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EXPLORING AN IMPAIRED SPHINGOSINE-1-PHOSPHATE RESPONSE TO
SKELETAL MUSCLE DAMAGE IN A MURINE MODEL OF TYPE 1 DIABETES
MELLITUS

By

Michael D. Mallender

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Kinesiology
in Partial Fulfillment of the Requirements for
the Degree of Master Human Kinetics at the
University of Windsor

Windsor, Ontario, Canada

2018

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EXPLORING AN IMPAIRED SPHINGOSINE-1-PHOSPHATE RESPONSE TO
SKELETAL MUSCLE DAMAGE IN A MURINE MODEL OF TYPE 1 DIABETES
MELLITUS

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DECLARATION OF ORIGINALITY

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ABSTRACT

Skeletal muscle is an adaptive tissue that possesses an innate ability to fully regenerate from a damaging stimulus. Type 1 diabetes mellitus (T1DM) elicits a pathophysiological environment that prevents normal skeletal muscle regeneration by dysregulating key events in the regenerative process. It has been shown that the sphingosine-1-phosphate (S1P) response to skeletal muscle damage is blunted in murine models of T1DM. S1P content normally increases in skeletal muscle acutely (within seven days) following damage to promote regeneration, and an absence of this response results in inadequate recovery. Thus, the lack of S1P accumulation seen in skeletal muscle of diabetic rodents following damage has the potential to contribute to impaired muscle regeneration. This investigation aimed to elucidate the mechanisms underlying this response by assessing: 1) S1P content via Liquid-Chromatography Mass-Spectrometry and 2) expression level of proteins that regulate S1P content via SDS-PAGE and Western Blot analysis. Results from this study show a blunted S1P response to skeletal muscle damage in a T1DM model as S1P content is reduced in Akita mice five days into regeneration. Furthermore, it was found that while sphingosine lyase (SPL) expression increased in both the T1DM models and WT mice following muscle damage, this expression was significantly greater in the diabetic condition. Total sphingosine kinase 1 content was also found to be increased five days following damage, but there was no significant effect of diabetes. Thus, the greater expression of SPL in the T1DM model suggests that S1P is degraded at a faster rate, preventing the normal accumulation of S1P following skeletal muscle damage. Future research should aim to identify the cause of this overexpression and the impact it has on skeletal muscle regeneration.

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LIST OF ABBREVIATIONS

ABCC1 – ATP-binding cassette C1

ATP – Adenosine triphosphate

CLIP – Chronic low-grade inflammatory profile

CM – CH – Control muscle in a control host environment

CM – DH – Control muscle in a diabetic host environment

CO₂ – Carbon dioxide

CTX – Cardiotoxin

CVD – Cardiovascular disease

DAPT - N-[2S-(3,5difluorophenyl)acetyl]-L-alanyl-2-phenyl-1,1-dimethylethyl ester glycine

DHEA – Dehydroepiandrosterone

DLL1 – Delta-like 1

DM-CH – Diabetic muscle in a control host environment

DM – DM – Diabetic muscle in a diabetic host environment

DMD – Duchenne muscular dystrophy

DMS - N, N-dimethylphingosine

ECM – Extracellular matrix

EDL – Extensor digitorum longus

EGF – Epidermal growth factor

ERK 1/2 - Extracellular signal-related kinase 1/2

GPS – Gastrocnemius-plantaris-soleus complex

H₂O₂ – Hydrogen peroxide

HGF – Hepatocyte growth factor

HNE – hydroxynonenal

IGF-1 – Insulin like growth factor- 1

IL-6 – Interleukin -6

IMCL – Intramyocellular lipid

LC-MS – Liquid chromatography-mass spectrometry

MMP – Matrix metalloproteinase

PAI-1 – Plasminogen activator inhibitor-1
ROS – Reactive oxygen species
S1P – Sphingosine-1-phosphate
S1PR₁₋₄ – S1P receptors 1-4
SPK1 – Sphingosine kinase 1
SPK2 – Sphingosine kinase 2
SPL – Sphingosine lyase
STZ – Streptozotocin
T1DM – Type 1 diabetes mellitus
TA – Tibialis anterior
THI - (tetrahydroxybutyl)imidazole
uPA – Urokinase plasminogen activator
WB – Western blot
WT – Wild type

1. Introduction

Maintaining adequate skeletal muscle health and mass is imperative for wellness and longevity (Ruiz et al., 2009; Studenski et al., 2011). To aid in this maintenance, skeletal muscle tissue is adaptive, illustrated by an innate ability to remodel itself in response to environmental stimuli (Breen et al., 2013; Glover et al., 2008). Included in this plasticity is a regenerative capacity, as skeletal muscle tissue can completely restore its structure and function within a few weeks of a severe damaging stimulus (Bentzinger et al., 2013). The skeletal muscle regeneration process is consistent across multiple mammalian species and muscle fibre types, and is regulated via numerous intracellular and extracellular mechanisms (Dumont, Sincennes, & Rudnicki, 2015; Mann et al., 2011; Hawke & Garry, 2001). Multiple events have been identified as key to this process, including a transient infiltration of inflammatory cells, the activation, proliferation, and differentiation of skeletal muscle satellite cells, and remodelling of the extracellular matrix (ECM); which all need to occur efficiently for skeletal muscle to fully regenerate following damage (Dumont et al., 2015; Mann et al., 2011; Hawke & Garry, 2001). When a pathophysiological environment is created by chronic disease(s), such as type 1 diabetes mellitus (T1DM), there is potential to negatively influence skeletal muscle regeneration via dysregulation of these key events, resulting in a disease complication known as myopathy (Krause et al., 2011b D'Souza, Al-Sajee, & Hawke, 2013; Monaco, Perry, Hawke, 2017).

The pathophysiological environment created by T1DM has been associated with the development of numerous complications, including: cardiovascular disease (CVD), neuropathy, nephropathy, and retinopathy (Pelletier et al., 2012; Soedamah-Muthu et al.,

2006). However, the adverse affects of a T1DM pathophysiological environment on skeletal muscle health have been overlooked and understudied, largely due to the erroneous assumption that exogenous insulin therapy would suffice to maintain adequate skeletal muscle health in this population (Andersen et al., 2005; Andersen et al., 1996; Andersen et al., 1997; Andersen et al., 2004; Cotter et al., 1989; Reske-Nielsen, Harmsen, & Vorre, 1977). Experiments utilizing animal models of T1DM have provided evidence that this negative environment exerts a significant influence on skeletal muscle regeneration, as T1DM models exhibit blunted skeletal muscle repair as compared to wild type (WT) counterparts in response to the same damaging stimulus (Aragno et al., 2004; D'Souza et al., 2016; Jeong, Conboy, & Conboy, 2013; Jerkovic et al., 2009; Krause et al., 2011a; Krause et al., 2013; Talesara & Vashishta, 2000; Vignaud et al., 2007). These impairments can be attributed to deviations from the normal skeletal muscle regeneration process, as T1DM skeletal muscle has been shown to exhibit attenuated ECM remodelling (Krause et al., 2011a; Krause et al., 2013), impaired satellite cell functionality (D'Souza et al., 2016; Jeong et al., 2013; Krause et al., 2013), and a blunted transient inflammatory response (Krause et al., 2013).

Characterization of a T1DM pathophysiological environment has identified a bevy of abnormalities with the potential to negatively influence skeletal muscle regeneration, including: an overexpression of the growth supressing protein myostatin (Jeong et al., 2013); oxidative stress induced by chronic hyperglycemia (Aragno et al., 2004; Carvalho et al., 2017); a chronic low grade inflammatory profile (CLIP) due to persistent elevation of interleukin (IL)-6 (Llauradó et al., 2012; Reis et al., 2012); overactivation of notch signalling (D'Souza et al., 2016); and overexpression of the blood clotting factor

plasminogen activator inhibitor (PAI)-1 (Krause et al., 2011a; Krause et al., 2013). In a recent attempt to further characterize the effects of a T1DM pathophysiological environment, a mass-spectrometry survey of 25 different skeletal muscle lipid species, using the Akita model of T1DM, was analyzed to identify if this pathophysiological environment influences the content of specific skeletal muscle lipid species at various time points during the regeneration process (Mallender, Trumble, Hawke, & Krause, unpublished results). While it was found that T1DM did not alter lipid content in undamaged skeletal muscle, the content of key lipids in the sphingolipid synthesis pathway were significantly lower in the Akita skeletal muscle five days following a cardiotoxin (CTX) injection (Mallender et al., unpublished results). As proper regulation of the sphingolipid synthesis pathway is important for adequate skeletal muscle regeneration (Loh et al., 2012; Nagata et al., 2006; Sassoli et al., 2011), an aberrant sphingolipid response to muscle damage could potentially be a previously unidentified contributor to the impaired regeneration seen in T1DM skeletal muscle.

The most notable dysregulated sphingolipid in this pathway was sphingosine-1-phosphate (S1P), which failed to significantly increase within the Akita skeletal muscle 5 days following CTX-induced muscle damage, despite an 11-fold increase in the WT skeletal at the same time point (Mallender et al., unpublished results). However, skeletal muscle S1P content then returned to their relative baseline in both the WT and Akita skeletal muscle 10 days following the CTX injection, indicating that this impairment only occurs acutely (within 10 days) following damage (Mallender et al., unpublished results). Multiple studies have reported *in vitro* and *in vivo* that skeletal muscle damage-induced a rise in S1P content (Loh et al., 2012; Nagata et al., 2006; Sassoli et al., 2011), and inhibition

of this response significantly attenuated proper skeletal muscle repair (Loh et al., 2012; Nagata et al., 2006). Furthermore, S1P has been shown to facilitate skeletal muscle regeneration in a dose-dependent manner; as exogenous administration of S1P *in vivo* following skeletal muscle damage to elicit supra-physiological levels significantly enhanced skeletal muscle regeneration (Danieli-Betto et al., 2009; Sassoli et al., 2011). S1P is known to promote regeneration by binding to one of four S1P G-protein coupled receptors (S1PR₁₋₄) present on satellite cells and within skeletal muscle tissue (Calise et al., 2012; Danieli-Betto et al., 2009), and depending on which receptor subtype is bound, promote satellite cell activation, migration, proliferation, and differentiation (Calise et al., 2012; Loh et al., 2012; Nagata et al., 2006). Furthermore, S1P facilitated ECM remodelling via activating effects on matrix metalloproteinase (MMP)-9 (Sassoli et al., 2011). Notably, both satellite cell functionality and ECM remodelling were dysregulated in T1DM (Krause et al., 2011a; Krause et al., 2013; D'Souza et al., 2016), which may have been related to the blunted S1P accumulation seen following skeletal muscle damage in this population (Mallender et al., unpublished results).

The skeletal muscle damage-induced rise in S1P content occurred via effects on two enzymes: i) sphingosine kinase 1 (SPK1), which phosphorylates sphingosine into S1P in an ATP-dependent manner, and ii) sphingosine lyase (SPL), which is responsible for irreversible degradation of S1P (Nagata et al., 2006). Therefore, S1P content was increased or decreased in skeletal muscle based upon the relative balance between active SPK1 (Sassoli et al., 2011; Bernacchioni, Cencetti, Donati, & Bruni, 2012) and total SPL expression (Loh et al., 2012; Saba & de le Garza-Roden, 2013). Thus, dysregulation of SPK1 activity and/or SPL expression that prevents S1P accumulation following skeletal

muscle damage has been shown to significantly hinder skeletal muscle regeneration (Loh et al., 2012; Nagata et al., 2006). This phenomenon has been observed to occur *in vivo* with murine *mdx* models of Duchenne muscular dystrophy (DMD), where a chronic overexpression of SPL prevents the damaged stimulated increase of skeletal muscle S1P content (Ieronimakis et al., 2013; Loh et al., 2012). Thus, precedent has been established that a pathophysiological environment created by a chronic disease can exert a negative influence on these S1P regulating enzymes, and subsequently contribute to the condition related myopathy.

Due to the importance of the sphingolipid (and S1P) response to skeletal muscle damage, it is pertinent to further explore the underlying mechanisms causing the dysregulation of the sphingolipid synthesis pathway in T1DM. Therefore, this investigation will have three primary objectives:

- 1) to better characterize the acute sphingolipid response to skeletal muscle damage in Akita models of T1DM by assessing for sphingolipid content at four separate time points (one day, three days, five days, and seven days) following the damage inducing stimulus; and,

- 2) to identify if a T1DM pathophysiological environment adversely affects SPK1 activity following a skeletal muscle damaging stimulus.

- 3) to identify if a T1DM pathophysiological environment adversely affects SPL expression following a skeletal muscle damaging stimulus.

The results of this investigation will advance knowledge surrounding T1DM-induced myopathy and help establish the role of S1P dysregulation in this condition.

Research Question #1

How does skeletal muscle content of key lipids in the sphingolipid synthesis pathway, including sphingomyelin, ceramide, sphingosine, and S1P, change in the Akita model of T1DM as compared to a WT control, one day, three days, five days, and seven days following CTX-induced skeletal muscle damage?

Hypothesis #1

If a T1DM pathophysiological environment has adverse effects on the sphingolipid response to skeletal muscle damage, then the skeletal muscle content of sphingomyelin, ceramide, sphingosine, and S1P will be lower in the Akita skeletal muscle as compared to the WT controls at all measured time points.

Research Question #2

How does a T1DM pathophysiological environment affect the activation state of skeletal muscle SPK1, one day, three days, five days, and seven days, following CTX-induced skeletal muscle damage?

Hypothesis #2

If a T1DM pathophysiological environment affects the activation state of SPK1 following CTX-induced muscle damage, then the Akita models will display lower SPK1 activity than WT controls at all measured time points.

Research Question #3

How does a T1DM pathophysiological environment affect the expression of skeletal muscle SPL, one day, three days, five days, and seven days, following CTX-induced skeletal muscle damage?

Hypothesis #3

If a T1DM pathophysiological environment affects the expression of SPL following CTX-induced muscle damage, then the Akita models will display greater SPL expression than WT controls at all measured time points.

2. Review of Literature

2.1. The Skeletal Muscle Regeneration Process

Skeletal muscle is an adaptive tissue, illustrated by an innate ability to remodel itself in response to environmental stimuli, such as the presence or absence of mechanical loading (Breen et al., 2013; Glover et al., 2008), or the relative availability of substrates for metabolism (Bohe, Low, Wolfe, & Rennie, 2003). In addition to this plasticity, skeletal muscle has a regenerative ability. Multiple stimuli have the capacity to induce mild-to-severe muscle damage, such as intense muscle contraction, sport injury, surgery, or exposure to a toxin or infection. When not affected by disease or abnormal environmental stimuli, skeletal muscle has the capacity to completely restore structure and function within a few weeks following a damaging stimulus (Bentzinger et al., 2013). To facilitate this recovery, mammalian skeletal muscle regeneration follows a consistent process. Any deviation from the typical skeletal muscle regeneration process may result in inadequate recovery and subsequently reduced skeletal muscle health. The events that facilitate skeletal muscle regeneration are numerous and complex, but most prominently include a transient infiltration of inflammatory cells, the activation, migration, proliferation, and differentiation of skeletal muscle satellite cells, and remodelling of the extracellular matrix (ECM); all of which are critical for the repair and growth of damaged and new myofibres, and ultimately allow for restored skeletal muscle function (Dumont, Sincennes, & Rudnicki, 2015; Hawke & Garry, 2001; Mann et al., 2011).

The first major event during skeletal muscle regeneration is the transient inflammatory response, which is triggered by localized activation of the innate immune

system at the site of damage, and results in an infiltration of a variety of resident macrophage populations that reside in the muscle perimysium (Brigitte et al., 2010; Green et al., 2009). This response also incorporates, albeit to a lesser functional importance, other inflammatory cells such as leukocytes and blood-derived macrophages (Tidball, 2005; Mann et al., 2011). Macrophage infiltration into damaged skeletal muscle occurs in two primary waves. The first wave initiates shortly following skeletal muscle damage and consists of classically activated (M1) macrophages which engage in phagocytic degradation of necrotic skeletal muscle tissue (Arnold et al., 2007; Mantovani et al., 2004). Following this, a second wave consisting of M2a and M2c macrophages infiltrates the damaged skeletal muscle, helping to promote healing by exerting an anti-inflammatory effect and deactivating the initial M1 wave (Arnold et al., 2007; Mantovani et al., 2004). Without this response, necrotic tissues will fail to be phagocytized, and will not be replaced by functional contractile tissue (Krause et al., 2013).

The activation, migration, proliferation, and differentiation of muscle satellite cells also occurs shortly following a damaging stimulus. Skeletal muscle satellite cells are a class of resident muscle stem cells which reside in a specific niche between the plasmalemmal and basement membrane in an inactivated state of quiescence (Dumont et al., 2015; Hawke & Garry, 2001). Following muscle damage, these satellite cells activate from their quiescent state and begin to proliferate, a process of cell-division and self-renewal, to increase the satellite cell population (Dumont et al., 2015; Hawke & Garry, 2001). A subset of the newly formed satellite cell population will return to quiescence and replenish the resting satellite cell pool, while the remaining satellite cells will continue down a path of differentiation into myoblasts (Dumont et al., 2015; Hawke & Garry, 2001). The

differentiated myoblasts will fuse to the damaged myofibres and donate their nuclei to the muscle cell, effectively replacing the necrotized tissue and add to the pre-existing myofibre (Dumont et al., 2015; McCarthy et al., 2011). Satellite cells can be identified by staining for the transcription factor Pax7, which is expressed in both quiescent and proliferating satellite cells (D'Souza, Al-Sajee, & Hawke, 2013; Dumont et al., 2015). To assess further down the myogenic lineage, the myogenic regulatory factors MyoD and Myogenin are often examined, as the relative expression of those myogenic factors and Pax7 gives an indication of the extent of activation, proliferation, or differentiation that has occurred in the resident satellite cell population following skeletal muscle damage (D'Souza et al., 2016; Dumont et al., 2015).

Finally, remodelling of the ECM acutely following skeletal muscle damage needs to occur to ensure an efficient regeneration process. ECM remodelling is primarily accomplished via the action of fibroblasts, which like satellite cells, activate, proliferate, and then subsequently migrate to the site of muscle damage (Mann et al., 2011). Activated fibroblasts will then produce structural proteins, including collagen type 1 and type 3, elastin, laminin, and fibronectin (Mann et al., 2011). The elevation in expression of these structural proteins is transient, as they stabilize the contractile elements during regeneration, act as a scaffolding system for new fibres, facilitate satellite cell migration, and help guide the formation of neuromuscular junctions (Mann et al., 2011; Uuri et al., 2006). The majority of this newly formed ECM is quickly degraded, allowing for adequate cell migration and tissue remodeling. This ECM degradation is largely accomplished by a variety of matrix metalloproteinases (MMPs), with over 15 different subtypes of MMPs

playing an important role in skeletal muscle regeneration (Chan & Ij, 2009; Mann et al., 2015).

Each of these events needs to commence properly for skeletal muscle regeneration to occur effectively. For example, inability to properly regulate the inflammatory response has functional consequences for skeletal muscle health, as chronic overactivation of this system blunts skeletal muscle regeneration (Gordon et al, 2013; Ieronimakis et al., 2013; Loh et al., 2012). Inadequate satellite cell functionality has significant negative effects on the ability of the skeletal muscle to properly form new myofibres after damage and effectively form new contractile tissue (D'Souza, et al., 2013; Krause et al., 2013; Loh et al., 2012). Finally, if MMPs fail to activate following skeletal muscle damage, the rate of collagen synthesis greatly exceeds the rate of collagen deposition (Krause et al., 2011a; Sassoli et al, 2011), resulting in a pathological condition known as fibrosis (Krause et al., 2011; Larcher et al., 2014; Loh et al., 2012; Mann et al., 2011). Therefore, dysregulation in any of these events will severely hinder the regenerative process and lead to reduced skeletal muscle health.

Pathophysiological states elicited by environmental stimuli or chronic disease have the capacity to cause dysregulation in these key events, resulting in a condition broadly known as myopathy. The aberrant skeletal muscle repair that occurs in some myopathies can be elicited by a wide variety of disease states, each exerting its negative influence on skeletal muscle health in a unique way. One of the best characterized myopathies is Duchenne muscular dystrophy (DMD). In DMD, a structural defect in the protein dystrophin results in a chronic cycle of muscle damage and regeneration, which eventually leads to a depleted satellite cell population, accumulated fibrosis, and a reduced capacity

for muscle regeneration (Larcher et al., 2014; Loh et al., 2012). In contrast to DMD, where the basis of the myopathy is through a skeletal muscle genetic defect, myopathy may occur in response to a disease state that is not primarily related to skeletal muscle. An example of such a condition is type 1 diabetes mellitus (T1DM), which elicits a pathophysiological state that impairs skeletal muscle development and regeneration, despite being an autoimmune disorder which targets the pancreas (D'Souza et al., 2013; Krause, Riddell, & Hawke, 2011b; Monaco, Perry, Hawke, 2017).

2.2. Myopathy in T1DM

T1DM is an autoimmune disorder that results in the body's immune system targeting and destroying pancreatic beta cells responsible for the production and secretion of insulin into the bloodstream; an essential function for the adequate maintenance of blood glucose levels, as it facilitates glucose uptake from the bloodstream into bodily tissues. The primary treatment for T1DM is exogenous insulin therapy, which is necessary to prevent a chronic state of hypoinsulinemia and hyperglycemia (Banting, Best, Collip, MacLeod, & Noble, 1922; Pelletier et al., 2012). While insulin therapy is vital for management of the disease, it is not a cure, and it cannot prevent the myriad of complications associated with T1DM, such as cardiovascular disease (CVD), neuropathy, nephropathy, and retinopathy (Pelletier et al., 2012; Soedamah-Muthu et al., 2006), all of which have the potential to contribute to the increased mortality rate (the number of individuals per thousand that pass away each year within a cohort) seen in this population (Soedamah-Muthu et al., 2006). However, a commonly overlooked and understudied complication of T1DM is the adverse affect the disease state has on skeletal muscle health (i.e., diabetic myopathy), which is apparent both with and without insulin therapy, and manifests itself independent of

neuropathy (D'Souza et al., 2013; Krause et al., 2011b; Monaco et al., 2017). As skeletal muscle health has been linked to overall health, wellbeing, and longevity of individuals (Cooper et al., 2010; Ruiz et al., 2009; Studenski et al., 2011), diabetic myopathy should be considered an important disease complication and treated accordingly, both with regards to scientific research and clinical treatment. Furthermore, because skeletal muscle is the largest organ in the body that can dispose of glucose in an insulin-dependent and insulin-independent manner, maintaining its health may be critical for long term glycemic control, leading to preservation of other organs that are sensitive to the diabetic milieu. Thus, elucidating the mechanisms underlying diabetic myopathy is critical to the development of treatment modalities to minimize the impact of this complication.

Prior to exploring myopathy in T1DM, it is important to note that although a variety of human studies have been conducted, the use of animal models of T1DM is necessary to fully examine the etiology of this complication. Thus, the applicability of different mammalian models to the human conditions must be investigated, as research has shown that inherent differences between animal models of T1DM do exist (Yoshioka, Kayo, Ikeda, & Koizumi, 1997; Wang et al., 1998), leading to issues in the transferability of these results to the human manifestation. While multiple models of T1DM have been used in myopathy research, two stand out due to the prevalence of their use throughout the literature. The most commonly used animal model is the streptozotocin (STZ)-induced rodent model (Krause et al., 2011b), which is created via a STZ injection to cause pancreatic beta cell death, and subsequently hypoinsulinemia (Armstrong & Ianuzzo, 1977; Elsner et al., 2000). Secondary to this, the genetic Akita model, in which mice spontaneously become diabetic at three to four weeks old due to a mutation of the insulin-

2 gene and subsequently develop hypoinsulinemia (Jackson Laboratory, 2018; Krause et al., 2009), has also been used to examine diabetic myopathy (Krause et al., 2009; Krause et al., 2011a; Krause et al., 2013; Vignaud et al., 2007). Despite a prevalence in the literature, the transferability of the STZ-induced model has been criticized for the adverse effects of STZ on skeletal muscle that occur independent of a T1DM pathophysiological environment (Krause et al., 2009; Johnston et al., 2007). Specifically, the STZ-induced model displays elevated intramyocellular lipid content, elevated type 1 muscle fibre cross sectional area, and increased relative muscle twitch and tetanic force as compared to the Akita model (Krause et al., 2009); that have been attributed to the direct effects of STZ on skeletal muscle, and not due to a T1DM pathophysiological environment (Krause et al., 2009). Thus, it has been suggested that results utilizing the STZ-induced model of T1DM be interpreted with caution when examining skeletal muscle tissue, and the type of model used should be kept in mind when drawing conclusions about myopathy in T1DM (Krause et al., 2009).

The skeletal muscle of individuals with T1DM is characterized by both structural and functional abnormalities as compared to a non-diabetic population. Detailed analysis of T1DM skeletal muscle was first accomplished in 1977, where the skeletal muscle ultrastructure was analyzed in a group of patients who had received a diagnosis of T1DM within the previous 28 weeks (Reske-Nielsen, Harmsen, & Vorre, 1977). Notably, it was found that diabetic skeletal muscle had displaced A and I bands, disrupted Z lines, yielded significant muscle fibre atrophy, and developed abnormalities in the mitochondria, all of which occurred prior to any morphological indications of neuropathy (Reske-Nielsen et al., 1977). These results have since been corroborated with other studies implicating a T1DM

pathophysiological environment with severe skeletal muscle structural impairments. For example, skeletal muscle atrophy (which will be used synonymously with attenuated skeletal muscle development) has been observed in individuals with both short-term and long-term exposure to a T1DM environment (Andersen et al., 1997; Andersen et al., 2004; Jakobsen & Reske-Nielsen, 1986; Reske-Nielsen, et al., 1977). The development of atrophy following acute exposure to a T1DM environment is particularly problematic, as T1DM is primarily diagnosed in childhood, and adolescent skeletal muscle has a limited capacity to recover from atrophic stimuli (Darr & Schultz, 1989; Mozdziak, Pulvermacher, & Schultz, 2000). Mitochondrial dysfunction has also been linked with diabetic myopathy (Cree-Green et al., 2015; Zabielski et al., 2016), and has been shown to result in an increased reliance on anaerobic metabolism in this population (Monaco et al., 2017; Krause et al., 2011b). Additionally, exposure to a T1DM pathophysiological environment appears to induce a fibre type shift towards glycolytic fibres in humans (Crowther et al., 2003; Fritzsche et al.; 2008), which despite producing more power than oxidative (type I) muscle fibres (Schiaffino & Reggiani, 1994), are more susceptible to atrophic stimuli (Cotter et al., 1993; Krause et al., 2009). Finally, it has been noted that humans and animal models with T1DM tend to display increased intramyocellular lipid (IMCL) content as compared to non-diabetic individuals (Bernroider et al., 2005; Dotzert et al., 2016; Kurek et al., 2016; Perseghin et al., 2003), although this finding has been challenged in the literature (Ling et al., 2003; Perseghin et al., 2003; Standl et al., 1980).

The bevy of structural abnormalities that can occur in diabetic myopathy are associated with a variety of skeletal muscle functional deficits. One such impairment is a reduction in absolute force production (Andersen et al., 2005; Andersen et al., 1996; Cotter

et al., 1989; Vignaud et al., 2007). However, assessment of contractile force relative to skeletal muscle mass has produced equivocal results (Cameron, Cotter & Robertson, 1990; Cotter et al., 1993; Krause et al., 2009; McGuire & McDermott, 1999; Vignaud et al., 2007). It is likely that these mixed findings are due to differences between diabetic models and experimental methods (Krause et al., 2011b), and it has been proposed that the loss of absolute contractile force is due to less contractile tissue, and not due to altered skeletal muscle fibre function (Krause et al., 2011b). Skeletal muscle endurance appears to be adversely affected by T1DM, as 12 week old Akita mice reach exhaustion significantly faster on a treadmill endurance test than their wild type (WT) counterparts (D'Souza et al., 2016), and contractile force is reduced quicker following 45 seconds of 50 Hz stimulation to the tibialis anterior muscle of STZ-induced rodents (Vignaud et al., 2007). However, the effect of a T1DM pathophysiological environment has on the exercise capacity of human skeletal muscle is still debated, but it generally appears as if there is a loss in exercise capacity in the human condition (Krause et al., 2011b). Furthermore, the potential for elevated IMCL content in T1DM puts individuals at risk for developing insulin resistance (Kraegen et al., 2006; Kraegen et al., 2008; Dotzert et al., 2016), and when combined with the insulin-dependent T1DM is known as “double diabetes” (Kilpatrick, Rigby, & Atkin, 2007), which has the potential to further exasperate the symptoms of diabetic myopathy and other diabetic complications.

The adverse effects of T1DM on skeletal muscle exist beyond basic structural and functional impairments, as it also exerts a negative influence on skeletal muscle regeneration following myotrauma (D'Souza et al., 2013; Krause et al., 2011b). This regenerative deficiency can be directly attributed to a pathophysiological environment

created by T1DM, and was first shown *in vivo* during a transplantation experiment using the extensor digitorum longus (EDL) muscle from STZ-induced diabetic rodents and WT controls (Gulati & Swamy, 1991). In this experiment, the EDL muscle of STZ rodents was transplanted into both a control (DM-CH) and diabetic host (DM-DH) environment, and the EDL muscle from WT controls was also transplanted into both a control (CM-CH) and a diabetic host (CM-DH) environment (Gulati & Swamy, 1991). Muscle regeneration was subsequently assessed in each group at 2, 4 and 12 weeks following transplantation (Gulati & Swamy, 1991). The results of this study were notable, as it directly implicated a T1DM pathophysiological environment as un conducive to skeletal muscle regeneration. The overall muscle mass and myofibre size in the EDL muscle of the DM-DH was significantly reduced as compared to the EDL muscle of the CM-CH all time points, illustrating that T1DM skeletal muscle does not regenerate as efficiently as WT control muscle (Gulati & Swamy, 1991). However, the regenerative capacity of the diabetic EDL muscle was rescued once transplanted into a control host environment, and the regenerative capacity of the WT control EDL was significantly blunted when transplanted into a T1DM host environment (Gulati & Swamy, 1991). Thus, it was not inherent differences between diabetic and non-diabetic skeletal muscle that was responsible for the observed impaired skeletal muscle regeneration, but rather exposure to the pathophysiological environment created by T1DM. Following this experiment, identifying how and why a T1DM pathophysiological environment impaired skeletal muscle regeneration became a focal point in the study of diabetic myopathy.

Since the first evidence of impaired skeletal muscle regeneration in T1DM was reported, multiple studies using primarily mammalian models of T1DM have been

conducted which support this finding (Aragno et al., 2004; D'Souza et al., 2016; Jeong, Conboy, & Conboy, 2013; Jerkovic et al., 2009; Krause et al., 2011a; Krause et al., 2013; Talesara & Vashishta, 2000; Vignaud et al., 2007). Notably, many indicators of inefficient skeletal muscle repair have been consistently identified across these studies. In experiments utilizing both STZ-induced and Akita models of T1DM, following either exercise- or pharmacologically-induced myotrauma, it has been shown that in comparison to WT counterparts at various times during regeneration, T1DM model skeletal muscle displays: an increased presence of muscle damage in response to the same damage inducing stimulus (D'Souza et al., 2016; Krause et al., 2013), reduced myofibre cross sectional area (Gulati & Swamy, 1991; Jerkovic et al., 2009; Krause et al., 2011a; Krause et al., 2013), reduced collagen degradation and an increased presence of fibrotic tissue (Krause et al., 2011a; Krause et al., 2013), a reduced number of regenerating myofibres (Jeong et al., 2013), reduced overall myonuclei content (Jeong et al., 2013), reduced production of key myogenic regulatory factors (Aragno et al., 2004; D'Souza et al., 2016; Krause et al., 2011a; Krause et al., 2013), and an attenuated inflammatory response (Krause et al., 2013). This regeneration phenotype can be attributed to an aberrant skeletal muscle regeneration response; thus, it is important to identify where a T1DM pathophysiological environment elicits deviations from this process. Currently, the two most notable aberrant characteristics of T1DM skeletal muscle regeneration appear to be attenuated ECM remodeling and impaired satellite cell functionality (D'Souza et al., 2016; Jeong et al., 2013; Krause et al., 2011a; Krause et al., 2013).

Attenuated ECM remodeling was first demonstrated in Akita models of T1DM, where skeletal muscle regeneration was assessed at 5, 10, 21, and 35 days following an

intramuscular cardiotoxin (CTX) injection to induce myotrauma. It was shown that Akita muscle displayed significantly increased collagen positive area as compared to wild type controls at 5 and 10 days following skeletal muscle damage (Krause et al., 2011a), which signalled an inability to properly degrade the collagen produced by fibroblasts during the early phases of regeneration (Mann et al., 2011). This impaired collagen breakdown was attributed to a reduction in the activation state of MMP-9 content, which is elevated in non-diabetic skeletal muscle acutely following injury to regulate collagen degradation (Krause et al., 2011a). A follow-up study identified that the attenuated ECM remodelling seen in these models had significant effects on other regeneration events, acting to prevent adequate inflammatory cell infiltration and satellite cell migration at these 5 and 10 day time points (Krause et al., 2013). Therefore, this inability to remodel the ECM led to fibrosis, as well as an increased presence of necrotic fibres and reduced embryonic myosin heavy chain content (Krause et al., 2013). The depressed MMP-9 activity was attributed to an overexpression of plasminogen activator inhibitor (PAI)-1, which depresses the plasminogen system and subsequently reduces MMP-9 activation (Krause et al., 2011a). When PAI-1 levels were pharmacologically inhibited to below normal physiological levels in both studies, the MMP-9 activation state was restored, collagen positive area decreased at 5 and 10 days following damage, satellite cell migration improved, and inflammatory cell infiltration increased (Krause et al., 2011a; Krause et al, 2013). Therefore, these results heavily implicate attenuated ECM remodelling as a major contributor to myopathy in T1DM.

Satellite cell functionality also appears to be impaired in T1DM. While it has been shown that attenuated ECM remodelling contributes to depressed satellite cell migration

(Krause et al., 2013), the impaired satellite cell functionality in T1DM goes beyond this reduced migratory ability. Specifically, a T1DM pathophysiological environment appears to prevent adequate satellite cell activation following a damaging stimulus (D'Souza et al., 2016; Jeong et al., 2013). This effect has been demonstrated *in vitro* utilizing isolated single fibres from Akita skeletal muscle, which induced satellite cells to enter an activation period (D'Souza et al., 2016). Following the activation period, the Akita muscle fibres displayed 40% less Pax7 content and significantly less BrdU incorporation 24 hours after isolation, indicating that satellite cells in the Akita muscle fibres failed to activate and proliferate as effectively as WT satellite cells (D'Souza et al., 2016). Furthermore, the Akita satellite cells which did activate displayed significantly less MyoD and Myogenin content than their WT counterparts, indicating that even when activated, a T1DM pathophysiological environment impairs progression down the myogenic lineage and subsequent transition into myoblasts (D'Souza et al., 2016).

This impaired satellite cell functionality has also been demonstrated *in vivo*. Following the administration of CTX to induce muscle damage, both STZ-induced and Akita models of T1DM display impaired satellite cell activation (Jeong et al., 2013). Furthermore, quantification of *in vivo* satellite cell content from humans with well controlled T1DM showed that chronic exposure to a pathophysiological environment resulted in a decreased presence of Pax7⁺ nuclei as compared to healthy aged-match controls (D'Souza et al., 2016), indicating the overall quiescent satellite cell population is reduced in T1DM (D'Souza et al., 2016). Thus, these findings, coupled with an impaired satellite cell migratory capacity induced via an attenuated ECM remodeling, implicates this impaired satellite cell functionality as a contributor to diabetic myopathy. However, while

it is important to unearth the way T1DM skeletal muscle deviates from the normal skeletal muscle regeneration process, it is equally important to consider what about a T1DM pathophysiological environment elicits these perturbations.

2.3. T1DM Pathophysiological Environment

While the impairments to skeletal muscle regeneration seen in T1DM have been well characterized, the underlying mechanisms causing the impaired skeletal muscle regeneration elicited by a pathophysiological environment have yet to be fully elucidated. Thus, understanding how a T1DM pathophysiological environment differs from a non-diabetic environment, and how any of these differences potentially influence the skeletal muscle regenerative process, is essential to the study of myopathy in T1DM. Due to the anabolic action of insulin on skeletal muscle, and potent positive impact on muscle protein turnover in both humans and murine models (Phillips, 2008), it has been suggested that this myopathy is a result of the hypoinsulinemia and hyperglycemia that occurs in uncontrolled T1DM. However, many aspects of T1DM myopathy are still present in humans when glycemic levels are well controlled with proper insulin therapy (Andersen et al., 2004; Andersen et al., 2005; D'Souza et al., 2016), therefore suggesting other aspects of a T1DM pathophysiological environment beyond insulin deficiency are contributing to the observed condition. Experimental evidence has implicated multiple differences in this pathophysiological environment that could contribute to the impaired regeneration, including overexpression of myostatin (Jeong et al., 2013), oxidative stress (Aragno et al., 2004), a chronic low grade inflammatory profile (CLIP) (Llauradó et al., 2012), overactivated notch signalling (D'Souza et al., 2016), and PAI-1 overexpression (Krause et al., 2011a; Krause et al., 2013).

2.3.1. Myostatin Overexpression

The transforming growth factor-beta family member myostatin is a potent suppresser of muscle growth (Taylor et al., 2001; Trendelenburg et al., 2009), and its overexpression in satellite cells is constitutently linked to impaired functionality (Langley et al., 2002; Trendelenburg et al., 2009). Overexpression of myostatin in T1DM satellite cells has been demonstrated *in vivo* with STZ-induced diabetic mice (Jeong et al., 2013), and was linked to the impaired satellite cell functionality displayed by the STZ-induced mice 72 hours following CTX-induced injury (Jeong et al., 2013). However, by restoring blood glucose to non-diabetic levels using a once daily intraperitoneal insulin injection, myostatin content within the STZ-induced murine satellite cells lowered to WT levels (Jeong et al., 2013). Thus, it appears the effect of insulin administration on skeletal muscle regeneration was quite potent, as the subsequent myostatin reduction led to a return of satellite cell functionality (Jeong et al., 2013). Therefore, the hyperglycemia induced by hypoinsulinemia in uncontrolled diabetes appears to cause an overexpression of myostatin in the satellite cells in T1DM models (Jeong et al., 2013).

2.3.2. Oxidative Stress

The hyperglycemia that accompanies uncontrolled T1DM has been implicated with increased skeletal muscle oxidative stress (Aragno et al., 2004; Bonnefont-Rousselot, 2002), caused by an elevation of reactive oxygen species (ROS); which have been shown *in vivo* to depress protein turnover (Li et al., 1998; Zhou et al., 2001), and *in vitro* to impair satellite cell functionality (Renault et al., 2002). Specifically, STZ-induced diabetic rodents display elevated skeletal muscle hydrogen peroxide (H₂O₂) and hydroxynonenal (HNE)

(Aragno et al., 2004; de Carvalho et al., 2017), which are both ROS with a potent ability to reduce cellular health, and potentially blunt effective skeletal muscle regeneration through their affect on satellite cells (Aragno et al., 2004). Both H₂O₂ and HNE are associated with a reduction in the expression of myogenic regulatory factors MyoD, Myogenin, and Jun D in STZ-induced rodents (Aragno et al., 2004). Reduction of these ROS with dehydroepiandrosterone (DHEA) helped recover the expression of these myogenic regulatory factors to near WT levels (Aragano et al., 2004); thereby suggesting that reducing oxidative stress in T1DM would help improve skeletal muscle regeneration. However, it should be noted that association between insulin therapy and skeletal muscle oxidative stress has yet to be examined *in vivo* with a T1DM model, nor has it been assessed in the human condition (D'Souza et al., 2013).

2.3.3. Chronic Low-Grade Inflammatory Profile (CLIP)

Another hyperglycemia-induced skeletal muscle complication is the development of CLIP, which has been commonly cited as a contributor to other forms of myopathy where the regenerative process is impaired, such as DMD (Loh et al., 2012; Vidal et al., 2008), or in aging skeletal muscle (McKay et al., 2013). Hyperglycemia appears to induce CLIP through the accumulation of advanced glycation end products (Ramasamy et al., 2005; Yan, Ramasamy, & Schmidt, 2008), which adversely affect skeletal muscle function (Snow et al., 2006; Snow, Fugere, Thompson, 2007). CLIP in T1DM has been characterized by chronic elevations of the inflammatory cytokine interleukin (IL)-6 (Llauradó et al., 2012; Reis et al., 2012). While acute elevation of IL-6 aids skeletal muscle regeneration following damage (Serrano et al., 2008), the chronic elevation seen in CLIP impairs the skeletal muscle regenerative process (Haddad et al., 2005; McKay et al., 2013;

Vidal et al., 2008). However, it should be noted that the implications of CLIP on skeletal muscle regeneration in T1DM has yet to be examined, nor has the potential impact of insulin therapy on this complication been investigated.

2.3.4. Overactivation of Notch Signalling

A recently identified contributor to T1DM-induced myopathy is dysregulation of notch signalling (D'Souza et al., 2016). Properly regulated notch signalling is imperative to skeletal muscle regeneration, as the activation of notch plays a key role in maintaining satellite cell quiescence (Mourikis & Tajbakhsh, 2014). An *in vitro* analysis using isolated muscle fibres from Akita models found that following skeletal muscle damage, notch signalling was significantly more active in the Akita muscle fibres as compared to WT counterparts (D'Souza et al., 2016), and contributed to an inability of the Akita satellite cells to activate (D'Souza et al., 2016). However, following the administration of N-[2S-(3,5difluorophenyl)acetyl]-L-alanyl-2-phenyl-1,1-dimethylethyl ester glycine (DAPT) to inhibit notch signalling in both models, a significant increase in Pax7 expression occurred in the Akita skeletal muscle while no significant change occurred in the WT models, indicating that overactivation of notch signalling is likely to be involved in the inability of Akita satellite cells to activate from quiescence (D'Souza et al., 2016). When assessed in human skeletal muscle samples *in vivo*, it was found that delta-like 1 (DLL1), a potent activator of the notch signalling pathway, was overexpressed in T1DM (D'Souza et al., 2016). This is notable, as all subjects were young adult, well controlled diabetics (D'Souza et al., 2016). Thus, skeletal muscle notch signalling may persist in an overactive state in T1DM, despite adequate insulin therapy (D'Souza et al., 2016). However, it should be

noted that this study did not examine notch signalling in human skeletal muscle samples during the regenerative process (D'Souza et al., 2016).

2.3.5. PAI-1 Overexpression

Overexpression of the blood clotting factor PAI-1, which has long been associated with the development of diabetic complications such as CVD or nephropathy (Bosnyak et al., 2003; Chen, Zhang, & Wang, 2006; Small et al., 1989), also appears to contribute to diabetic myopathy. PAI-1 acts to inhibit the activity of urokinase plasminogen activator (uPA), an important upstream regulator of the MMP system, and is critical for effective ECM remodelling (Koh et al., 2005; Sission et al., 2009). This has been shown *in vivo*, as uPA-deficient mice display an inability to properly remodel the ECM following skeletal muscle damage, ultimately resulting in inefficient skeletal muscle regeneration (Koh et al., 2005; Luis et al., 2001; Sisson et al., 2009; Suelves et al., 2007). Conversely, when PAI-1 expression was inhibited and uPA activity was increased above normal physiological levels, many markers of skeletal muscle regeneration improved beyond what was seen in WT mice (Koh et al., 2005; Sission et al., 2009). Studies utilizing the Akita model of T1DM have demonstrated that the overexpression of PAI-1 elicited by the T1DM pathophysiological environment is large enough to significantly sequester uPA activity following skeletal muscle damage, and that the Akita skeletal muscle displayed a similar regenerative phenotype as uPA knockout mice from previous studies (Koh et al., 2005; Krause et al., 2011a; Krause et al., 2013; Sission et al., 2009). When PAI-1 levels were pharmacologically reduced in the Akita mice, effective ECM remodelling was restored via increases of uPA and MMP-9 activity (Krause et al., 2011a), and in turn improved satellite cell migration and inflammatory cell infiltration (Krause et al., 2013).

2.4. Role of IMCL in T1DM Myopathy

Despite the bevy of characteristics which have been hypothesized or shown to influence the skeletal muscle regenerative process in T1DM, there are multiple aspects of a pathophysiological environment that could contribute to this impaired response that have yet to be explored in the literature. One such unexplored avenue is the role of altered IMCL accumulation. It is well established in both humans and animal models that T1DM elicits changes to plasma lipid content (Fox et al., 2010; Laaksonen et al., 1999; Sorensen et al., 2010), while its effect on skeletal muscle lipid content has been more controversial (Dotzert et al., 2016; Ling et al., 2003; Krause et al., 2009; Krause et al., 2011b; Kurek et al., 2016; Perseghin et al., 2003; Standl et al., 1980). The results of these analyses typically indicate that T1DM induces an elevation in plasma and skeletal muscle fatty acid levels (Dotzert et al., 2016; Fox et al., 2010; Kurek et al., 2016), although evidence has indicated that these differences may be dependent upon the lipid species (Sorensen et al., 2010), muscle fibre type (Krause et al., 2009; Kurek et al., 2016), and T1DM model (Krause et al., 2009). Most skeletal muscle lipid species analysis in T1DM have been conducted with relation to the development of insulin resistance (Dotzert et al., 2016; Kurek et al., 2016), but a paucity of research has examined the role of these altered skeletal muscle lipid profiles on skeletal muscle regeneration. Multiple lines of evidence have indicated that IMCL lipid content levels change following skeletal muscle damage (Loh et al., 2012; Nagata et al., 2006), and that the presence or absence of specific IMCL species is critical for proper skeletal muscle regeneration (Loh et al., 2012; Nagata et al., 2006; Sassoli et al., 2011). Therefore, evaluating how IMCL levels change during the skeletal muscle regeneration process in T1DM is a worthy topic of exploration.

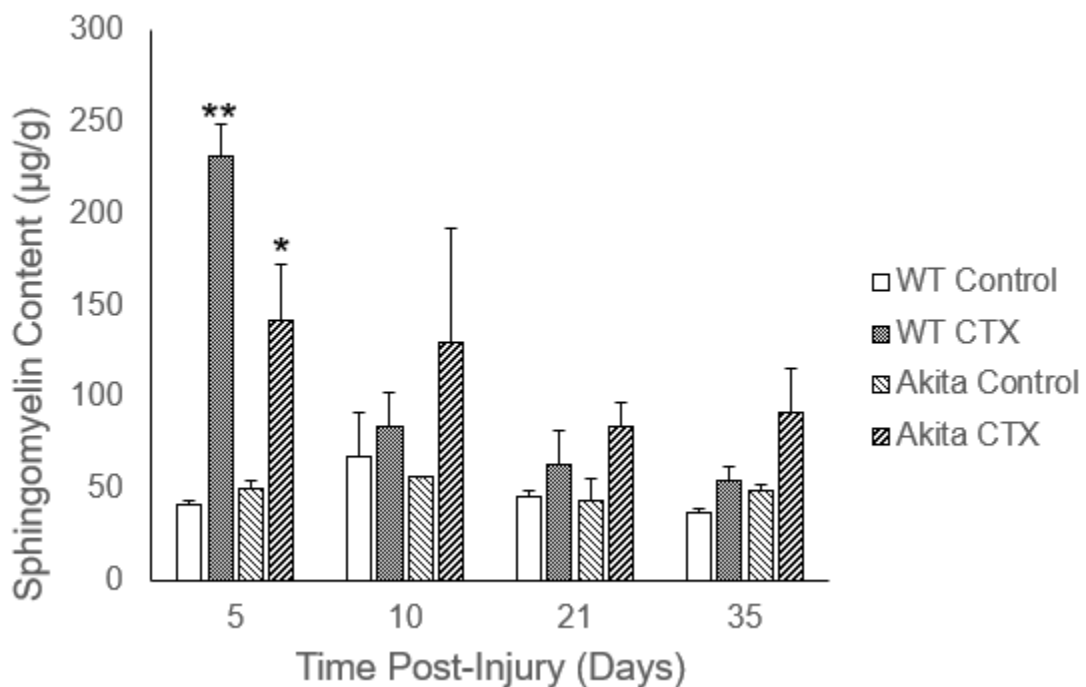
A recent, unpublished, exploratory analysis sought to examine this question by conducting a mass-spectrometry survey to identify how the accumulation of 25 distinct skeletal muscle lipid species differed in the Akita mice and WT controls during skeletal muscle regeneration (Mallender, Trumble, Hawke, & Krause., unpublished results). Samples from the quadriceps muscle were taken in both an undamaged state, and 5, 10, 21, and 35 days following a CTX injection to induce damage, to gain perspective of the lipid species content at various time points in the skeletal muscle regeneration process (Mallender et al., unpublished results). The mass-spectrometry survey indicated that none of these 25 lipid species consistently differed in content between the WT and Akita skeletal muscle in an undamaged state, which is in direct contrast to previous skeletal muscle lipid species surveys in T1DM models, where specific lipid species were found to be elevated (Dotzert et al., 2016; Kurek et al., 2016). This discrepancy can likely be attributed to the use of different T1DM models, as the previous analyses both utilized the STZ-induced model (Dotzert et al., 2016; Kurek et al., 2016), whereas the unpublished results utilized the Akita model. Previous work has shown that the STZ-induced model of T1DM displays elevated lipid content as compared to the Akita model (Krause et al., 2009), and it has been hypothesized that the direct effects of STZ on skeletal muscle elicits this elevation due to a depression of oxidative enzyme activity, and not a T1DM pathophysiological environment (Krause et al., 2009).

Notably, while the content of most skeletal muscle lipid species did not differ between WT and Akita mice in an undamaged state, this was not found during the skeletal muscle regeneration process (Mallender et al., unpublished results). The exploratory analysis identified that 11 of the 25 (44%) analyzed lipid species yielded statistically

significant differences in content between the WT and Akita skeletal muscle during at least one measured time point in the regeneration process, despite no differences between the groups in the undamaged state (Mallender et al., unpublished results). While most of the lipid species identified in the analysis have not been highlighted in the literature as impactful to skeletal muscle regeneration, it was found that five days following skeletal muscle damage, the content of multiple sphingolipid species were significantly depressed in the Akita mice as compared to the WT controls (Mallender et al., unpublished results *Figures 1,2,&3*). Specifically, the lipid species sphingomyelin, ceramide, and sphingosine-1-phosphate (S1P) were significantly increased in content five days following damage in the WT skeletal muscle (Mallender et al., unpublished results), and this post-damage content increase appeared to be attenuated in the Akita models.

A depressed sphingolipid synthesis pathway during skeletal muscle regeneration is notable, as the end product of this pathway, S1P, has been shown to be an essential regulator of multiple regeneration events (Danieli-Betto et al., 2009; Loh et al., 2012; Nagata et al., 2006; Sassoli et al., 2011), and dysregulation of this pathway in skeletal muscle is detrimental to repair following damage (Ieronimakis et al., 2013; Loh et al., 2012; Nagata et al., 2006; Sassoli et al., 2011). Like the WT muscle, both sphingomyelin (*Figure 1*) and ceramide (*Figure 2*) content significantly increased in the Akita skeletal muscle five days following damage, but this increase was significantly lower in magnitude than in the WT counterparts (Mallender et al., unpublished results). Furthermore, unlike sphingomyelin and ceramide, S1P content (*Figure 3*) remained statistically unchanged in the Akita mice at this same five day time point, despite a significant elevation in the WT skeletal muscle (Mallender et al., unpublished results). By 10 days following the CTX injection and

beyond, the content of all three-sphingolipid species returned to pre-damaged levels in both the WT and Akita muscle, indicating the differences in lipid content occurs early in the regenerative process (Mallender et al., unpublished results). Therefore, this altered sphingolipid response, particularly the blunted accumulation of S1P, following skeletal muscle damage in T1DM has the potential to greatly influence the skeletal muscle regeneration process.



*Figure 1. Skeletal muscle sphingomyelin content in WT and Akita models at 5 (WT n=4, Akita n=4), 10 (WT n=4, Akita n=4), 21 (WT n=4, Akita n=4), and 35 (WT n=4, Akita n=4) days post-CTX injection. ** indicates significantly ($p < 0.05$) more skeletal muscle sphingomyelin content than all other groups at that time point. * indicates significantly ($p < 0.05$) more sphingomyelin content than the within-group counterpart.*

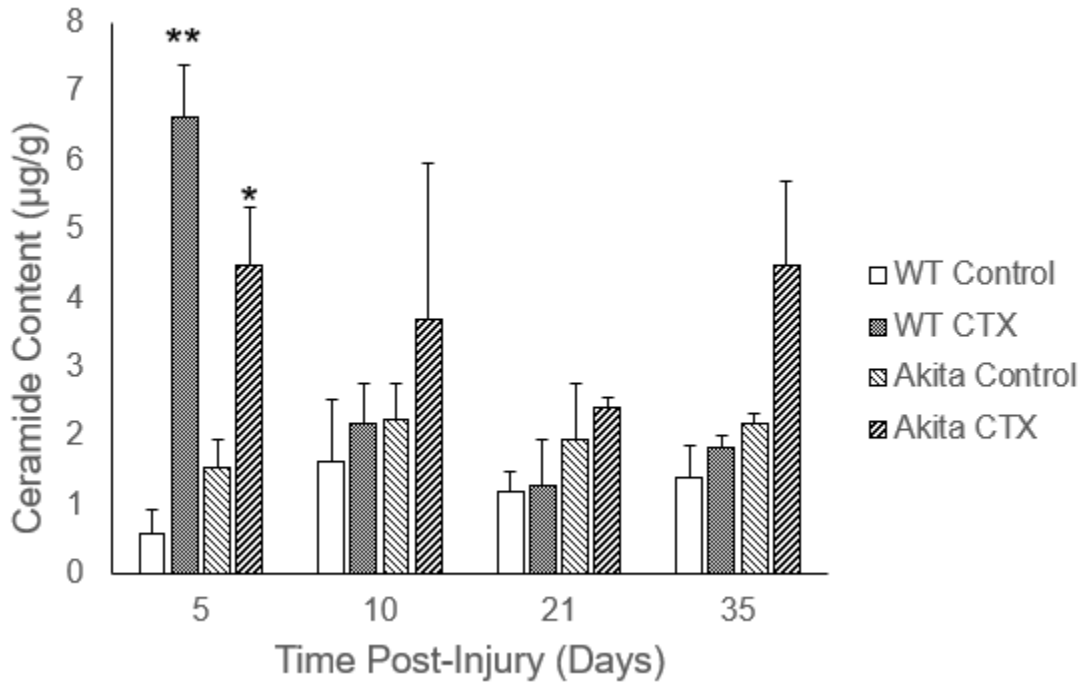


Figure 2. Skeletal muscle ceramide content in WT and Akita models at 5 (WT n=4, Akita n=4), 10 (WT n=4, Akita n=4), 21 (WT n=4, Akita n=4), and 35 (WT n=4, Akita n=4) days post-CTX injection. ** indicates significantly ($p < 0.05$) more skeletal muscle ceramide content than all other groups at that time point. * indicates significantly ($p < 0.05$) more ceramide content than the within-group counterpart.

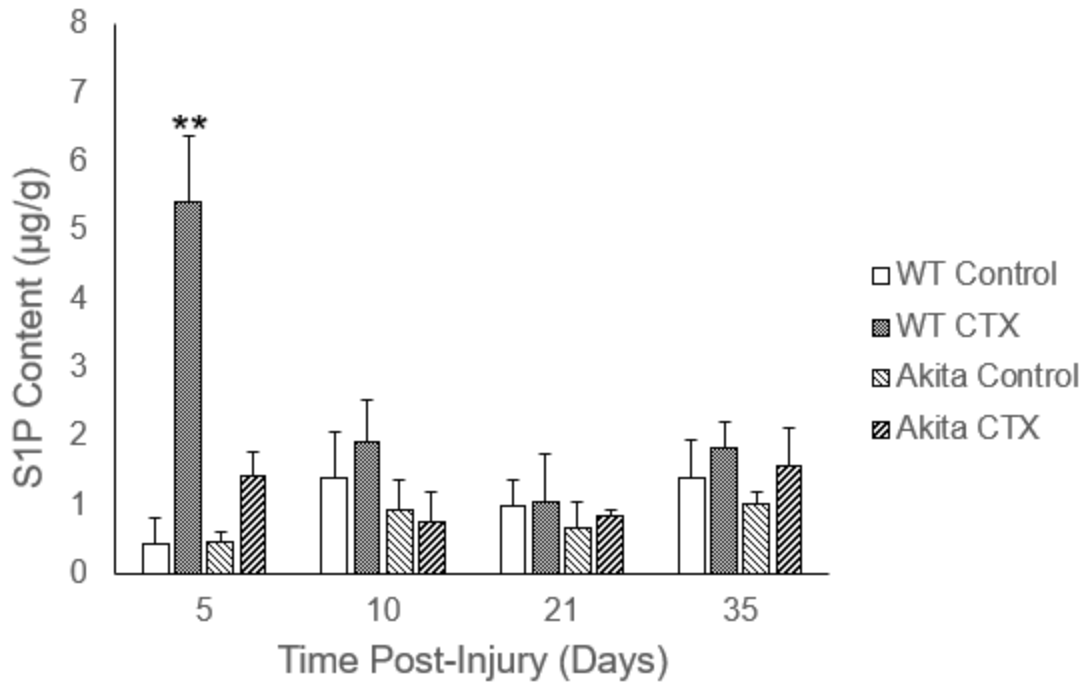


Figure 3. Skeletal muscle S1P content in WT and Akita models at 5 (WT n=4, Akita n=4), 10 (WT n=4, Akita n=4), 21 (WT n=4, Akita n=4), and 35 (WT n=4, Akita n=4) days post-CTX injection. ** indicates significantly ($p < 0.05$) more skeletal muscle S1P content than all other groups at that time point.

2.5. Role of S1P in Skeletal Muscle Regeneration

S1P is a bioactive sphingolipid that is produced endogenously in a variety of bodily tissues, and functions to promote cell survival and growth (Lebman & Spiegel, 2008; Takabe et al., 2008). One such tissue is skeletal muscle, where S1P is synthesized at a rapid rate following a damaging stimulus (Danieli-Betto et al., 2009; Loh et al., 2012; Nagata et al., 2006; Sassoli et al., 2011). The synthesis of S1P in response to damage has been demonstrated both *in vitro* and *in vivo*, and is necessary for adequate skeletal muscle repair (Calise et al., 2012; Danieli-Betto et al., 2009; Loh et al., 2012; Nagata et al., 2006; Sassoli et al., 2011). While no one site of skeletal muscle S1P synthesis has been identified, it has been speculated that following skeletal muscle

damage, synthesis of S1P within the satellite cell is critical (Nagata et al., 2006; Calise et al., 2012). This pathway starts with plasma membrane-derived sphingomyelin, which is subsequently converted into ceramide via N-SMase. Ceramide is then converted into sphingosine via ceramidase activity. The produced sphingosine can then be phosphorylated by SPK1 in an ATP-dependent manner to produce S1P. Following production, S1P can be irreversibly degraded by SPL, dephosphorylated into sphingosine, or exert its bioactive influence within skeletal muscle (*Figure 4*) (Nagata et al., 2006). Following synthesis within the satellite cell, if S1P is not degraded, it will engage in inside-out signalling in an autocrine/paracrine manner, where S1P is shuttled out of the satellite cell via an ATP-binding cassette C1 (ABCC1) transporter, and subsequently binds to one of four transmembrane S1P G-protein coupled receptors (S1PR₁, S1PR₂, S1PR₃, S1PR₄) present on the satellite cell membrane (Calise et al., 2012; Danieli-Betto et al., 2009; Takabe et al., 2008). The influence of S1P on skeletal muscle regeneration has been shown to be receptor-dependent. For example, it has been shown that binding to S1PR₁ & S1PR₄ promoted satellite cell migration, while binding to S1PR₂ & S1PR₃ promoted satellite cell proliferation (Calise et al., 2012). Furthermore, the expression of these receptors on the satellite cell is variable throughout the skeletal muscle regeneration process, therefore signifying that the effects of these receptors can be promoted or depressed to facilitate various phases of satellite cell activity (Calise et al., 2012; Danieli-Betto et al., 2009). In addition, S1P has been demonstrated to exert a trophic action on skeletal muscle, as S1PR₁ & S1PR₃ receptors have been localized at the neuromuscular junction, sarcolemma, and T-tubule membrane in rodent skeletal muscle, and S1P binding to these receptors helps attenuate muscle atrophy following denervation of the soleus muscle (Zanin et al., 2008).

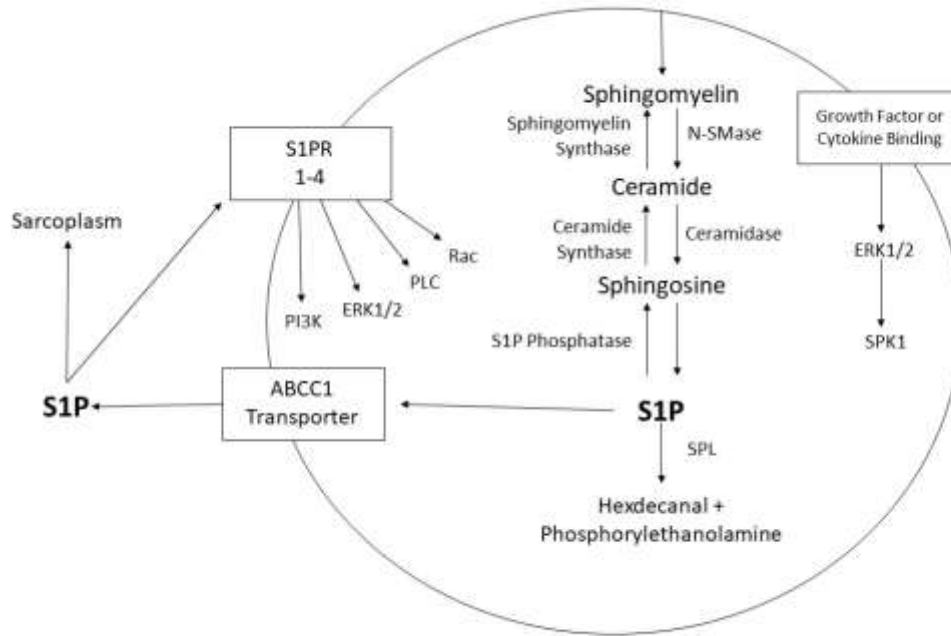


Figure 4. The sphingolipid synthesis pathway and downstream signalling as it occurs in a skeletal muscle satellite cell following skeletal muscle damage. Adapted from Nagata et al., 2006.

The beneficial role of S1P in skeletal muscle regeneration has been experimentally implicated both *in vitro* and *in vivo* utilizing a variety of animal models. Incubation of isolated skeletal muscle satellite cells in a S1P-rich solution resulted in significantly increased satellite cell proliferation and myotube formation as compared to a vehicle solution (Calise et al., 2012; Nagata et al., 2006). Furthermore, incubation in a S1P-rich solution had a similar stimulatory effect on satellite cell proliferation as did incubation in insulin like growth factor -1 (IGF-1) or epidermal growth factor (EGF)-rich solutions (Calise et al., 2012), indicating that S1P may be as potent as well-known growth factors at promoting skeletal muscle regeneration. Injection of exogenous S1P to elicit supra-physiological levels in skeletal muscle following damage improves regeneration *in vivo*, as this resulted in a larger muscle fibre cross sectional area, reduced collagen content, and increased expression of myogenic regulatory factors than in untreated controls at the same time

point in the skeletal muscle regeneration process (Danieli-Betto et al., 2009; Sassoli et al., 2011). In contrast, pharmacological inhibition of S1P production, induced by a SPK1 inhibitor (Nagata et al., 2006; Sassoli et al., 2011), SPK1 knockout (Loh et al., 2012), or via S1P receptor blocking (Calise et al., 2012), significantly attenuated skeletal muscle regeneration in otherwise healthy animal models. Thus, these results indicate that the S1P accumulation that occurs following skeletal muscle damage appears to be critical to facilitate proper skeletal muscle repair.

Due to the detrimental effect that blocking the action of S1P has on skeletal muscle regeneration, the blunted accumulation of S1P acutely following skeletal muscle damage in T1DM has the potential to be a major contributor to this myopathy. Reduced skeletal muscle S1P content has been experimentally implicated to contribute to other forms of myopathy, such as in the murine *mdx* model of DMD, where depressed skeletal muscle S1P content has been identified as compared to WT counterparts, both in the undamaged state and following skeletal muscle damage (Ieronimakis et al., 2013; Loh et al., 2012). Implementing a pharmacological intervention in the *mdx* model that allowed for the adaptive accumulation of S1P following skeletal muscle damage vastly improved regeneration, as there were significant increases in the muscle fibre cross sectional area, satellite cell activation, and reduced fibrosis (Ieronimakis et al., 2013; Loh et al., 2012). Thus, precedent exists that dysregulation of S1P accumulation induced by a pathophysiological environment can contribute to impaired skeletal muscle regeneration, and therefore, provides a basis to further study this phenomenon in T1DM.

2.6. Potential Role of S1P in T1DM Myopathy

Due to the prominent role S1P plays in skeletal muscle regeneration, it is a distinct possibility that certain aspects of a T1DM pathophysiological environment which have already been associated with myopathy in this population could be exerting this negative

influence through an S1P-dependent mechanism. Based upon this assumption, further examination of the skeletal muscle regeneration phenotype in animal models of T1DM and in animal models where S1P action has been blocked reveals a high degree of overlap. The skeletal muscle of both Akita mice and S1P blocked mice display attenuated ECM remodelling following myotoxin or exercise-induced damage (Loh et al., 2012; Krause et al., 2011a; Krause et al., 2013; Sassoli et al., 2011). When S1P content and action was restored in the S1P blocked models, ECM remodelling was restored as indicated by a reduced collagen positive area (Loh et al., 2012; Sassoli et al., 2011), in large part due to the stimulatory effect of S1P on MMP-9 activity (Sassoli et al., 2011). Furthermore, animal models with blocked S1P action also have impaired satellite cell functionality (Calise et al., 2012; Loh et al., 2012; Nagata et al., 2006), much like models of T1DM (D'Souza et al., 2016; Krause et al., 2013). When S1P synthesis was prevented either via a knockout model or via a pharmacological method, *in vitro* analysis identified that isolated muscle satellite cells failed to adequately proliferate by five days following induction (Nagata et al., 2006), similar to findings in T1DM (D'Souza et al., 2016). Furthermore, *in vivo* analysis shows that an absence of S1P following myotoxin-induced damage results in significantly less expression of Pax7, MyoD, & Myogenin at various time points within 7 days of the skeletal muscle regeneration process (Danielia-Betto et al., 2009; Loh et al., 2012; Nagata et al., 2006), which is again a similar phenotype displayed by Akita skeletal muscle during regeneration (D'Souza et al., 2016; Krause et al., 2011a; Krause et al., 2013). Therefore, it could be hypothesized that the reduced S1P content acutely (within 10 days) following skeletal muscle damage in the Akita skeletal muscle may contribute to both the attenuated ECM remodelling and impaired satellite cell functionality in this population.

Therefore, as previous research has indicated that S1P accumulation following skeletal muscle damage is essential for an adequate regeneration response, the finding of impaired S1P accumulation during the acute phase of skeletal muscle regeneration in T1DM models provides possible evidence of a previously unidentified contributor to myopathy in T1DM. This finding, when coupled with the similar skeletal muscle regeneration phenotypes observed in S1P deficient animals and T1DM models, provides the basis for further exploration of the sphingolipid synthesis pathway in T1DM skeletal muscle. However, prior to exploring what about a T1DM pathophysiological environment is preventing the accumulation of S1P in the acute phase of skeletal muscle regeneration, it will be important to characterize sphingolipid content at multiple acute regeneration time points, which is being defined as less than seven days (within one week) from the damage-inducing stimulus. This is suggested due to the previous analysis by Mallender et al. (unpublished results), where the WT skeletal muscle sphingolipid content was significantly elevated only at 5 days following damage, and returned to undamaged muscle content levels after 10 days and beyond. That result, coupled with the finding that Akita skeletal muscle only differed from the WT in sphingolipid content at 5 days following skeletal muscle damage, indicates that the sphingolipid response to skeletal muscle damage occurs within the first 10 days of the regeneration process, and a T1DM pathophysiological environment could act to depress this response within the same acute time frame (Mallender et al., unpublished results). Furthermore, better characterizing the skeletal muscle sphingolipid changes acutely following damage in this population is important, as S1P appears to exert its influential effects on skeletal muscle regeneration within seven days of the damage-inducing stimulus (Danielia-Betto et al., 2009; Loh et al., 2012; Nagata et al., 2006; Sassoli et al., 2011). Thus, examining skeletal muscle sphingolipid species content (most importantly S1P), at multiple acute time points prior to the return to control

levels at 10 days post skeletal muscle damage will help provide further insight into the observed aberrant sphingolipid response to skeletal muscle damage in T1DM.

2.7. Key Enzymes in the Sphingolipid Response to Skeletal Muscle Damage

Skeletal muscle S1P content is regulated via the activity and expression of two major enzymes. SPK1 is the primary facilitator of S1P synthesis in skeletal muscle following a damaging stimulus (Nagata et al., 2006a; Loh et al., 2012; Sassoli et al., 2011), as it performs ATP-dependent phosphorylation of sphingosine to produce S1P (Nagata et al., 2006a; Loh et al., 2012; Piston et al., 2003; Piston., 2011). In contrast to SPK1, SPL is responsible for the irreversible degradation of S1P into hexadecenal and phosphorylethanolamine (Nagata et al., 2006a; Loh et al., 2012; Saba & de la Garza-Rodea, 2013), and is the primary means of S1P degradation in skeletal muscle. Therefore, skeletal muscle S1P content is carefully regulated by the activation and expression of both SPK1 and SPL, and the balance of activity between the two determines whether S1P content increases, decreases, or remains constant within the muscle. Skeletal muscle damage has potent effects on the activation and/or expression of both SPK1 and SPL (Nagata et al., 2006; Loh et al 2012; Sassoli et al., 2011), and dysregulation of these enzymes could result in an inability to adequately produce S1P. Therefore, a pathophysiological environment may exert influence on either of these enzymes to induce the observed aberrant sphingolipid response to skeletal muscle damage. Thus, in order to examine the role of S1P in T1DM myopathy, how SPK1 and SPL are regulated in response to a damaging stimulus should be characterized.

2.7.1. Regulation of SPK1 Activity

Sphingosine kinase has two primary isoforms present in skeletal muscle, the aforementioned SPK1, and an additional isoform known as sphingosine kinase 2 (SPK2) (Piston et al., 2003; Piston et al., 2011). While both SPK1 and SPK2 have been localized to skeletal muscle tissue (Loh et al., 2012; Sassoli et al., 2011), it appears that only SPK1 regulates S1P synthesis following skeletal muscle damage. It has been shown *in vivo* that when all SPK activity is inhibited via a N, N-dimethylsphingosine (DMS) injection, S1P content is significantly reduced seven days following CTX-induced skeletal muscle damage (Nagata et al., 2006). However, when these effects are isolated to the SPK1 enzyme *in vivo* via the use of a SPK1 murine knockout, S1P content is still reduced by as much as 57% in when compared to WT controls, despite having a viable and intact SPK2 gene (Loh et al., 2012). Finally, experiments looking at the activation state of skeletal muscle SPK1 and SPK2 have identified that only SPK1 activity increases after skeletal muscle damage, and that SPK2 activity remains unchanged in the same physiological environment (Sassoli et al., 2011). Therefore, evidence implicates SPK1 as the critical kinase involved in S1P synthesis following skeletal muscle damage, and that SPK2 appears to be dispensable to this process.

It has been suggested that the increase in SPK1 action following skeletal muscle damage is due either to an increase in its activation state, an increase of kinase expression, or a combination of these two events (Sassoli et al., 2011). Activation of SPK1 has been shown to require extracellular signal-related kinase (ERK) 1/2-mediated phosphorylation at the Ser225 binding site present on SPK1 (Piston et al., 2003; Piston, 2011). Following phosphorylation, SPK1 migrates from its normal centralized niche within the cytoplasm

towards the cellular membrane, where it can subsequently phosphorylate sphingosine into S1P (Piston et al., 2003; Piston, 2011). Therefore, the observed increase of SPK1 phosphorylation *in vivo* following exercised-induced skeletal muscle damage is logically consistent (Sassoli et al., 2011), as an increase in ERK1/2 activity is a hallmark of normal skeletal muscle regeneration (Yin, Price, & Rudnicki, 2013; Jones, Federov, Rosenthal, & Olwin, 2001; Kramer & Goodyear, 2007; Rennie et al, 2004; Bennet & Tonks, 1997). With regards to the overall expression of SPK1 following skeletal muscle damage, evidence has shown that gene expression of SPK1 is increased following myotoxin-induced skeletal muscle damage *in vivo* (Loh et al., 2012). However, the same study failed to provide any evidence pertaining to the activation state of SPK1 (Loh et al., 2012). In contrast, it has been shown that while the phosphorylation and activation state of SPK1 increase following exercise-induced skeletal muscle damage *in vivo*, the overall expression of SPK1 remains unchanged (Sassoli et al, 2011), and that IGF-1 binding to C2C12 myoblast *in vitro* enhanced the activity of SPK, but failed to exert any effects on its transcriptional regulation (Bernacchioni, Cencetti, Donati, & Bruni, 2012). Thus, while evidence would suggest that skeletal muscle damage results in an increase of SPK1 activity, the effect of skeletal muscle damage on overall SPK1 expression is still unclear.

While ERK1/2-mediated phosphorylation at Ser225 is the key event needed for SPK1 activation, several events upstream of this phosphorylation need to occur in order to stimulate S1P synthesis. The primary means of stimulating ERK1/2-mediated activation of SPK1 occurs via the effects of multiple growth factors and cytokines (Piston, 2011; Lebman & Spiegel, 2008; Piston et al., 2003; Donati et al., 2013). Though not always studied directly in skeletal muscle tissues, many of the identified growth factors have also

been implicated within the skeletal muscle regeneration process. However, due to the paucity of research examining the effects of growth factor activity and S1P synthesis directly in skeletal muscle, it is difficult to confidently ascertain what, if any, individual growth factor primarily regulates SPK1 activity following damage, or if this activity is dependent on the action of multiple growth factors acting simultaneously. Therefore, as the list of growth factors with the potential to influence S1P synthesis during skeletal muscle regeneration is quite extensive, this review will focus on three primary growth factors, hepatocyte growth factor (HGF), IGF-1, and EGF, due to the strong evidence that they contribute to SPK1 regulation in multiple bodily tissues (Duan et al., 2004; El-Shewy et al., 2006), preliminary evidence of this regulation in skeletal muscle tissue (Bernacchioni et al., 2012; Nagata et al., 2014), and/or due to the well-established positive influence each growth factor has on satellite cell activity during skeletal muscle regeneration (Adams & McCue, 1998; Chakravarthy, Davis, & Booth, 2000; Halevy & Cantley, 2004; Nagata et al., 2014; O'Reily, McKay, Phillips, Tarnapolsky, & Praise, 2008).

HGF, IGF-1, and EGF have been experimentally shown to act directly on and regulate satellite cell activity throughout the skeletal muscle regeneration process, and the downstream signalling of each growth factor includes activation of ERK1/2 signalling (Nagata et al., 2014; Halevy & Cantley, 2004). Thus, each growth factor has the potential to stimulate S1P synthesis via SPK1 activation. IGF-1 is arguably the most well recognized hypertrophy-inducing growth factor within skeletal muscle, as it has long been implicated as a positive regulator of satellite cell proliferation and differentiation following skeletal muscle damage, due to its ability to activate the PI3K/Akt/mTOR pathway (Adams & McCue, 1998; Allen & Boxhorn, 1989; Chakravarthy et al., 2000; Halevy & Cantley, 2004;

Hill & Goldspink, 2003; Machida & Booth, 2004). Preliminary evidence *in vitro* has shown that IGF-1 increases SPK1 activity in an ERK1/2-dependent manner (El-Shewy et al., 2006; Bernacchioni et al., 2012), and that pharmacologically inhibiting SPK1 activity significantly blunted an IGF-1-induced increase in myogenin content (Bernacchioni et al., 2012); thereby signalling that at least some of IGF-1's positive effects on satellite cell functionality may be mediated by S1P production. Furthermore, EGF binding to C2C12 myoblasts has been shown *in vitro* to significantly upregulate SPK1 activity and S1P content, an outcome which is dependent upon EGFs stimulatory effect on ERK1/2 signalling (Nagata et al., 2014). However, EGFs ability to activate C2C12 myoblasts was significantly reduced in the presence of an SPK1 inhibitor, indicating that EGFs activating effects on muscle satellite cells may also be mediated by S1P production (Nagata et al., 2014).

While the effects of HGF activity on SPK1 activation have yet to be directly tested within skeletal muscle tissue, it has been demonstrated *in vitro* that HGF-induced migration of endothelial cells is dependent upon HGF stimulated S1P production (Duan et al., 2004). This was identified, as pharmacologically inhibiting either ERK1/2 or SPK activity significantly reduced S1P production, and ablated HGFs ability to induce endothelial cell migration (Duan et al., 2004). As skeletal muscle HGF content and activity is significantly upregulated following skeletal muscle damage (Sission et al., 2009; O'Reily et al., 2008) and strongly promotes satellite cell functionality (Allen, Sheehan, Taylor, Kendall, & Rice, 1995; Sisson et al., 2009; O'Reilly et al., 2008), in conjunction with HGFs potent ability to increase ERK1/2 signalling both in variety of tissues (Halevy & Cantley, 2004; Duan et al., 2004; Walker, Kahamba, Woudberg, Goetsch, & Nisler, 2015), the potential exists that

SPK1 activation and S1P production are also necessary for HGF-induced satellite cell proliferation. Thus, all three of these growth factors are likely to contribute to sphingolipid response to skeletal muscle regeneration via their potential stimulating effects on SPK1 activity and S1P production.

2.7.2. Regulation of SPL Expression

In contrast to the S1P production elicited by SPK1 activation, SPL is the primary enzyme responsible for irreversible S1P degradation (Saba & de la Garza-Roden, 2013; Loh et al., 2012; Nagata et al., 2006). Therefore, skeletal muscle S1P content is regulated via the relative balance of SPK1 activity and SPL expression within the skeletal muscle (Saba & de la Garza-Roden, 2013; Loh et al., 2012; Nagata et al., 2006). However, unlike SPK1, the overall activity of SPL is highly correlated to its expression, and not by a phosphorylation state (Saba & de la Garza-Roden, 2013; Loh et al., 2012). In relation to SPK1, there is a paucity of research examining how SPL expression changes following skeletal muscle damage. Nonetheless, a few studies have indicated that SPL expression does change in response to a damaging stimulus, and that dysregulation of this response can severely inhibit proper skeletal muscle regeneration (Saba & de la Garza-Roden, 2013; Ieronimakis et al., 2013; Loh et al., 2012).

Counterintuitive to the observed increases in skeletal muscle S1P content that occurs following a damage-inducing stimulus, SPL expression, much like phosphorylated SPK1, appears to significantly increase 3 days following myotoxin-induced muscle damage *in vivo* (Loh et al., 2012). In an attempt to identify the cellular source of SPL production, *in vitro* analysis of isolated satellite cells derived from *mdx* myoblasts revealed a significant number of cells co-expressing SPL and Pax7; meaning that SPL may be

endogenously produced within activated skeletal muscle satellite cells (Saba & de la Garza-Roden, 2013). However, SPL content was notably absent in WT Pax7 positive satellite cells at rest and 10 days following skeletal muscle damage *in vivo*, suggesting that satellite cells only express SPL acutely following activation, or this phenomenon is limited to *mdx* myoblasts (Saba & de la Garza-Roden, 2013). It has also been proposed that SPL is produced by and secreted from inflammatory cells that infiltrate skeletal muscle tissues in the early phases of regeneration (Loh et al., 2012). This possibility was supported *in vivo*, where a significant number of the mononucleated infiltration inflammatory cells were found to express SPL, indicating that the upregulation of SPL content seen in response to skeletal muscle damage can be at least partly attributed to inflammatory cell infiltration (Saba & de la Garza-Roden, 2013).

Therefore, the increased skeletal muscle S1P content seen in response to skeletal muscle damage is regulated by multiple mechanisms. SPK1 activity has been observed to increase acutely following skeletal muscle damage, resulting in an increased production of S1P (Sassoli et al., 2011; Loh et al., 2012), and it has further been noted that SPL expression is also increased during this same time period (Loh et al., 2012; Saba & de la Garza-Roden, 2013). Thus, while it seems relative SPK1 activity would be larger than relative SPL expression following skeletal muscle damage to facilitate a net increase in skeletal muscle S1P content, it is clear that both enzymes play critical roles in the sphingolipid response to skeletal muscle damage.

2.8 Effect of T1DM on the Sphingolipid Response to Skeletal Muscle Damage

Due to the key role that SPK1 and SPL play in regulating skeletal muscle S1P content in response to damage, the potential exists that a T1DM pathophysiological

environment could dysregulate one or both enzymes during regeneration, resulting in the aberrant sphingolipid response seen in this population (Mallender et al., unpublished results). It has been proposed that the reduced S1P content observed in DMD is a result of SPL overexpression induced by CLIP in this population (Ieronimakis et al., 2013; Loh et al., 2012), much like the CLIP seen in T1DM. Furthermore, while it has yet to be shown that a pathophysiological environment can influence SPK1 activity, many of its activating growth factors, such as IGF-1, EGF, or HGF, may be adversely affected by T1DM (Nagata et al., 2014; Sission et al., 2009; Wedrychowicz, Dziatkowiak, Nazim, Sztefko, 2005). Therefore, multiple aspects of a T1DM pathophysiological environment have the potential to affect normal sphingolipid signalling through SPK1- and SPL-dependent mechanisms (*Figure 5*). However, due to the exploratory nature of this study, and the sparse literature examining S1P in T1DM skeletal muscle, further speculation as to how a T1DM pathophysiological environment could induce effects on these enzymes would be inappropriate, at least until the activity and expression of these enzymes is quantified. Therefore, the activity of SPK1 and expression of SPL following skeletal muscle damage in a T1DM pathophysiological environment must first be examined to see if these enzymes are adversely affected by this condition. The results of such an investigation could then guide future research questions attempting to unearth the mechanisms behind the dysregulated sphingolipid response to skeletal muscle damage in T1DM myopathy. Therefore, SPK1 activations and SPL expression will be examined.

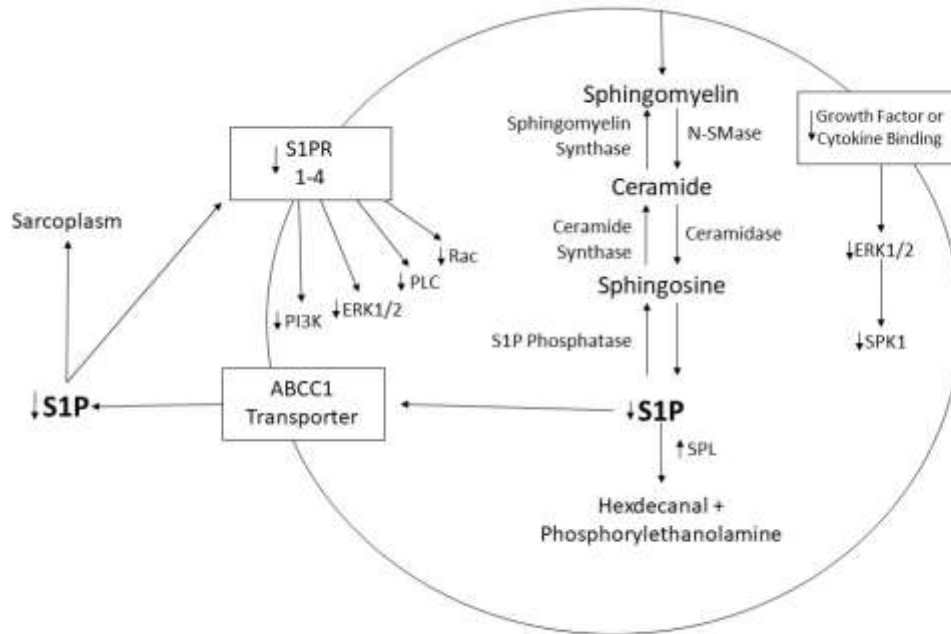


Figure 5. T1DM may potentially affect sphingolipid signalling via SPK- and SPL-dependent mechanisms. The arrows inserted beside different lipids/enzymes/lyases indicate how their activity or expression may change due to a T1DM pathophysiological environment. Adapted from Nagata et al., 2006.

2.9. Clinical Significance

There are numerous complications induced by a pathophysiological environment created by T1DM which have a significant impact on health, wellness, and longevity (Pelletier et al., 2012; Soedamah-Muthu et al., 2006). Some of the most notable complications include CVD, nephropathy, neuropathy, retinopathy (Pelletier et al., 2012; Soedamah-Muthu et al., 2006), and myopathy (Monaco et al., 2017; D'Souza et al., 2013; Krause et al., 2011b); which all contribute to the 3.7-fold higher mortality rate observed in the T1DM population (Soedamah-Muthu et al., 2006). Furthermore, having a diagnosis of diabetes increases the risk of developing multiple co-morbidities, including hypertension, heart disease, chronic obstructive pulmonary disease, and arthritis (Pelletier et al., 2012).

As a result, almost 40% of adult Canadians diagnosed with diabetes self-report their health as being “fair” to “poor” when compared to a non-diabetic population (Pelletier et al., 2012). The impact of these complications should not be underestimated, as it has been stated that diabetes itself rarely leads directly to death, but the complications which accompany the disease often result in mortality (Pelletier et al., 2012).

Myopathy in T1DM has historically been understudied and clinically overlooked as compared to other more recognized complications. However, adequate skeletal muscle health has been identified as an important contributor to overall health and well-being, thus, myopathy in this population is likely to contribute to the observed increased morbidity and mortality rate (Pelletier et al., 2012; Soedamah-Muthu et al., 2006). A meta-analysis utilizing results from 23 studies assessing objective measures of physical capacity (and indirectly muscle function) identified that poor performance on tests of grip strength, walking speed, chair rising, and standing balance times were all associated with an increased mortality rate (Cooper et al., 2010). Furthermore, maintaining muscular strength as measured by one-repetition maximal measures for bench press and leg press was associated with lower cancer mortality in men, independent of potential cofounders (Ruiz et al., 2009); and lower limb strength, as assessed through gait speed testing, has been significantly associated with a higher mortality rate in community dwelling older adults (Studenski et al., 2011).

This impaired skeletal muscle health and regeneration also contributes to poor health outcomes in tandem with other T1DM complications. For example, patients with diabetic neuropathy are two-three times more likely to fall than those without neuropathy (Agrawal, Carey, Della Santina, Schubert, & Minor, 2010). Not only would an individual

with T1DM neuropathy and myopathy be more susceptible to falling, but they would also have a limited ability to recover from a fall event due to impaired skeletal muscle regenerative capacity. Furthermore, as skeletal muscle is the largest organ in the human body which acts as a glucose repository, reduced skeletal muscle mass may significantly hinder an individual's ability to regulate blood glucose content. This could further contribute to dysregulated blood glucose control above what normally occurs in T1DM and act to amplify an induced pathophysiological environment, exasperating the action of other complications and affects to other tissue susceptible to T1DM. Therefore, these findings would indicate that myopathy in T1DM is likely to have a greater clinical relevance than initially thought, and it is imperative that more attention be paid to this complication in both the academic and clinical domains.

3. Methods

3.1. Animal Handling

3.1.1. Animal Care

Twenty, 4-week-old male mice heterozygous for the $Ins2^{Akita}$ (Akita) gene and 22 WT littermates ($C57Bl/6J$) were purchased from Jackson Laboratory (Bar Harbor, ME). Akita mice developed diabetes spontaneously at approximately four weeks of age (Jackson Laboratory, 2018). Hyperglycemia was confirmed at time of death with a blood glucose measurement (Freestyle Freedom Lite Blood Glucose Monitor, Abbot Diabetes Care, Alameda, California). Male mice were utilized as they display a more severe diabetic phenotype than their female counterparts (Jackson Laboratory, 2018). Mice were stored in an animal room at 21°C, at 50% humidity, with a 12:12h light dark cycle. All mice were provided access to a standard diet and water ad libitum, and enrichment was provided via nesting material and cardboard tubing. All experimental protocols were approved by the University of Windsor Animal Care Committee (AUPP 16-06).

3.1.2. Skeletal Muscle Injury

The Akita mice were subjected to eight weeks of uncontrolled diabetes. Akita mice heterozygote for the $Ins2^{Akita}$ gene exhibit a median lifespan of 305 days, contrasted to an average lifespan of 690 days of the within strain ($C57Bl/6J$) counterparts (Jackson Laboratory, 2018). Thus by 12 weeks of age, the Akita mice had lived approximately one third of their expected lifespan. At twelve weeks from date of birth, total body mass was collected, and skeletal muscle damage (injury) was induced via a 50 μ l intramuscular injection of 10 μ M cardiotoxin (CTX) (Latoxan, France) in the left tibialis anterior (TA); three evenly spaced

50 μ l injections into the gastrocnemius-plantaris-soleus (GPS) complex; and, three evenly spaced 50 μ l injections into the left quadriceps muscle of all mice in both groups. A subset of mice from each group (n=5 or n=6) were sacrificed via carbon dioxide (CO₂)-induced euthanasia and cervical dislocation one day, three days, five days, or seven days following CTX-induced muscle damage. The TA, GPS, and quadriceps muscle from both the left (damaged) and right (undamaged) legs were harvested from the euthanized mice and muscle mass was collected. However, only the quadriceps muscles were analyzed in the present study. Samples were snap frozen in liquid nitrogen and stored at -80°C. Euthanasia time points were chosen to best approximate the early phase of the skeletal muscle regeneration process, which was defined for this study as within one week of the skeletal muscle damage stimulus. As it has previously been shown that skeletal muscle sphingolipid content returns to basal levels in undamaged muscle as soon as 10 days following CTX-induced injury (Mallender et al., unpublished results), these selected euthanasia time points were expected to provide an adequate representation of skeletal muscle sphingolipid dynamics after skeletal muscle damage.

3.2. Research Questions and Procedures

3.2.1. Objective #1

Research Question #1

How does skeletal muscle content of key lipids in the sphingolipid synthesis pathway, including sphingomyelin, ceramide, sphingosine, and SIP, change in the Akita model of T1DM as compared to a WT control, one day, three days, five days, and seven days following CTX-induced skeletal muscle damage?

Experiment #1

Sphingomyelin, ceramide, sphingosine, and S1P, were selected due to their implication within the skeletal muscle sphingolipid synthesis pathway (Nagata et al., 2006). Sphingomyelin, ceramide, and S1P have been identified to be dysregulated in T1DM model skeletal muscle five days following CTX-induced muscle damage (Mallender et al., unpublished results). Sphingosine was not examined in the previous analysis (Mallender et al., unpublished results), but was included in the present study due to its role as the immediate precursor to S1P in the sphingolipid synthesis pathway (Nagata et al., 2006), and the role that this function plays in skeletal muscle regeneration (Danielia-Betto et al., 2009; Nagata et al., 2006). Liquid chromatography-mass spectrometry (LC-MS) was performed to measure the skeletal muscle sphingolipid content of muscle samples, due to its status as the gold standard of skeletal muscle sphingolipid measurement (Meacci, Bini, & Battistini, 2012). The proximal half of both control and damaged quadriceps muscles were sent to the Genome BC Proteomics Centre (University of Victoria, Victoria, British Columbia) through affiliation with The Metabolic Innovation Centre (University of Alberta, Edmonton, Alberta) for LC-MS analysis. A detailed description of the LC-MS procedure can be found in Appendix A.

Hypothesis #1

If a T1DM pathophysiological environment has adverse effects on the sphingolipid response to skeletal muscle damage, then the skeletal muscle content of sphingomyelin, ceramide, sphingosine, and S1P will be lower in the Akita skeletal muscle as compared to the WT controls at all measured time points.

3.2.2. Objective #2

Research Question #2

How does a T1DM pathophysiological environment affect the activation state of skeletal muscle SPK1, one day, three days, five days, and seven days, following CTX-induced skeletal muscle damage?

Experiment #2

In order to test for changes in SPK1 activity, anti-SPK1 and anti-SPK1-Ser225 phospho-specific antibodies were obtained from ECM Biosciences (Cat. #SP5421 & Cat. #SP1641, Versailles, KY). SDS-PAGE and Western Blot was performed with the distal portion of both control and damaged quadriceps muscle samples from each time point in both groups with both the SPK1 and SPK1-Ser225 phospho-specific antibodies. A description of all components in the SDS-PAGE and Western Blot protocol can be found in Appendix B-E.

Hypothesis #2

If a T1DM pathophysiological affects the activation state of SPK1 following CTX-induced muscle damage, then the Akita models will display lower SPK1 activity than WT controls at all measured time points.

2.2.3. Objective #3

Research Question #3

How does a T1DM pathophysiological environment affect the expression of skeletal muscle SPL, one day, three days, five days, and seven days, following CTX-induced skeletal muscle damage?

Experiment #3

In order to test for SPL expression, anti-SPL antibodies were obtained from Millipore Sigma (Cat. #ABS528, Etobicoke, ON). SDS-PAGE and Western blot was performed with the anti-SPL antibody on the distal portion of all control and damaged quadriceps muscle samples from all time points in both the WT and Akita groups. A description of all components in the SDS-PAGE and Western Blot protocol can be found in Appendix B-E.

Hypothesis #3

If a T1DM pathophysiological environment affects the expression of SPL following CTX-induced muscle damage, then the Akita models will display greater SPL expression than WT controls at all measured time points.

3.3. Data Analysis

A three-way (2x2x4) multifactorial ANOVA was conducted to compare the four groups of WT, Akita, WT (CTX), and Akita (CTX) mice at each measured time point (one day, three days, five days, and seven days) during the skeletal muscle regeneration process. When a variable of interest exhibited a significant interaction in the main analysis, a post-hoc simple main effects analysis was performed to determine where differences existed, utilizing a Bonferroni adjustment for multiple comparisons. All analyses were conducted using the statistical software package SPSS (Version 22). All statistical tests were considered significant at an alpha level of 0.05.

4. Results

4.1 Physiological Characteristics

The physiological characteristics of body mass and blood glucose were analyzed via an one-way ANOVA to confirm the presence of a type 1 diabetes mellitus (T1DM) pathophysiological environment in the Akita mice. Analysis showed that the wild type (WT) mean body mass was significantly more than their Akita counterparts ($p < 0.05$) prior to muscle injury at 25.49 g vs 22.63 g respectively (*Figure 6*). This is consistent with the described T1DM phenotype (Jackson Laboratory, 2018). Furthermore, Akita mice displayed significantly elevated blood glucose levels compared to their WT counterparts ($p < 0.05$) with a mean blood glucose of 20.32 mM in the Akita mice compared to 8.55 mM in the WT mice (*Figure 6*). Therefore, these findings confirm that the Akita mice were experiencing the hyperglycemia associated with T1DM, and were exposed to a pathophysiological environment not observed in the WT mice.

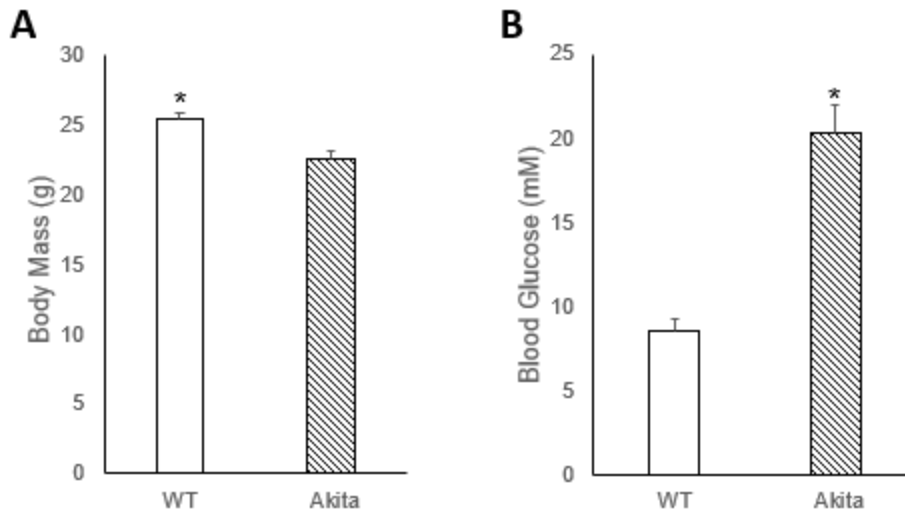


Figure 6. A) Body mass of WT (n=22) and Akita (n=20) mice in grams prior to CTX muscle injection. B) Blood glucose of WT and Akita mice in mM just prior to euthanization. * Indicates a significant difference between the WT and Akita populations at the $p=0.05$ level.

4.2 Lipid Profile

All muscle samples were sent to the University of Victoria Genome BC Proteomics Centre for liquid chromatography-mass spectrometry (LC-MS) analysis to quantify skeletal muscle sphingolipid content. Due to time limitations prior to submission, only data regarding skeletal muscle S1P content will be presented in the following analysis. A three-way (2x2x4) ANOVA was performed to assess for changes in skeletal muscle S1P content across the three experimental conditions: T1DM, muscle damage, and regeneration time point. Statistical analysis indicated a statistically significant effect of T1DM ($p=0.013$), muscle damage ($p<0.05$), and regeneration time point ($p=0.042$) on skeletal muscle S1P content. Furthermore, a statistically significant interaction between T1DM x regeneration time point was observed ($p=0.017$), but not for muscle damage x regeneration time point ($p=0.645$), T1DM x muscle injury ($p=0.111$), or T1DM x muscle damage x regeneration

time point ($p=0.745$). A post-hoc simple main effects analysis was conducted to identify the source of the T1DM x regeneration time point interaction. A statistically significant difference in skeletal muscle S1P content occurred between the WT and Akita mice five days ($p<0.05$), but not at one day ($p=0.632$), three days ($p=0.544$), or seven days ($p=0.170$) post-damage.

Therefore, results from the primary analysis and T1DM x regeneration time point post-hoc test combined with visual analysis of *Figure 7* would suggest that both the WT and Akita mice experience increased skeletal muscle S1P content following skeletal muscle damage, and do not differ in this response until five days into the regenerative process. At this point, it appears the WT mice display greater skeletal muscle S1P content than their Akita counterparts; but this difference returns to negligible levels seven days post-damage. Thus, these results are consistent with pilot data from Mallender et al., (Unpublished results) which illustrated that WT mice have greater skeletal muscle S1P content than Akita mice five days following a damage inducing stimulus. Therefore, the key enzymes regulating skeletal muscle S1P content, SPK1 and SPL, were analyzed to elucidate the mechanisms underlying this impaired response.

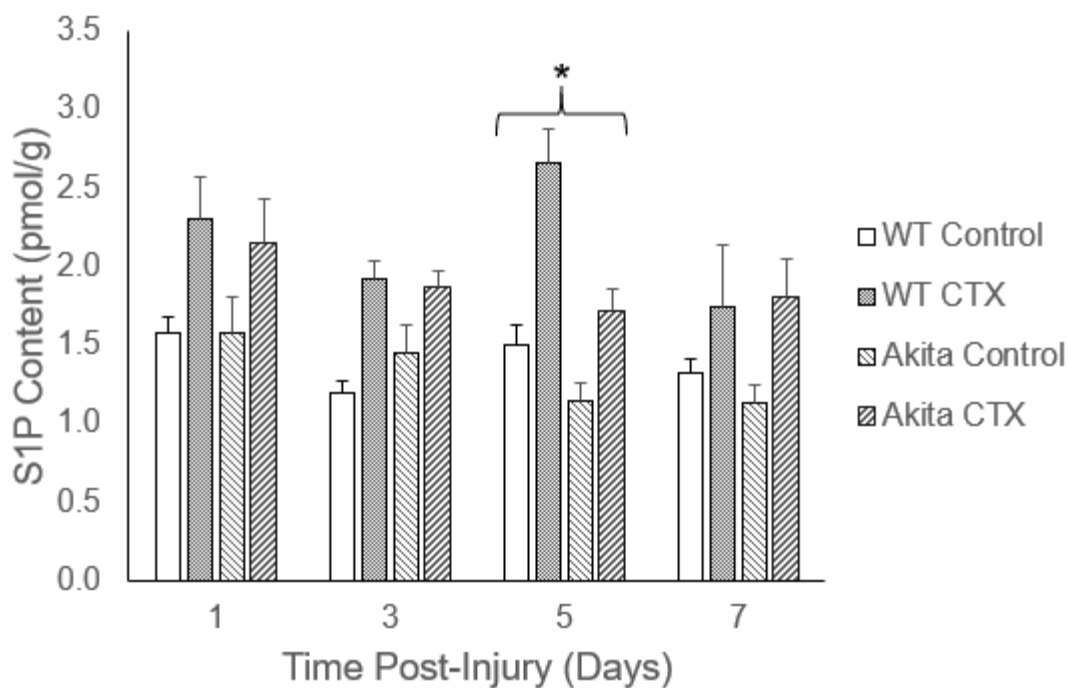


Figure 7. Skeletal muscle SIP content in the WT and Akita models at one (WT n=5, Akita n=5), three (WT n=6, Akita n=5), five (WT n=6, Akita n=5), & seven days (WT n=5, Akita n=5) post-CTX injection. * Indicates a statistically significant difference in skeletal muscle SIP content between the WT and Akita mice ($p < 0.05$).

4.3 SPK1 Expression & Activity

SDS-PAGE and Western Blot analysis was used to quantify skeletal muscle sphingosine kinase (SPK)1 and phospho-SPK1 content under both the control and CTX condition at all regeneration time points. However, the phospho-SPK1 antibody displayed strong non-specific binding that was not alleviated through troubleshooting efforts, such as utilizing different blocking solutions and concentrations. Therefore, the present study was unable to confidently identify phospho-SPK1 on the membrane, which prevented quantitative analysis. Thus, only results pertaining to total SPK1 content will be presented

in this section. Further details about SPK1 activity analysis can be found in the discussion section.

A three-way (2x2x4) multifactorial ANOVA was used to assess for changes in SPK1 content across the three experimental conditions: T1DM, muscle damage, and regeneration time point. Statistical analysis indicated a statistically significant effect of muscle damage ($p=0.001$), regeneration time point ($p<0.05$), and interaction between muscle damage x regeneration time point ($p<0.05$). However, the analysis did not find any effect for T1DM ($p=0.321$), or interaction between muscle damage x T1DM ($p=0.442$), regeneration time point x T1DM ($p=0.293$), or muscle damage x T1DM x regeneration time point ($p=0.235$). A post-hoc simple main effects analysis of the interaction between muscle damage x regeneration time point revealed that control and CTX muscle significantly differed in SPK1 content at one ($p=0.013$), five ($p<0.05$), seven ($p<0.05$), but not three ($p=0.563$) days post-damage inducing stimulus. Interpretation of these results would suggest that there is a statistically significant drop in total SPK1 content one day following a damage inducing stimulus in both WT and Akita skeletal muscle, but that this difference becomes negligible by three days following damage (*Figure 8*). After five days of muscle regeneration, there is a statistically significant increase of skeletal muscle SPK1 content for both WT and Akita mice, and that this increase is maintained to at least seven days following the damage inducing stimulus.

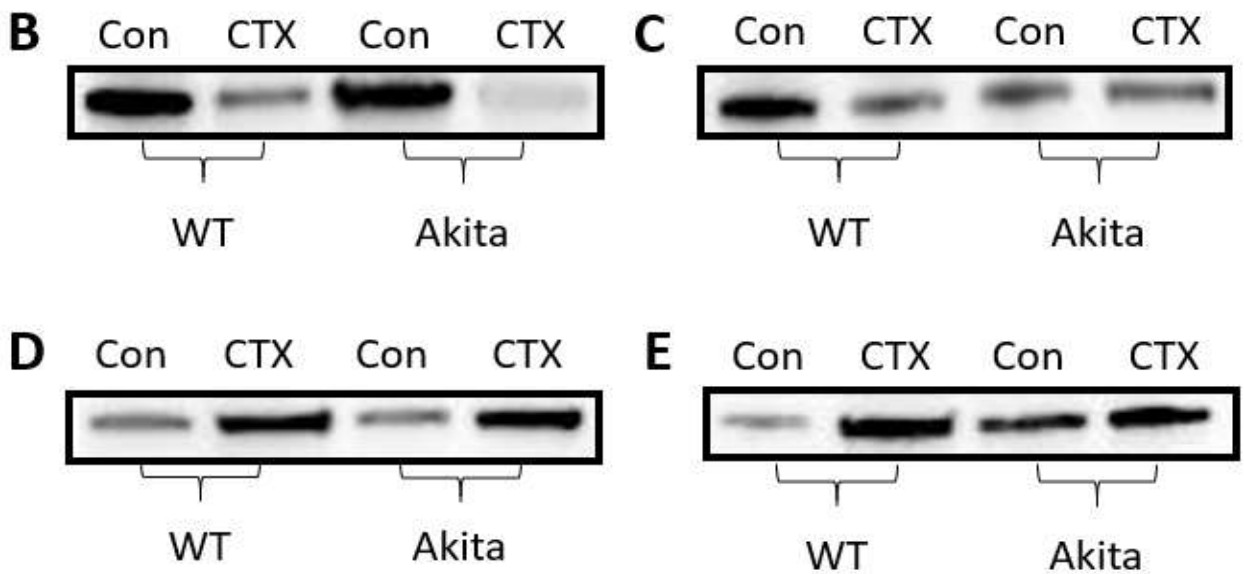
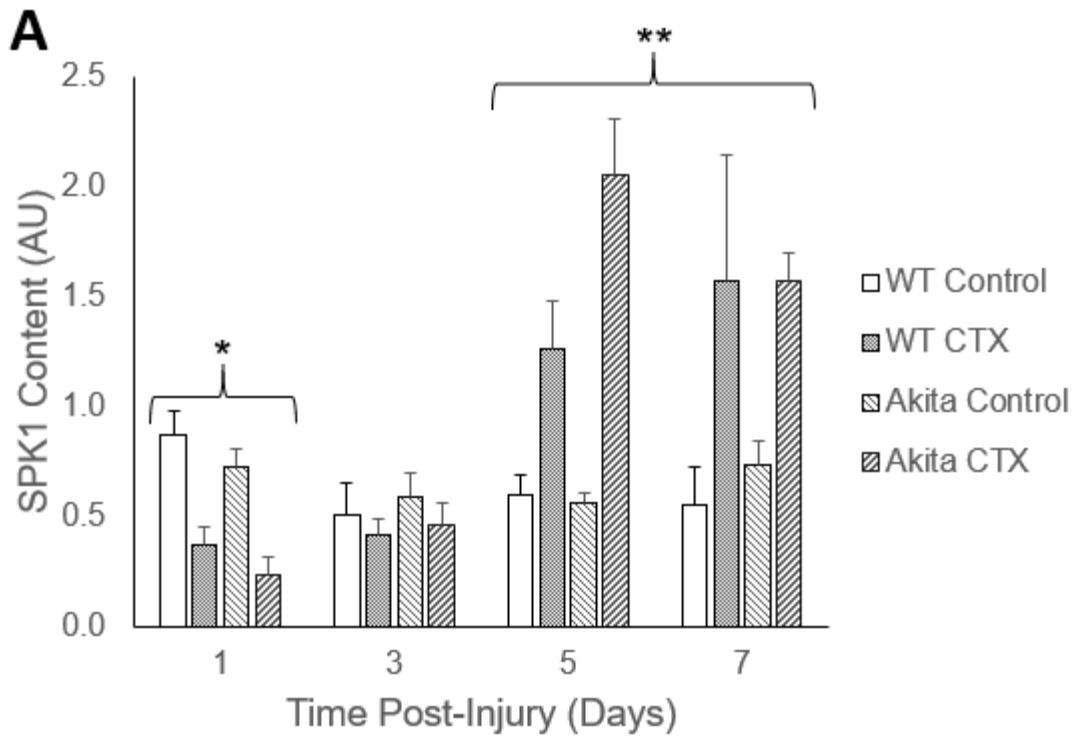


Figure 8. A) Skeletal muscle SPK1 content in both WT and Akita mice at one (WT n=5, Akita n=5), three (WT n=6, Akita n=5), five (WT n=6, Akita n=5), and seven (WT n=5, Akita n=5) days post-skeletal muscle damage. Data is presented in arbitrary units (AU). * Indicates significantly less SPK1 content in the CTX condition than control condition regardless of T1DM ($p < 0.05$). ** Indicates significantly more SPK1 content in the CTX condition than control condition, regardless of T1DM ($p < 0.05$). B) Western Blot analysis showing skeletal muscle SPK1 content one day, C) three days, D) five days, and E) seven days into the regenerative process.

4.4 SPL Expression

Skeletal muscle sphingosine lyase (SPL) content was quantified using the same Western Blot protocol, under both the control and CTX condition at all regeneration time points. A three-way (2x2x4) multifactorial ANOVA was used to assess for changes in SPL content across the three experimental conditions: T1DM, muscle damage, and regeneration time point. A statistically significant main effect was found for T1DM ($p=0.024$), muscle damage ($p<0.05$), and regeneration time point ($p<0.05$), indicating that SPL content differed based on these experimental conditions. Furthermore, there was a statistically significant interaction effect between T1DM x muscle damage ($p=0.036$), and muscle damage x regeneration time point ($p<0.05$), but not for T1DM x regeneration time point ($p=0.227$), nor for the three-way interaction between all conditions ($p=0.188$).

Two post-hoc simple main effects analyses were conducted to parse the T1DM x muscle damage and muscle damage x regeneration time point interactions. It was seen through the T1DM x muscle damage simple main effects analysis that no statistically significant differences existed between the WT and Akita mice with respect to skeletal muscle SPL content in the control condition ($p=0.903$). However, following muscle damage in the CTX condition, the Akita skeletal muscle displays significantly elevated SPL content when compared to the WT mice ($p=0.002$, *Figure 9*). Furthermore, the muscle damage x regeneration time point simple main effects analysis indicated that SPL content was significantly elevated in CTX muscle three ($p<0.05$), five ($p<0.05$), and seven ($p=0.002$) following the damage inducing stimulus. SPL levels did not differ between CTX and control muscle one day following damage ($p=0.807$). It can be suggested based on these results that SPL content was significantly upregulated in skeletal muscle tissue three

to seven days following muscle damage, which is consistent with previous literature (Saba & de la Garza-Roden, 2013). This upregulation happens regardless of T1DM, but according to the results of this study it would suggest the Akita mice display a larger SPL response to muscle damage than their WT counterparts. This difference is most pronounced three days post-damage during the peak expression of SPL in both models (*Figure 9*). While the difference in SPL content appears to lessen five and seven days post-damage, the Akita mice still display more SPL content. Therefore, it appears that the T1DM pathophysiological acts to enhance SPL expression above the normal SPL response to skeletal muscle damage three to seven days post-muscle damage.

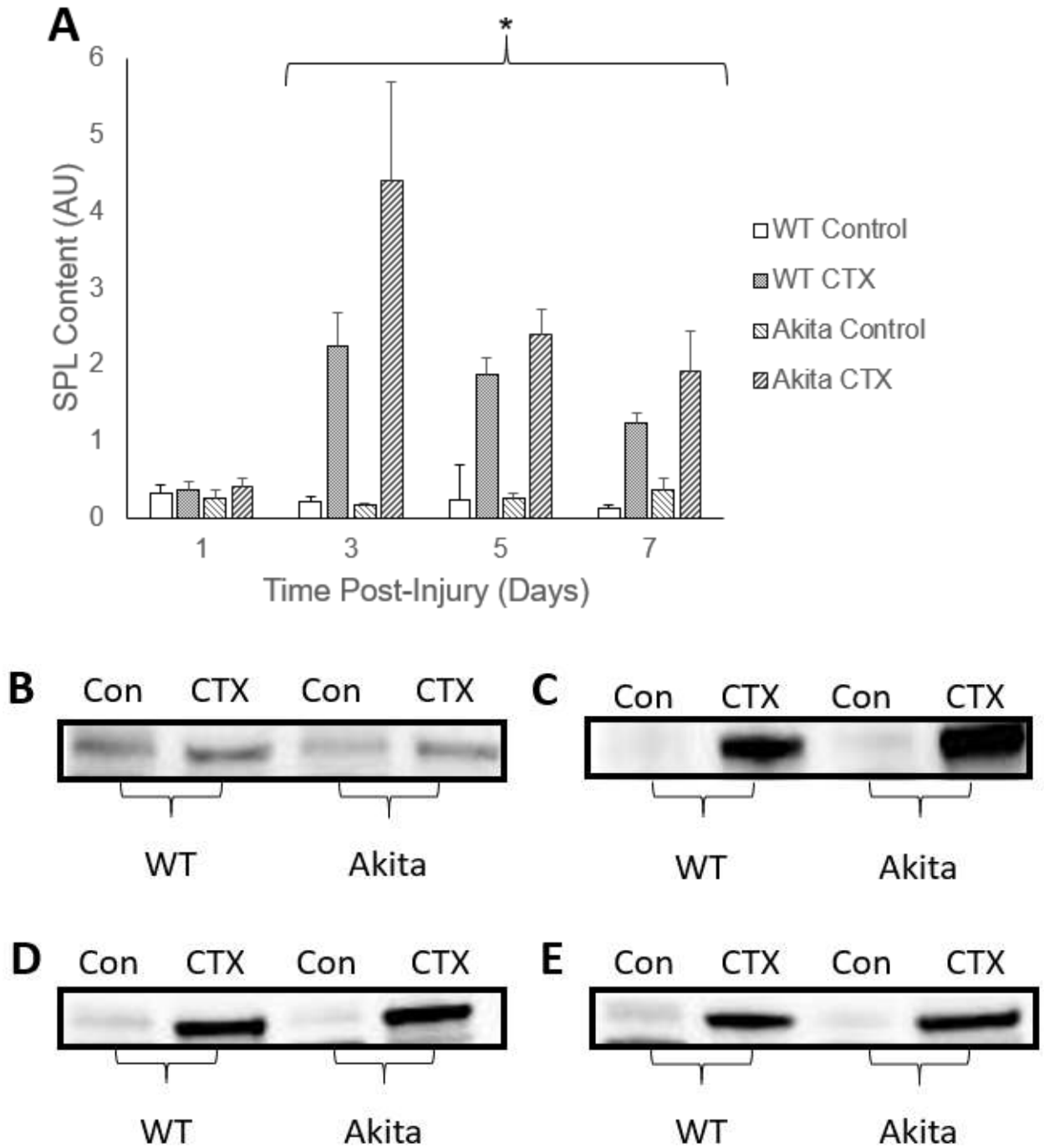


Figure 9. A) Skeletal muscle SPL content in both WT and Akita mice at one (WT n=5, Akita n=5), three (WT n=6, Akita n=5), five (WT n=6, Akita n=5), and seven days (WT n=5, Akita n=5) post-skeletal muscle damage. Data is presented in arbitrary units (AU). * Indicates significantly elevated SPL content in the CTX muscle compared to the control muscle, and Akita CTX muscle compared to WT control muscle ($p < 0.05$). Western Blot analysis revealing skeletal muscle SPL content B) one, C) three, D) five, and E) seven day(s) into the regenerative process.

5. Discussion

The purpose of this study was to examine differences between the Akita and Wild Type (WT) sphingolipid response to skeletal muscle damage. It was found that a key lipid mediator of skeletal muscle regeneration, sphingosine 1-phosphate (S1P), did not follow a regular pattern of accumulation in Akita skeletal muscle post-damage. The sphingolipid response in Akita skeletal muscle damage was shown to significantly diverge from the control mice five days post-damage, resulting in decreased skeletal muscle S1P content. This finding is consistent with pilot data, which found a blunted S1P response to skeletal muscle damage in Akita mice five days into regeneration (Mallender et al., unpublished results). Thus, this result lends support *Hypothesis #1* by showing the T1DM pathophysiological environment dysregulates the S1P response to skeletal muscle damage five days into the regenerative process, but not one, three, or seven days following damage.

The activity and expression of two S1P-regulating proteins, sphingosine kinase (SPK)1 and sphingosine lyase (SPL), were also assessed. Although limitations prevented the accurate assessment of SPK1 activity, SPK1 expression was successfully assessed. There were no statistically significant differences observed between the WT and Akita mice with respect to total SPK1 expression, as they both followed the same pattern of SPK1 expression following damage. However, since SPK1 activity was not successfully assessed, *Hypothesis #2* remains inconclusive.

With respect to the other S1P-regulating protein, it was observed that Akita mice overexpress SPL from three to seven days after skeletal muscle damage when compared to WT counterparts, which may contribute to the lower skeletal muscle S1P content in the Akita mice five days into regeneration. This finding provided support for *Hypothesis #3* as

it was shown that the T1DM pathophysiological environment-induced overexpression of skeletal muscle SPL from three to seven days following cardiotoxin (CTX)-induced muscle damage. Each of these primary themes, 1) changes in skeletal muscle S1P content; 2) overexpression of SPL in Akita model skeletal muscle; and 3) Temporal changes in SPK1 content, will be explored in greater depth below.

5.1 Changes in skeletal muscle S1P content

S1P has previously been shown to facilitate the regenerative process following skeletal muscle damage (Nagata et al., 2006; Danieli-Betto et al., 2009), and endogenous S1P production within skeletal muscle has been observed to increase following a damage inducing stimulus (Loh et al., 2012; Sassoli et al., 2011). The significant effect of muscle injury found in the present study further adds to this consensus in the literature, illustrating that skeletal muscle S1P content does increase following a damage inducing stimulus. Additionally, this increase in skeletal muscle S1P content was observed to occur in both a control and T1DM environment, and was present at all measured time points. It can be inferred from the results that the S1P response to skeletal muscle damage is dysregulated in the Akita mice, due to the significant effect of T1DM on skeletal muscle S1P content, and the finding of significantly less skeletal muscle S1P five days into regeneration. This is consistent with pilot data that originally highlighted lower skeletal muscle S1P content at five days of regeneration (Mallender et al., unpublished results). Therefore, these observations support the notion that a T1DM pathophysiological environment prevents a normal accumulation of skeletal muscle S1P following a damage inducing stimulus, and this may contribute to the impaired regeneration seen in the population.

The finding in the present study of negligible differences between WT and Akita mice with respect to skeletal muscle S1P content one and three days into regeneration was unexpected, as it was predicted S1P content would be reduced in the Akita models at these time points. Therefore, based on the results of this study it can be suggested that the dysregulation of skeletal muscle S1P content between the WT and Akita mice does not occur until after three days of regeneration. The skeletal muscle S1P gap between the WT and Akita mice lessens by 7 days post-damage, which follows the trend observed in pilot data, where the differences were negligible 10 days into regeneration (Mallender et al., unpublished results). However, what impact this delayed dysregulation would have on the ability of S1P to promote proper skeletal muscle regeneration is unclear.

The observed impaired S1P response to skeletal muscle damage in Akita mice should be further explored in future work. Foremost, the effect of lower skeletal muscle S1P content has on the effectiveness of skeletal muscle regeneration should be explored to determine the relevance of this dysregulation to the observed myopathy. This could be accomplished by artificially increasing skeletal muscle S1P to near WT amounts through exogenous S1P administration and observe if this alleviates previously noted impairments seen in T1DM skeletal muscle regeneration (Krause et al., 2011a; 2013). Further, once the cellular mechanism(s) for the impaired S1P response has been identified, future investigators could remedy the pathophysiological mechanism(s) via pharmacological intervention (or another modality) and observe the effects this has on skeletal muscle S1P content and thus regeneration in this population.

5.2 Overexpression of SPL in Akita model Skeletal Muscle

The regulatory effect of SPL on skeletal muscle S1P content has been well documented. The primary function of SPL is the irreversible degradation of S1P, thus a greater expression of skeletal muscle SPL is associated with decreased skeletal muscle S1P content (Loh et al., 2012; Ieronimakis et al., 2013; Saba & de la Garza-Roden, 2013). It has been reported in the literature that SPL expression is at its peak within skeletal muscle three to five days following a damage inducing stimulus (Loh et al., 2012; Saba & de la Garza-Roden, 2013). The results from the present study further support the literature suggesting SPL content is elevated in regenerating skeletal muscle, and shows this occurs in both the WT and Akita mice three, five, and seven day time points. It has been shown that SPL expression beyond what is expressed in a control model during regeneration can act to blunt the S1P response (Loh et al., 2012; Saba & de la Garza-Roden, 2013). Therefore, the nearly two-fold increase in SPL expression seen in the Akita skeletal muscle three days post-CTX injection, coupled with elevations in SPL expression that were sustained throughout the first week of regeneration could be contributing the reduced S1P content observed within the Akita skeletal muscle.

Interestingly, this pattern of SPL dysregulation does not follow the same patterns seen with skeletal muscle S1P content during regeneration. Specially, while it was identified in the present study that SPL content is upregulated in the Akita models from three to seven days of regeneration, the peak of this difference was observed three days into regeneration. This occurs despite no differences in S1P content between the WT and Akita models at three days into regeneration, suggesting that an acute overexpression of SPL is not enough to blunt S1P accumulation. Due to the overexpression of SPL throughout

the acute regenerative phase, it is possible that the cumulative effect of increased SPL expression across this acute time course in the Akita mice prevents the increase in S1P content seen in the WT mice by five days post-damage, and the non-significant reduction seen by seven days. However, it should be noted that S1P accumulation is dependent upon the activity of SPK1 and expression of SPL, thus without SPK1 activity data it is difficult to draw definitive conclusions. Therefore, these results lead to speculation that dysregulated SPL expression is a contributor to the depressed skeletal muscle S1P content in the Akita mice. The potential mechanisms for this overexpression will be further explored.

The depression of skeletal muscle S1P via SPL overexpression has been previously observed in the *mdx* model of Duchene Muscular Dystrophy (DMD) (Loh et al., 2012; Ieronimakis et al., 2013). However, based on the results from the present study would, key differences exist in the etiology of skeletal muscle S1P deficiency observed between the Akita model and *mdx* model. Mainly, it was noted that SPL dysregulation only occurred during regeneration in the Akita mice, as opposed to dysregulation being present in a basal state within the *mdx* model (Loh et al., 2012). This indicates that the trigger in the pathophysiological environment inducing this dysregulation of the sphingolipid synthesis pathway is only present after muscle damage in Akita mice and not in the basal state.

The post-damage-induced rise in SPL content is derived from two primary sources: i) activated satellite cells, and, ii) a variety of inflammatory cells, such as bone-marrow derived macrophages (Saba & de la Garza-Roden, 2013). However, the overexpression of SPL in the Akita skeletal muscle tissue acutely post-damage appears paradoxical when considering the effects of the T1DM pathophysiological environment on skeletal muscle

regeneration. The activation and infiltration of satellite cells, and the infiltration of various macrophage populations, is impaired in T1DM model skeletal muscle due to poor ECM remodeling (D'Souza et al., 2016; Krause et al., 2013). Coupled with the temporal trends of SPL expression, it appears unlikely that this increased expression of SPL is due to an overabundance of inflammatory cells secreting SPL as seen in *mdx* models; it is more likely that regenerative factors specific to the T1DM pathophysiological environment may be influencing this observed response. However, it should be noted that this impaired infiltration was observed five days post-damage (Krause et al., 2013), and that discrepancies between WT and Akita skeletal muscle morphology may be different during one to three days of regeneration, which could influence inflammation and subsequent SPL expression.

While there is a paucity of literature surrounding SPL transcription in damaged skeletal muscle tissue, SPL has been identified as a transcriptional target of platelet-derived growth factor (PDGF) (Chen, Delrow, Corrin, Fraizer, & Soriano, 2004), a growth factor involved in the skeletal muscle regeneration process (Husmann, Soulet, Gautron, Martelly, & Barritault, 1996; Sugg et al., 2017). Interestingly, PDGF has been found to be upregulated in the T1DM pathophysiological environment. Specifically, it has been noted that chronic hyperglycemia results in elevated circulating PDGF (Guillausseau et al., 1989; Harrison, Dunbar, & Neale, 1994), and this altered expression has direct effects on other diabetic complications such as diabetic neuropathy and wound healing (Thomson, McLennan, & Twigg, 2006). While PDGF and its role in T1DM skeletal muscle regeneration has yet to be examined, an overexpression of this growth factor early in the regenerative phase could induce the increased expression of SPL observed in Akita skeletal

muscle seen in the present study. Furthermore, it could potentially explain how this increased expression is occurring despite a reduction in cells capable of secreting SPL (D'Souza et al., 2016; Krause et al., 2013).

Based upon the findings from the present study and connections to previous literature, two major areas of focus are recommended for future research examining SPL in T1DM skeletal muscle tissue. First, it is important to determine how the increased expression of SPL in Akita skeletal muscle compared to WT skeletal muscle from three to seven days post-damage affects S1P content and skeletal muscle regeneration. This would explain if this overexpression is directly related to the impaired S1P accumulation observed in Akita skeletal muscle five days into regeneration. This could be accomplished via an inhibition experiment. For example, SPL function would be blocked by the administration of a pharmacological compound such as (tetrahydroxybutyl)imidazole (THI), an inhibitor of SPL activity (Loh et al., 2012; Ieronimakis et al., 2013), with the same muscle injury protocol performed in the present study. Examining the same outcome measures would allow for an accurate comparison between studies and delineation of the effect of SPL expression on Akita skeletal muscle S1P content and regeneration. Second, if the increased expression of SPL is determined to be influential to the impaired sphingolipid response, the cellular location of SPL and factors regulating its expression should be explored. Specifically, analysis of the potential altered PDGF expression and its role in diabetic skeletal muscle regeneration and SPL transcription would be recommended.

5.3 Temporal Changes in SPK1 Content

Previous literature has suggested that SPK1 activity increases in response to skeletal muscle damage (Loh et al., 2012; Sassoli et al., 2011). However, the effect of

muscle injury on overall SPK1 expression has been less definitive. One study indicated an upregulation of SPK mRNA following myotoxin-induced muscle damage three to five days into regeneration (Loh et al., 2012). In contrast, a study utilizing eccentric contraction to induce muscle damage found no change in SPK1 protein content following damage, although the elapsed regeneration time was not specified (Sassoli et al., 2011). Therefore, findings from the present study appear to be the first to show that SPK1 protein content increases five days following skeletal muscle damage, and that this increase is maintained until at least seven days post-damage. However, what regenerative processes could be driving this increased expression in skeletal muscle tissues is unclear. As SPK1 is ubiquitously expressed in the many cell types (Piston et al., 2011), the simple increase in infiltrating cells that occurs during the skeletal muscle regeneration process (i.e., satellite cells, inflammatory cells) may be responsible for the observed overall increase in SPK1 content, but further research into this topic is necessary.

The lack of significant effect or interaction for T1DM would suggest that this pattern of SPK1 expression is the same between the WT and Akita mice during the first week of skeletal muscle regeneration. Despite this lack of interaction, it is worth noting that skeletal muscle SPK1 content is greater in the Akita mice than the WT mice five days into muscle regeneration, and then subsequently returns to near WT levels seven days following muscle damage. However, due to the lack of difference in skeletal muscle SPK1 content at other regeneration time points between the two models, it appears that this difference was not large enough to elicit a statistical effect, and thus prohibits the use of a post-hoc test to determine if the difference between WT and Akita mice is significant five days into the regenerative process.

The observation of increased SPK1 content in the present study unfortunately cannot adequately contribute to an explanation of changing S1P dynamics in both WT and Akita skeletal muscle tissues without accurate data pertaining to its activation state. While one may speculate that total SPK1 would correlate with S1P production, findings from the present study indicate that this does not appear to be the case. This was highlighted by the observed drop in total SPK1 content one post-damage, while skeletal muscle S1P content increased in both the WT and Akita mice. Furthermore, the Akita mice had significantly less skeletal muscle S1P five days into regeneration, despite greater overall SPK1 expression. As it prevents accurate interpretation of SPK1 activity in the dysregulated S1P response to skeletal muscle damage in T1DM, the inability to confidently identify phospho-SPK1 expression via Western Blot analysis is a limitation for the present study. Therefore, it is suggested that future research utilize alternative methods of SPK1 activity analysis to avoid the experimental limitations that occurred within the present study.

Alternative experimental methods to evaluate SPK1 activity proposed have included a radio-labelled assay procedure, which has been considered the gold standard of SPK1 activity measurement (Pitman, Pham, & Piston, 2012), and has been used in many studies examining SPK1 activity (Sassoli et al., 2011; Loh et al., 2012; Nagata et al., 2006; Piston et al., 2003). Due to the lack of necessary equipment, this procedure was not an option for the present study. Another alternative experimental method to assess SPK1 activity would be immunohistochemistry (IHC) analysis with an SPK1 selective antibody. While techniques utilizing IHC to identify the SPK1 isoform have been well defined (Visentin, Reynolds, & Sabbadini, 2012), IHC does not appear to be a commonly used method to assess SPK1 activity due to the prevalence of the radio-labelled assay procedure

(Pitman et al., 2012). However, the strong relationship between SPK1 cellular location and activity would allow an investigator to quantify the relative density of SPK1 located centrally within and at the membrane of the cell via IHC. As SPK1 is only transported to the cellular membrane following activation via phosphorylation (Piston et al., 2011), a higher proportion of SPK1 at the membrane would indicate a greater overall rate of SPK1 activity. However, this method of SPK1 activity assessment has not been validated nor well defined, and thus if used, results should be interpreted with caution.

Despite the inability of the present study to elucidate differences in SPK1 activity between WT and Akita skeletal muscle following a damage inducing stimulus, there are a variety of characteristics within the T1DM pathophysiological environment that could blunt SPK1 activity. Four potential mechanisms with the strongest theoretical basis will be further explored below, including: impaired hepatocyte growth factor (HGF) infiltration and activation, reduced circulating levels of insulin-like growth factor (IGF)-1, insulin-mediated epidermal growth factor (EGF) activity, and enhanced protein phosphatase 2A (PP2A) activity. All of these proteins directly or indirectly affect the SPK1 phosphorylation state, and thus could potentially contribute to the impaired sphingolipid response to skeletal muscle damage seen in the Akita mice.

5.3.1 Impaired HGF infiltration and activation

HGF is found embedded within the ECM of skeletal muscle in a basal state and is released from this niche during the remodeling process that occurs following damage (Sisson et al., 2009; Tatsumi et al., 1998; Tatsumi & Allen, 2004). However, the release and activation of HGF is dependent on the plasminogen activator inhibitor (PAI)-1/urokinase plasminogen activator (uPA)/matrix metalloproteinase (MMP)-9 axis (Sisson

et al., 2009), which is notably impaired in T1DM (Krause et al., 2011a; Krause et al., 2013). Specifically, HGF release from the ECM and its activation requires active uPA in the skeletal muscle (Sission et al., 2009); without this uPA-mediated activation, regeneration is markedly impaired (Sission et al., 2009). PAI-1 inhibits uPA activity, which is notable as elevated levels of PAI-1 have been shown to be a common feature of the T1DM pathophysiological environment and impairs regeneration via uPA inhibition (Krause et al., 2011a;., 2013). Thus, the possibility exists that the elevated levels of PAI-1 seen in T1DM are preventing uPA mediated HGF release and activation from the ECM. This is relevant, as HGF induces its effect on satellite cell activation and migration via a downstream signalling cascade involving ERK1/2, an upstream regulator of SPK1 activity (Halevy & Cantley, 2004; Miller, Thaloor, Matteson, & Pavlath, 2000; Tatsumi et al., 1998; Tatsumi & Allen, 2004). Inhibition of SPK1 activity *in vitro* within endothelial cells blunted HGF-induced cell migration, suggesting that HGF exerts its cellular effects via a S1P-dependent mechanism (Duan et al., 2004). Therefore, it is likely that HGF binding to contributes to the S1P response to skeletal muscle damage via activating effects on SPK1. Thus, the possibility exists that the elevated levels of PAI-1 seen in T1DM are preventing uPA mediated HGF release and activation from the ECM. If this proves true, then reduced skeletal muscle HGF activity in T1DM could help explain the impaired S1P accumulation post-damage, as a lack of active HGF would likely coincide with reduced SPK1 activity via reduced ERK1/2 phosphorylation.

5.3.2 Decreased IGF-1 availability

IGF-1 is one of the most well studied and recognized growth factors in musculoskeletal physiology due to its role in the PI3K/Akt/mTOR pathway and muscle

protein synthesis (Rennie, 2004). However, there is evidence to indicate that IGF-1 may act, at least in part, through an S1P-dependent mechanism to promote skeletal muscle regeneration. A study conducted *in vitro* indicated that SPK1 was a downstream target of IGF-1 following satellite cell binding (El-Shewy et al., 2006; Bernacchioni et al., 2012), and that blocking SPK1 activity with a pharmacological inhibitor significantly blunted an IGF-1-induced increase in myogenin content (Bernacchioni et al., 2012). Notably, the T1DM pathophysiological environment appears to display a reduced basal expression of IGF-1 in both human (Moyer-Mileur, Slater, Jordan, & Murray 2008; Jehle, Jehle, Mohan, & Bohm, 1998) and rodent models (Bink, Zapf, & Froesch, 1989; Dehoux et al., 2004), even with insulin therapy (Scheiwiller et al., 1986). Therefore, it is conceivable that a lack of available IGF-1 following skeletal muscle damage may contribute to the blunted S1P accumulation observed in the present study. However, how skeletal muscle IGF-1 levels change in response to damage in a T1DM pathophysiological environment has yet to be explored, thus, it is difficult to interpret if IGF-1 levels are reduced following skeletal muscle damage in T1DM as they are in a basal state.

5.3.3 Insulin-mediated EGF activity

EGF has been implicated in the skeletal muscle regeneration process due to its ability to activate satellite cells from quiescence (Husmann et al., 1996; Nagata et al., 2014). The ability of EGF to activate satellite cells has been shown *in vitro* to be dependent upon ERK1/2-induced SPK1 phosphorylation and subsequent S1P production (Nagata et al., 2014). However, not only does EGF appear to activate satellite cells via an SPK1-dependent mechanism, but that the activation only occurred in the presence of insulin (Nagata et al., 2014). Therefore, the chronic hypoinsulinemia that is present in the T1DM

pathophysiological environment may prevent skeletal muscle EGF from exerting its influence on satellite cells during skeletal muscle regeneration and would subsequently result in reduced S1P content due to inadequate SPK1 activation. Thus, future work could explore the potential role of EGF in the aberrant sphingolipid signalling after skeletal muscle damage, and explore if this impaired response could be alleviated with exogenous insulin treatment.

5.3.4 Enhanced PP2A activity

SPK1 phosphorylation is induced in an ERK1/2-dependent manner, but its dephosphorylation is regulated by PP2A (Piston et al., 2011). This is notable, as PP2A expression appears to be tightly associated with insulin deprivation; insulin administration was shown to reduce skeletal muscle PP2A expression in healthy human subjects (Hojlund, Poulsen, Staehr, Brusgaard, & Beck-Nielsen, 2002). Furthermore, studies examining rodent models of T1DM have found that PP2A is overexpressed in cardiac tissue (Rastogi, Sentex, Elimban, Dhalla, & Netticadan, 2003), and hyperglycemia induces PP2A activity (Arora et al., 2014). Therefore, even if skeletal muscle damage persists in the ability to activate SPK1 in Akita skeletal muscle, the possibility exists that the potential overexpression and enhanced activity of PP2A in these T1DM models may prevent SPK1 activity from reaching a level necessary to elicit the full S1P response to skeletal muscle damage. Thus, future work could aim to explore how PP2A expression and activity is regulated in Akita skeletal muscle, and examine what impact it could have on SPK1 phosphorylation status.

Therefore, there are multiple indirect lines of evidence in the literature that suggest the T1DM pathophysiological environment could suppress S1P production by impinging

on SPK1 activity. However, it is important to reiterate that limitations in the present study prevented adequate assessment of SPK1 activity following skeletal muscle damage in the Akita models. Thus, implementing the discussed alternative methods of SPK1 activity measurement should be prioritized over experiments assessing the potential impact of the T1DM pathophysiological environment on SPK1 phosphorylation. If future work determines that SPK1 activity is indeed blunted by the T1DM pathophysiological environment and contributes to the impaired sphingolipid response to skeletal muscle injury in the Akita mice, then the discussed lines of inquiry should be investigated.

5.4 Alternative explanations for sphingolipid dysregulation

The experiments conducted in the present study focused on proteins that have been most commonly cited in the literature as regulators of sphingosine-1-phosphate content in skeletal muscle. Despite the evidence surrounding SPK1 and SPL regulation of skeletal muscle S1P, it is possible that other aspects of the sphingolipid synthesis pathway are dysregulated in a T1DM pathophysiological environment and are worthy of exploration. Specifically, it is worth mentioning the S1P phosphatases (SPP1 & SPP2), which function to dephosphorylate S1P into sphingosine (Fyrst & Saba, 2010; Johnson et al., 2003). However, at least in the context of the sphingolipid response to skeletal muscle damage, most view SPL as the primary protein responsible for breaking down S1P (Saba & de la Garza-Roden, 2013). Nevertheless, it would be inappropriate to completely discount the potential role of S1P phosphatases in this altered sphingolipid response to skeletal muscle damage, simply due to the limited amount of literature examining the topic. Therefore, future research should look to identify how the activity of the S1P phosphatases changes in response to skeletal muscle damage and the role they play in the sphingolipid response.

Once the role of the S1P phosphatases following skeletal muscle damage is well defined, an examination of how the T1DM pathophysiological environment could influence the activity of the S1P phosphatases post-damage.

6. Conclusion

The present study provided further evidence of a dysregulated S1P skeletal muscle damage in murine models of T1DM and identified an overexpression of SPL in the acute phase of regeneration as a potential contributor to this impaired response. It was also shown for the first time that SPK1 protein content increases during the acute phase of skeletal muscle regeneration, but how T1DM affects the activity of SPK1 during regeneration remains unknown. Therefore, while the results from the present study provide important new insights to the literature, they also highlight the need for future research to further define this phenomenon and delineate its overall impact on skeletal muscle health/regeneration in a T1DM population.

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APPENDICES

Appendix A: Liquid Chromatography-Mass Spectrometry

Purpose: To quantify sphingolipid content within all skeletal muscle samples.

1. The proximal portion of murine quadriceps muscle samples were sent to the Genome BC Proteomics Centre for analysis.
2. Muscle samples were weighed out to 2 ml homogenizing tubes.
3. Water containing 0.01% TFA at 2 μ l per mg tissue and two 4-mm metal balls were added to each tube.
4. After the tubes were capped, the samples were homogenized on a MM 400 mill mixer at a shaking frequency of 25 Hz for 1 minute three times.
5. The samples were placed on ice to cool down for one minute between each homogenization cycle.
6. After a 3 second spin down, a mixed solvent of methanol-chloroform (5:2, v/v) at 8 μ l per mg of tissue was added to each tube, followed by homogenizing the tubes again for one minute three times using the same setup.
7. The samples were then ultra-sonicated in an ice-water bath for five minutes to further extract the lipids.
8. The solutions were clarified by centrifugation of the sample tubes to precipitate proteins in an Eppendorf 5420 R centrifuge for 15 minutes at 21,000 x g and 10 oC.
9. 100ul of the supernatant was mixed with 50 μ l of an internal standard solution containing four 17-carbon number sphingolipids (not detected in the samples as tested) prepared in water-methanol-chloroform (1:4:1).
10. 7.5 μ L of the clear supernatants was injected to measure the detected sphingolipids by LC-MS.
11. Sample analyses was carried out on a Waters UPLC system coupled to a 4000 QTRAP mass spectrometer operated in the multiple-reaction monitoring (MRM) mode with positive-ion detection.
12. Chromatographic separation was performed on a C8 UPLC column (2.1 x 50mm, 1.7 μ m) and with 0.1% formic acid in water and mixed acetonitrile-isopropanol as the mobile phase for binary-solvent gradient elution.
13. The ion transitions for the MRM detection for each sphingolipid were optimized by direction infusion of their standard solutions, with two ion transitions per compound for UPLC-MRM/MS runs.
14. Serially diluted standard solutions of sphingolipids, which were individually mixed with the internal standard solution at 2:1 (v/v) were injected to prepare the linear calibration curves with internal standard calibration for calculating the concentrations of the sphingolipids detected in the mouse muscle samples.
15. The concentrations of each sphingolipid in the standard solutions ranged from 0.6 pmol/ml to 10 nmol/ml.

Appendix B: Sample Homogenization

Purpose: To prepare skeletal muscle samples for SDS-PAGE and Western Blot analysis.

1. The distal portion of all quadriceps muscle samples were placed into a 2 ml homogenization tube and suspended in homogenization buffer at a 1:1500 ratio (1g :1500 μ l) with four plastic beads.
2. Samples were homogenized with the Omni Bead Rupter 12 (Omni International, Kennesaw, GA) for three homogenization cycles of 20s at 6.0 m/s and were placed on ice for 20s between cycles.
3. Homogenized muscle samples underwent centrifugation at 4500G for 30 minutes in a temperature-controlled centrifuge at 4°C (Eppendorf, Hamburg, Germany).
4. Supernatant was removed and placed into 200 μ l aliquots which were subsequently stored at -80°C.

Appendix C: Bradford Protein Assay

Purpose: To identify protein content of all skeletal muscle samples.

1. Each well of a Flat bottom, 96 well tissue culture plate (Falcon, Teksbury, MA) was loaded with 200 μ l of Ready-To-Use Bradford Reagent (Alfa Aesar, Haverhill, MA)
2. 10 mg/ml Bovine Serum Albumin (BSA) solution was used to create a protein standard curve.
3. BSA and muscle samples were added to the respective lanes in triplicate with ddH₂O as described in the loading scheme below.
4. The 96 well tissue culture plate was read with a Synergy HT multi-mode microplate reader (BioTek, Winooski, VT).
5. Absorbance values were recorded at 595 nm and transferred to Microsoft Excel.
6. A standard curve was created and protein concentrations for each sample were obtained.

| Standard Lane* | | | Muscle Samples** | | | | | | | | |
|----------------|------|------|------------------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 0+20 | 0+20 | 0+20 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 |
| 1+19 | 1+19 | 1+19 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 |
| 2+18 | 2+18 | 2+18 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 |
| 4+16 | 4+16 | 4+16 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 |
| 8+12 | 8+12 | 8+12 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 |
| 16+4 | 16+4 | 16+4 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 |
| 20+0 | 20+0 | 20+0 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 |
| | | | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 | 10+10 |

* DDH₂O + 10 mg/ml BSA (μ l)

** DDH₂O + muscle sample (μ l)

All samples were measured in triplicate

Appendix D: SDS-PAGE and Western Blot

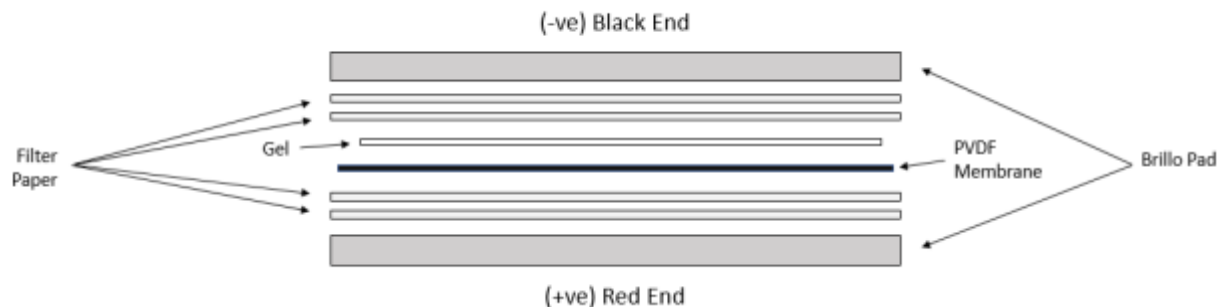
Purpose: To identify and quantify specific proteins of interest within skeletal muscle samples.

Gel Electrophoresis

1. Muscle homogenates were mixed with 6x laemmli buffer (Alfa Aesar, Haverhill, MA) in a 5:1 ratio and boiled at 95-100⁰ C for 5 minutes.
2. Mini-Protean TGX Precast Gels (4-20%) (Bio-Rad, Hercules, CA) were used to assemble a cassette in a Bio-Rad Mini-Protean Vertical Electrophoresis Chamber (Bio-Rad, Hercules, CA).
3. The chamber was filled with 1x running buffer.
4. 10 μ l of Precision Plus Protein Standard (Bio-Rad, Hercules, CA) was loaded into the first lane of the gel.
5. Lanes 2-10 were loaded with approximately 20 μ g of protein sample as determined through the Bradford assay.
6. The second lane of every gel was loaded with an identical loading standard.
7. Lanes three through ten were each loaded with muscle samples of interest for the analysis.
8. Gels were run at 200 mV with the Bio-Rad Power Pack Basic system (Bio-Rad, Hercules, CA), until samples reached the reference line at the bottom of the gel (approx. 30 minutes).

Transfer to PVDF Membrane

1. PVDF membrane was cut to the same size as the gel.
2. PVDF membranes were activated in methanol for two minutes prior to sandwich assembly.
3. Filter paper was cut to be slightly larger than the gel.
4. Brillo pads, filter paper, and PVDF membrane were pre-soaked in chilled 1x transfer buffer.
5. Mini-Protean TGX Precast Gels (4-20%) were open and a sandwich was created using the Bio-Rad Mini Trans-Blot Cell (Bio-Rad, Hercules, CA) as seen below:



6. The sandwich was placed within the Bio-Rad Mini Trans-Blot Cell, which was filled to the brim with pre-chilled 1x transfer buffer and connected to the Bio-Rad Power Pack Basic system. An ice pack was placed inside the cell to provide extra cooling throughout the transfer period.

7. Transfer was run at 100mV for 60 minutes. Ice packs were changed every 20 minutes to ensure the transfer buffer remained chilled.
8. Following the completion of the transfer, the sandwich was de-assembled and the PVDF membrane was suspended in a 1x Tris-Buffered Saline – Tween (TBST) solution and stored at 4°C until further use.

Ponceau Stain

1. Membranes were suspended in 10ml of Ponceau solution for 15 minutes on a gyrating platform.
2. Stained membranes were washed three times with ddH₂O for five minutes to reduce background.
3. Ponceau images were acquired using the Bio-Rad Chemi-Doc Imaging system (Bio-Rad, Hercules, CA).
4. Ponceau solution was removed by multiple washes in TBST on a gyrating platform until all signs of the Ponceau stain were removed.
5. Membranes were re-activated in methanol for one minute and re-washed in TBST.
6. Membranes were stored at 4°C in 1x TBST until further use.

Block and Incubate Membrane

1. Membranes were blocked with 10 ml of blocking solution at room temperature for 1 hour followed by three, 5 minute washes with 1x TBST.
2. Membranes were incubated with primary antibody solution for one hour at room temperature, followed by three, five minute washes with 1x TBST.
3. Membranes were incubated with secondary antibody solution (Cat.# 7074P2, Cell Signalling Technology, Danvers, MA) for one hour at room temperature, followed by three, five minute washes with 1x TBST.
4. Membranes were stored at 4°C until further use.

Chemiluminescent Imaging and Analysis

1. 1 ml of Amersham ECL Prime Western Blotting detection agent solutions A & B (GE Healthcare, Chicago, IL) were mixed in a 2 ml Eppendorf tube.
2. 2ml of the ECL solution was pipetted onto the membrane and exposed to light for one minute prior to imaging.
3. Chemiluminescent analysis was conducted using the Alpha Innotech Flurochem IS-8900 (Azure Biosystems, Dublin, CA). Images were captured prior to the point of band saturation.
4. Bands were quantified using the Bio-Rad ImageLab software (Version 6.0).
5. All bands were standardized to the loading standard and total protein acquired from the Ponceau Stain was used to confirm equal loading between lanes (Romero-Calvo et al., 2010).

Appendix E: List of Lab Prepared SDS-PAGE and Western Blot Buffers and Solutions

| Name | Components/Concentrations | Example Amounts | Storage |
|-----------------------------|---|---|--------------------------------------|
| 1x Homogenization Buffer | 150mM NaCl 50 mM Tris Base pH 8.0 | 8.76 g – NaCl 6.06 g – Tris Top up to 1 L DD H ₂ O | Room Temperature (4°C for use) |
| 10x Running Buffer | 25 mM Tris Base 190 mM Glycine 0.1% SDS pH 8.3 | 30.28 g – Tris Base 142.6 g – Glycine 10 ml – 10% SDS Top up to 1 L DD H ₂ O | Room Temperature |
| 1x Running Buffer | 10x Running Buffer – 10% (v/v) | 100 ml 10X Running Buffer Top up to 1 L DD H ₂ O | Room Temperature |
| 10x Transfer Buffer | 25 mM Tris Base 190 mM Glycine pH 8.3 | 30.28 g – Tris Base 142.6 g – Glycine Top up to 1 L DD H ₂ O | Room Temperature |
| 1x Transfer Buffer | 10x Transfer Buffer – 10% (v/v) | 100 ml 10X Transfer Buffer Top up to 1 L DD H ₂ O | Room Temperature (4°C for use) |
| 10x TBS | 15mM Tris-HCL 150mMol NaCL pH 7.6 | 24 g – Tris HCL 88 g- NaCl Top up to 1 L DD H ₂ O | Room Temperature |
| 1x TBST | 10X TBS- 10% (v/v) 0.1% Tween 20 (v/v) | 100 ml – 10X TBS 1 ml – Tween 20 Top up to 1 L DD H ₂ O | Room Temperature |
| Primary Antibody Solution | 1:1000 Antibody against protein of interest:Blocking solution | 10 ml - Blocking Solution 10 µl - Primary antibody | N/A |
| Secondary Antibody Solution | 1:1000 Antibody against Primary Antibody:Blocking solution | 10 ml - Blocking Solution 10 µl - Secondary antibody | N/A |
| Blocking Solution | Bovine Serum Albumin 5% (w/v) 1x TBST 95% | 25 g – Bovine Serum Albumin Top up to 500 ml 1x TBST | Room Temperature |

| | | | |
|------------------|--|--|---------------------|
| SDS Solution | 10% SDS (w/v) | 10 g SDS Top up to 1 L DD H ₂ O | Room Temperature |
| Ponceau Solution | Ponceau S 0.1% (w/v) Acetic Acid 1% (v/v) | 0.5 g- Ponceau S 2.5 ml – Acetic Acid Top up to 50 ml DD H ₂ O | 4°C |

Appendix F: Western Blot and Ponceau Stain Images

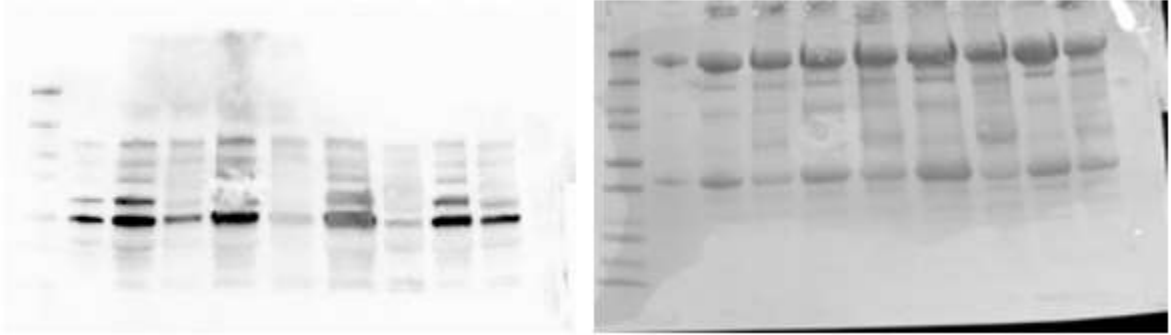
Interpretation Key:

- Left image: Chemiluminescent Western Blot Image
- Right image: Ponceau Stain (Total Protein Content)
- Grid legend:
 - W= Wild Type, A= Akita
 - Number= Regeneration time point (one day, three days, five days, or seven days)
 - C= Control (no muscle damage), X= CTX injection (muscle damage)
 - Ex. W1-X = Wild Type, 1 day since CTX injection, muscle sample received a CTX injection
 - Ex. A7-C = Akita, 7 days since CTX injection, muscle sample did not receive a CTX injection

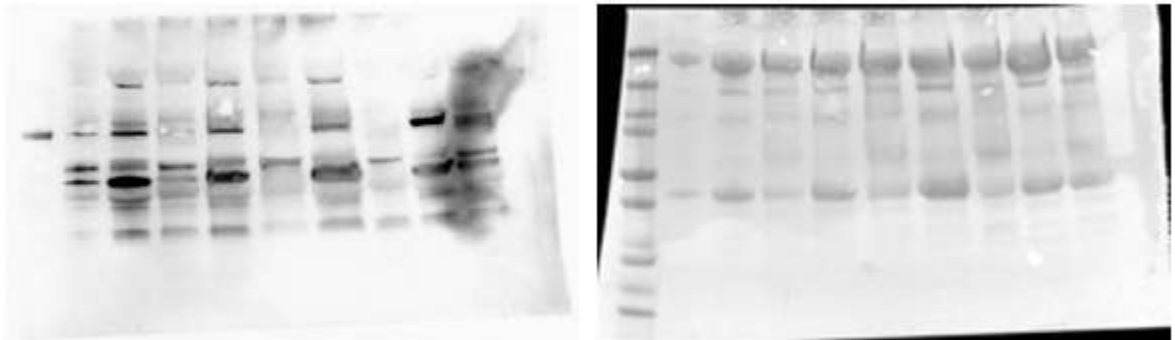
Blot 1

| | | | | | | | | | |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| <u>Lane 1</u> | <u>Lane 2</u> | <u>Lane 3</u> | <u>Lane 4</u> | <u>Lane 5</u> | <u>Lane 6</u> | <u>Lane 7</u> | <u>Lane 8</u> | <u>Lane 9</u> | <u>Lane 10</u> |
| W1-C | W1-X | A1-C | A1-X | W1-C | A1-X | A1-C | W1-X | W1-C | W1-X |

SPK



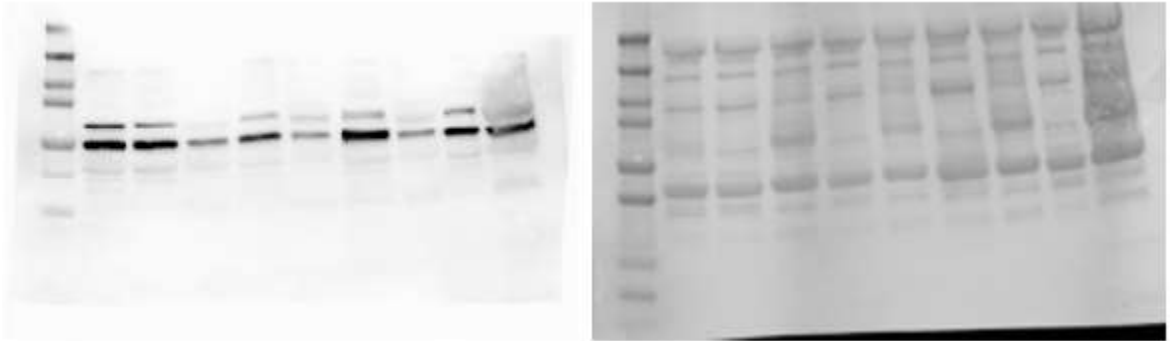
SPL



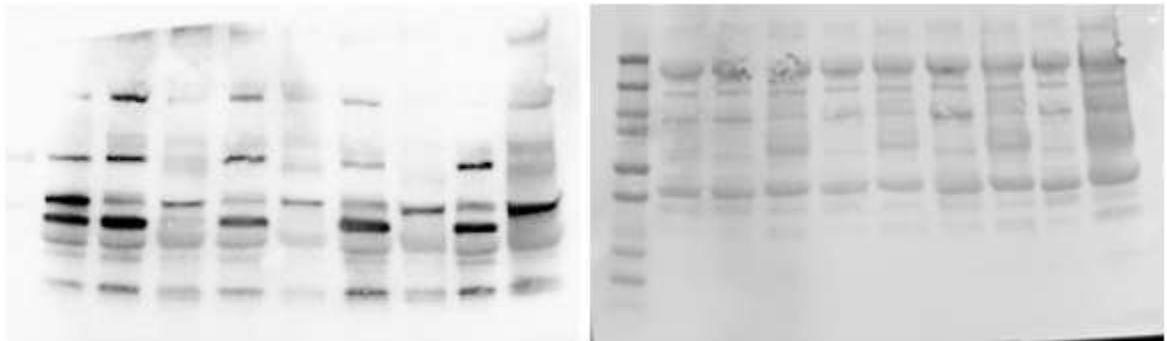
Blot 2

| | | | | | | | | | |
|-------------------------|---------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|------------------------|
| <u>Lane 1</u> Ladder | <u>Lane 2</u> LS | <u>Lane 3</u> W1-C | <u>Lane 4</u> W1-X | <u>Lane 5</u> A1-C | <u>Lane 6</u> A1-X | <u>Lane 7</u> W1-C | <u>Lane 8</u> W1-X | <u>Lane 9</u> A1-C | <u>Lane 10</u> A1-X |
|-------------------------|---------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|------------------------|

SPK



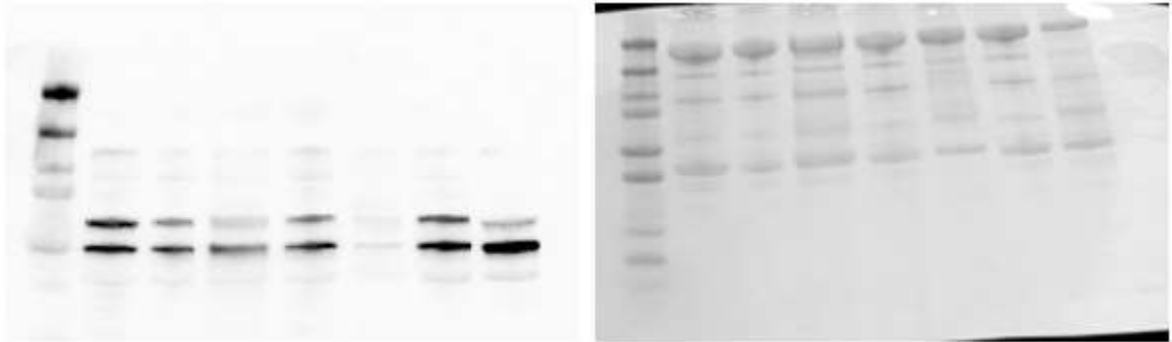
SPL



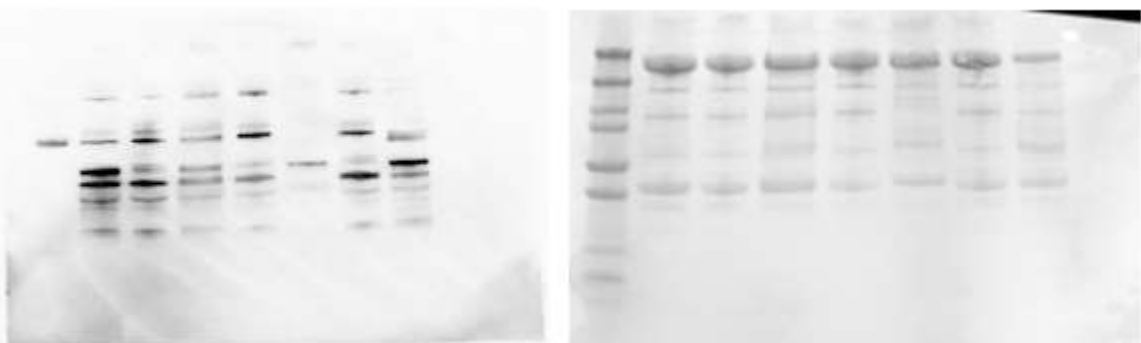
Blot 3

| | | | | | | | | | |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| <u>Lane 1</u> | <u>Lane 2</u> | <u>Lane 3</u> | <u>Lane 4</u> | <u>Lane 5</u> | <u>Lane 6</u> | <u>Lane 7</u> | <u>Lane 8</u> | <u>Lane 9</u> | <u>Lane 10</u> |
| Ladder | LS | W1-C | W1-X | A1-C | A1-X | W7-C | W7-X | Empty | Empty |

SPK



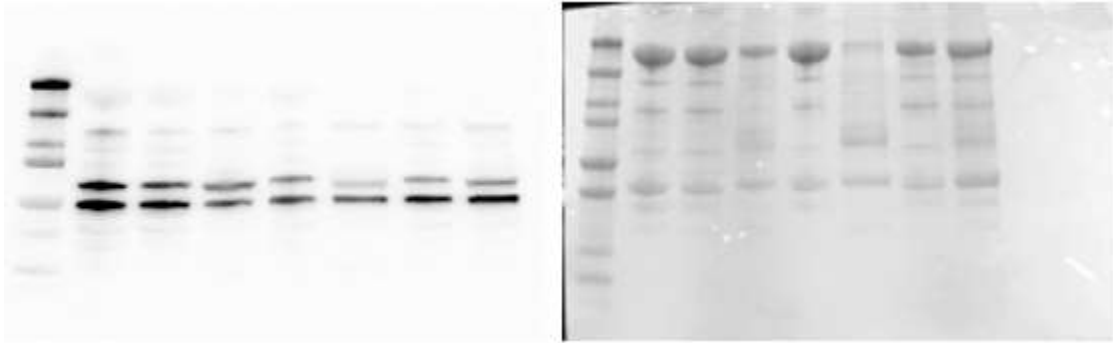
SPL



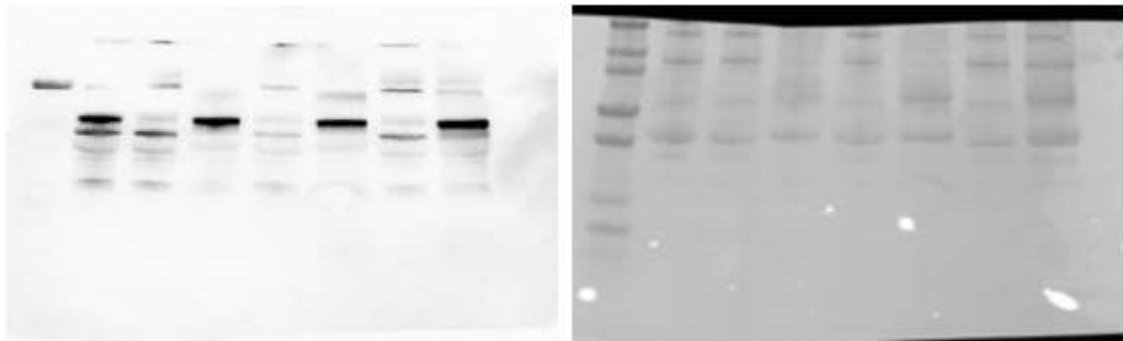
Blot 4

| | | | | | | | | | |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| <u>Lane 1</u> | <u>Lane 2</u> | <u>Lane 3</u> | <u>Lane 4</u> | <u>Lane 5</u> | <u>Lane 6</u> | <u>Lane 7</u> | <u>Lane 8</u> | <u>Lane 9</u> | <u>Lane 10</u> |
| Ladder | LS | W3-C | W3-X | A3-C | A3-X | W3- C | W3- X | Empty | Empty |

SPK



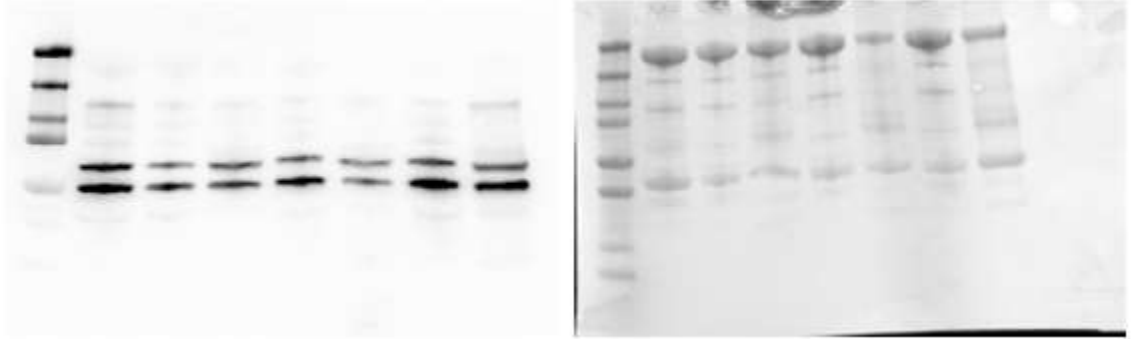
SPL



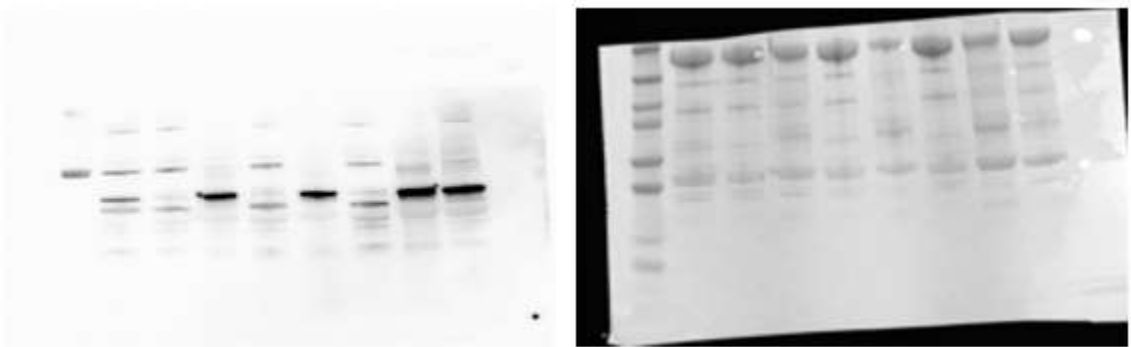
Blot 5

| | | | | | | | | | |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| <u>Lane 1</u> | <u>Lane 2</u> | <u>Lane 3</u> | <u>Lane 4</u> | <u>Lane 5</u> | <u>Lane 6</u> | <u>Lane 7</u> | <u>Lane 8</u> | <u>Lane 9</u> | <u>Lane 10</u> |
| Ladder | LS | A3-C | A3-X | W3-C | W3-X | A3-C | A3-X | Empty* | Empty |

SPK



SPL

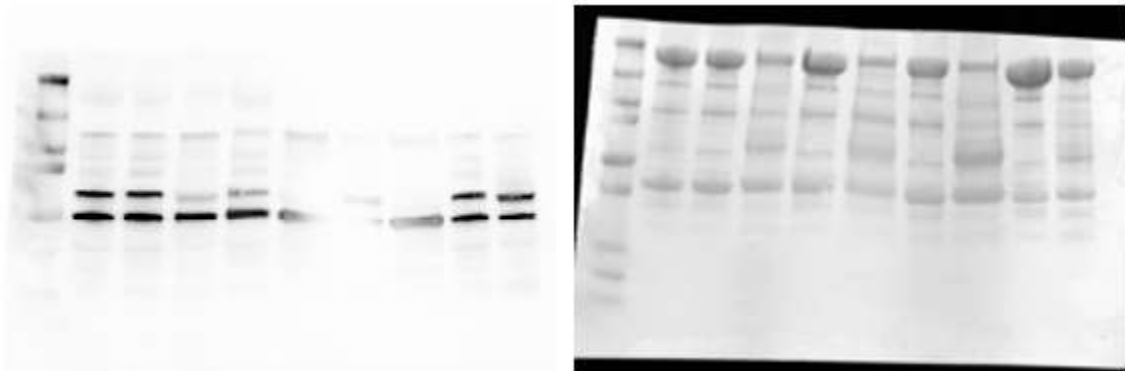


* Ignore lane 9, performed for trouble shooting reasons and not included in analysis.

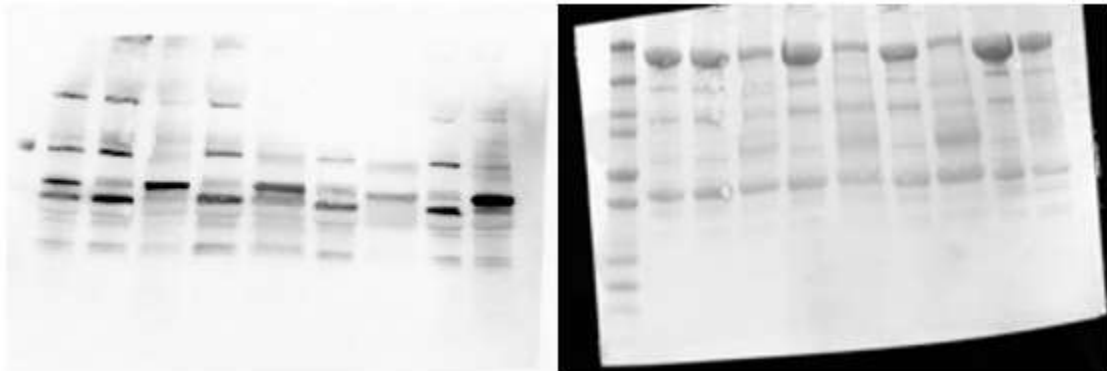
Blot 6

| <u>Lane 1</u> | <u>Lane 2</u> | <u>Lane 3</u> | <u>Lane 4</u> | <u>Lane 5</u> | <u>Lane 6</u> | <u>Lane 7</u> | <u>Lane 8</u> | <u>Lane 9</u> | <u>Lane 10</u> |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| Ladder | LS | W3-C | W3-X | A3-C | A3-X | W3- C | W3- X | A3-C | A3-X |

SPK



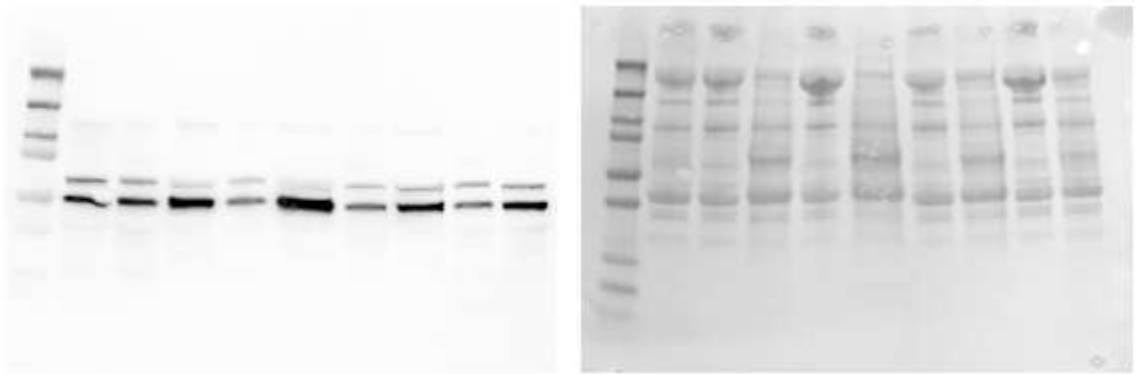
SPL



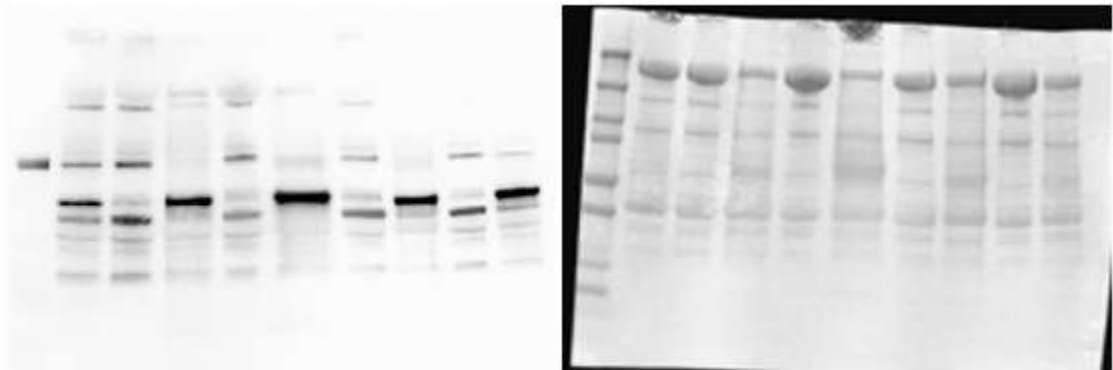
Blot 7

| <u>Lane 1</u> | <u>Lane 2</u> | <u>Lane 3</u> | <u>Lane 4</u> | <u>Lane 5</u> | <u>Lane 6</u> | <u>Lane 7</u> | <u>Lane 8</u> | <u>Lane 9</u> | <u>Lane 10</u> |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| Ladder | LS | W5-C | W5-X | A5-C | A5-X | W5-C | W5-X | A5-C | A5-X |

SPK



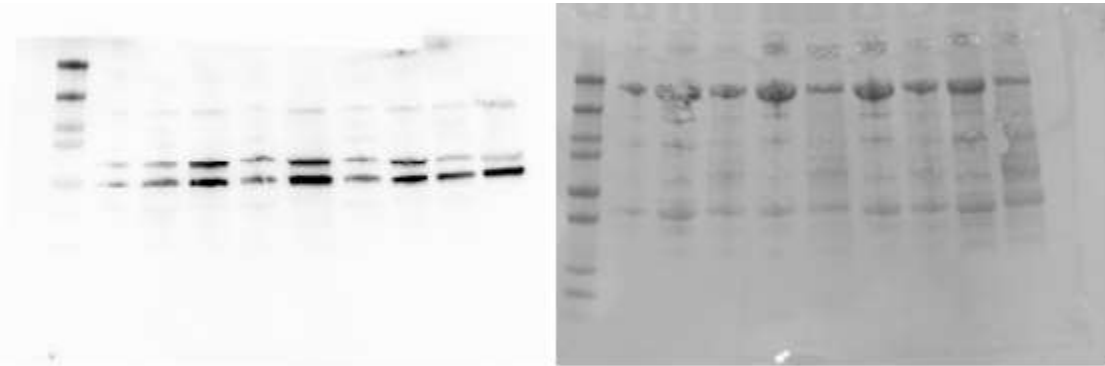
SPL



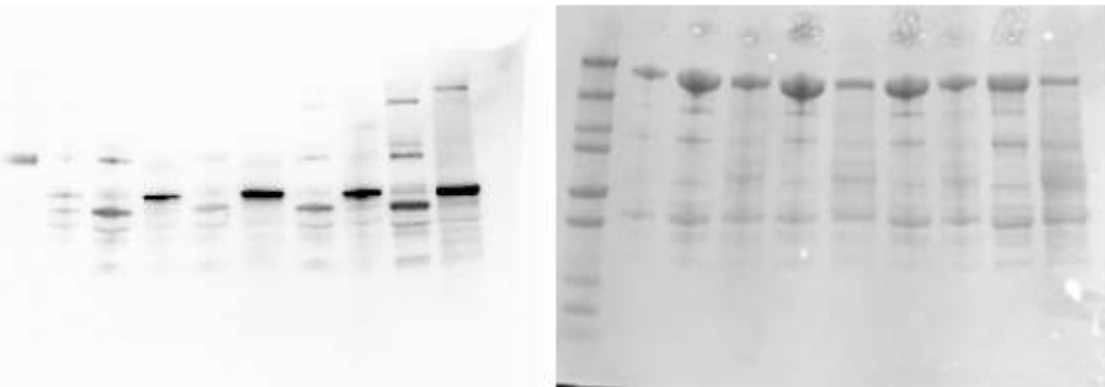
Blot 8

| <u>Lane 1</u> | <u>Lane 2</u> | <u>Lane 3</u> | <u>Lane 4</u> | <u>Lane 5</u> | <u>Lane 6</u> | <u>Lane 7</u> | <u>Lane 8</u> | <u>Lane 9</u> | <u>Lane 10</u> |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| Ladder | LS | W5-C | W5-X | A5-C | A5-X | W5-C | W5-X | A5-C | A5-X |

SPK



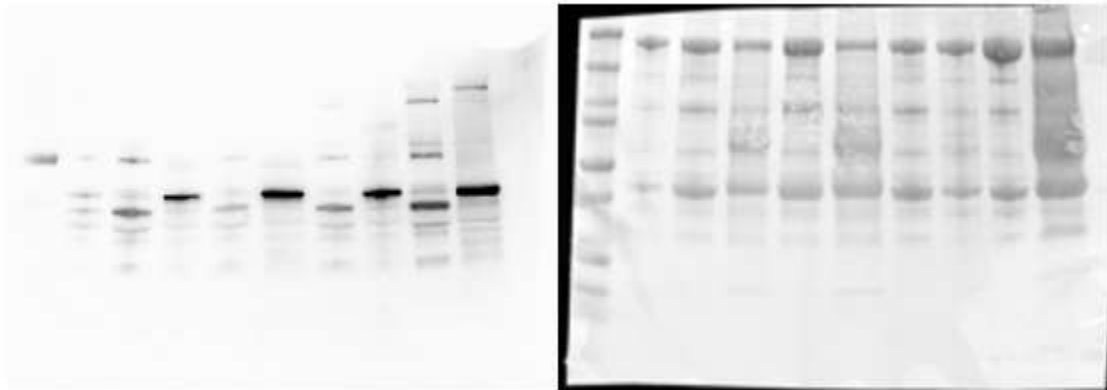
SPL



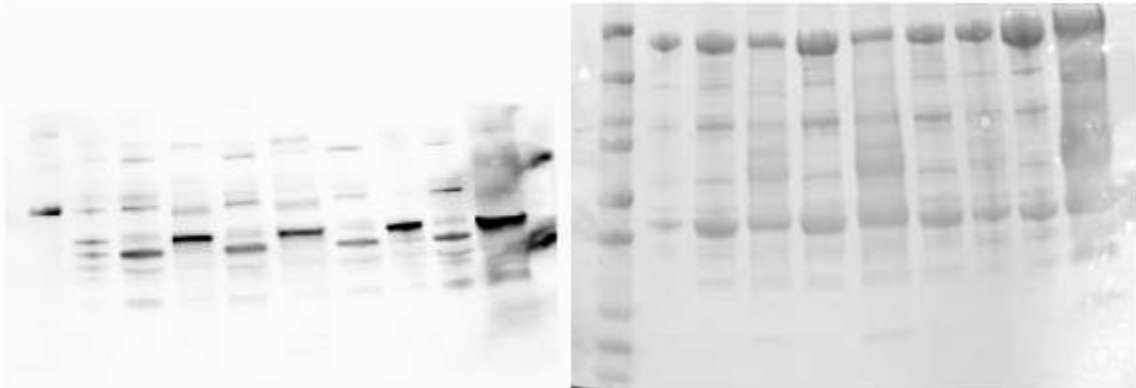
Blot 9

| <u>Lane 1</u> | <u>Lane 2</u> | <u>Lane 3</u> | <u>Lane 4</u> | <u>Lane 5</u> | <u>Lane 6</u> | <u>Lane 7</u> | <u>Lane 8</u> | <u>Lane 9</u> | <u>Lane 10</u> |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| Ladder | LS | W5-C | W5-X | A5-C | A5-X | W5-C | W5-X | W3-C | W3-X |

SPK



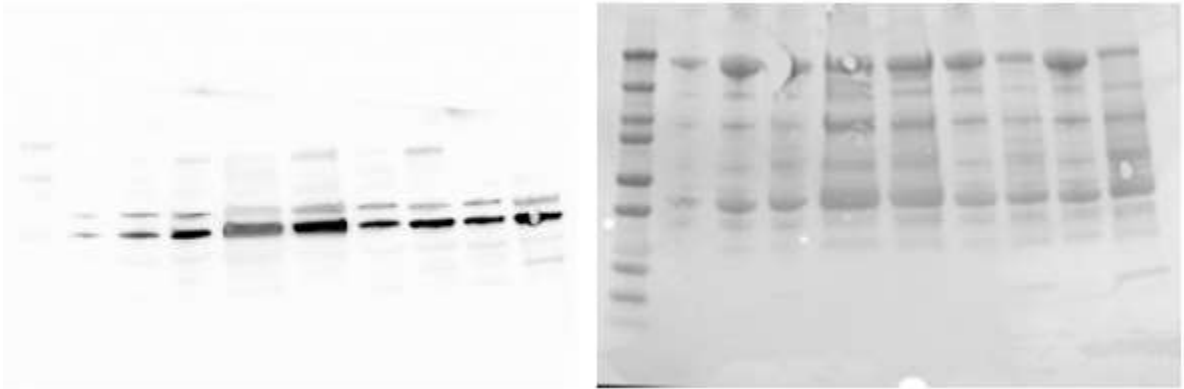
SPL



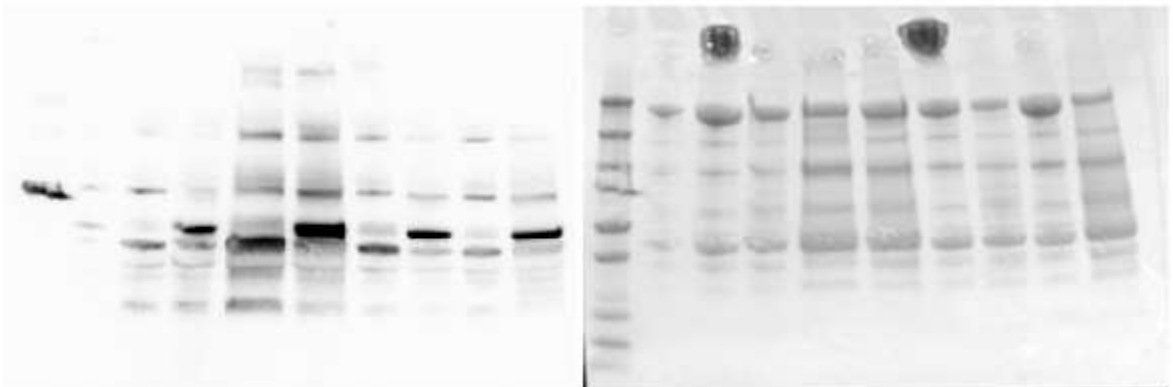
Blot 10

| <u>Lane 1</u> | <u>Lane 2</u> | <u>Lane 3</u> | <u>Lane 4</u> | <u>Lane 5</u> | <u>Lane 6</u> | <u>Lane 7</u> | <u>Lane 8</u> | <u>Lane 9</u> | <u>Lane 10</u> |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| Ladder | LS | W7-C | W7-X | A7-C | A7-X | W7-C | W7-X | A7-C | A7-X |

SPK



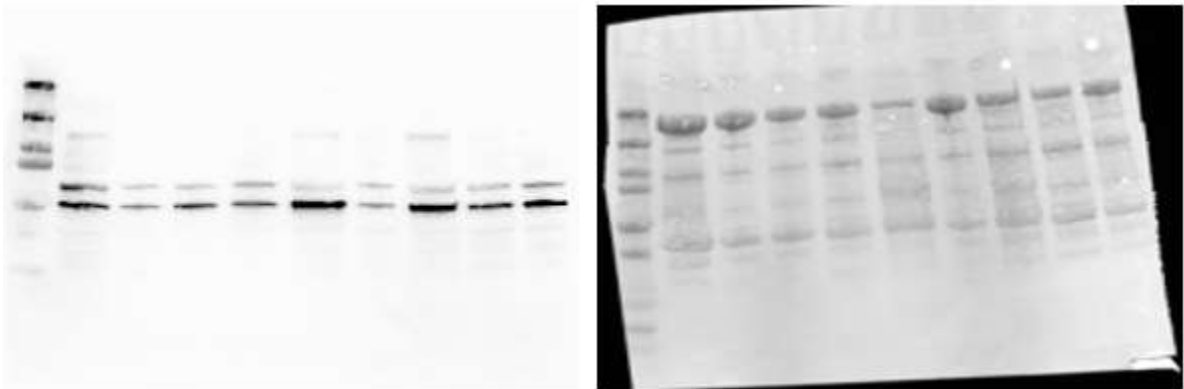
SPL



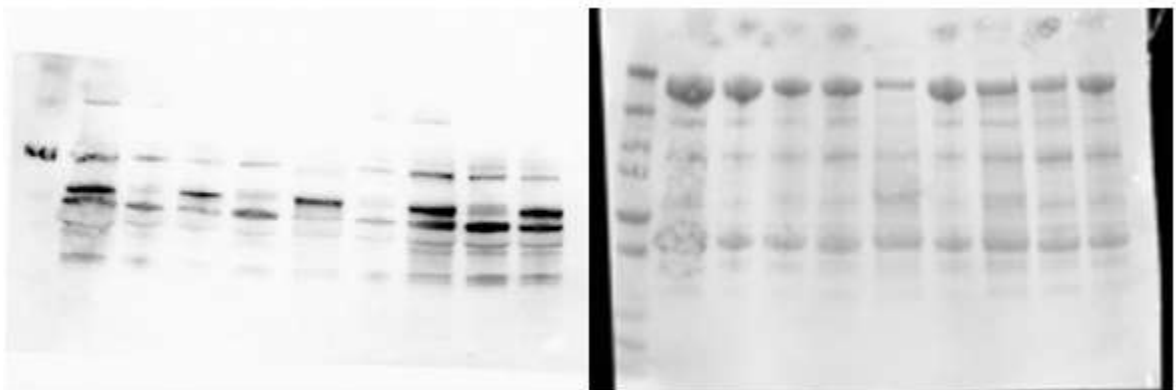
Blot 11

| <u>Lane 1</u> | <u>Lane 2</u> | <u>Lane 3</u> | <u>Lane 4</u> | <u>Lane 5</u> | <u>Lane 6</u> | <u>Lane 7</u> | <u>Lane 8</u> | <u>Lane 9</u> | <u>Lane 10</u> |
|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|----------------|
| Ladder | LS | W7-C | W7-X | A7-C | A7-X | W7-C | W7-X | A7-C | A7-X |

SPK



SPL



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