atg7* cells. Representative immunoblots (A) and quantitative analysis (B-E) of CYCS, PPARGC1A, TFAM and MYOG in sh*Atg7* cells treated with leucine (or vehicle) during differentiation. Representative immunoblots (F) and quantitative analysis (G-J) of CYCS, PPARGC1A, TFAM and MYOG in caffeine- or vehicle-treated sh*Atg7* cells during differentiation. Also shown are representative GAPDH loading control blots. *p<0.05 compared to D0 (within group). †p<0.05 between groups at the same time point.

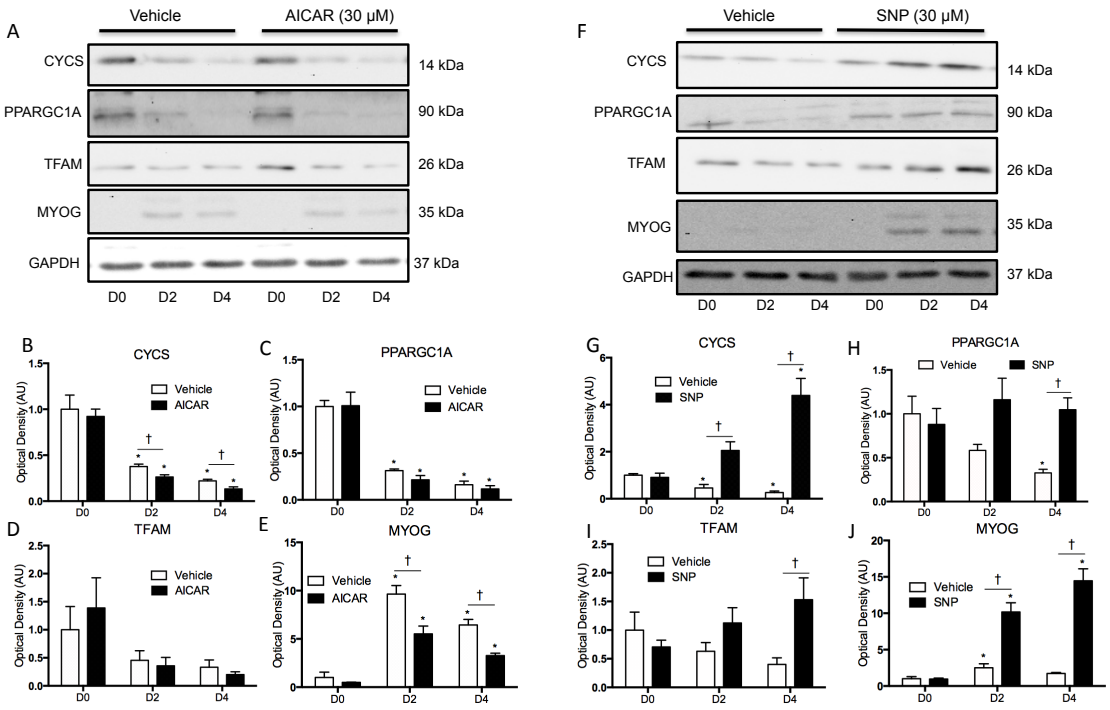

Figure 2. The effect of AICAR and SNP treatment on mitochondrial biogenesis- and myogenesis-related protein expression in sh*Atg7* cells.
Representative immunoblots (A) and quantitative analysis (B-E) of CYCS, PPARGC1A, TFAM and MYOG in sh*Atg7* cells treated with AICAR or vehicle during differentiation. Representative immunoblots (F) and quantitative analysis (G-J) of CYCS, PPARGC1A, TFAM and MYOG in sh*Atg7* cells treated with SNP (or vehicle) during differentiation. Also shown are representative GAPDH loading control blots. *$p<0.05$ compared to D0 (within group). $^{\dagger}p<0.05$ between groups at the same time point.

SNP treatment enhances mitochondrial network rebuilding in both shAtg7 and bnip3$^{-/-}$ cells

Based on the results of our initial screen (Figure 1 and Figure 2), SNP was selected for further experiments to determine if it could enhance mitochondrial biogenesis and improve myogenic differentiation in sh*Atg7* as well as *bnip3*$^{-/-}$ cells. Moreover, consultation with the literature suggested that high doses of SNP are toxic to C2C12 myoblasts (Lee et al., 2005); therefore, the dosage was reduced from 30 μm to 15 μm for subsequent experiments. Interestingly, the lower dose of SNP (15 μm) produced a similar effect and sh*Atg7* cells showed increased PPARGC1A levels after D2. Moreover,

PPARGC1A levels were significantly higher in SNP-treated sh*Atg7* cells at D4 and D5 (Figure 3A, B), and TFAM expression was also elevated (Figure 3A, C). Additionally, SNP-treated cells had significantly higher CYCS levels than vehicle-treated cells throughout differentiation (Figure 3A, D), and VDAC1 was significantly elevated at D4 and D5 in SNP-treated cells relative to controls (Figure 3A, E). This demonstrates that mitochondrial biogenesis and mitochondrial content increased in SNP-treated cells. Similar results were observed in *bnip3*$^{-/-}$ cells treated with SNP. As shown in Figure 3F-J, SNP-treated cells showed increases in PPARGC1A, TFAM, CYCS, and VDAC1, with all showing significantly elevated levels relative to vehicle-treated cells at D5.

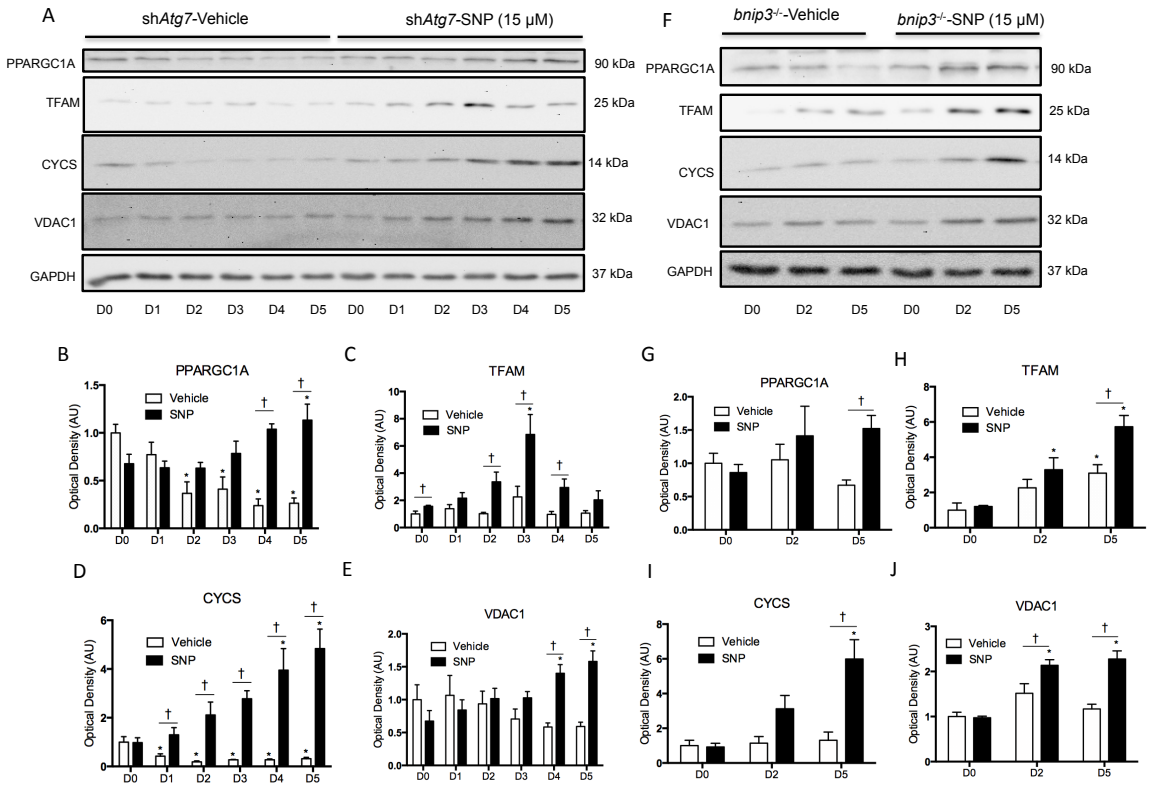

Figure 3. SNP causes increased mitochondrial biogenesis- and mitochondria-related protein expression in sh*Atg7* cells and *bnip3*$^{-/-}$ cells. Representative immunoblots (A) and quantitative analysis (B-E) of PPARGC1A, TFAM, CYCS, and VDAC1 in sh*Atg7* cells treated with SNP (or vehicle) during differentiation. Representative immunoblots (F) and quantitative analysis (G-J) of PPARGC1A, TFAM, CYCS, and VDAC1 in *bnip3*$^{-/-}$ cells treated with SNP or vehicle during myogenic differentiation. Also shown are representative GAPDH loading control blots. *$p<0.05$ compared to D0 (within group). $^{\dagger}p<0.05$ between groups at the same time point.

SNP treatment can partially recover myogenic differentiation in shAtg7 cells

Given the improvements we observed with respect to mitochondrial biogenesis and mitochondrial protein content in sh*Atg7* cells treated with SNP (Figure 3A-E), we next wanted to evaluate myogenic differentiation. First, sh*Atg7* cells treated with either vehicle or SNP, were grown and differentiated on glass coverslips for 4 days, and immunofluorescent staining was performed to visualize myotube formation and measure the fusion and differentiation index. As shown in Figure 4A, MYH-positive cells were rarely observed in vehicle-treated sh*Atg7* cells resulting in a low differentiation index (Figure 4C) and the complete absence of fusion events (Figure 4D). In contrast, sh*Atg7* cells treated with SNP displayed a striking increase in MYH-positive myotubes (Figure 4B), and a significant increase in both the differentiation index (Figure 4C) and fusion index (Figure 4D) relative to the vehicle-treated cells. Similar to the improvements observed previously (Figure 2F, 2J), treatment with a lower concentration of SNP was sufficient to cause a significant increase in MYOG expression (Figure 4E-F).

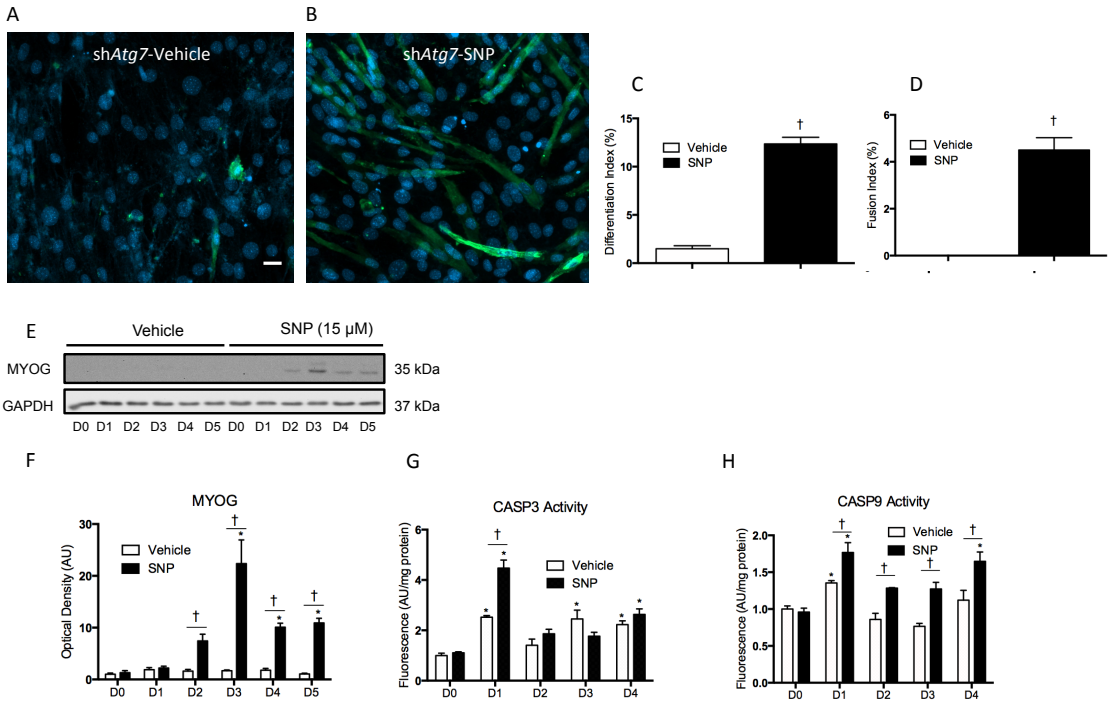

Figure 4. SNP treatment partially restores myogenic differentiation in sh*Atg7* cells. Representative images of sh*Atg7* cells treated with vehicle (A) or SNP (B) at D4 of differentiation. Anti-MYH (green) was used to visualize myotube formation, and nuclei are labelled with DAPI. Scale bar=20 μm. Quantitative analysis of differentiation index (C) and fusion index (D) in vehicle- and SNP-treated sh*Atg7* cells. Representative immunoblots (E) and quantitative analysis (F) of MYOG in sh*Atg7* cells treated with vehicle or SNP during differentiation. Quantitative analysis of CASP3 (G) and CASP9 (H) activity during myogenic differentiation. *$p<0.05$ compared to D0 (within group). †$p<0.05$ between groups at the same time point.

Myogenic differentiation is improved in bnip3$^{-/-}$ cells following SNP treatment

Given that SNP treatment was able to partially restore differentiation in sh*Atg7* cells, we speculated that treating *bnip3*$^{-/-}$ cells with SNP might also recover myogenic differentiation. *bnip3*$^{-/-}$ myoblasts were treated with SNP (or vehicle) throughout differentiation, and immunofluorescent staining revealed that MYH-positive, fused myotubes were rarely observed in vehicle-treated cells (Figure 5A, C-D), while SNP-treated cells showed a significant increase in the number of MYH-positive cells (Figure 5B, C) and fusion events (Figure 5D). Moreover, Western blot analysis showed that SNP

caused a significant increase in both MYH (Figure 5E, F) and MYOG (Figure 5E, G) expression, further supporting that SNP treatment enhances myogenesis in *bnip3*$^{-/-}$ cells.

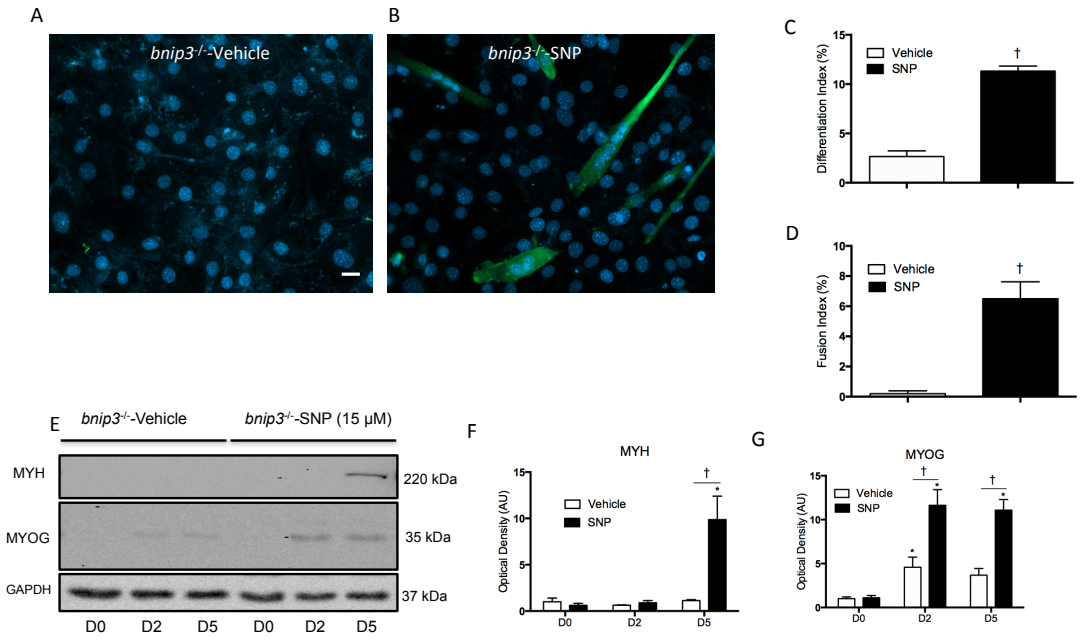

Figure 5. SNP treatment improves myogenic differentiation in *bnip3*$^{-/-}$ cells. Representative images of *bnip3*$^{-/-}$ cells treated with vehicle (A) or SNP (B) at D4 of differentiation. Anti-MYH (green) was used to visualize myotube formation, and nuclei are labelled with DAPI. Scale bar=20 μm. Quantitative analysis of the differentiation index (C) and fusion index (D) in vehicle- and SNP-treated cells. Representative immunoblots (E) and quantitative analysis (F-G) of MYH and MYOG in *bnip3*$^{-/-}$ cells treated with vehicle or SNP during differentiation. *$p<0.05$ compared to D0 (within group). †$p<0.05$ between groups at the same time point.

CASP activation is not reduced in SNP-treated cells

It is known that autophagy-deficiency is associated with increased CASP activation (McMillan & Quadrilatero, 2014), and that a partial recovery of myogenesis is observed in sh*Atg7* cells treated with CASP inhibitors (McMillan, 2015; Baechler et al., 2019). Thus, CASP3 and CASP9 activity were measured in both vehicle- and SNP-treated sh*Atg7* cells (Figure 4G-H). Interestingly, SNP treatment did not reduce CASP activation and actually caused an increase in CASP3 activity at D1 (Figure 4G), and increased

CASP9 activity throughout differentiation (Figure 4H). Therefore, the enhanced myogenic differentiation observed in response to SNP treatment does not result from a decrease in CASP activity. Further, given that CASP3 activation occurs and is required during myogenic differentiation (Fernando et al., 2002), it is possible that the elevated CASP activation in SNP-treated cells could accompany the enhanced differentiation in these cells.

SNP treatment improves oxidative capacity but does not improve mitochondrial integrity

Previous studies have shown that increased oxidative capacity can accompany an increase in mitochondrial biogenesis (Vaughan et al., 2013; Barbieri et al., 2011), and previous work in our lab has determined that oxygen consumption is reduced in sh*Atg7* and *bnip3*$^{-/-}$ cells relative to SCR/Scram cells (Bloemberg, 2017). Therefore, we used the OROBOROS O_2k to determine the maximal oxygen consumption rate in sh*Atg7* and *bnip3*$^{-/-}$ myotubes (D4) treated with vehicle or SNP. Maximal ADP-stimulated respiration was determined by first adding complex I substrates, followed by the addition of a complex II substrate. As shown in Figure 6, SNP-treated sh*Atg7* cells showed a slight increase in respiration following the addition of succinate; however, this was not statistically significant (Figure 6A). Interestingly, *bnip3*$^{-/-}$ cells treated with SNP did show a statistically significant increase in complex I/II-supported respiration relative to controls (Figure 6B). After measuring complex I- and complex II-supported respiration, cytochrome c was added to each chamber to evaluate mitochondrial membrane integrity (Garcia-Roche et al., 2018). Interestingly, the addition of cytochrome c caused increased

oxygen consumption in SNP-treated sh*Atg7* cells (Figure 6A) and this effect was even more pronounced in SNP-treated *bnip3*$^{-/-}$ cells relative to the vehicle-treated controls (Figure 6B). Therefore, it is likely that the overall structural integrity of the mitochondria does not improve in sh*Atg7* and *bnip3*$^{-/-}$ cells in response to SNP treatment. To further support this interpretation, we used flow cytometry to measure calcein fluorescence in the presence of $CoCl_2$ (Figure 6C-D). The calcein AM/$CoCl_2$ assay is used to evaluate mitochondrial permeability transition pore (mPTP) formation (Dam et al., 2013). The calcein AM dye accumulates in mitochondria, which are impermeable to cobalt chloride ($CoCl_2$). Therefore, if mitochondria are leaky/permeable then $CoCl_2$ is able to enter the mitochondria and quench the calcein fluorescence signal (Dam et al., 2013). Thus, a decrease in calcein fluorescence is associated with increased mPTP formation. Although we saw elevated calcein fluorescence in SNP-treated sh*Atg7* and *bnip3*$^{-/-}$ cells relative to controls in the absence of $CoCl_2$ (Figure 6C-D), the addition of $CoCl_2$ caused calcein fluorescence levels to drop to similar levels in both SNP and vehicle-treated sh*Atg7* cells (Figure 6C). Similarly, $CoCl_2$ caused a reduction in calcein fluorescence in *bnip3*$^{-/-}$ cells; however, the SNP-treated cells did maintain a higher level of calcein fluorescence relative to controls (Figure 6D). Thus, although enhancing mitochondrial biogenesis could have increased mitochondrial content, it is likely that the clearance of damaged/dysfunctional mitochondria is still impaired in SNP-treated sh*Atg7* and *bnip3*$^{-/-}$ cells.

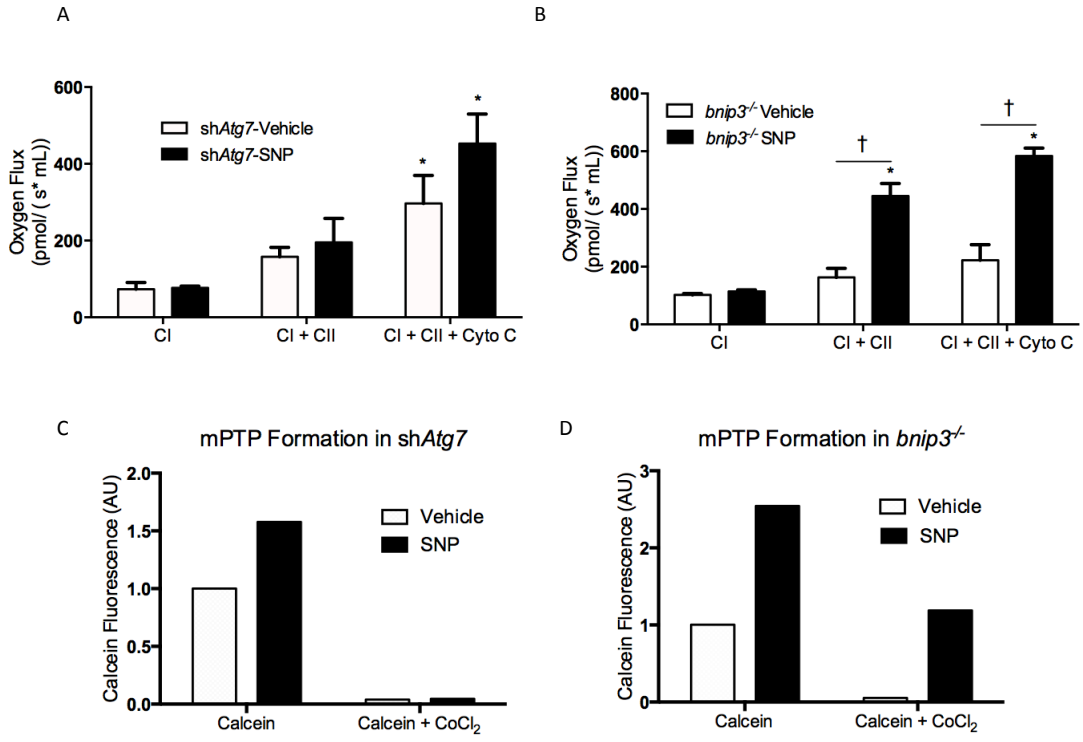

Figure 6. SNP treatment increases mitochondrial respiration in sh*Atg7* and *bnip3*$^{-/-}$ cells, but does not improve mitochondrial health.
(A) Maximal oxygen consumption with complex I substrates, complex I/II substrates, and cytochrome c in SNP- and vehicle-treated sh*Atg7* cells. (B) Maximal oxygen consumption with complex I substrates, complex I/II substrates, and cytochrome c (Cyto c) in *bnip3*$^{-/-}$ cells treated with SNP or vehicle. Quantitative analysis of calcein fluorescence in the presence/absence of $CoCl_2$ as a measure of mPTP formation in sh*Atg7* cells (C) and *bnip3*$^{-/-}$ cells (D), where a decrease in calcein fluorescence is indicative of increased mPTP formation. *$p<0.05$ compared to D0 (within group). $^{\dagger}p<0.05$ between groups at the same time point.

Discussion

The results demonstrate that treating ATG7- and BNIP3-deficient cells with SNP, an inducer of mitochondrial biogenesis (Wang et al., 2015), can partially restore myogenic differentiation. sh*Atg7* cells were initially treated with leucine, AICAR, caffeine, and SNP, all of which have previously been shown to promote PPARGC1A expression and/or mitochondrial biogenesis (Liang et al., 2014; Schnuck et al., 2018; Wang et al., 2015); however, we only saw positive changes in mitochondria-related and myogenesis-

related protein expression in SNP-treated cells (Figure 1 and Figure 2). Further, we demonstrated that SNP partially restores myogenic protein expression, myotube formation and fusion in sh*Atg7* cells (Figure 4) and *bnip3*$^{-/-}$ cells (Figure 5). Moreover, this was accompanied by increased expression of mitochondrial biogenesis-promoting proteins PPARGC1A and TFAM, increases in mitochondrial proteins CYCS and VDAC1 (Figure 3), as well as enhanced mitochondrial respiration (Figure 6A, B). Based on these results, we speculate that increased mitochondrial biogenesis induced by SNP treatment might facilitate myogenic differentiation.

Mitochondrial biogenesis and mitophagy, two opposing processes, are both required and important in regulating cell differentiation (Palikaras et al., 2015; Naik et al., 2018; Wanet et al., 2015). As myoblasts differentiate, they require additional energy to initiate and power through the differentiation process, and must build a mitochondrial network that is better suited to support the OXPHOS-based metabolism associated with differentiated myotubes (Wagatsuma & Sakuma, 2013; Sin et al., 2016). Interestingly, work done by Tang and Rando (2014) suggested that autophagy is induced when muscle satellite cells (SCs) switch from being quiescent to being activated, and that autophagy helps to generate the metabolic substrates needed to produce energy during this transition (Tang & Rando, 2014). Further, they found that SIRT1 regulates this induction of autophagy during SC activation, and that SC activation can be partially rescued in autophagy-deficient cells by providing cells with exogenous pyruvate to support the increased energy requirements (Tang & Rando, 2014). It would therefore be interesting to explore if supplying exogenous energy sources can also rescue the muscle regeneration and differentiation impairments observed in ATG7-deficient cells (Garcia-Prat et al.,

2016; McMillan & Quadrilatero, 2014). Therefore, given that a deficiency in macroautophagy could prevent sh*Atg7* cells from generating sufficient energy and metabolic substrates to support cell differentiation, this likely contributes to the impairment in myogenesis observed in sh*Atg7* cells. Interestingly, although SIRT1 is required for muscle satellite cell activation and can promote myogenic differentiation (Tang & Rando, 2014; Zhou et al., 2015), some studies report that it inhibits myogenesis (Fulco et al., 2008; Pardo & Boriek, 2011). Moreover, SIRT1 has been shown to regulate autophagy and more specifically mitophagy (Tang & Rando, 2014; Jang et al., 2012; Di Sante et al., 2015; Tang, 2016), processes required/induced during myogenesis (McMillan & Quadrilatero; 2014; Sin et al., 2016; Fortini et al., 2016). To further complicate things, SIRT1 is an NAD-dependent histone deacetylase that can deacetylate PPARGC1A to increase its activity, which can therefore promote mitochondrial biogenesis (Tang, 2016; Gerhart-Hines et al., 2007; Cameron et al., 2016). Thus, SIRT1 can mediate both mitochondrial degradation as well as mitochondrial biogenesis, opposing processes that are critical and must be tightly regulated during myogenic differentiation (Tang, 2016; Sin et al., 2016; Wagatsuma & Sakuma, 2013). Interestingly, SNP treatment has not only been shown to increase PPARGC1A levels, but it can also increase SIRT1 expression and activity which could lead to enhanced PPARGC1A activity and mitochondrial biogenesis (Wang et al., 2015; Engel & Mahlknecht, 2008). Although we found that PPARGC1A and its downstream target TFAM (Wu et al., 1999) were upregulated in sh*Atg7* and *bnip3*$^{-/-}$ cells in response to SNP treatment (Figure 3), we did not measure SIRT1 levels or activity. Thus, given that SIRT1 plays numerous roles in regulating myogenic differentiation, future experiments

should address whether SIRT1 expression/activity is impaired in sh*Atg7* and *bnip3*$^{-/-}$ myoblasts, and if the improvement in myogenic differentiation observed in sh*Atg7* and *bnip3*$^{-/-}$ cells in response to SNP treatment is caused by a restoration of SIRT1 levels/activity.

Given that we observed an increase in mitochondrial biogenesis markers in SNP treated cells (Figure 3) along with improvements in myogenic differentiation (Figure 4 and Figure 5), it is interesting to hypothesize that mitochondrial biogenesis is required for and/or promotes myogenic differentiation. Therefore, in order to determine if the rescue we obtained in response to SNP treatment is mediated by the SNP-dependent up-regulation of PPARGC1A and/or TFAM, rescue experiments should be repeated using constructs to overexpress PPARGC1A and TFAM in sh*Atg7* cells and *bnip3*$^{-/-}$ cells. If we are able to obtain a similar recovery in myogenic differentiation by overexpressing PPARGC1A and TFAM, then this would further support our interpretation that mitochondrial biogenesis is both necessary and sufficient to promote myogenic differentiation in sh*Atg7* cells and *bnip3*$^{-/-}$ cells.

Although we were able to improve myogenic differentiation and mitochondria-related protein expression in both sh*Atg7* and *bnip3*$^{-/-}$ cells, the respirometry data (cytochrome c addition) and flow cytometry data (calcein) indicates that the overall mitochondrial health in the SNP-treated cells is compromised (Figure 6). Given our previous findings that mitochondrial degradation does occur in ATG7-deficient cells (Chapter 2) and *bnip3*$^{-/-}$ cells (Chapter 3) it seems reasonable to speculate that it might be an impairment in the specific targeting of damaged/dysfunctional mitochondria rather than bulk/non-selective mitochondrial degradation that disrupts myogenesis. In support

of this, we demonstrated that sh*Atg7* cells release pro-apoptotic factors into the cytosol (Chapter 2) and have previously shown an increase in mitochondria-mediated apoptotic signaling in sh*Atg7* cells (McMillan, 2015; Baechler et al., 2019). Similarly, mitochondria-mediated apoptotic signaling is also elevated in *bnip3*$^{-/-}$ cells (Chapter 3), suggesting that dysfunctional mitochondria persist in sh*Atg7* and *bnip3*$^{-/-}$ cells. Moreover, our previous work has demonstrated that myogenic differentiation can be partially restored in sh*Atg7* cells by inhibiting mitochondrial-mediated CASP activity (McMillan, 2015; Baechler et al., 2019), which demonstrates that mitochondrial dysfunction and the associated cell death signaling can inhibit myogenesis. Thus, given that mitochondrial integrity was still compromised in sh*Atg7* and *bnip3*$^{-/-}$ cells following treatment with a mitochondrial biogenesis inducer (Figure 6), it is likely that these cells are unable to identify and eliminate damaged mitochondria specifically. Although additional experiments are needed to determine why mitochondrial dysfunction persists in SNP-treated cells, we have identified several possible contributing factors that might be disrupting mitochondrial homeostasis. First, we observed a significant increase in PPARGC1A expression in SNP-treated cells, which is expected to promote mitochondrial biogenesis (Palikaras et al., 2015) as well as an increase mitochondrial respiration (Wu et al., 1999). However, others have reported that one role of PPARGC1A during myogenic differentiation is to limit mitophagy (Baldelli et al., 2014). Therefore, if we are causing PPARGC1A levels to rise above a certain threshold, then it is possible that we are further reducing mitochondrial clearance in cells that already have a decreased ability to eliminate damaged/dysfunctional mitochondria. Moreover, given that mitophagy is required during myogenic differentiation (Sin et al., 2016), inhibiting

mitophagy by augmenting PPARGC1A expression could also have negative impacts on differentiation. Thus, it would be beneficial to repeat these experiments using the p-mito-mRFP-EGFP fluorescent mitophagy reporter to determine if mitochondrial degradation is reduced or impaired following SNP treatment.

Our previous work has shown that apoptotic signaling is elevated in both ATG7- and BNIP3-deficient cells during differentiation, and is likely to account for some of the observed impairments in myogenesis (Chapter 2; Chapter 3; Baechler et al., 2019; McMillan & Quadrilatero, 2014). Moreover, in Chapter 2 we showed that there is increased mitochondrial release of pro-apoptotic factors in sh*Atg7* cells, which is likely to contribute to CASP activation and cell death. Further, we determined that mitochondrial membrane integrity does not improve in cells treated with SNP so it is not surprising that CASP activation was not lower in SNP-treated cells (Figure 4G-H). Interestingly, previous work in our lab has demonstrated that increasing mitochondrial biogenesis protects against CASP-dependent and CASP-independent cell death (Dam et al., 2013). However, in that study, cells were treated with mitochondrial biogenesis inducers prior to being treated with apoptotic stressors (Dam et al., 2013). This differs from the current study in that we attempted to enhance mitochondrial biogenesis in cells that were already stressed and unhealthy due to autophagy/mitophagy impairments. This suggests that although mitochondrial biogenesis can offer protection against pro-apoptotic stimuli (Dam et al., 2013), it might not be sufficient to limit cell death or apoptotic signaling in cells that are already unhealthy or dysfunctional. Further, studies have shown that SNP treatment can cause an increase in CASP activation and apoptosis in C2C12 myoblasts (Lee et al., 2005). Although we did see a higher level of CASP activity in SNP-treated

sh*Atg7* cells relative to controls (Figure 4G, H), we speculate that the lower CASP activity in the controls might have occurred because they are already dead or employing CASP-independent cell death mechanisms. Additionally, the elevated CASP activation in SNP-treated cells might occur because the cells contain a greater number of mitochondria than controls, and these mitochondria are leaky and releasing pro-apoptotic factors into the cytosol resulting in a greater induction of CASP activation. Further, studies have shown that high doses of SNP are associated with mitochondrial damage and mitochondria-mediated cell death signaling (Liu et al., 2016). Although we employed a low dose treatment, it is possible that the continuous treatment used might have had a negative impact on mitochondrial quality. Therefore, it would be interesting to try using a more intermittent SNP treatment protocol to see if we can enhance mitochondrial biogenesis and survival, while also limiting the potential for mitochondrial damage and cell death.

Conclusion

Overall these experiments have demonstrated that the impairments in myogenesis observed in sh*Atg7* and *bnip3*$^{-/-}$ cells can be partially overcome by treating cells with SNP. Further investigation is required to determine the mechanism by which SNP treatment restores myogenic differentiation; however, due to the increased mitochondrial-related protein expression we observed in SNP treated cells, and our knowledge of how important mitochondrial biogenesis is for promoting cell differentiation (Wagatsuma & Sakuma, 2013; Sin et al., 2016), we speculate that SNP treatment can partially restore myogenic differentiation by enhancing mitochondrial biogenesis.

Materials and Methods

Cell culture

Culturing conditions have been described previously (McMillan & Quadrilatero, 2014). Briefly, low pass C2C12 myoblasts (A.T.C.C.) were plated in polystyrene cell culture dishes or on Cultrex BME-coated coverslips in growth media (GM) composed of low-glucose Dulbecco's modified Eagle's medium (DMEM), 10% FBS, and 1% penicillin/streptomycin (P/S), and incubated at 37 °C in 5% CO_2. Media was replaced every 24-48 hours. To induce differentiation, GM was replaced with differentiation media (DM) comprised of low-glucose DMEM, 2% horse serum, and 1% P/S, when cells were 80-90% confluent. Cells were collected at day 0 (D0) prior to switching from GM to DM, and collected at appropriate time-points after switching to DM (D1, D2, etc.). Cells were trypsinized (0.25% trypsin with 0.2g/l EDTA), centrifuged (1000g for 5 min), and stored at -80°C.

sh*Atg7* and *bnip3*$^{-/-}$ cell lines were generated previously in our lab (Bloemberg, 2017). Briefly, C2C12 cells were grown in 12-well plates and transfected with vectors encoding an shRNA against *Atg7* (Origene TG504956) or a *bnip3* CRISPR 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 hours. 24 hours 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 and BNIP3 protein levels in selected clones.

Chemical treatments

Chemicals/reagents were dissolved in autoclaved H_2O and fresh solutions were made each day. Chemical solutions were diluted in DM to achieve the appropriate treatment concentrations and were added each day throughout differentiation. The following chemicals were used: 5-Aminoimidazole-4-carboxamide-ribonucleoside/AICAR (30 μM; Toronto Research Chemicals), sodium nitroprusside dihydrate/SNP (15 μM and 30 μM; Sigma–Aldrich), leucine (0.5 mM; Sigma-Aldrich), caffeine (100 μM; Sigma–Aldrich).

Immunoblotting

Immunoblotting was performed as described previously (McMillan & Quadrilatero, 2011; McMillan & Quadrilatero, 2014). 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 (Roche Applied Sciences). Equal amounts of protein were loaded and separated on 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°C in primary antibodies against: GAPDH (2118; Cell Signaling), MYOG, MYH, (F5D, MF20; Developmental Studies Hybridoma Bank), CYCS, PPARGC1A, TFAM, VDAC1 (sc-13156, sc-13067, sc-166965, sc-390996;Santa Cruz Biotechnology). 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 and bands were visualized using the Clarity Western ECL substrate (Bio-Rad) and the ChemiGenius 2 Bio-Imaging System

(Syngene).

Caspase assays

CASP3 and CASP9 assays were performed as described previously (McMillan et al., 2015; Baechler et al., 2019). Cells were sonicated in lysis buffer without protease inhibitors. CASP3 and CASP9 activity were determined using the fluorogenic substrates Ac-DEVD-AFC and Ac-LEHD-AFC, respectively (AAT Bioquest, 13401; Tocris Bioscience, 1575), and fluorescence was measured at the appropriate wavelength using a Synergy H1 microplate reader (BioTek) in duplicate. CASP activity measures were normalized to total protein content which was determined using the BCA method.

Immunofluorescence and microscopy

Immunofluorescent staining of cells was performed as described previously (McMillan & Quadrilatero, 2014). Cells were grown on glass coverslips in culture dishes and the coverslips were removed from the dishes at D4. Coverslips were transferred to a new dish and washed with PBS (3x 5 min washes). Cells were fixed with 4% formaldehyde in PBS for 10 minutes at room temperature, and PBS washes were repeated. Cells were permeablized with 0.5% Triton X-100 for 10 minutes and washed in PBS. Blocking to reduce non-specific binding involved incubating coverslips in 10% goat serum (Sigma–Aldrich) in PBS for 30 minutes. After blocking, coverslips were incubated with anti-MYH primary antibody (MF20; DSHB) at the appropriate dilution in fresh blocking solution for 1 hour. PBS washes were then repeated. Next, the coverslips were incubated for 1 hour in the appropriate fluorochrome-conjugated secondary antibody diluted in blocking solution. Subsequently, PBS washes were repeated, cells were stained with DAPI nuclear stain (Life Technologies; D3571) for 5 minutes, and then washed with

PBS. Coverslips were placed on slides and Prolong Gold Antifade Reagent was used for mounting. Slides were imaged the following day using an Axio Observer Z1 microscope equipped with an AxioCam HRm camera and AxioVision software (Carl Zeiss). Fusion index was calculated as the percentage of nuclei in multinucleated cells (two or more nuclei) relative to total nuclei, and differentiation index was determined by counting the percentage of MYH-positive cells relative to the total number of cells per field.

Respirometry

High-resolution respirometry was performed using the OROBOROS O_2k and measurements of oxidative capacity were performed using previously developed protocols (Bloemberg, 2017; Bradley et al., 2017). Briefly, $1.0x10^6$ cells/group were collected and centrifuged at 100g, permeabilized in digitonin/sucrose buffer (PBS with 250 mM sucrose, 80 mM KCl, and 50 µg/mL digitonin) for 3 min at room temperature, then centrifuged again at 200g. The digitonin/sucrose buffer was aspirated and cells were re-suspended in mitochondrial respiration buffer (Mir05: 0.5 mM EGTA, 3 mM $MgCL_2$-$6H_2O$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/L fatty acid-free BSA). The re-suspended cells were then added to the O_2K chambers and respiration was performed at 37°C under oxygenated conditions (one chamber was used for control cells and one chamber was used for SNP-treated cells). Subsequently, the following complex I substrates were added to each chamber: glutamate (10 mM), pyruvate (5 mM), and malate (2 mM). ADP-stimulated respiration was then determined by adding increasing concentrations of ADP to the chambers, up to a maximum concentration of 1 mM. In order to determine maximal complex-II supported

respiration, succinate was added to each chamber (10 mM). After reaching maximal respiration, cytochrome c (10 μM) was added to each chamber to evaluate mitochondrial membrane integrity (Bloemberg, 2017). Cytochrome c is released into the cytosol if the mitochondrial outer membrane is damaged or leaky, and this is shown by increased oxygen consumption following the addition of cytochrome c (Garcia-Roche et al., 2018).

Flow cytometry

Calcein AM/$CoCl_2$ was used to assess mitochondrial permeability transition pore (mPTP) formation, which can result in the release of pro-apoptotic factors from the mitochondria. Calcein AM is a fluorescent dye that will accumulate in mitochondria and can be quenched by $CoCl_2$. Mitochondria are impermeable to $CoCl_2$ but if mPTP formation occurs, $CoCl_2$ can enter the mitochondria and quench calcein fluorescence. Therefore, a decrease in calcein fluorescence is indicative of mPTP formation (Dam et al., 2013). Cells were collected and incubated with 1 μM calcein AM (Enzo Life Sciences, ALX-610-026) only or with 1 mM $CoCl_2$ dissolved in HBSS for 15 min at 37°C. Following incubation, cells were washed by centrifugation, and resuspended in 500 μl HBSS. All analyses were performed using a BD FACSCalibur flow cytometer equipped with Cell Quest Pro software (BD Bioscience).

Statistics

Statistical analysis was performed using GraphPad PRISM. A one-way ANOVA was used to assess the effect of differentiation within groups, with Bonferroni's multiple comparison test to compare differences from D0. Differences between time-matched

treated and control groups were assessed using a Students T-test. For all experiments, n = 3 or more independent trials.

Chapter 5: Summary and Discussion

Chapter 2 Summary

The major goals/hypotheses of Chapter 2 were:
1) To determine if mitophagy is completely blocked in ATG7-deficient myoblasts/myotubes. We hypothesized that mitophagy would be reduced but not absent in sh*Atg7* cells.

2) To determine if overexpression of a mitophagy-related protein is sufficient to reduce cell death and improve differentiation in ATG7-deficient cells. We predicted that overexpression of mitophagy-related proteins would enhance mitophagy in sh*Atg7* cells to reduce cell death but that it would not fully restore myogenic differentiation.

Major findings:

- Mitophagy increases during C2C12 myogenic differentiation, which was validated using several fluorescence-based mitophagy reporters and confocal microscopy.
- Mitochondrial biogenesis, fission, and fusion proteins were reduced in sh*Atg7* cells, and the expression of mitochondrial proteins was also lower in sh*Atg7* cells, suggesting that mitochondria-related signaling and content is reduced in sh*Atg7* cells.
- Mitochondrial release of AIFM1 and CYCS was elevated in sh*Atg7* cells, suggesting that sh*Atg7* cells contain more leaky/damaged mitochondria, which can contribute to elevated mitochondria-mediated apoptotic signaling.
- LC3-dependent mitophagy is almost absent in sh*Atg7* cells as demonstrated by DsRed-Mito/LC3-GFP co-localization.
- LC3-independent/alternative mechanisms of mitochondrial degradation occur in sh*Atg7* cells, as demonstrated using a p-mito-mRFP-EGFP fluorescent mitophagy reporter.
- Treating sh*Atg7* cells with an alternative mitophagy inhibitor, BFA, reduces mitochondrial degradation in sh*Atg7* cells and exacerbates myogenic differentiation and myogenesis impairments in sh*Atg7* cells.
- Alternative mitophagy is not required for myogenic differentiation in healthy C2C12 myoblasts.
- Overexpression of BNIP3 in sh*Atg7* cells can delay cell loss/death, resulting in a partial recovery of myotube formation.
- Overexpression of PRKN and BECN1 in sh*Atg7* cells does not improve myogenic differentiation.

Chapter 3 Summary

The major objectives for Chapter 3 were:
1) To determine if the mitophagy-related proteins BNIP3 and BNIP3L/NIX are required for myogenic differentiation. We predicted that myogenic differentiation would be impaired in BNIP3L- and BNIP3-deficient myoblasts.

2) To determine if autophagy and mitophagy are altered in BNIP3-deficient cells. We hypothesized that mitophagy would be disrupted in BNIP3-deficient cells and that this would cause an increase in (macro)autophagy in an attempt to degrade accumulating mitochondria.

Major findings:

- Myogenic differentiation is impaired in myoblasts deficient in BNIP3L or BNIP3.
- There is a compensatory increase in the expression of BNIP3L in BNIP3-deficient cells, and conversely a compensatory increase in the expression of BNIP3 in BNIP3L-deficient cells.
- Mitophagy does occur in *bnip3*$^{-/-}$ cells during differentiation as demonstrated by DsRed-Mito/LC3-GFP co-localization as well as the p-mito-mRFP-EGFP mitophagy reporter. However, we suspect that there is impairment in the specific targeting and degradation of dysfunctional mitochondria in *bnip3*$^{-/-}$ cells, which results in increased CASP9 and CASP3 activity.
- Mitochondrial signaling- and mitochondria-related protein expression was generally lower in *bnip3*$^{-/-}$ cells relative to Scrams.
- Autophagy-related protein expression is elevated in *bnip3*$^{-/-}$ cells.
- Overexpression of ATG7 or treatment with the autophagy inducer rapamycin disrupts myogenesis, as demonstrated by reduced levels of MYOG and MYH expression.
- Overexpression of autophagy-related proteins or rapamycin treatment generally caused a reduction in CASP activation during differentiation.

Chapter 4 Summary

The main objectives for Chapter 4 were:
1) To determine if increased mitochondrial biogenesis can restore myogenic differentiation in sh*Atg7* cells and *bnip3*$^{-/-}$ cells. We had hypothesized that enhancing mitochondrial biogenesis might reduce cell death, given that previous work in our lab has shown that increasing mitochondrial biogenesis can protect against cell death (Dam et al., 2013), which could then result in increased myotube formation.

2) To determine if mitochondrial function improves in sh*Atg7* and *bnip3*$^{-/-}$ cells following treatment with an inducer of mitochondrial biogenesis. We had predicted that mitochondrial function and health would improve in sh*Atg7* cells and *bnip3*$^{-/-}$ cells treated with mitochondrial biogenesis inducers.

Major findings:

- SNP treatment increased mitochondrial biogenesis- and mitochondria-related protein expression in sh*Atg7* and *bnip3*$^{-/-}$ cells.
- SNP treatment enhances differentiation and myotube formation in sh*Atg7* and *bnip3*$^{-/-}$ cells.
- SNP treatment resulted in a small increase in mitochondrial respiration in sh*Atg7* cells.
- SNP treatment significantly enhanced mitochondrial respiration in *bnip3*$^{-/-}$ cells.
- SNP treatment did not improve mitochondrial integrity in sh*Atg7* and *bnip3*$^{-/-}$ cells.

Mitophagy plays a role in supporting myogenic differentiation

We among others have shown that mitophagy is required during myogenic differentiation, with its role being to limit/control apoptotic signaling and to support mitochondrial biogenesis and remodelling (Sin et al., 2016; Baechler et al., 2019; Chapter 2; Chapter 3). Interestingly, Sin et al (2016) had used immunoblotting/immunostaining to show that there is a decrease in the level of the mitochondrial protein TOMM70A during early myogenic differentiation. Moreover, they also reported increased SQSTM1 levels in mitochondrial fractions, to suggest that mitochondrial targeting and degradation increases during early differentiation (Sin et al., 2016). In support of this interpretation, we have shown that LC3-puncta (autophagosomes) and mitochondria co-localize during differentiation (Baechler et al., 2019; Chapter 2; Chapter 3). Moreover, this thesis was the first study that used a p-mito-mRFP-EGFP fluorescent mitophagy reporter (Kim et al., 2013) to demonstrate that mitophagy increases when C2C12 myoblasts are induced to differentiate (Chapter 2 - Figure 1). Further, this reporter shows that mitochondria are not only targeted for degradation, but that they are also successfully delivered to lysosomes (Kim et al., 2013). Although we had assumed that co-localization of autophagosomes and mitochondria is indicative of mitophagy, the LC3-GFP and DsRed-Mito method does not allow us to specifically determine if the autophagosomes and lysosomes ever fuse or if the mitochondria are degraded. Therefore, we have provided sufficient evidence to support that mitophagy increases during myogenic differentiation, as it does during differentiation in other cell types (Sandoval et al., 2008; Ney, 2015). Thus, it is likely that the induction of mitophagy plays a role in mediating myogenic differentiation and myotube formation.

Although we have demonstrated that mitophagy occurs during myogenesis, we among others have used cells deficient in autophagy-related proteins, such as ATG7, to investigate the requirement for mitophagy during differentiation. Given that LC3 lipidation is disrupted in these cells (Chapter 2 - Figure 6), we had assumed that autophagosomes do not form and thus that mitophagy cannot occur. However, previous work has determined that mitophagy and mitochondrial degradation can occur using alternative pathways that do not require LC3 (Nishida et al., 2009; Hirota et al., 2015; Wang et al., 2016; Hammerling et al., 2017; Oliveira et al., 2015). In support of this, when we examined sh*Atg7* cells expressing the tandem p-mito-mRFP-EGFP mitophagy reporter we found that mitochondrial degradation was happening in sh*Atg7* cells during myogenic differentiation (Chapter 2 - Figure 7). This result was somewhat surprising because our previous work has indicated that mitochondrial membrane potential is lower and mitochondrial membrane permeability is higher in sh*Atg7* cells, which is indicative of mitochondrial dysfunction (McMillan, 2015; Baechler et al., 2019). Additionally, mitochondria-mediated apoptotic signaling is elevated in sh*Atg7* cells (McMillan, 2015; Baechler et al., 2019). In support of this, we demonstrated that there were increased cytosolic levels of CYCS and AIFM1 in sh*Atg7* cells (Chapter 2 - Figure 3), which would occur as a result of mitochondrial damage and could induce CASP-dependent and CASP-independent cell death signaling (Wang, 2001). This is in agreement with previous work in our lab which showed that CASP9 and CASP3 activity is elevated in sh*Atg7* cells, and is indicative of mitochondria-mediated CASP activation (McMillan, 2015). Moreover, sh*Atg7* cells had higher levels of the mitochondrial antioxidant SOD2 (Bresciani, 2015; Candas & Li, 2014), which could indicate that there is increased mitochondrial oxidative

stress in sh*Atg7* cells. In agreement with this interpretation, previous studies in our lab have shown that sh*Atg7* myoblasts have elevated levels of mitochondrial 4-HNE (McMillan, 2015; Baechler et al., 2019), which is a known marker of oxidative stress (Uchida, 2003; Xiao et al., 2017). Further, our previous work has demonstrated that reducing mitochondria-mediated apoptotic signaling through CASP9 inhibition can partially rescue myogenic differentiation in sh*Atg7* cells (McMillan, 2015; Baechler et al., 2019). Therefore, these results would suggest that the specific degradation of damaged or dysfunctional mitochondria is somewhat impaired in sh*Atg7* cells and that this impairment disrupts proper myogenesis. Therefore, although mitochondria can be degraded in sh*Atg7* cells, it is possible that alternative mitophagy or other forms of mitochondrial degradation are not sufficient to selectively degrade damaged mitochondria during myogenic differentiation. Studies using erythroleukemia cells have shown that ATG7-deficient cells use alternative mitophagy to limit ROS and apoptosis; however, cell differentiation is still impaired (Wang et al., 2016). This is similar to our work in that sh*Atg7* myoblasts fail to differentiate; however, in contrast to the work of Wang et al (2016), we have found that apoptotic signaling and ROS levels are elevated in *shAtg7* cells (Chapter 2 - Figure 3; McMillan, 2015; Baechler et al., 2019; McMillan & Quadrilatero, 2014). Interestingly, it has been suggested that alternative/LC3-independent mitophagy is used to eliminate mitochondria during erythrocyte differentiation (Nishida et al., 2009; Honda et al., 2014). However, one major difference between erythrocyte differentiation and the differentiation of myoblasts is that mitochondria are eliminated and not replaced in erythrocytes. In contrast, mitophagy during myogenic differentiation is accompanied by mitochondrial biogenesis and the

formation of a new mitochondrial network (Sin et al., 2016). Therefore, it is possible that alternative mitophagy might effectively degrade mitochondria but that canonical autophagy/mitophagy is required to trigger mitochondrial biogenesis. In support of this hypothesis, we demonstrated that mitochondrial degradation occurs in sh*Atg7* cells during myogenic differentiation (Chapter 2; Figure 7), but that mitochondrial biogenesis is impaired (Chapter 2 - Figure 2). Therefore, future work is needed to address the differences among canonical and non-canonical mitophagy as well as other forms of mitochondrial degradation to determine if any of the proteins that are specific to mediating canonical mitophagy are also involved in promoting mitochondrial biogenesis during myogenic differentiation. Identifying differences among the different pathways that mediate mitochondrial degradation could help to determine why one pathway is dominant or crucial in certain contexts, such as during myogenesis. Another important consideration is that specific mitophagy pathways may be more/less important for degrading damaged mitochondria. Thus, if alternative mitophagy is used to degrade mitochondria during erythrocyte differentiation (Nishida et al., 2009; Honda et al., 2014), then this would be an example of developmentally programmed mitochondrial removal and might not require that mitochondria be damaged/dysfunction. Our previous work and the results of this thesis have demonstrated that sh*Atg7* cells have more damaged mitochondria and elevated ROS levels, suggesting that the mitophagy-mediated removal of dysfunctional mitochondria is compromised (Chapter 2; McMillan, 2015; Baechler et al., 2019). Thus, although we observed mitochondria being degraded in sh*Atg7* cells, these events may represent the removal of non-damaged mitochondria for the purpose of mitochondrial remodelling and differentiation, and might not distinguish between

damaged and healthy mitochondria. Therefore, it is possible that the LC3-independent/alternative mitophagy occurring in sh*Atg7* cells might not be specific to degrading damaged mitochondria, which could then accumulate and outnumber the population of healthy mitochondria, and thus explain the increased apoptotic signaling that is characteristic of sh*Atg7* cells during differentiation (McMillan & Quadrilatero, 2014; McMillan, 2015; Baechler et al., 2019). Further, basal levels of mitochondrial degradation might be low in undifferentiated myoblasts, while the stress associated with differentiation could result in increased mitochondrial damage and the need to eliminate dysfunctional mitochondria. If this is the case, then sh*Atg7* cells might fail to differentiate and die (McMillan & Quadrilater, 2014) because they cannot identify and eliminate damaged mitochondria specifically using alternative (autophagy-independent) mechanisms to degrade mitochondria. In Chapter 2, we demonstrated that healthy C2C12 myoblasts do not require alternative mitophagy to generate myotubes (Chapter 2 - Figure 8), suggesting that alternative mitophagy is likely not the dominant pathway for eliminating mitochondria during myogenesis. Thus, it is possible that alternative mitophagy serves as a compensatory mechanism to degrade mitochondria in autophagy-deficient cells only, but that it may not be specific to the removal of damaged mitochondria, or sufficient to support the required mitophagy and mitochondrial remodelling during myogenic differentiation. Therefore, future experiments might involve treating sh*Atg7* cells with chemicals known to cause mitochondrial damage to evaluate if damage-induced mitochondrial degradation occurs via the alternate mitophagy pathway in sh*Atg7* cells.

Additionally, we used the p-mito-mRFP-EGFP mitophagy reporter as a qualitative measure of mitochondrial degradation. Although it would have been ideal to quantify the number of mitophagy/mitochondrial degradation events in sh*Atg7* cells in comparison to SCR cells to determine if overall mitochondrial degradation was reduced in sh*Atg7* cells, the transient nature of the fluorescent reporter made it difficult to quantify events. Further, as cells fused, the strength of the fluorescent signal seemed to diminish. Therefore, it would be useful to generate a stable p-mito-mRFP-EGFP reporter cell line for future microscopy experiments and mitophagy-related measures; however, this would have been challenging to do in our sh*Atg7* cell lines. Further, although this method could provide a measure of gross mitochondrial degradation, it does not allow us to determine if mitochondrial degradation is selective/specific or if the mitochondria being degraded are damaged/dysfunctional. Therefore, it could be that although mitochondrial degradation does occur in sh*Atg7* cells, it could happen less frequently/rapidly than in SCR cells, and more importantly, it is possible that there is less specificity and targeting of damaged/dysfunctional mitochondria in sh*Atg7* cells.

In an attempt to compensate for a deficiency in autophagy/mitophagy and recover myogenic differentiation in sh*Atg7* cells, we overexpressed the autophagy/mitophagy-related proteins BECN1, PRKN, and BNIP3 (Chapter 2 - Figure 4). Moreover, we had speculated that overexpressing these proteins might enhance mitophagy in sh*Atg7* cells, which could limit cell death in addition to promoting differentiation. Interestingly, we found that BECN1 and PRKN did not improve myogenic differentiation in sh*Atg7* cells (Chapter 2 - Figure 4), while BNIP3 seemed to improve myogenesis by delaying cell death (Chapter 2 - Figure 4; Figure 5), which supports a role for BNIP3 and mitophagy in

regulating differentiation and cell death. We suspect that PRKN might not have recovered myogenic differentiation for the following reasons: 1) we have never detected PRKN expression in C2C12 myoblasts (Bloemberg, 2017; Chapter 2 - Figure 4), which might indicate that it has no mitophagy-related role in C2C12 cells; 2) PRKN is generally associated with canonical/LC3-dependent mitophagy, which does not occur in sh*Atg7* cells (Chapter 2 - Figure 6); and 3) there may not have been sufficient PINK1 levels or activity to activate PRKN and trigger PINK1/PRKN-mediated mitophagy. PINK1 is required to activate PRKN E3 ligase activity and recruit PRKN to damaged mitochondria (Kane et al., 2014; Gladkova et al., 2018; Pickles et al., 2018). Thus, although we overexpressed PRKN in an attempt to increase PINK1/PRKN-mediated mitophagy, PRKN might have remained in an inactive form. Therefore it might be necessary to also increase PINK1 levels or activity in order to activate PRKN activity and/or identify mitochondria to be degraded. The failure to recover myogenesis by overexpressing BECN1 may have resulted because: 1) canonical autophagy/mitophagy cannot be upregulated because the ATG7-deficiency would disrupt LC3 lipidation; 2) too much BECN1 can have negative consequences with respect to cell health/survival (Zhu et al., 2018). For example, overexpression of BECN1 in sarcoma cells causes an increase in CASP9 and CASP3 activation, resulting in increased cell death (Zhu et al., 2018). Therefore, given that CASP9 and CASP3 activity is elevated in sh*Atg7* cells (McMillan, 2015; Baechler et al., 2019), elevated levels of BECN1 could further augment apoptotic signaling. We did; however, achieve a partial recovery of myogenic differentiation by overexpressing BNIP3 in sh*Atg7* cells. Overexpression of BNIP3 delayed cell loss/death in sh*Atg7* cells, so there were more cells available to fuse and form myotubes (Chapter 2

- Figure 5). Moreover, overexpression of BNIP3 caused a reduction in p-H2AFX levels in sh*Atg7* cells at D2 of differentiation (Chapter 2 - Figure 4). Although we did not see a significant reduction in CASP3 activity in sh*Atg7* cells overexpressing BNIP3 (Chapter 2 - Figure 5I), the elevated cytosolic CYCS and AIFM1 levels in sh*Atg7* cells (Chapter 2 - Figure 3) would suggest that sh*Atg7* cells are more susceptible to both CASP-dependent and CASP-independent cell death (Wang, 2001). Therefore, we speculate that the reduction in cell death may have occurred because BNIP3 can limit cell death signaling by eliminating damaged mitochondria (Zhu et al., 2013), and can mediate LC3-independent mitophagy and mitochondrial degradation (Zhang et al., 2012; Hammerling et al., 2017). Interestingly, we had found that overexpression of BNIP3 was able to induce mitochondrial degradation in proliferating C2C12 myoblasts (Chapter 2 - Figure 4D). Additionally, BNIP3 has been shown to transcriptionally repress several death-promoting proteins, and thus it is possible that BNIP3 can reduce cell death in a mitophagy-independent manner (Burton et al., 2009; Burton et al., 2013). Future work should therefore address the localization of BNIP3 when it is overexpressed to determine if it is localized to the nucleus or mitochondria, which might assist with understanding its ability to partially rescue and restore myogenic differentiation in sh*Atg7* cells.

To further support our results demonstrating that impairment in the degradation of dysfunctional or damaged mitochondria inhibits myogenic differentiation, we generated cells deficient in the mitophagy receptor proteins BNIP3L and BNIP3 (Chapter 3). Moreover, this was the first study to knockdown/knockout these mitophagy-related proteins in C2C12 myoblasts to investigate their roles during myogenic differentiation, as previous work has relied upon using cells deficient in general autophagy-related proteins

such as SQSTM1 and ATG5 (Sin et al., 2016). Interestingly, we found that myogenic differentiation was impaired in both BNIP3L-deficient (Chapter 3 - Figure 1) and BNIP3-deficient cells (Chapter 3 - Figure 2). Moreover, and similar to sh*Atg7* cells, we found that *bnip3*$^{-/-}$ cells had elevated CASP9 and CASP3 activity as well as increased p-H2AFX levels (Chapter 3 - Figure 2E-G), suggesting that mitochondria-mediated apoptotic signaling is elevated in *bnip3*$^{-/-}$ cells. Further, *bnip3*$^{-/-}$ cells had higher levels of SOD2 expression early during differentiation, which could suggest an increase in oxidative stress or mitochondrial accumulation. Thus, the combination of elevated mitochondria-mediated apoptotic signaling and increased expression of a marker of mitochondrial oxidative stress would suggest that *bnip3*$^{-/-}$ cells contain more dysfunctional/damaged mitochondria. Although this is similar to what we have observed in sh*Atg7* cells, one major difference is that LC3-dependent autophagy/mitophagy occurs during myogenic differentiation in *bnip3*$^{-/-}$ cells (Chapter 3 - Figure 4A-C), but does not occur in sh*Atg7* cells (Chapter 2 – Figure 6). However, the DsRed-Mito and GFP-LC3 method can only demonstrate that mitochondria co-localize with autophagosomes and does not allow us to determine if mitochondria are ever delivered to and degraded by the lysosome. Therefore, we also used the p-mito-mRFP-EGFP reporter to show that mitochondrial degradation does occur in *bnip3*$^{-/-}$ cells during differentiation (Chapter 3 - Figure 4). Interestingly, previous studies have suggested that BNIP3 can mediate both LC3-dependent and LC3-independent mitochondrial degradation (Hanna et al., 2012; Zhang et al., 2012; Hammerling et al., 2017). Moreover, recent work has shown that BNIP3 can promote endosome-mediated mitochondrial degradation, in addition to its established role in promoting mitophagy (Hammerling et al., 2017). Thus, if BNIP3 is involved in

regulating multiple pathways involved in regulating mitochondrial degradation, then several mechanisms of mitochondrial removal could be compromised in *bnip3*$^{-/-}$ cells. Further, if one of these pathways, such as mitophagy-mediated degradation, is compromised, then there could be a compensatory upregulation or need for an alternate pathway to degrade mitochondria, like in the sh*Atg7* cells. Moreover, given that endosome-mediated mitochondrial degradation might occur more rapidly than autophagosome-mediated mitophagy (Hammerling et al., 2017), it seems reasonable to speculate that this mechanism for mitochondrial elimination might be less specific to targeting and removing damaged/ dysfunctional mitochondria, and instead might increase the overall elimination of mitochondria (both healthy and unhealthy). If this is the case, then an increase in gross/non-specific mitochondrial removal in BNIP3-deficient cells could result in a higher ratio of unhealthy to healthy mitochondria, which would then cause elevated mitochondria-mediated cell death signaling (Chapter 3 - Figure 2E-F). Interestingly, we found that BNIP3L expression was elevated in *bnip3*$^{-/-}$ cells (Chapter 3 - Figure 5A-B), which has been reported in other BNIP3-deficient cell lines (Chourasia et al., 2015; Shi et al., 2014). Therefore, it is possible that BNIP3L expression was upregulated to compensate for a deficiency in BNIP3 to eliminate mitochondria; however, as others have reported, it is likely that elevated BNIP3L expression cannot fully compensate for having insufficient levels of BNIP3. Taken together, these results demonstrate that ATG7, BNIP3, and BNIP3L are required during myogenic differentiation. Moreover, the mitochondrial dysfunction and mitochondria-mediated apoptotic signaling observed in sh*Atg7* and *bnip3*$^{-/-}$ cells suggests that damaged

mitochondria are not being eliminated, which likely contributes to the observed impairment in myogenesis observed in these cell lines.

Inappropriate levels of autophagy can disrupt myogenic differentiation

Previous work in our lab and the work of others, has established an important role for autophagy in supporting satellite cell quiescence, activation, as well as myogenic differentiation. Interestingly, these studies have all shown that autophagy is necessary and that insufficient levels of autophagy have a negative impact on some element of cell maintenance and/or differentiation (Garcia-Prat et al., 2016; Tang & Rando, 2014; McMillan & Quadrilatero, 2014; Fortini et al., 2016). However, in this thesis, we examined the potential impact of having too much autophagy, given that that there was elevated autophagy in *bnip3*$^{-/-}$ cells (Chapter 3 - Figure 5), and found that overexpression of some autophagy-related proteins can have negative consequences with respect to myogenic differentiation (Chapter 3 - Figure 6). This is in agreement with mouse studies that have shown that overactivation of autophagy can be damaging to muscle (Chrisam et al., 2015). In support of these interpretations, we found that overexpression of ATG1/ULK1 in muscle progenitors in *Drosophila* was disruptive to development and resulted in abnormal wing postures (Chapter 3 - Figure 8). Interestingly, similar phenotypes have been reported in flies that are deficient in PINK1, and these muscle abnormalities are associated with mitochondrial dysfunction and apoptosis (Clark et al., 2006; Park et al., 2006). Our results have demonstrated that myogenesis is impaired and mitochondrial dysfunction is elevated in BNIP3-deficient cells, and that autophagy-related protein expression is higher in *bnip3*$^{-/-}$ cells relative to Scram cells. Therefore, it

is interesting to speculate that PINK1-deficient flies might also upregulate macroautophagy in an attempt to eliminate damaged mitochondria, and that this might contribute to the reported muscle dysfunction.

One interesting finding was that CASP activity was generally lower in cells overexpressing autophagy-related proteins (Chapter 3 - Figure 7). Our lab has previously shown that CASP activation and apoptotic signaling is elevated in autophagy-deficient cells, suggesting that autophagy may be required to limit CASP activity (McMillan & Quadrilatero, 2014). Therefore, it is possible that overexpression of autophagy-related proteins or treatment with an autophagy inducer can suppress CASP activation. A similar effect has been reported in animal studies demonstrating that enhancing autophagy suppresses CASP activation in skeletal muscle (Li et al., 2018). Although this effect could be beneficial for preventing cell death, it is known that CASP3 activation is required during myogenic differentiation (Fernando et al., 2002). Therefore, if the timing or level of CASP3 activation is altered due to elevated expression of autophagy-related proteins, then this could prevent cells from differentiating properly.

Interestingly, studies have shown that autophagy can actually promote both CASP-dependent and CASP-independent cell death (Scott et al., 2007; Liu & Levine, 2015). Moreover, as mentioned in Chapter 3, a form of autophagy-mediated cell death termed "autosis" has recently been described (Liu et al., 2013). Interestingly, autotic cell death is not associated with CASP activation and often results in increased cell adhesion (Liu et al., 2013; Liu & Levine, 2015). This could potentially explain why we do not observe an increase in CASP activity in cells overexpressing autophagy-related proteins. Additionally, we found that although differentiation was impaired in cells overexpressing

ATG7 and in cells treated with rapamycin, the cells were not eliminated. Moreover, a similar phenotype was observed in *bnip3*$^{-/-}$ cells, which also showed elevated expression of autophagy-related proteins (Chapter 3 - Figure 5). Therefore, it is important to consider that autophagy levels must be tightly regulated during myogenic differentiation, given that insufficient levels disrupt differentiation and induce cell death, while similar consequences can result from having too much autophagy.

Autophagy is required to limit apoptosis

The requirement for autophagy during myogenesis in C2C12s has been supported by animal studies and studies using primary myoblasts (Garcia-Prat et al., 2016; Fortini et al., 2016). Our previous studies and the experiments comprising this thesis would suggest that differentiation is impaired in autophagy/mitophagy-deficient cells due to increased cell death signaling (Chapter 2; Chapter 3; McMillan & Quadrilatero, 2014; Baechler et al., 2019) and insufficient mitochondrial biogenesis (Chapter 2; Chapter 3; Sin et al., 2016; Baechler et al., 2019). Previous work in our lab has demonstrated that stable knockdown of ATG7 in C2C12 myoblasts or treatment with the autophagy inhibitor 3MA, causes increased apoptosis and cell death during differentiation (McMillan & Quadrilatero, 2014). Interestingly, studies using autophagy-deficient SCs *in vivo* or primary myoblasts have reported higher p-H2AFX levels in ATG7-deficient cells, and suggest that autophagy-deficiency results in senescence (Garcia-Prat et al., 2016); however, they did not evaluate apoptotic signaling. Moreover, Garcia-Prat et al (2016) had suggested that autophagy is required to protect against cell senescence, and that cell senescence results from dysfunctional mitochondria and elevated ROS levels

(Garcia-Prat et al, 2016). We have also demonstrated that ATG7-deficient cells display mitochondrial dysfunction and have elevated ROS levels; however, we did not measure markers of senescence and instead found that these factors resulted in the induction of apoptosis (McMillan, 2015; Baechler et al., 2019). Interestingly, we have not been able to detect the major regulator and marker of cell senescence, p16 (Sousa-Victor et al., 2014) in C2C12 myoblasts in our lab. Thus, it is also possible that there are differences between our studies and those mentioned previously because we are using C2C12 myoblasts, which differ from SCs (Cornelison, 2008). Further, others have shown that *p16* is not expressed in C2C12 myoblasts (Pajcini et al., 2010). Therefore, an inability to become senescent could potentially contribute to the increased cell death that we observe in ATG7-deficient C2C12 myoblasts (Munoz-Espin et al., 2011; McMillan & Quadrilatero, 2014; Baechler et al., 2019), which differs from the increased senescence reported in autophagy-deficient SCs (Garcia-Prat et al., 2016). Similar to our work, Sin et al (2016) reported that autophagy-deficient cells fail to differentiate and regenerate the mitochondrial network; however, they did not report increased apoptosis (Sin et al., 2016). Interestingly; however, they did not perform multiple measures of apoptosis and strictly relied on measuring activated CASP3 protein levels (Sin et al., 2016). In contrast, we have shown that there is increased CASP-dependent and CASP-independent cell death signaling in sh*Atg7* cells by measuring ANXA5-propidium iodide staining, DNA fragmentation, cytosolic AIFM1 and CYCS, apoptotic nuclei, p-H2AFX levels, the BAX:BCL2 ratio, and CASP3 and CASP9 activity (McMillan & Quadrilatero, 2014; McMillan, 2015; Baechler et al., 2019; Chapter 2). Additionally, we have demonstrated that differentiation can be partially recovered by treating sh*Atg7* cells with a chemical

inhibitor of CASP3 and CASP9, or a dominant-negative CASP9 (ad-DNCASP9), which further supports that elevated mitochondria-mediated apoptotic signaling disrupts myogenic differentiation in sh*Atg7* cells (McMillan, 2015; Baechler et al., 2019). Moreover, *in vivo* animal studies (Pare et al., 2017; Masiero et al., 2009) have supported our results demonstrating that cell death signaling is elevated in ATG7-deficient muscle. More specifically, Pare et al (2017) had demonstrated that there is increased mitochondrial release of AIFM1 and CYCS in muscle-specific, ATG7 knockdown mice (Pare et al., 2017), while increased CYCS release has also been reported in miR-378 KO mice, which also show impaired autophagy (Li et al., 2018). This is similar to what we observed in sh*Atg7* cells and would contribute to increased apoptotic signaling. Additionally, Li et al (2018) demonstrated that CASP9 activity is elevated in miR-378 KO mice, and that overexpression of miR-378 can enhance autophagy and also reduce CASP9 activation and apoptosis (Li et al., 2018). Further, other studies have shown increased DNA fragmentation and apoptotic nuclei in autophagy-deficient mouse models (Masiero et al., 2009; Chrisam et al., 2015; Grumati et al., 2011), which can be suppressed through restoration of autophagy levels (Chrisam et al., 2015; Castagnaro et al., 2016). Moreover, we also observed increased apoptotic signaling in *bnip3*$^{-/-}$ cells (Chapter 3 - Figure 2), similar to sh*Atg7* cells, which suggests that the controlled regulation of autophagy/mitophagy, in addition to the elimination of dysfunctional/leaky mitochondria, is crucial to limit cell death signaling during myogenic differentiation.

Mitochondrial biogenesis is necessary during myogenic differentiation

One of the initial goals of this thesis was to address our hypothesis that mitophagy is disrupted in ATG7-deficient cells, and then attempt to rescue myogenesis and reduce apoptotic signaling by correcting the mitophagy deficiency. Thus, we overexpressed the autophagy/mitophagy-related proteins BECN1, PRKN, and BNIP3 in sh*Atg7* cells and observed no myogenic improvements in cells expressing BECN1 and PRKN, while overexpression of BNIP3 caused a partial rescue due to a delay in cell loss (Chapter 2-Figure 4/5). Interestingly, we found that LC3-dependent mitophagy does not occur in sh*Atg7* cells (Chapter 2 - Figure 6); however, we also determined that mitochondria are degraded in sh*Atg7* myoblasts (Chapter 2; Figure 7). Therefore, we wondered if our attempts to rescue sh*Atg7* cells by enhancing mitophagy were unsuccessful because we were trying to enhance overall mitophagy/autophagy levels in cells that can already degrade mitochondria. Additionally, given that ATG7 plays a critical role in regulating autophagy, it is unlikely that overexpression of another mitophagy-related protein can fully compensate for having insufficient levels of ATG7. Given that sh*Atg7* cells have more mitochondrial damage and show elevated mitochondria-mediated apoptotic signaling (Chapter 2 - Figure 3; McMillan, 2015; Baechler et al., 2019), the best approach would be to enhance the degradation of damaged mitochondria specifically. Although BECN1 is involved in regulating canonical and alternative autophagy/mitophagy (Hirota et al., 2015), a role for BECN1 in the specific targeting and elimination of damaged mitochondria has not been identified. Therefore, BECN1 overexpression is unlikely to correct a defect in the identification and degradation of dysfunctional mitochondria specifically. In contrast, PRKN is known to identify and remove damaged mitochondria;

however, given our inability to detect endogenous PRKN expression in C2C12 myoblasts (Chapter 2 - Figure 4F; Bloemberg, 2017), we speculate that PRKN is not involved in regulating damage-induced mitophagy in C2C12 myoblasts. BNIP3 expression increases during myogenic differentiation (Chapter 2 – Figure 2K; Chapter 3- Figure 2B) and could therefore be an important regulator of mitophagy during differentiation. BNIP3 has been shown to limit the accumulation of damaged mitochondria (Zhu et al., 2013; Glick et al, 2012; Hamacher-Brady & Brady, 2016), and can facilitate mitochondrial degradation in both an LC3-dependent and LC3-independent manner (Hanna et al., 2012; Hammerling et al., 2017). Therefore, we suspect that the reduced cell loss observed in sh*Atg7* cells overexpressing BNIP3 (Chapter 2; Figure 5) resulted because BNIP3 could have promoted the degradation of dysfunctional mitochondria to reduce cell death, and could have accomplished this without requiring LC3.

Our results suggest that an accumulation of damaged mitochondria results in increased cell death signaling in sh*Atg7* and *bnip3*$^{-/-}$ cells, and likely contributes to the impairment in myogenic differentiation observed in these cell lines. Moreover, Sin et al (2016) had suggested that mitophagy is needed during myogenesis to eliminate old mitochondria and to initiate mitochondrial biogenesis to generate a new mitochondrial network. Further, a recent study demonstrated that overexpression of PRKN in skeletal muscle can promote mitochondrial biogenesis and function and reduce apoptosis (Leduc-Gaudet et al., 2019), which suggests that mitophagy-related proteins are not only required for mitochondrial removal but that they also regulate overall mitochondrial health and homeostasis. In support of this, we found that the expression of the mitochondrial biogenesis-promoting protein PPARGC1A was lower in both sh*Atg7* and *bnip3*$^{-/-}$ cells.

Moreover, sh*Atg7* and *bnip3*$^{-/-}$ cells had depressed levels of the mitochondrial proteins VDAC1, CYCS, and SLC25A4 relative to SCR/Scram cells, suggesting that the rebuilding of the mitochondrial network does not occur in sh*Atg7* and *bnip3*$^{-/-}$ cells. Therefore, this compliments the work performed by Sin et al (2016) in that it demonstrates the importance of autophagy/mitophagy-related proteins in eliminating mitochondria to trigger/promote mitochondrial biogenesis during myogenic differentiation.

Given that mitochondrial biogenesis was impaired in both sh*Atg7* and *bnip3*$^{-/-}$ cells, we hypothesized that the cells might be failing to thrive and differentiate due to failure to generate a sufficient mitochondrial network. Mitochondria play multiple roles including: responding to changes in cellular energy requirements, generating energy, regulating Ca^{2+} signaling, and regulating apoptosis (Hood et al., 2019; Xu et al., 2013; Jacobson & Duchen, 2004; Wagatsuma & Sakuma, 2013). Thus, it is not surprising that an inability to rebuild and maintain a functional mitochondrial network would be detrimental to cell function and survival. Therefore, we speculated that enhancing mitochondrial biogenesis could improve myogenic differentiation in sh*Atg7* and *bnip3*$^{-/-}$ cells (Chapter 4). In order to test this, we treated sh*Atg7* and *bnip3*$^{-/-}$ cells with SNP, a nitric oxide (NO) donor that has previously been reported to enhance mitochondrial biogenesis (Wang et al., 2015), and found that enhancing mitochondrial biogenesis improved myogenic differentiation in sh*Atg7* and *bnip3*$^{-/-}$ cells. More specifically, we observed increased expression of the mitochondrial biogenesis-related proteins PPARGC1A and TFAM, increased mitochondrial protein levels, as well as increased myotube formation and myogenic protein expression in cells treated with SNP (Chapter 4

- Figure 3-5). Thus, SNP treatment promoted mitochondrial biogenesis in sh*Atg7* and *bnip3*$^{-/-}$ cells, which likely allowed the cells to generate a sufficient mitochondrial network to support myogenesis. In support of this, mitochondrial respiration also increased in cells treated with SNP (Chapter 4 - Figure 6). However, the addition of cytochrome c to evaluate mitochondrial membrane integrity, combined with the flow cytometry data (Chapter 4 - Figure 6), indicated that mitochondrial health is still compromised in SNP-treated cells. We speculate that this occurred because although SNP treatment would cause sh*Atg7* and *bnip3*$^{-/-}$ cells to generate new and healthy mitochondria, the treatment would not have corrected the reduced elimination of damaged mitochondria in sh*Atg7* and *bnip3*$^{-/-}$ cells. Therefore, SNP-treated sh*Atg7* and *bnip3*$^{-/-}$ cells would have an increased number of healthy mitochondria but would also be retaining a large population of dysfunctional mitochondria. Thus, a better approach might be to rescue sh*Atg7* cells by enhancing the degradation of damaged mitochondria in addition to enhancing mitochondrial biogenesis. This could potentially be accomplished by overexpressing BNIP3 in sh*Atg7* cells and then treating the cells with SNP throughout differentiation, and this might result in an additive improvement in myogenic differentiation and mitochondrial homeostasis in sh*Atg7* cells.

SNP is a drug that is commonly used to treat hypertension (Cobb & Thornton, 2018), which makes it an attractive candidate for *in vivo* muscle regeneration studies. Moreover, SNP was used as an NO donor in an earlier muscle differentiation study, which found that SNP treatment promoted myoblast fusion (Lee et al., 1994). Although these authors did not investigate mitochondrial remodelling or function, it would be interesting to investigate if the increased fusion is influenced by an increase in

mitochondrial content. Further, if SNP has the potential to enhance muscle differentiation/regeneration, then it would be interesting to use an *in vivo* model to test if short-term SNP treatment can improve muscle recovery following an injury.

Given that any chemical treatment could have non-specific or off-target effects, it would be important for future experiments to determine if the elevated expression of the mitochondrial biogenesis-related proteins PPARGC1A and TFAM in SNP-treated cells is contributing to myogenesis. In other words, we should determine if PPARGC1A and/or TFAM can recover myogenic differentiation in sh*Atg7* and *bnip3*$^{-/-}$ cells, as SNP treatment did. Therefore, sh*Atg7* and *bnip3*$^{-/-}$ cells should be transfected with constructs to overexpress PPARGC1A and TFAM to determine if there is an improvement in myogenesis. These experiments would allow us to evaluate if mitochondrial biogenesis is causing myogenic differentiation to occur, or if mitochondrial biogenesis happens because the cells are already differentiating. Moreover, this would further support our interpretation that SNP treatment is enhancing mitochondrial biogenesis to restore myogenic differentiation.

Limitations

For these experiments we used knockdown/knockout cell lines and found that *bnip3*$^{-/-}$ cells had higher levels of BNIP3L, a compensatory effect that has been reported in other cell types (Chourasia et al., 2015; Shi et al., 2014). Although BNIP3L is structurally and functionally similar to BNIP3 and is a known regulator of mitophagy (Zhang et al., 2012; Hamacher-Brady & Brady, 2016; Ney, 2015), the differentiation impairments observed in *bnip3*$^{-/-}$ cells, despite having elevated BNIP3L levels, would

suggest that BNIP3L cannot compensate for a lack of BNIP3 with respect to eliminating damaged mitochondria. Thus, it is important to consider that any compensatory upregulations in autophagy/mitophagy-related proteins would have impacted our ability to generate a truly mitophagy-deficient cell line. However, the myogenic and mitochondrial impairments that we observed in *bnip3*$^{-/-}$ cells despite them having elevated expression of other mitophagy/autophagy-related proteins, further demonstrates the importance of BNIP3 specifically in supporting myogenic differentiation. Moreover, the stable knockout/knockdown of any protein is likely to have some off-target effects or compensatory changes in the expression of other proteins, which could impact myogenesis and cell survival. However, we have demonstrated similar effects with respect to impaired myogenesis and cell death signaling in ATG7-, BNIP3-, and BNIP3L-deficient cells, each generated using a different knockout/knockout approach, which is in agreement with studies using chemical inhibitors of autophagy (McMillan & Quadrilatero, 2014; Sin et al., 2016). Therefore, we suggest that the reported effects likely result from insufficient levels of autophagy and impairment in the elimination of damaged mitochondria, rather than from off-target or autophagy/mitophagy-independent effects.

Summary and Conclusions

The purpose of this thesis was to evaluate the importance of autophagy/mitophagy-related proteins during myogenic differentiation. The results comprising this thesis demonstrated that knocking down the autophagy-related protein ATG7 or the mitophagy receptor proteins BNIP3 and BNIP3L disrupted myogenic differentiation. Further,

mitochondria-mediated apoptotic signaling was elevated in both sh*Atg7* and *bnip3*$^{-/-}$ cells, which suggests impairment in the elimination of damaged/dysfunctional mitochondria. Interestingly, we found that mitochondrial degradation does occur in both sh*Atg7* and *bnip3*$^{-/-}$ cells; however, we speculate that it is the specific removal of damaged mitochondria that is compromised in sh*Atg7* and *bnip3*$^{-/-}$ cells. Moreover, we found that mitochondrial biogenesis was reduced in both sh*Atg7* and *bnip3*$^{-/-}$ cells, and that treating cells with an inducer of mitochondrial biogenesis can partially recover myogenic differentiation. However, mitochondrial integrity was still compromised in sh*Atg7* and *bnip3*$^{-/-}$ cells even though mitochondrial content increased, which further suggests that damaged mitochondria are not properly eliminated in sh*Atg7* and *bnip3*$^{-/-}$ cells. Further, we found that elevated levels of autophagy-related proteins can suppress apoptotic signaling. Moreover, we found that elevated autophagy levels can be just as damaging as insufficient levels during myogenesis, which emphasizes the need for careful and controlled regulation of autophagy/mitophagy during differentiation.

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