Notch, Numb and Numb-Like Responses to Exercise Induced Muscle Damage in Human Skeletal Muscle

By

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Abstract

Introduction: Notch proteins are a single-pass type 1 transmembrane protein that regulates cellular proliferation and inhibits myogenic differentiation. Numb and Numb-Like are adaptor proteins. Among their functions is control of cell fate determination and progression of cell differentiation via inhibition of Notch. While no role for Numb-Like has been found in cells of the myogenic lineage, Numb promotes myogenic differentiation of satellite cells. The roles these proteins in human skeletal muscle in response to exercise-induced muscle damage have yet to be examined. **Purpose:** The purpose of this investigation is to examine changes in the expression of Notch, Numb and Numb-Like in human skeletal muscle after a bout of muscle damage via eccentric exercise. **Methods**: Seventeen, recreationally trained, male subjects signed an informed consent approved by The University of Kansas's Institutional Review Board and were randomly assigned to one of two groups: a control group (n = 5) or a damage group (n = 12). Subjects completed a one repetition maximum (1RM) in leg extension followed by seven sets of ten repetitions of eccentric leg extension at %120 of 1RM with a two minutes of rest period between sets. Four muscle biopsies of the *vastus lateralis* were collected at baseline, 3-hours post- two days post-, and five days post-muscle damage and analyzed utilizing Western blot and quantitative reverse transcription polymerase chain reaction analyses. The results were analyzed using two separate analyses. The first being a 2X3 (Group X Time) Two-Way Repeated Measures ANOVA for the baseline measures, two days post- and five days post-muscle damage time points with the exclusion of the 3-hour post damage time point. The second being a One-Way Repeated Measures ANOVA with only the experimental group. **Results**: There were no significant main effects of time for the damage group with the inclusion of the three hour time point from baseline for Numb ($p > 0.05$: 3-Hour $p = 0.22$, Day 2 $p = 0.89$, and Day 5 $p = 0.17$) and Numb-Like (3-Hour $p = 0.63$,

Day 2 p = 0.30, and Day 5 p = 0.77). Additionally, no significant differences in mRNA expression were observed for *Numb* between groups two days post- and five days post-muscle damage for Numb (p = 0.13 and p = 0.74, respectively) and Numb-Like (p = 0.28 and p = 0.60, respectively). There was no significant main effect of time for Notch1 among the damage group with the inclusion of the three-hour time point from baseline (3-Hour p = 0.84, Day 2 p = 0.28, and Day 5 p = 0.20). Additionally, no significant differences in mRNA expression were observed for *Notch1* between groups at two days post-muscle damage (p = 0.56). However, there was a significant increase in *Notch1* at five days post-muscle damage between the exercise group (1.91 ± 1.29 fold change) and the control group (0.52 ± 0.38 fold change) from baseline measures (p = 0.04). **Conclusion**: Numb and Numb-Like expression was unaltered post-muscle damage, while Notch mRNA expression was increased after exercise-induced muscle damage. These results indicate that Notch and Numb-Like may have a greater role in muscle repair after strenuous exercise in humans than previously thought. Funding provided by NIA grant 5R01AG060341-02 to CPC and the CSACSM Doctoral Grant.

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Chapter I: Introduction

Human skeletal muscle is a stable, post-mitotic tissue with infrequent turnover of myonuclei under normal physiological conditions. However, turnover does occur in response to myofibril damage resulting from day-to-day activity or exercise. Extensive myofibril damage may occur during resistance exercise. In particular unaccustomed, heavy, or eccentric exercise results in deterioration in the Z-line, thick myofilaments, mitochondria content, and A-band disturbances, predominately in type II fibers (1). Extensive damage to myofibrils has the capacity to alter skeletal muscle structure and function from one to three days (2) and possibly longer. Recent evidence has suggested that muscle specific progenerator cells, satellite cells, are necessary for the adaptations and repair process produced from exercise induced muscle damage (3).

This myogenic process requires highly specific signaling pathways in order to activate satellite cell proliferation, differentiation and fusion. Notch, Numb and Numb-Like, a homolog of Numb, have been shown to participate in neurogenesis, embryonic myogenesis and somite formation with regulatory roles in cell proliferation and differentiation in numerous tissues (4-7). Notch receptors (Notch1, 2, 3 and 4) are type I transmembrane proteins that bind several ligands and induce intracellular signaling. Once Notch binds to a ligand, two proteolytic cleavages occur to produce the Notch Intracellular Domain (NICD) which allows for the translocation of the NICD to the nucleus to induce transcription. The translocation of the NICD stimulates the proliferation of cells via increasing the gene expression of Hairy-Enhancer of Spilt (HES) proteins (4, 8, 9), potentially increasing the number of satellite cells available for myonuclei production and myofibril regeneration. Numb and Numb-Like are hypothesized to have redundant functions that inhibit the translocation of the NICD, altering the satellite cell's linage (10, 11).

There are four isoforms of Numb with molecular masses of 65, 66, 71 and 72 kDa while Numb-Like only has one isoform. Numb and Numb-Like function as regulators of cellular progression. Numb in particular is asymmetrically segregated into one daughter cell, while Numb-Like is symmetrically segregated, indicating that Numb-Like may have redundant and independent functions of Numb (4). Both of these homologs are derived from the same family of proteins (4) and appear to have redundant functions inhibiting the NICD to induce a transition in satellite cells from proliferation to myogenic differentiation (12). Numb and Numb-Like inhibit signaling via sequestering the NICD in the cytoplasm and inducing ubiquitination (13). The exclusion of the NICD from the nucleus is hypothesized to transition the satellite cell from a proliferative state to a differentiated state and promotes the development of myoblasts (10, 11). These molecular signaling pathways suggest that Numb and Numb-Like inhibit the downstream signaling of Notch progressing and promoting myogenic differentiation and promote muscle repair.

The majority of research conducted has focused on Numb in *Drosophila* and rodent models (4, 12, 14-16). To our knowledge, only one study, by Carey et al. (2007), has been conducted in humans investigating Notch and Numb after exercise. The findings of this study indicate that *Notch1* and *Numb* mRNA were significantly decreased with age in comparison to younger individuals. However, two hours after an acute bout of exercise, there were no differences seen between younger and older individuals. Therefore, exercise may have the capacity to increase the muscle regenerative capacity of aging skeletal muscle. Also, Carey et al. (2007) determined that *Notch3* mRNA expression peaks 48 hours after induced differentiation in vitro, with increases in expression myogenin, a marker of differentiation. These data indicate that Notch3 may have a role in differentiation (10). Interestingly, in mice Notch1 and Notch2 activation increases and maintains

the self-renewal capacity of the satellite cell pool (17). However, this study was conducted in mice in non-physiological conditions. Therefore, understanding the temporal regulation and the role of Notch, in conjunction with the negative regulators of Notch (Numb and Numb-Like), after an acute bout of exercise in human satellite cells is necessary (skeletal muscle has yet to be determined). To our knowledge, no studies have investigated the temporal expression of Notch, Numb and Numb-Like in human muscle after an acute bout of eccentric exercise.

This study examined the changes in Notch, Numb and Numb-Like expression in human skeletal muscle after a bout of eccentric exercise. Twelve subjects performed an eccentric bout of leg extensions that targeted the quadriceps (n = 12) and five subjects served as a control group (n = 5). Skeletal muscle biopsies of the *vastus lateralis* were obtained pre- (Pre), 3-hours post- (3-Hour), two days post- (Day 2) and five days post-muscle damage (Day 5). Analysis of skeletal muscle tissue was done for *Notch*, *Numb* and *Numb-Like* mRNA and their gene products, which are essential for the regulation of satellite cell proliferation and differentiation that may regulate skeletal muscle growth after exercise. We hypothesized that Notch expression will increase immediately after muscle damage occurs, Numb gene expression will increase in the later stages of muscle repair with corresponding increases in protein and non-significant increases in Numb-Like mRNA and protein. These hypotheses are based on prior research of Numb having the dominate role in comparison to Numb-Like.

Chapter II: Literature Review

2.1 Introduction

During exercise induced muscle growth and regeneration, satellite cells are vital for myofiber development and renewal. These progenitor cells require a highly specific signaling pathway in order to activate satellite cell proliferation, differentiation and fusion. In skeletal muscle, paired box transcription factor (Pax-7) is required for the development and signaling pathway of satellite cell function (18). In addition to Pax-7, myoblast determination protein (MyoD) and Myogenin are two members of a family of myogenic regulatory factors (MRFs) involved in the regulation and coordination of myogenic differentiation. During satellite cell activation, MyoD initiates cycling of myoblasts increasing the number of undifferentiated cells. Downstream of MyoD, Myogenin induces the transition from myoblasts to myocytes and myotubes (19).

In conjunction with Pax-7 and MRFs, Notch, Numb and Numb-Like are potential regulators of satellite cell activity and will be the primary focus of this review. Notch contributes to muscle development, regenerative properties and proliferation of satellite cells. In contrast, Numb and Numb-Like proteins are hypothesized to inhibit Notch signaling and the expression of some myogenic genes, decreasing proliferation and promoting differentiation (10). The balance and timing of gene expression among these genes determines the activity levels of satellite cells as well as muscle repair and growth after exercise.

2.2 Satellite Cells

2.2.1 Introduction to Satellite Cells

Skeletal muscle has extraordinary ability to respond to environmental stimuli. The type of stimuli can result in muscle specific adaptations. For example, being sedentary leads to muscle atrophy while participating in exercise can induce in muscle injury that ultimately leads to growth and regeneration. This remarkable renewable capacity in response to exercise is thought to be attributed to satellite cells due to the majority of evidence indicating that that skeletal muscle nuclei are post-mitotic. Therefore, muscle is unable to go through cellular replication to produce two identical daughter cells. Skeletal muscle thus depends upon satellite cells, which undergo cellular division via mitosis, to replicate muscle tissue in response to damaged cells.

Satellite cell were discovered via electron microscopy by Dr. Mauro in 1961 (20) and are skeletal muscle specific stem cells involved in maintenance, repair, remodeling and growth during muscular stress. Specifically, these primary stem cells are responsible for maintaining a homeostatic environment and the induction of new myonuclei during muscle growth and regeneration in response to stimuli. Evidence suggest that satellite cells are essential for muscle fiber regeneration after extensive injuries are sustained via exercise or mechanical injury. Abolition of satellite cells results in muscle dysfunction and the complete absence of muscle regeneration after injury (21). However, some evidence suggest that satellite cells are not necessary after minor muscle damage occurs (3). Since the function of satellite cells is to repair muscle tissue, they are in close proximity to sites of damage between the sarcolemma and basal lamina of the muscle fibers (22) which enable them to be readily available for repair.

As stated earlier, muscle repair can occur in the absence of satellite cells. For example, during everyday muscle damage, intracellular vesicles containing essential RNAs and extracellular proteins are recruited to the site of myofibril damage and repaired (23). During extensive myofibril damage, e.g. exercise, there is an increase in necrosis, apoptosis, inflammation which in turn activates satellite cell proliferation and differentiation (3). The abolition of satellite cells via expression of diphtheria toxin receptor under control of the Pax-7 locus, severely reduces the regenerative capacity of skeletal muscle in response to exercise induced damage (24). However, other cell types, such as fibroblasts and adipocytes, are also critical for skeletal muscle regeneration. For instance, the abolition of satellite cells results in a complete loss in muscle regeneration and dysregulation of fibroblasts with an increase in connective tissue. Whereas an ablation of fibroblasts results in early differentiation and reduction in the satellite cell pool (21). Therefore, muscle regeneration is likely that a dynamic interaction among multiple cell types.

2.2.2 Satellite Cell Symmetrical and Asymmetrical Division

Satellite cells are specialized cells that have two fates during activation: 1) differentiation into myoblasts (a form of progenitor cells, a biological cell that has a more specific destination than a stem cell, that gives rise to an immature muscle cell or myofiber) or 2) clonogenicity (renewal of the satellite cell populace or pool via proliferation) (22, 25). The process of proliferation involves cellular division to form new cells, while differentiation is the specialization of the cells into a definitive state as a mature muscle cell.

During proliferation satellite cells have the capacity to undergo symmetric or asymmetric division. This balance is critical for satellite cell and skeletal muscle maintenance and homeostasis. During symmetrical division, two identical undifferentiated daughter cells are generated. Throughout asymmetrical division, one daughter cell maintains self-renewal while the other cell progresses through differentiation (25, 26). Evidence has shown that the template chromosomes, during DNA replication, in the S phase are segregated to the stem like daughter cell while the newly formed copies were segregated to the differentiating daughter cell during asymmetrical cellular division (25, 26). Therefore, it is hypothesized that the reserve pool of satellite cells are adherent to the immortal DNA hypothesis proposed by Dr. John Cairns (1975) (27), potentially reducing mutations. This cellular proliferation and differentiation are under a strict temporal regulation. For example, satellite cell number in the G0 (quiescent state of a cell) and G1 (interphase – also known as the growth stage) phase increases 24 and 48 hrs post-resistance exercise, indicating that satellite cells have gone through one cellular cycle and are undergoing proliferation (28, 29). Therefore, several factors contribute to the regulation of satellite cells and determine the cellular fate of these highly specialized cells.

Proliferation and differentiation of satellite cells is also reversible and has the capacity to change the lineage of a cell. Upon activation during quiescence, satellite cells progress through proliferation. At this junction the cells can continue to proliferate, differentiate into myoblasts, or return to a state of quiescence (30). Myoblasts then have the ability to proliferate or differentiate (31). This flexibility and reversibility of skeletal muscle tissue and lineage specification is referred to as tissue plasticity. Skeletal muscle has a high level of plasticity and responds well to anabolic and catabolic stimuli, which is in part due to satellite cells activity.

2.2.3 Satellite Cell Populations

Previously, satellite cells were considered to be homogeneous as myogenic progenitor cells indicating that all satellite cells were similar in genetic composition. However, recent evidence has suggested that during differentiation, satellite cells exhibit heterogeneity, the quality of being diverse in the cellular outcome. Distinct populations of satellite cells undergo mitosis at different rates. Approximately 80% of total satellite cells are readily available to enter the cell cycle (responsive population), while approximately 20% enter the cell cycle at a much slower rate (reserve population). The reserve population is hypothesized to remain in the quiescent state while the responsive population mobilizes to repair tissue. The reserve population is activated after extensive damage when a greater level of muscle regeneration is necessary (32). Although both the responsive and reserve populations are in the same quiescent state, these two populations have differing genetic compositions that make one population able to respond quicker. Therefore, indicating the satellite cells in the same state have a degree of heterogeneity.

Another subpopulation of satellite cells are "true" stem cells that differentiate into multiple mesenchymal lineages which may include fibroblasts, myocytes, adipocytes, or osteocytes (25, 26, 33). This is dependent upon the expression of genes that activate different molecular pathways. For example, during the progression through the myogenic lineage, the majority of satellite cells do not express CD45 and Sca-1 within the cellular membrane and do not differentiate into adipocytes or fibroblasts. However, satellite cells that do not express CD45 and express Sca-1 can differentiation into adipocytes and fibroblasts (3). Again, this alludes to the ability of satellite cells to alter the cell line lineage and their high level of plasticity as well as their heterogeneity.

However, in skeletal muscle, satellite cells predominately give rise to myoblasts which then form multinucleated myotubes.

2.2.4 Satellite Cells Vary Upon Cellular Environment

In adult skeletal muscle satellite cells represent approximately 2%-7% of the nuclei (34). This can vary dependent upon the fiber type, with fast twitch fibers having fewer satellite cells than slow twitch fibers (35). However, this is also muscle group dependent. Gibson and Schultz (1982) indicated that the *extensor digitorum longus*, in adult mice, had a greater quantity of satellite cells in the Type IIA fibers with fewer, but equal, quantity of satellite cells surrounding Type IIB and Type I fiber. Surrounding the *soleus*, satellite cells were rarely found on the Type IIA fibers but high quantities were located near Type I fibers (36). However, in humans there are greater number of satellite cells clustered around Type I muscle fibers (37). These data indicate satellite cell quantity may vary dependent upon fiber type, muscle type, and species. Additionally, satellite cells are found adjacent to capillaries regardless of fiber type (38), at the ends of myofibers during embryonic, longitudinal muscle growth (39), and at presynaptic regions of skeletal muscles (40). These data indicate that the localization of satellite cells is nonrandom, and it is likely that their location makes them readily available for muscle regeneration in response to injury or damage. It should be noted that the location and number of satellite cells may vary due to the mythologies utilized by the researchers.

2.2.5 Satellite Cells Response to Exercise

Additionally, muscle contractile activity has the capacity to alter satellite cell number and activation. After a single bout of 15 sets of 20 repetitions of high-force eccentric knee extensions on an isokinetic dynamometer, the number and activation of satellite cells increases 24 hours post-muscle damage in type II muscle fibers accompanied by a 73% increase in satellite cell number, with no change in type I muscle fibers (41). This suggests that either muscle damage occurs in a fiber type specific manner or satellite cells associated with type II are stimulated to a greater extent in response to eccentric exercise induced muscle damage. After electrical stimulation of the gastrocnemius medialis muscle, in vivo satellite cell activity increased 7-fold. Seattleite cell activity was measure via immunohistochemical labeling with CD56, Pax-7, NCAM and Ki67 (37). In addition to fiber type, age and training status can also alter the quantity of satellite cells available. Crameri et al. (2004) demonstrated that aging populations have a decrease quantity of satellite cells but resistance training was able to stimulate an increase the satellite cell pool as early as four days following a single bout of high intensity exercise (42). In healthy males, satellite cell number has been shown to increase beginning at 24 hrs post-exercise, peaks at 72 hrs post-exercise and is detectable for up to five days post-exercise (22). In rat skeletal muscle, satellite cell differentiation peaked seven days after cells were placed in cell culture (43). These data indicate that satellite cells activity and number are highly dependent upon several key factors such as muscle composition, fiber type, muscle activity, and age.

When muscle damage occurs, satellite cells migrate to the periphery of the muscle fiber while increasing Pax-7 and MyoD expression (19, 44). Then, the immature myogenic cells are replaced with mature myoblasts that undergo several rounds of cell cycling decreasing Pax-7 expression

while increasing myogenin expression. These cells then differentiate to form myocytes, a mature muscle cell (19, 45, 46). In response to exercise, satellite cells proliferate and differentiate becoming myoblasts in a similar fashion. This process is controlled by MRFs such as myogenin, MyoD, Myf-5, and Myf-6 (11). All of these regulator factors are basic helix loop helix (bHLH) transcriptions factors present during myogenic differentiation. MRFs and other essential proteins are responsible for the transcription of myosin heavy chain proteins and creatine phosphokinase enzymes that are imperative for muscle function. Furthermore, this family of transcription factors is believed to be regulated by Notch signaling, which is an essential component of satellite cell proliferation.

2.2.6 Pax-7

Pax-7 is a transcription factor that has a critical role in satellite cells proliferation. A transcription factor is a protein that controls the rate of transcription. It does this by binding to a specific target sequence in the DNA and promoting or repressing the binding of RNA polymerase. *Pax-7* is exclusively expressed in satellite cells of developed muscle cells, primarily in the nuclei of myofibers and ablation of Pax-7 results in reduced regenerative capacity of skeletal muscle (24). Pax-7 is also considered a canonical biomarker for satellite cells because it is expressed across multiple species and in all quiescent and proliferating cells and therefore is utilized to determine the location of satellite cells in vivo. Previous research hypothesized that satellite cell regulation was coordinated via the expression of Pax-7 and the MRFs (18). However, current literature hypothesizes the muscle repair pathway to be controlled by the Notch signaling pathway. In adult males that underwent an acute, eccentric only, plyometric exercise bout, *Pax-7* expression was significantly increased in relation to satellite cell activity. The increases in Pax-7 positive satellite

cells were shown to increase proliferation but not differentiation. Additionally, in mice that had over expression of *Notch1* there was an increase in *Pax-7* expression. In contrast, mice that had impaired *Notch1* expression had decreased *Pax-7* and impaired regeneration of muscle. It should be noted that this study was done in mice cell culture but this pathway has been shown to be conserved across species (47). Therefore, these results indicate that Pax-7 has a role satellite cell proliferating but not differentiating (48) and are potentially regulated by Notch.

2.2.7 MyoD

MyoD is expressed during satellite cell differentiation, but not proliferation and is considered the master regulator of myogenesis (49, 50). During satellite cell activation and entrance to the cell cycle, MyoD is upregulated followed by increases in Myf-5 and MRF4 (51). This indicates that MyoD is one of the earliest markers of myogenic commitment. In cell culture and frog embryos, high levels of *MyoD* expression induced terminal differentiation of myoblasts, indicating key regulatory roles during muscle development. *Notch1* expression antagonizes MyoD, via its downstream product Hairy-Enhancer of Split proteins (HES) 1 and HES Related Family BHLH Transcription Factor With YRPW (HEY) 1, which binds to MyoD at the bHLH region, decreasing satellite cell differentiation and limiting myogenesis in these cells (52-54). Therefore, it is hypothesized that Notch regulates MyoD expression during the activation of satellite cell during muscle growth. This hypothesis has yet to be investigated in humans, but due to the conservation of the canonical Notch pathway it is hypothesized that Notch regulates MyoD expression in humans.

2.2.8 Myogenin

Myogenin is another bHLH transcription factor that is involved in the coordination of myogenesis and satellite cell regulation. The primary role of Myogenin is to control the transition from myoblasts into myocytes and subsequently myotubes and is expressed last during the final stages of fusion and differentiation (51, 55). In human skeletal muscle homogenate after an acute bout of endurance- and resistance-type exercise, Myogenin and MRF4 increased expression 3.4 and 2.6 fold, respectively, nine hours post exercise (56), a key timing for myogenic differentiation. This process particularly involves the fusion of myoblasts. The primary role of the fusion of myoblasts is to synthesize the formation of myotubes and mature muscle fibers (57). Figure 1 illustrates the hierarchy of transcription factors regulating skeletal muscle (58).

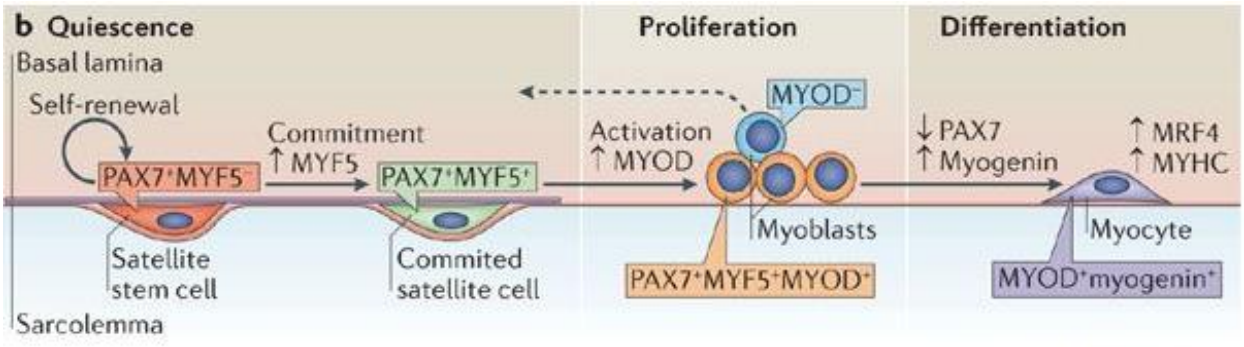

Figure 1: These MRFs transcriptionally and epigenetically control myogenic linage and satellite cells. Illustration from Figure 1b of Wang and Rudnicki, 2012 (58). These MRFs act in a sequential manner in order to induce satellite cell quiescence, proliferation and differentiation.

2.2.9 Myostatin

Myostatin is a myokine and a transforming growth factor-b family member that inhibits myogenesis, specifically the proliferation of satellite cells. Myostatin has been shown to induce satellite cell quiescence and negatively regulates self-renewal capacity. Mice lacking the

Myostatin gene, muscle fibers undergo hyperplasia and hypertrophy increasing overall muscle mass by approximately double and increasing the overall quality of the muscle (59). These adaptations are not limited to knockout models of mice. In mice that underwent immobilization of the hind leg and received a Myostatin inhibitor, the mice were able to maintain muscle mass, fiber size and force production (60). These data also indicate that Myostatin potentially regulates satellite cell activation and fusion to myofibers. Interestingly, in satellite cells that are Myostatin deficient, there is an increase in the number of activated satellite cells in comparison to wild type in mice during embryogenesis (61). Therefore, Myostatin has been shown to be a key regulator in satellite cell function and may be a key factor in the regulation of Numb, Numb-Like and Notch.

2.3 Electron Microscopy

Electron microscopy is a technique used to obtain high resolution images of both biological and non-biological specimens. As the name implies, electron microscopes utilize electrons to charge a sample instead of light such as an epifluorescences or confocal microscope. The basic principle of electron microscopy is that electrons are shot, via an electron gun, toward a sample. Those electrons are focused via magnetic lenses and metal apertures to from a single beam. The beam is further focused with more and more magnetic lenses onto the sample which interrupts the electron beam. The interaction of the electron beam and the sample then scatters the electrons which are detected and transformed into an image (62).

This analytical tool yields information about the topography, morphology, composition and crystallographic information of a sample down to one angstrom in size. In general, there are two types of electron microscopes: 1) transmission electron microscope (TEM) or 2) scanning electron

microscope (SEM). TEM focuses a narrow beam through relatively thin specimens at a specific location and permits the study of the inner structures and delineations of objects. SEM focuses an electron beam onto a thicker specimen where the electrons are then scattered. The electron beam is then scanned across the specimen to generate an image that characterizes the surface of an object or sample. The third type of electron microscope is a scanning transmission electron microscope (STEM) that utilizes principles from TEM and SEM. STEM requires the use of thin samples, like TEM, and the electron beam scans across the sample, like SEM.

Satellite cell were discovered via electron microscopy by Dr. Mauro in 1961 by utilizing the sartorius and ileofibularis of a frog and of the sartorius and tongue muscle a white rat. Based upon the location, beneath the basal lamina membrane on the periphery of the myofiber plasma membrane, it was hypothesized that they were post-natal myofibril repair and regeneration. The anatomical location gives the satellite cell a "wedge" morphology when observed via electron miscopy in a longitudinal section (20) and a circular or oval appearance in a correctional section. In addition to their distinct shape, satellite cells have a large nuclear-to-cytoplasmic ratio, a reduced number of organelles, small nucleus, and condensed interphase chromatin (3).

2.4 Notch

2.4.1 Notch Structure and Function

The Notch signaling pathway is highly conversed throughout multiple organisms (63). In mammals, there are four Notch proteins (Notch1, -2, -3 and -4), which are single-pass type 1 transmembrane proteins that contain epidermal growth factor like repeats (10). Therefore, Notch

proteins function as signaling proteins during short-range communication between cells (64). In skeletal muscle, Notch proteins have several roles in myogenesis which have predominantly been explored in *Drosophila* and mouse models, illustrating the role of Notch in inhibiting the formation of mature muscle cells (65). Primarily, Notch proteins are involved in signaling that enhance satellite cell and myoblast proliferation and inhibit myogenic differentiation (66). Notch is hypothesized to have a regulatory role in maintaining satellite cell quiescence, regulation in cell proliferation and cell determination in symmetrical cell division (63). Notch is essential for the early phases of muscle development and promoting proliferation (26).

However, some evidence suggest that the different Notch isoforms may have independent functions. For example, Cui and colleagues (2019) demonstrated that Notch1 expression is upregulated five days after upstream stimulation, and Notch1 is vital for differentiation of Mesenchymal stem cells (67). Carey et al. (2007) found Notch3 mRNA expression increase 48-72 hours post myoblast differentiation in cell cultures. They hypothesized that Notch may have dual roles in proliferation and differentiation of satellite cells and myoblasts (10). In addition, the increase in Notch3 expression seen in culture corresponds with an increase in Myogenin expression. Therefore, Notch expression may be low during the early stages of myogenesis and expressed later during differentiation of myoblasts (10). These data, in conjunction with previous research, indicate Notch may stimulate proliferation and differentiation in progenitor cells, depending on temporal regulation and levels of expression. Alternatively, the activation of Notch in the later stages of muscle growth could indicate a return to quiescence and inhibition of myogenesis. Bi and colleagues (2019) demonstrated that activation of Notch1, via stage-specific Cre alleles, in myocytes results in dedifferentiation to Pax-7 positive, quiescent satellite cells. This

resulted in decreased muscle growth and in postnatal lethality. However, Notch1 expression in myotubes improves muscle regeneration and increases exercise performance in age and diseased models (68). These data represent non-physiological states and therefore Notch signaling in vivo, in myofibers warrants further investigation.

2.4.2 Notch Ligands

Notch proteins have five ligands that bind to the extracellular domain: Jagged-1 and -2 and Delta-like-1, -3, and -4 (10). These ligands are also transmembrane proteins with epidermal growth factor like repeats. These ligands are a member of the Delta/serrate/jagged family of proteins that interact with the membrane protein Notch and regulate cell fate decision by activating and phosphorylating Notch receptors which stimulate a cascade of proteolytic cleavages (69). Upon activation Delta and Jagged are cleaved via metalloproteases when stimulated via Notch binding. The ligands are can compete with the cleavage of Notch by brining with γ-secretases. The inner portions of the proteins are released and translocated to the nucleus, indicating that Notch and its ligands are processed by the same machinery (70). However, it should be noted that in skeletal muscle, Delta-like seems to be the primary affecter in Notch signaling (71).

During skeletal muscle injury, Delta-like is necessary for regeneration (72) and is upregulated, potentially to induce the expansion of satellite cells via Notch signaling (73). In aged skeletal muscle Delta-like ligand upregulation in response to injury is limited, potentially altering the ability of aged skeletal muscle leading to impaired regeneration (74). Delta-like 1 is the primary ligand for Notch signaling and has been shown to induce p53 transcriptional activity in an RBP-J

dependent manner in vitro in a Delta-like 1 treated satellite cells. This was further demonstrated by the abolition of Delta-like 1 and the induction of Notch downstream target genes HEY1, HEY2 and HEYL which induced p53 transcriptional activity (75). It should be noted that Notch activation did not increase the transcription of p53. Delta-like 1 is asymmetrically expressed in dividing satellite cells, with higher expression in the cell that is destined for differentiation (64), potentially initiating differentiation of neighboring satellite cells.

2.4.3 Notch Canonical Pathway

Notch proteins must be translated and inserted into the cellular membrane in order to be functional. In order for Notch to become functional it first must undergo several rounds of cleavage. Frist, the Notch receptor protein is proteolitcally cleaved by PC5/furin at site one (S1) in the cytoplasm. The first cleavage causes translocation and endocytosis of Notch to the cellular membrane. The second site (S2) is flanked by the C-terminus and the negative regulatory region (NRR), which is toward the N-terminus of the protein. The NRR prevents the cleavage at S2 (76). However, upon Notch binding to its ligand, a conformation change occurs, and ADAM metalloproteases cleaves the extracellular portion of the protein at the second site (S2). Metalloproteases assist in the breakdown of extracellular matrixes (77) and up regulates following muscle damage (78). The cleavage by metalloproteases produces the Notch extracellular truncation (NEXT) fragment. The NEXT fragment is short lived and then cleaved at site three (S3) and four (S4) via γ-secretase at the inner plasma membrane and near the middle of the transmembrane domain, synthesizing and activating the NICD (52). This cleavage can be blocked via γ-secretase inhibitors (79, 80). The primary from of Notch, Notch1, is cleaved between the amino acids Gly 1743 and Val 1744 (81). The NICD then translocates to the nucleus to bind to CBF1/Su(H)/Lag-1 (CSL) in *Drosophila* or the

mammalian homolog of CBF1, RBP-J kappa in mice, which is a transcriptional repressor. RPBJ-kappa is a Notch coactivator imperative for activating downstream targets of Notch (75). These repressors are converted to transcriptional activators via binding of the NICD. The protein complex recruits transcriptional coactivators to induce gene expression of Hairy-Enhancer of Split proteins (HES) and HES Related Family BHLH Transcription Factor With YRPW (HEY) genes, which inhibits *MyoD* expression (82). The expression of HES and HEY transcription factors and the inhibition of MyoD results in the blockage of myogenesis and differentiation, primarily via HEY1 in C2C12 myoblast cell cultures (83). It should be noted that evidence has been shown that Notch is able to inhibit myoblast differentiation during development via CSL independent pathways but further investigations in Notch signaling post-exercise are warranted (8).

2.4.4 Downstream Signaling of Notch

Notch activation induces the expression of the HES genes, a family of transcriptional repressors. HES genes have a bHLH domain, which is imperative to DNA binding and dimerization, and an Orange domain that is involved in the selection of bHLH heterodimer partner. This allows for the HES repressors to inhibit differentiation and maintain progenitor cells via both active and passive repression (84). It is unclear if HES genes are involved in satellite cell proliferation. However, in the absence of HES1 and HES5, Notch is unable to inhibit neurogenesis in the nervous system in mice (85), therefore indicating that the downstream factors of Notch are necessary for function. This process of inactivation of differentiation is essential for embryogenesis, neurogenesis and myogenesis to allow for the maintenance of stem cell and satellite cell pools.

2.4.5 Notch PEST Domain

After the induction of the NICD to the nucleus and transcription of HES genes occurs, the NICD becomes degraded. The NICD has a short half-life due to the phosphorylation of the PEST domain located at the C-terminus. A PEST domain is a portion of the protein that is rich in proline, gluatime acid, serine and threonine-rich regions, and has been shown to be involved in protein turnover signaling (86). In healthy tissue, the phosphorylation of the PEST domain causes ubiquitination and subsequently proteasome dependent degradation. Interestingly, upon the removal of the PEST domain in T-cells, impaired degradation of NICD follows and in some cases leads to acute lymphoblastic leukemia (87). Also, mutations in the PEST domain region of Notch1 half been shown to increase the half-life of the NICD and increase cellular proliferation and oncogenesis (76), indicating the importance of the regulation of the PEST domain of the NICD. Upon deletion or inactivation of the PEST domain from Notch, there is a reduced repressive capacity for β-catenin, which will be discussed later on (81). This illustrates the importance of the PEST domain in the functional capacity of Notch. Figure 2 illustrates the current pathway of Notch activation and transcription signaling.

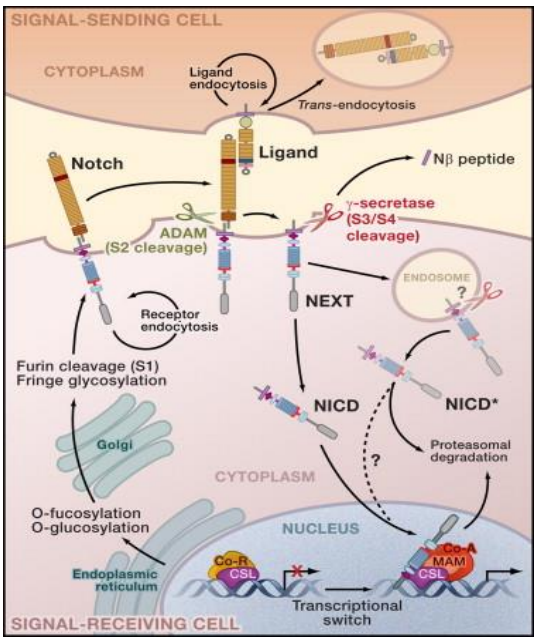

Figure 2: The core Notch signaling pathway is mediated by regulated proteolysis and extracellular signaling pathways. Notch is cleaved from the intracellular membrane and translocates to the nucleus to increase satellite cell proliferation and inhibit differentiation. Illustration from Figure 1 of Kopan & Ilagan (64).

2.5 Numb and Numb-Like

2.5.1 Numb and Numb-Like Inhibition of Notch

Numb and the Numb homolog, Numb-Like which shares 76% homology (88), interact with Notch proteins to determine cellular fates. *Numb* encodes for four protein isoforms produced via splicing of two exons (Ex3 and Ex9), while *Numb-Like* only has one, that are involved in the determination of cellular fates by participating in protein ubiquitination (89-91). Ubiquitination is the process of tagging a protein with ubiquitin, a small regulator protein, to mark it primarily for degradation, but also translocation, activation, or promote interactions. Specifically Numb and Numb-Like inhibit Notch signaling in one daughter cell via inhibition of the nuclear translocation of the Notch and cotransactivator suppressor of hairless (Su(H)) in *Drosophila* or recombining binding protein suppressor of hairless (RBP-J kappa), the mammalian homolog (92, 93). Notch binds to RBP-J kappa at the RBP-J kappa associated module (RAM) domain, which is a crucial portion of the NICD that interacts with RPB-J. The RAM domain is necessary for the ability of Notch to interact with β-catenin (81).

Due to the conservation of amino acids at the amino-terminal of *Drosophila* and mammalian Numb in conjunction with in vitro studies, the N-terminus directly interacts with the NICD (5). Indicating that the N-terminus is an essential component of a functional Numb protein. Numb is activated by the binding at the N-terminus with the Acyl-CoA Binding Domain Containing 3 (ACBD3) in *Drosophila* neuronal cells (mammalian homolog: Golgi resident protein GCP60). ACBD3 is synthesized in the Golgi Apparatus and is released during mitosis and then binds to other protein targets of Numb to form a protein complex. This portion of the Numb complex is the antagonist

to Notch and inhibits the lateral signaling between cells (94). Recent evidence has also suggested that in HeLa cell lines the interaction between the Numb in conjunction with the ACBD3 complex, associates with the RAM and PEST domains of the NICD to stabilize the NICD and not degrade it (5, 95). This discrepancy between Numb positively or negatively regulating the NICD could be due to the tissue being utilized in each study, therefore indicating that Numb's role may be tissue dependent. However, further research is needed in order to determine the role of Numb and Numb-Like in other tissues. Figure 3 below illustrates the pathway of Notch inhibition via Numb activation:

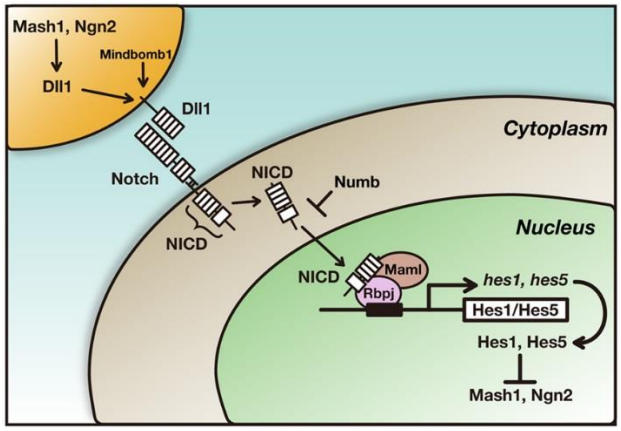

Figure 3: The Notch signaling pathway and the role of Numb inhibition on downstream gene expression of HES mRNA. Numb interacts with NICD and degrades it to inhibit proliferation and increase differentiation. Illustration is from Figure 2 of Shimojo et al. (7).

A secondary hypothesis for Notch inhibition is that Numb decreases the quantity of Notch receptors in the membrane via endocytosis. The C-terminus of Numb and Numb-Like interact with the EH-binding domain, necessary for protein-protein interactions, of epidermal growth factor receptor substrate 15 (Eps15), a protein involved in the endocytosis of epidermal growth factors (96). Since structure of a protein determines the function, the EH domains potentially explain one of the hypothesized mechanisms of inhibiting Notch via receptor mediated endocytosis. A third hypothesis states, Numb potentially inhibits the interaction between Notch and its ligands. This can occur in two different methods, the first is via endocytosis of the Notch receptor and the second is that Numb can bind to the intracellular portion of Notch and alter the binding properties to

decrease affinity for its ligand. Therefore, Notch is unable to bind with its ligands. A fourth hypothesis for Notch inhibition via Numb is the *Numb* mRNA inhibits Notch transcription meaning that *Numb* is acting as RNA interference (RNAi) (97). These hypotheses are currently under investigation and have primarily been conducted in *Drosophila* neuronal and sensory organ precursor cells.

2.5.2 Numb: PTB Domain and PRR Region

Numb protein contains a phosphotyrosine-binding domain (PTB) domain (92) for protein scaffolding and a C-terminal proline rich domain (PRR, containing putative Src homology 3 binding sites) (13). Numb proteins also contain acid-prline-phenylalanine (DPF) and asparagine-proline-phenylalanine (NPF) motifs, which are essential for binding to AP-2 complex (responsible for clathrine mediated endocytosis) and EH domain containing proteins necessary for the regulation of endocytosis (98), at the carboxyl-terminus for regulation of endocytosis for protein-protein interactions (95). Alternative splicing of *Numb* mRNA at allows for the formation of the different isoforms of Numb. The four isoforms of Numb contain differing lengths of PTB domains and PRR regions. The PTB (exon3) and PRR (exon9) regions can be denoted as long or short, depending upon the length of the amino acids in the particular regions. The Numb isoforms can be a combination of the two regions producing four isoforms: p72 (Numb+exon3+exon9), p71(NumbΔexon3+exon9), p66 (Numb+exon3Δexon9) and p65(NumbΔexon3Δexon9) (99, 100). The p72 and p71 isoforms are primarily expressed in progenitor tissues where the p66 and p65 isoforms are expressed in adult tissues (91, 100). Overexpression of the p72/p71 isoforms has been shown to increase proliferation while the overexpression of p66/65 isoforms promote differentiation in neuronal cell lines (100). The expression of different isoforms could be a key

factor in understanding why some evidence indicates that Numb antagonize Notch signaling to promote proliferation and while others indicate that Numb does not affect Notch signaling and promotes differentiation. For example, in cancer cells containing the p72/p71 isoforms, there was increased notch signaling (101) and the p66/p65 isoforms decreased Notch target gene expression (102). The greater inclusion of Exon9 are corresponding to increased proliferation in cancer cells (100).

The PTB regions have been shown to have several different functions involving calcium release, reactive oxygen species (ROS) production and apoptotic signals. The isoforms expressing the short PTB domains have been shown to have greater amounts of calcium in the cells in comparison to the cells with the long PTB domains. However, the short PTB domain has been shown to make PC12 Cells more susceptible to calcium imbalances and susceptible to amyloid β-peptide, a key membrane protein, induced apoptosis (103). Therefore, Numb may regulate calcium release from the endoplasmic reticulum with the short PTB domain increasing calcium release to a greater extent in comparison to the long PTB domain to promote differentiation of neuronal cells. The longer PTB domains however, had increased production of mitochondrial ROS production in comparison to the short PTB domain (99). Lastly, the Numb isoforms with short PTB domains are more codependent upon tropic factors for survival in PC12 cells, which are pheocharmocytoma cancer cells, in basil conditions (6). These data indicate that the length of the PTB domain has functional consequences for the function of Numb.

The PTD domain Numb targets LNX a RING finger, E3 ubiquitin-protein ligase responsible for ubiquitination of Numb isoform p66 and p72, which difference by 11 amino acids inserted into the PTD domain, but not p71 and p65. The PTB domain of Numb interacts with the first PDZ domain 1 (PDZ1) of LNX at amino acids ^{183}LDNPAY^{188} between the PDZ1 and the RING finger (88) to ubiquitinate Numb and cause degradation. The deletion of PDZ1 of LNX results in the loss of ubiquitination of Numb and therefore no degradation of Numb occurs (104). A single point mutation of Y188A resulted in the no interaction between Numb and LNX therefore inhibiting the interactions between the proteins (88). Therefore, the regulation of certain isoforms of Numb is dependent upon functional PDZ1 domain and PTB domain interactions. Some evidence suggested that the PTB domain required tyrosine phosphorylation for the binding of Numb to LNX (105). However, Dho et al., (1999) demonstrated that tyrosine phosphorylation was not necessary for protein-protein interaction indicating that tyrosine phosphorylation is required only under specific conditions (88). This evidence also demonstrates that the Numb PTD domain way have diverse binding abilities adding to the complexity of Numb protein interactions.

The PRR region is a 48 amino acid region that's functions as a SH3-binding domain, in which the SH3 proteins regulate the cytoskeleton, RAS protein, and Src Kinases in conjunction with many others. Verdi et al. (1999) demonstrated that the varying lengths of PRR regions are responsible for the function of Numb. The mammalian Numb isoforms with longer PRR regions cause cellular proliferation while the shorter PRR isoforms promote differentiation during neurogenesis in *Dropshila* (6). Therefore, the Numb isoform present may alter the cellular outcome in skeletal muscle. However, at this time it is unclear which Numb isoforms are present and functional in human skeletal muscle. It should be noted that Verdi at al. (1999) hypothesized that the different

isoforms of Numb could potentially interact with the different isoforms of mammalian Notch (6), but this has not been conducted in humans.

2.5.3 Numb Isoforms and Numb-Like Interaction

Numb and Numb-Like have the ability to interact with themselves. Numb-Like interacts with Numb isoform p65 and p71 via the PTB domain and proline rich region. While Numb p65 also interacted with itself preferentially with the same isoform via its PTB domain, indicating that Numb may interact as a heterodimer or homodimer with Numb-Like (106). This additionally, the p72 isoform has the capacity to antagonize the p65 isoform and increase Notch target gene expression (102), HEY1 and HEY2. This was also correlated with an increase in the MAPK/ERK signaling (100). It should be noted that p72 expression alone does not increase downstream transcriptional targets of Notch in cancer cells (100). The ability of the four isoforms of Numb, Numb-Like and Notch to bind and regulate one another adds to the complexity of satellite cell regulation. Therefore, the ratio of which each of these isoforms are present within the cell should be taken into consideration. Also, the ability of these proteins to from complexes may alter their regulator role in skeletal muscle. Table 1 indicates the interactions possible between the different isoforms of Numb and they regulation of Notch.

Table 1: The table indicates which isoform of numb contains a PTB and PRR region. Also, the table indicates if the isoform is able to interact with other isoforms of Numb and the effect on Notch expression.

Numb Isoform	Exon3 (PTB)	Exon9 (PRR)	Interaction	Influence on Notch Expression
p72	Yes	Yes	p65	↑Notch
			p66	↓Notch
p71	No	Yes	NA	↑Notch
p66	Yes	No	p72	↓Notch
p65	No	No	p65	↓Notch
			p72	↑Notch

2.5.4 Notch, Numb and Numb-Like

While, the majority of research has been conducted in neuronal cells and several different animal models, Numb and Numb-Like have been limitedly investigated in human subjects, namely skeletal muscle. The mechanism of Numb inhibition of Notch is semi-conserved across cell types and species (63). This is imperative for the understanding of current body of research. For example, in chickens Numb modulates neurogenesis by binding to Notch1 in a similar manner as discussed earlier and induces proliferation and suppresses differentiation (107). In mouse neuronal and skeletal muscle cells, Numb has been shown to antagonize Notch signaling to determine cellular fates (9, 11, 83, 97). In mouse skeletal muscle, Numb positive cells enter into cell differentiation, while Numb negative cells do not differentiate becoming satellite cells and maintaining clonogenicity (11), potentially through the inhibition of Notch signaling. Lastly, the Numb and Notch signaling pathway has been shown to be conserved in humans (10) as well, which will be discussed later.

2.5.5 Notch, Numb, and Numb-Like: Redundancy

The conserved pathway of inhibition of Notch via Numb allows for asymmetrical division, where there are decreases in Notch signaling in one daughter cell and the other daughter cell can go through myogenic differentiation (97, 108). Asymmetrical division maintains the satellite cell pool at a constant capacity while still permitting differentiation. Numb also interacts with the E3 ubiquitin ligase, Itch, to down-regulate Notch1 receptors by degrading the NICD (90), effectively decreasing satellite cell proliferation and increasing differentiation. In addition, high levels of Notch, specifically the NICD, can potentially cause a decrease in Numb and Numb-Like indicating that these proteins have reciprocal negative regulation (109). However, there is controversial evidence that states Numb stabilizes the NICD1 at the post-transcriptional level via association with deubiquitinating enzymes in Hela Cell lines (95).

Satellite cells have the capability to divide asymmetrically and symmetrically. During symmetrical division, satellite cells produce two daughter cells that are similar to the paternal cell. During asymmetrical division, one cell is similar to the parent cell and a second cell different. In *Drosophila* neuronal precursor cells, Numb is asymmetrically divided to the daughter cells while Numb-Like is symmetrically distributed (4). Interestingly, when loss of Numb occurred, the cells failed to divide asymmetrically. However, the expression of Numb-Like produces two daughter cells with similar results as Numb, causing asymmetrically division, therefore adding to the redundancy of these two homologs. It should be noted that this was only seen in neocortex of *Drosophila* (4). For example, mice that contain knockouts of *Numb* and *Numb-Like* phenotypes have more sever impairments of neurogenesis than knockouts of *Numb* or *Numb-Like*, independently (110). Within cells that undergo cellular division such as satellite cells or neuronal

cells, Numb is asymmetrically localized to one pole of the daughter cells. This induces distinctive fates for each daughter cell.

In myocardial cell, the loss of Numb and Numb/Numb-Like, via troponin T-Cre meditated knockouts, causes ventricular noncompaction, which is an increase in the thickness and spongy appearance of the left ventricular wall. The Numb/Numb-Like knockouts presented a greater phenotypic severity of ventricular noncompaction in comparison to the Numb knockouts (106). This result is consistent with a previous study conducted by Yang et al. (2012) in that double knockouts promote a more severity lethality in embryonic mice. However, conversely to previous studies, in mouse myocardial cells, NICD1 did not increase in Numb/Numb-Like double knockouts during proliferations (106). Therefore, Numb and Numb-Like's negative regulation of NICD1 may be redundant, tissue specific or other levels transcriptional regulation may be primary.

2.5.6 Notch, Numb, and Numb-Like: Independence

In contrast, evidence has shown that Numb and Numb-Like have distinctive functions. For example, Numb is expressed in most tissues while Numb-Like is primarily expressed in nervous system. In addition, Numb is a membrane associated protein, but Numb-Like is a cytoplasmic protein in mice neuronal cells (4). As previously stated, Numb and Numb-Like are both negative regulators of Notch, but the results of negatively regulating Notch are dissimilar. For example, in an embryonic stem cell lines, Numb expression inhibits Sonic Hedgehog and Notch while inducing p53 (12, 111). Numb is required to prevent p53-dependent cessation of cellular division (112). However, Numb-Like expression does not alter p53 levels and induces Sonic Hedgehog signaling

(12). Sonic Hedgehog has a regulatory role in differentiation, specifically the expression of several families of growth factors (113). Another example of the independent functions of Numb and Numb-Like can be observed in mouse myocardial cells. Myocardial cells that underwent Cre-mediated deletion of Numb died at day 11.5 during embryotic life. Deletion of Numb-Like did not cause lethality but did induce low fertility in females. Double deletion of Numb and Numb-Like caused increased lethality and the embryos died at day 9 of embryotic life, thus suggesting a redundancy of Numb and Numb-Like (114). Lastly, in three cancer cell lines: cervix Hela, breast T47D and sarcoma AX, down regulation of Numb-Like is sufficient to increase NICD translocation to the nucleus and active the Notch signaling pathway (115). These data indicate that Numb and Numb-Like may have redundant and independent functions.

2.5.7 Animal Models

In adult mice that underwent CRE-mediated knockouts of Numb and then muscle damage via $BaCl_2$ injury, there was an upregulation of Myostatin, decreased number of satellite cells and impaired myofiber repair. Due to decreased Numb expression in satellite cells this also leads to decreased proliferation. Inversely, when a Myostatin specific siRNA was introduced into the cell cultures, satellite cell proliferation was salvaged. In cells that contained overexpression of Numb, Myostatin was down regulated and Notch was unaffected, potentially indicating that Notch is not the primary target of Numb in cells undergoing proliferation. Lastly, the loss of Numb-Like did not affect the function of skeletal muscle in mice and therefore was hypothesized not to have a significant role in muscle repair (108). These data indicate that Myostatin and Numb work inversely without affecting Notch signaling.

This is in partial agreement with previous research indicating that Numb is the primary regulator of proliferation and not the homolog Numb-Like. However, this research is dissimilar in that the authors hypothesize that Myostatin is the inhibitor of Numb, and Numb does not alter Notch levels. In aging skeletal muscle there is an upregulation of Myostatin and decreased expression Numb, suggesting that these proteins have a key regulatory role in sarcopenia and age related muscle loss (10). The balance of Myostatin, Notch, Numb and potentially Numb-Like are integral for healthy skeletal muscle growth and repair. Therefore, any disturbance in expression patterns could potentially alter skeletal muscle performance, satellite cell proliferation and differentiation.

In mice that underwent spinal denervation, NICD and HEY1 expression increased leading to Notch signaling increases in skeletal muscle 7 to 56 days, after denervation. In addition, satellite cell numbers increased significantly during denervation. Interestingly, in mice that underwent denervation and anabolic steroid supplementation (Nandrolone), NICD and HEY1 expression was reduced. This was potentially due to increases Numb RNA and protein levels, which indicates that Numb might be androgen responsive in skeletal muscle (116). This study concluded that the alterations made in Notch, Numb and HEY1 did not affect the degree to which denervation muscle atrophy occurs. In resemblance to the previous study, adult males, age 60-75 years old, were treated with monthly injections of a GnRH agonist (Lupron depot, 7.5 mg) to suppress endogenous testosterone production while simultaneously receiving weekly testosterone (25, 50, 125, 300 or 600 mgs) via intramuscular injections. The participants had significant, dose dependent increases in muscle cross-sectional area and increases in satellite cells positive for proliferating antigens. Additionally, there was an increase in Notch expression in satellite cells with no differences in Numb protein after 20 weeks of treatment. The investigation of Notch and Numb was only

conducted in the 300 mg injection group. These data, in conjunction with previous studies, indicate that human skeletal muscle may react differently when undergoing neurological muscle atrophy compared to pharmacological interventions (117). Thus, the method of muscle damage may alter the expression levels of Notch, Numb, Numb-Like and their downstream factors. Further research is warranted in order to understand muscle damage methodologies and Numb and Notch responses.

2.5.8 Human Models

Most studies involving Numb, Numb-Like and Notch have been conducted in animal and cell culture models. Only one study investigating the responses of Numb and Notch to exercise has been conducted in human skeletal muscle to my knowledge. This study conducted by Carey et al. (2007) utilized older and younger men who underwent 12-weeks of resistance training. Skeletal muscle biopsies were taken pre-12-week training, prior to and two hours after an acute exercise bout (3 sets of 12 repetitions of maximal knee extension on a Cybex Norm dynamometer with two minutes of rest between sets) and post-12 week training, prior to and two hours after an acute exercise bout. Carey et al. also investigated human primary skeletal muscle cell cultures to induce differentiation and synthesize a timeline of the expression of Notch and Numb. Notch mRNA expression significantly increased at 24-, 48- and 72-hours post differentiation and peaked at 48-hours post-differentiation in myoblast cells obtained from the *vastus lateralis* young healthy individuals. There was no alteration in the levels of Numb mRNA in cell culture. If the study had been carried out past 72-hours there may be poetical alterations in Numb mRNA expression. However, future research is needed in order to determine if Numb expression occurs past three days. These findings indicated that these genes played a significant role in myoblast differentiation and proliferation in human skeletal muscle.

At rest, prior to training older males had significantly less *Notch1* mRNA expression compared to younger males. After an acute bout of exercise, prior to training, there was a non-significant decrease in *Numb* mRNA two hours post-exercise in both groups. In addition, *Numb* expression was decreased in both older males and younger males compared to pre-training, indicating a significant main training effect. There was also a main effect for age, the older group had decreased Numb expression in comparison to the younger group. After the 12-week training program, older males had significant increases in *Notch1* mRNA expression from baseline measures taken prior to training. *Notch1* mRNA also increased after a single bout of exercise after 12 weeks of training. Interestingly, after training the older group had a significant decrease in Numb mRNA compared to the younger group. There was no difference in Numb mRNA expression two hours after an acute bout of exercise. These findings indicate that age and training status have the capacity to alter the expression of Notch and Numb potentially altering satellite cell proliferation and differentiation. Interestingly, the authors also indicate that this phenomenon is a hypothesized mechanism of impaired regenerative capacity of aging skeletal muscle (10).

The role of Numb during myogenesis has yet to be fully elucidated. However, there is evidence to suggest that Numb and Numb-Like promote satellite cell differentiation and decrease proliferation by inhibiting Notch signaling. During decreased or the absence of Numb and Numb-Like, Notch increases satellite cells proliferation and increase the satellite cell pool. Therefore, Numb is involved in the balancing of muscle cell proliferation and differentiation. Previous research indicates that Numb may have different roles of regulation during different strategies of

myogenesis. However, further research is needed to understand the exact interaction between Numb and Notch and how these proteins are affected after exercise.

2.6 Protein Interactions and Regulators of Notch, Numb and Numb-Like

Although the scope of this review is to illuminate the function and interaction of Notch, Numb and Numb-Like, it is important to understand potential regulators of these genes. This allows for increased understanding of skeletal muscle in response to exercise and potentially other environmental signals.

2.6.1 Sirt1

Notch, Numb and Numb-Like are intricately regulated and are affected by other regulatory mechanisms. Sirtuin 1 (SIRT1) is a NAD-dependent deacetylase that has the capacity to regulate protein function and epigenetic control of gene expression via the removal of acetyl groups (118). Guarani et al., (2011) found that in endothelial cells, SIRT1 deacetylates the NICD thus destabilizing the protein and promoting proteasomal degradation. When SIRT1 is deactivated via siRNA, Notch activity was not altered but the sensitivity to Notch signaling increased, measured via increases in downstream gene expression. Interestingly, during siRNA mediated knockout of SIRT1, there was decreased the levels of ubiquitin tagged NICD indicating that acetylation may interfere with the ability of the NICD to be degraded (119). This could potentially alter the function of Numb/Numb-Like complexes to bind and ubiquitinylate the NICD. However, this has yet to be investigated.

2.6.2 microRNAs

Numb inhibition occurs by the recruitment of microRNA-146a (miRNA-146a), a class of non-coding RNAs that has a fundamental role in negatively regulating posttranscriptional gene expression (120). This particular miRNA is believed to be regulated by transcriptional activator NFkappaB, which is released from the Golgi organ during cellular division. NFkappaB is stimulated during cellular stretching and inflammatory states (120, 121), which is associated with exercise. During cyclic stretching of mouse C2C12 cells via BioFlex plates, miRNA-146a gene expression increased. miRNA-146a was also found to be bound to the 3' untranslated region (UTR) of *Numb,* preventing translation and reducing satellite cell differentiation. Therefore, miRNA-146a may alter the expression and activation of cellular process associated with satellite cell proliferation and differentiation.

2.6.3 Itch

Itch (mouse homolog of Su(dx)) proteins have been shown to ubiquitinate Notch1 proteins, however the consequences of the ubiquitination are not yet known. It is hypothesized that increases in Itch increase the ability to bring the Notch receptors toward lysosomal degradation (122). Increased expression of either *Numb* or *Itch* has been shown to increase the ubiquitination of Notch1. When both *Numb* and *Itch* are expressed in conjunction, there is a greater ubiquitination of Notch1 suggesting a cooperation between Numb and Itch proteins (90). It should be noted that this study was conducted in *Drosophila* (fruit flies). However, this pathway is relatively conserved across *Drosophila*, mice, and human models.

2.6.4 Ligand of Numb Protein X

An additional regulator of Numb is ligand of Numb-Protein X (LNX). LNX contains an amino terminal RING finger, a zinc-binding cysteine rich sequence motif found in an abundance of proteins (123), and four PDZ domains. A PDZ domain is a string of 80-90 amino acids involved in anchoring receptor proteins to the membranes. LNX is hypothesized to ubiquitinate and degrade Numb therefore lowing the levels of Numb which increases Notch signaling. The PTB domain of Numb and only the first PDZ domain of LNX are required for Numb ubquitlylation and subsequently increasing Notch signaling (124).

2.6.5 MyoD

MyoD is a regulator of myoblast regulation and muscle development. MyoD, in the presence of nandrolone (an anabolic steroid), has been shown to form protein complexes with Numb CHIP in addition to androgen receptors. In addition to MyoD binding to Numb, Numb was shown to bind to bind to the promoter at the E-box of Myosin Heavy Chain 7 (MyH7) gene, where MyoD binds, indicating there may be some potential regulatory role of Numb (125).

2.6.6 MDM2 and P53

MDM2 (HDM2) is an oncogenic that gene encodes for a nuclear localized E3 ubiquitin ligase. This protein targets proteins such as p53 for proteasomal degradation therefore promoting tumor production. In addition to binding to p53, MDM2 can also bind to Numb forming a trimeric complex (126). P53 is a transcription factor that acts as a tumor suppressor and has been implicated in mitochondrial biogenesis and apoptosis in skeletal muscle (127). Numb binds to p53 and MDM2

to form a trimeric complex in epithelial cells which increases the stability of p53 preventing ubiquitination thus prolonging the half-life of the protein. Utilizing short harpin and short interfering RNA for Numb, effectively reducing Numb protein 80-90%, the half-life of p53 was decreased from ~60 minutes to ~20 minutes. In addition the quantity of p53 decreased two-fold. This effect was only seen in Numb and no effect for Numb-Like knockouts on the levels of p53 were seen. Conversely, during overexpression of Numb the half-life of p53 increased from ~60 minutes to ~120 minutes and increased transcriptional activity of p53 (126). However, it is unclear if Numb's protective function stems from the interaction between MDM2 or p53, therefore more research is warranted. It has also been shown that activated Notch stabilizes p53 *in vitro* via HEY1 binding to a *MDM2* enhancer box therefore decreasing transcription (75). Colaluca et al. (2008) found that Notch did not participate in the regulation of p53 via Numb mechanisms (126) indicating that p53 regulation can occur in a Notch-dependent and -independent manner. Interestingly, in isolated satellite cells of aged skeletal muscle there was a decreased Notch signaling, increased MDM2 and decreased p53 (75). In the absence of p53 via MDM2 inhibition, abnormal spindle assembly can occur and cause mitotic catastrophe and cellular death of satellite cells. Mitotic catastrophe occurs when proper mitosis fails to produce viable progeny and is typically used in cancer research. It typically occurs during anaphase and the cell does not survive long after. Interestingly in satellite cells from older mice, pharmacological activation of p53 via Nutlin-3, which inhibits the interaction between p53 and MDM2, decreased cell death via mitotic catastrophe and the proliferation of satellite cells indicating p53 activity was able to restore the youthfulness of satellite cells in older skeletal muscle (75). These studies indicate that p53 regulation occurs via a complex interaction among Numb, Notch, HES and HEY proteins and genes.

2.6.7 Wnt

Wnt proteins are a family of 21 glycoproitens that are potent regulators of satellite cells. The role of Wnt proteins in skeletal muscle regeneration after injury is controversial. However, evidence suggests that Wnt is highly involved in the self-renewal regulation of satellite cells. In particular, Wnt is involved in inducing myogenesis via satellite cells during muscle regeneration, while it has also been shown to suppress myogenesis and induce fibrosis in aging skeletal muscle (128, 129). These data determine that more research is needed in order to understand Wnt signaling in satellite cells in response to muscle damage.

Otto et al. (2008) demonstrated that Wnt signaling, in isolated muscle fibers, is activate during proliferation of satellite cells in conjunction with activated-β-catenin (Act-β-Cat), a downstream transcriptional coactivator. In particular Wnt1, Wnt3a and Wnt5a overexpression induced satellite cell proliferation, where as Wnt4 and Wnt6 hindered proliferation (130), indicating that only certain Wnt proteins are involved in satellite cell regulation. It should be noted that Wnt1, Wnt3a and Wnt5a as well as Wnt4 and Wnt6 caused the induction of Act-β-Cat, however the canonical wnt signaling pathway of Wnt1, Wnt3a, and Wnt5a induced the translocation of Act-β-Cat, allowing it to act as a transcriptional activator (130). Brack et al., (2007) found that induction of Wnt3a in young mouse skeletal muscle, resulted in increased myogenic to fibrogenic conversion in vitro thus increasing the deposition of connective tissue deposition in skeletal muscle. This was also supported via in vivo injection of Wnt3a one day post injury which resulted in connective tissue deposition (128). Wnt4 and Wnt6 cause Act-β-Cat to from a dimer and interact with

cadherins and α-catenin which localizes in the cellular membrane and not the nucleus. MyoD expression occurs prior to Act-β-Cat translocation to the nucleus therefore, it was concluded that satellite cell activation was Act-β-Cat independent, but proliferation is dependent upon the canonical Wnt signaling pathway (130).

In young and aged mice that underwent steady-state activation of Wnt signaling, there was an upregulation of Axin2, which plays a role in stabilizing β-Catenin in uninjured muscle. Furthermore, in purified satellite cells, aged cells had more expression of Axin2 in comparison to younger muscle indicating that there was an increase in Wnt signaling during ageing. Also during Wnt activation, active glycogen synthase kinase 3β (GSK3β) decreased and Act-β-Cat increased. Also in contrast to Otto et al., (2008), expression of Wnt3a expression in younger muscle resulted in a phenotype similar to aging skeletal muscle and reduced cellular proliferation in vivo. Upon the induction of Dickkopf-1 (DKK1), a Wnt inhibitor, no alterations were observed in the young muscle (128). Therefore, these results indicate that increased Wnt signaling results in the progression of myogenic to fibrogenic phenotype in skeletal muscle. However, other research has shown that Wnt signaling is essential for the progression of myogenic lineage during development (131), therefore indicating that Wnt may have specific roles during myogenesis.

2.6.8 β-catenin

Notch has been shown to post-translationally regulate β-catenin in progenitor cells. Specifically, Notch reduces levels of β-catenin in cardiac progenitor cells, therefore limiting cellular proliferation. During Notch1 knockdown models, dephosphorylated β-catenin levels increased, which is the transcriptionally active form of β-catenin protein, without decreasing the total amount

of protein or amino-terminal phosphorylated Ser-37. Interestingly, during implementation of siRNA of Notch1-4, there was a mild increase of β-catenin activation, indicating that Notch 1 is the predominating Notch receptor in embryonic stem cells (81). The overexpression of the Notch1 intracellular domain (N1ICD) in particular decreases overall and active levels of β-catenin but does not modulate the level of mRNA present in embryonic stem cells (81). These data indicate that Notch1 may potentially antagonistically regulate β-catenin phosphorylation in stem cells. However, to the research has been conducted on the role of Notch1 and β-catenin in skeletal muscle, therefore the outcome of Notch on β-catenin phosphorylation in muscle unknown.

Kwon et al. (2011) demonstrated that Numb and Notch together have the capacity to regulate β-catenin activity. Numb and Numb-Like bind to the β-catenin-Notch complex and traffic the complex to the lysosome for degradation. Kwon et al. observed that Numb was found to co-immunoprecipitated in conjunction with membrane bound Notch. In Numb and Numb-Like knockdown embryonic stem cells, membrane bound Notch was unable to repress β-catenin activity. Interestingly in Numb knockdown cells (via siRNAs), Numb-Like did have the capacity to bind to membrane bound notch and decrease active β-catenin protein levels (81). This another redundant function of Numb and Numb-Like indicating that both have the capacity to alter gene transcription. Lastly, Kown et al. investigated the effects of ibuprofen on these signaling pathways. Ibuprofen lowered the levels of active β-catenin via the notch RBP-J kappa luciferase activity in SW480 cancer cells. Therefore indicating that ibuprofen decreases the activity of the Notch signaling pathway and potentially contribute to the shielding effects of NSAIDS on induction of cancer cells (81).

2.6.9 Bone Morphogenetic Protein

Notch has been shown to interact with bone morphogenetic protein (BPM) to inhibit myogenic differentiation in C2C12 cells. BMP is a part of the transforming growth factor beta superfamily of ligands that inhibits differentiation independently of Notch signaling in satellite cells (132, 133). During BMP4 induced differentiation of C2C12 cells induced upregulation of both *HES1* and *HEY1* expression and increases in *HEY1* in satellite cells, which are downstream factors of canonical Notch signaling. This occurs due to BMP4 signaling situating mothers against decapentaplegic homolog 1 (SMAD1), a signal transducer and transcriptional modulator, which can activate the *HEY1* promoter. SMAD1 then binds to the promoter in a NICD dependent manner to induce the transcription of the target gene (132). Therefore, the potential cross talk between these two signaling pathways may further inhibit differentiation in myoblasts and satellite cells altering skeletal muscle repair.

2.6.10 Other interactions and regulators

Several inflammatory cytokines, myokines, growth factors and other gene pathways are suggested to have a critical role in the regulation of satellite cells function, proliferation and differentiation in response to exercise. However, it cannot be ruled out that other pathways are responsible for the proliferation and differentiation of satellite cells in human skeletal muscles. These genes in particular have key regulator roles in muscle damage, growth and repair. Therefore, the expression of these genes could potentially interact with Numb and Notch proteins to alter satellite cell proliferation and differentiation. Therefore, if given the opportunity these genes should be analyzed in conjunction with Numb and Notch genes and gene products.

2.8 Conclusion

Notch, Numb and Numb-Like as well as the potential modulators may have potential a role in skeletal muscle cell damage, repair and growth. The pathways involved are closely regulated in association with satellite cells. Previous research investigating the physiological pathway of Notch, Numb, and Numb-Like has been conducted in *Drosophila* and mouse models with limited research conducted studies in humans. To the researcher's knowledge there are limited studies that investigate the role of these genes and gene products after an acute bout of eccentric exercise. Also, the localization and interactions of the gene products has yet to be investigated in human skeletal muscle. The potential for these genes to mediate satellite cell activity could potentially lead to better understand of the physiologic mechanisms driving muscle adaptions to exercise, muscle damage and muscle atrophy.

Chapter III: Methods

3.1 Study design

This study utilized a randomized, counter balanced study design. Initially, 12 male subjects were randomly assigned to one of two groups: a control group that did not participate in any exercise (n = 6, age: 24 ± 4 years, height: 181.2 ± 5.8 cm, weight: 84.57 ± 12.92 kg, BMI: 25.67 ± 3.06) or an exercise group (n = 6, age: 21 ± 3, height: 177.8 ± 6.2 cm, weight: 76.13 ± 6.8 kg, BMI: 23.81 ± 3.06, 1RM: 99 ± 7 kg). Due to the acceptance of a grant provided by the Central States Chapter of the American College of Sports Medicine (CSACSM), an additional seven subjects were added to the damage group. Additionally, one damage and one control subject were removed due to being statistical outliers (control group: n = 5, age: 23 ± 4 years, height: 180.3 ± 6.1 cm, weight: 84.5 ± 14.5 kg BMI: 25.86 ± 3.38, and damage group: n = 12, age: 27 ± 6 years, height: 180.0 ± 6.8 cm, weight: 84.07 ± 8.80 kg, BMI: 27.08 ± 6.01 1RM: 107 ± 12 kg). Subjects were recruited from the University of Kansas and the surrounding area. The following inclusion and exclusion criteria were used to determine the subjects' eligibility for this study: Inclusion: These criteria includes being a healthy male, between the ages of 18-40, non-smoking, and free of metabolic or cardiovascular diseases. Exclusion: Exclusion criteria included unhealed fractures, thrombophlebitis (blood clots), any foot or ankle surgeries within the past 6 months, any hip or knee surgeries within the past year, recent uncontrolled bruising, recent muscle, bone or joint damage, osteomyelitis (acute or chronic bone infection), or myositis ossificans (hardened scarring in muscle tissue of the thigh). Subjects freely and willing signed an informed consent approved by The University of Kansas's Institutional Review Board. The participants freely and willingly signed an Institutional Review Board (IRB) approved informed consent document before participation and the research study abided by the Declaration of Helsinki (134).

3.2 Diet and Exercise Control Restrictions

Subjects were asked to maintain their current diet and refrain from alcohol, dietary supplements of any kind, and exercise during the course of the study. In addition, subjects were asked to refrain from over counter, non-steroidal, anti-inflammatory drugs (eg. Advil, Tylenol, Aspirin, or generic ibuprofen) due to their potential to alter the expression of genes and proteins under investigation.

3.3 One Repetition Maximum and Exercise Protocol

The one repetition maximum (1RM) protocol for leg extension (Universal Power Circuit, Leg Extension Model: EN11306/09132) consisted of an initial warmup of ten repetitions at a self-selected resistance performing. After one minute of rest the resistance was increased, and the participants were asked to complete six to eight repetitions. After two minutes of rest, the resistance was increased, and the subjects were asked to complete four to six repetitions. After three minutes of rest, participants completed two to three repetitions at a near maximal load. Single repetition maximum attempts were completed with increasing resistance, no more that 5-10% of previous resistance, until failure with three minutes of rest in-between sets. Immediately following the 1RM participants completed seven sets of ten repetitions of eccentric leg extension at 120% of 1RM with a two-minute rest period between sets. The lever arm of the knee extension machine was raised for each repetition and the subject lowered the lever arm at a controlled descent for approximately two to four seconds.

3.4 Muscle Biopsies

Three muscle biopsies of the *vastus lateralis* were performed via percutaneous needle biopsy technique with the aid of suction (135) Pre-, Day 2, and Day 5. Again, with the acceptance of the CSACSM grant and IRB approval of the modified protocol, a 3-Hour post-muscle damage time point was added with the additional seven subjects. The initial biopsy was performed on a randomized leg approximately 12 cm proximal to the patella from the *vastus lateralis*. The subsequent biopsy was taken from the alternate leg in previously mentioned manner. The third biopsy was taken two centimeters proximal to the initial biopsy incision site on the first leg. The samples were immediately cleared of excess blood and connective tissue and a portion of each sample was placed in RNA later solution (Ambion, Grand Island, NY). Another portion was flash frozen in liquid nitrogen and subsequently stored at -80°C for later analysis.

3.5 Gene Expression

Muscle biopsy samples were analyzed for the gene expression for Numb, Numb-Like and Notch1 (Applied Biosystems, Foster City, CA). Approximately 21.4 ± 3.6 mg of skeletal muscle was homogenized using an electric homogenizer (Fischer Scientific, Waltham, MA) in 600 ul of Trizol Reagent (Invitrogen, Carlsbad, CA). The RNA was further cleaned using a RNeasy mini kit (Qiagen, Valencia, CA). The RNA was reverse transcribed to cDNA via a High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA) per manufacturer protocol to produce cDNA. The cDNA served as a template to run the RT-qPCR (Rotor-Gene, Qiagen, Valencia, CA) fusing TaqMan real-time PCR assays (Applied Biosystems, Foster City, CA) for Numb, Numb-Like and Notch as well as a housekeeping gene: Beta-2-microglobuin (B2M) (Applied Biosystems, Foster City, CA), which has demonstrated stability in response to exercise.

Parameters were set at one hold 50 °C for two minutes, then a cycle at 95 °C for five minutes, then 40 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds. Analysis was completed with the corresponding software using the $2^{-\Delta\Delta Ct}$ model with the housekeeping gene (136). Gene expression was measured 3-Hour (control: n = 0, and experimental: n = 7) and Day 2 (control: n = 5, and experimental: n = 12) and Day 5 post-muscle damage (control: n = 5 and experimental: n = 12) for Numb, Numb-Like and Notch and normalized to the geometric mean of the stable reference genes and the pre-damage (control: n = 5 and experimental: n = 12) condition for each participant.

3.6 Protein Analysis

Muscle samples (15.2 ± 1.8 mg) were homogenized via tissue grind tube (Kimble Kontes LLC, Vineland, NJ) and lysed via TPERS (Fischer Scientific, Waltham, MA). A Bicinchoninic Acid Protein Assay (Pierce, Rockford, IL) was used to determine protein concentrations for each sample per manufacturer protocol. Approximately 3.88 ± 1.4 µL of protein were loaded into 12% acrylamide gels with a buffer solution and separated based on size of proteins via SDS-PAGE (Mini Protean 3, Bio-Rad Laboratories, Hercules, CA). The proteins were transferred to a polyvinylidene difluoride membrane (Thermo Fischer Scientific, Waltham, MA) via electrophoresis (Mini Protean 3, Bio-Rad Laboratories, Hercules, CA). After blocking overnight in 5% milk solution (Carnation Instant Powder, Nestle, Arlington, VA) in a solution containing a primary antibody specific for Numb (dilution: 1:1000, Cell Signaling Technology, Inc., Beverly, MA), Numb-Like (dilution: 1:1000, Santa Cruz Biotechnology, Inc., Dallas, TX) and Notch1 (dilution: 1:1000, eBioscience™, San Diego, CA) independently, the membranes were washed and incubated in secondary IgG antibodies anti-mouse (dilution: 1:2000, Cell Signaling Technology, Inc., Beverly, MA), anti-rabbit (dilution: 1:2000, Cell Signaling Technology, Inc.,

Beverly, MA) or (anti-goat dilution: 1:5000, Enzo Life Sciences, Plattsmouth Meeting, PA) respectively. Following more washes, the membranes were incubated with enhanced horseradish peroxidase chemiluminescent substrate (GE Healthcare Bio-sciences Corp., Piscataway, NJ) for five minutes. Finally, the membranes were imaged using a digital camera imaging system (FluorChem SP, Protein Simple, Santa Clara, CA) and Western blots were quantified using AlphaView software (Protein Simple, Santa Clara, CA). Protein expression was measured Pre- (control: n = 5, and experimental: n = 12), 3-Hours (control: n = 0, and experimental: n = 7) and Day 2 (control: n = 5, and experimental: n = 12) and Day 5 post-muscle damage (control: n = 5 and experimental: n = 12) for Numb and Numb-Like. Notch protein analysis utilized an n = 3 for the control group due to COVID-19 interruption of data analysis and upon reopening of The University of Campus, two additional subjects will be analyzed.

3.7 Statistical Analysis

The muscle biopsy samples were analyzed to determine the gene expression of Notch, Numb and Numb-Like mRNA using RT-qPCR, and the protein products were analyzed using Western blotting and immunohistochemistry staining. Due to the addition of seven subjects to the damage group and the 3-hour post-damage time point, the results were analyzed using two separate analyses. The first being a 2X3 (Group X Time) Two-Way Repeated Measures ANOVA for Pre, Day 2 and Day 5 time points with the exclusion of the 3-Hour post damage time point. The second being a One-Way Repeated Measures ANOVA with only the experimental group at Pre, 3-Hour Day 2, and Day 5 post-muscle damage. If significance was detected, a Fisher protected LSD post-hoc analysis was performed to detect where differences occurred. The significance level was set

at $p < 0.05$. All data was analyzed using Statistical Package for Social Sciences software (SPSS 25.0) (Chicago, IL) and reported as mean ± SE.

Chapter IV: Results

Due to the additional grant funding awarded later in the project timeline, there were no samples collected for the control group at three hours post-muscle damage.

4.1 Notch1

The Two-Way Repeated Measures ANOVA revealed no significant differences in mRNA expression were observed for *Notch1* between groups at Day 2 ($p = 0.56$). However, there was a significant increase in *Notch1* at Day 5 between the damage group (1.91 ± 1.29 fold change) and the control group (0.52 ± 0.38 fold change) from baseline measures ($p = 0.04$, Figure 4A). There were no significant differences detected in the Notch1 protein between groups (Pre = 0.17, Day 2 $p = 0.77$, and Day 5 $p = 0.66$, Figure 4B). Additionally, there was no significant differences in mRNA (3-Hour $p = 0.84$, Day 2 $p = 0.28$, and Day 5 $p = 0.20$, Figure 4A) or protein (3-Hour $p = 0.82$, Day 2 $p = 0.87$, and Day 5 $p = 0.30$, Figure 4B) expression from baseline when the One-Way Repeated Measure ANOVA was utilized.

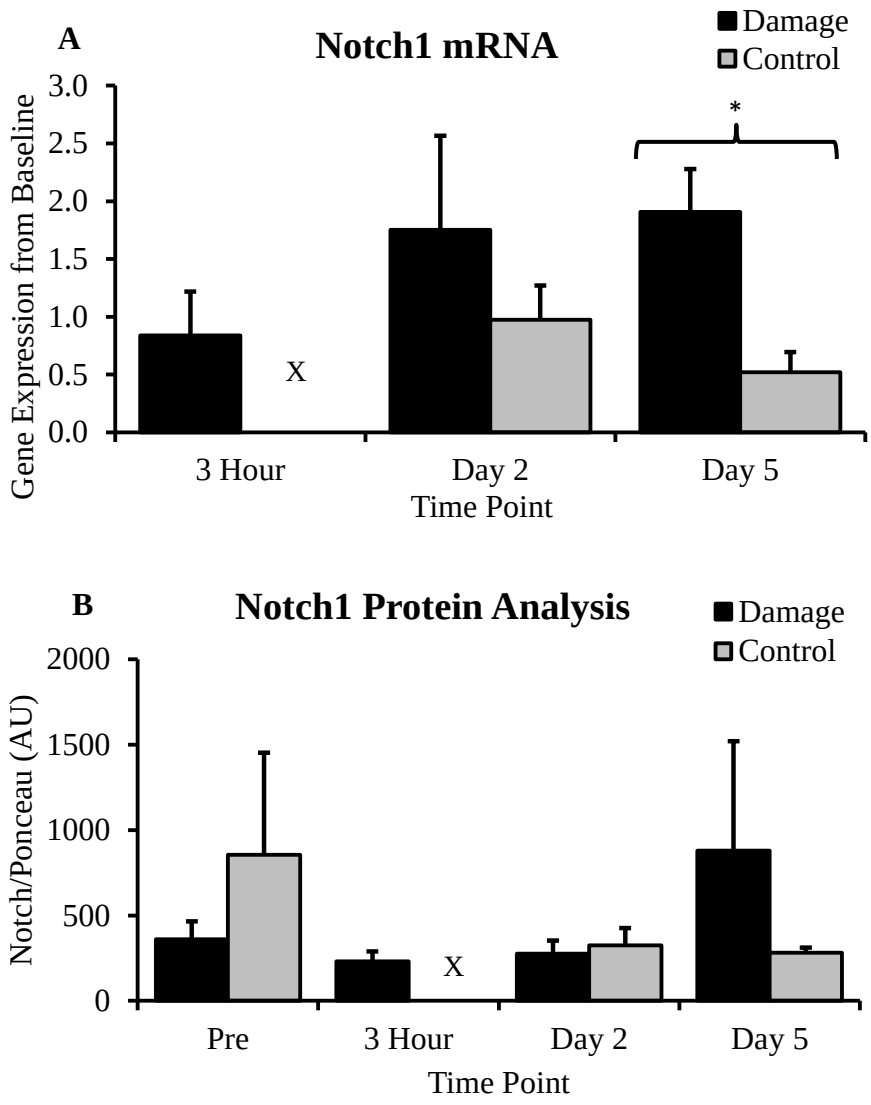

Figure 4: Notch mRNA (A) and protein (B) expression, * $p < 0.05$ and X = no data obtained. Notch mRNA expression is significantly greater at Day 2 in the damage group compared to the control group from baseline measures. Data are mean ± SE.

4.2 Numb

The Two-Way Repeated Measures ANOVA revealed no significant differences in *Numb* mRNA expression between groups Day 2 and Day 5 from baseline measures ($p = 0.74$ and $p = 0.13$,

respectively, Figure 5A). There were no significant differences detected in the Numb protein between groups (Pre = 0.21, Day 2 p = 0.75, and Day 5 p = 0.55, Figure 5B). Additionally, there was no significant differences in *Numb* mRNA (3-Hour p = 0.22, Day 2 p = 0.89, and Day 5 p = 0.15, Figure 5A) or protein (3-Hour p = 0.68, Day 2 p = 0.78, and Day 5 p = 0.24, Figure 5B) expression from baseline when the One-Way Repeated Measure ANOVA was utilized.

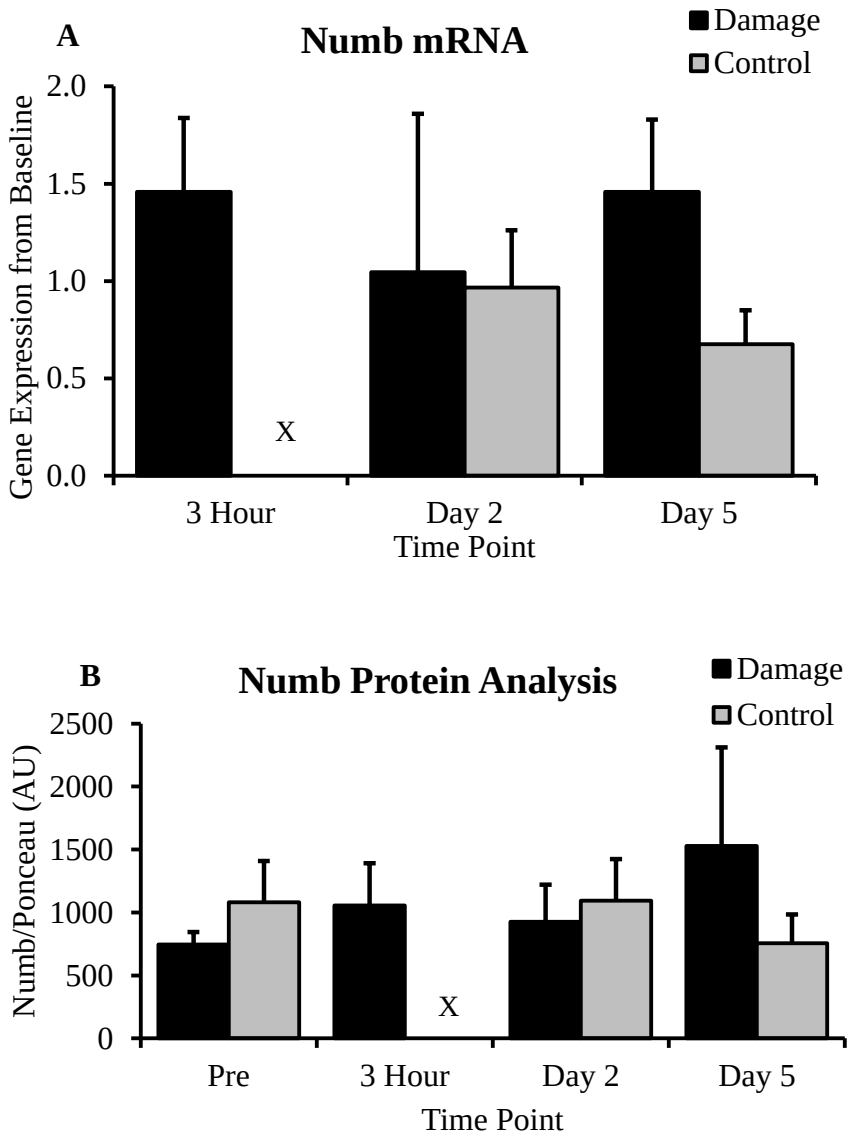

Figure 5: Numb mRNA (A) and protein (B) expression, * $p < 0.05$ and X = no data obtained. Data are mean ± SE.

4.3 Numb-Like

The Two-Way Repeated Measures ANOVA revealed no significant differences in *Numb-Like* mRNA expression between groups Day 2 and Day 5 from baseline measures ($p = 0.60$ and $p = 0.23$, respectively, Figure 6B). There were no significant differences detected in the Numb-Like protein between groups (Pre = 0.81, Day 2 $p = 0.68$, and Day 5 $p = 0.87$, Figure 6B). Additionally, there was no significant differences in *Numb-Like* mRNA (3-Hour $p = 0.63$, Day 2 $p = 0.30$, and Day 5 $p = 0.77$, Figure 6B) or protein (3-Hour $p = 0.52$, Day 2 $p = 0.75$, and Day 5 $p = 0.61$, Figure 6B) expression from baseline when the One-Way Repeated Measure ANOVA was utilized.

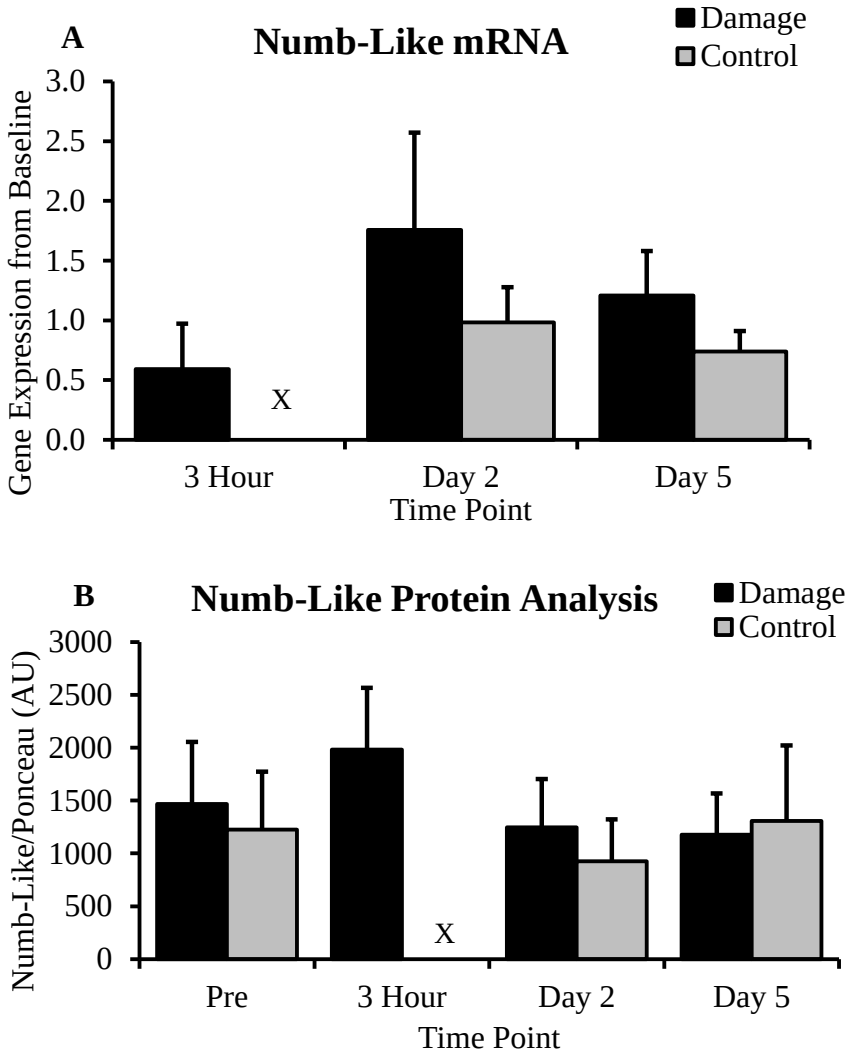

Figure 6: Numb-Like mRNA (A) and protein (B) expression, * $p < 0.05$ and X = no data obtained. Data are mean ± SE.

Chapter V: Discussion

The roles of Notch, Numb and Numb-Like as potential regulators of satellite cell proliferation and differentiation in human skeletal muscle are poorly understood. Eccentric exercise can induce muscle damage, altering the activity of satellite cells (137) and the expression of Notch, Numb and Numb-Like. Only one study has investigated the responses of Numb and Notch to an acute bout of resistance exercise (10). However, no studies have determined the responses of Notch, Numb and Numb-Like to an acute bout of eccentric resistance exercise. Thus, we aimed to determine the responses of Notch, Numb and Numb-Like to eccentric-exercise induced muscle damage.

Our results demonstrate a significant increase in *Notch1* mRNA at Day 5 post-muscle damage between the exercise group (1.91± 0.37 fold change) and the control group (0.52 ± 0.17 fold change) from baseline measures. These findings are in congruence with Carey et al. (2007), which demonstrated a significant increase in Notch3 mRNA expression over the course of a 72-hour differentiation time course, peaking at 48-hours, in human primary skeletal muscle cell cultures from the *vastus lateralis* of young, healthy males. Thus, indicating Notch may have a potential role in satellite cell differentiation, which is a contradiction to previous research in animal models and neuronal tissues (138-140). However, in vivo, there were no significant differences in *Notch1* mRNA expression after 3 sets of 12, maximal single leg extension (10). The lack of change in the control group indicates the differences observed were a result of the exercise intervention and not the repeated percutaneous needle biopsies. Collectively, these data suggest that *Notch1* mRNA expression occurred later in the muscle repair process, indicating Notch1 may have a role in satellite cell differentiation. There are several possibilities that could explain the contradictory evidence. The constitutive expression and repression of genes may result in deviations from physiological norms, therefore altering the regulator role of genes and proteins in question (141).

This theory could also explain the differing results seen in cell culture and in vivo. Cui and colleagues (2019) demonstrated that *Notch1* expression is upregulated five days after upstream stimulation, and *Notch1* is vital for differentiation of Mesenchymal stem cells (67). Carey et al. suggested that the increase in *Notch3* expression seen in culture corresponds with an increase in myogenin expression, a marker of differentiation. Therefore, Notch expression may be low during the early stages of myogenesis and expressed later during differentiation of myoblasts (10). These data, in conjunction with previous research, indicate Notch may stimulate proliferation and differentiation in progenitor cells, depending on temporal regulation and levels of expression. Alternatively, the activation of Notch in the later stages of muscle growth could indicate a return to quiescence and inhibition of myogenesis. Bi and colleagues (2019) demonstrated that activation of Notch1, via stage-specific Cre alleles, in myocytes results in dedifferentiation to Pax-7 positive, quiescent satellite cells. This resulted in decreased muscle growth and resulted in postnatal lethality. However, Notch1 expression in myotubes improves muscle regeneration and increases exercise performance in age and diseased models (68). These data represent non-physiological states and therefore Notch signaling in vivo, in myofibers warrants further investigation.

Based on previous research it was hypothesized that Numb mRNA and protein would increase significantly post-muscle damage with little to no change in Numb-Like expression. This investigation utilized antibodies that bound to all four isoforms of Numb, and contrary to this hypothesis, the main findings were that there were no significant changes in Numb or Numb-Like expression. The exact effect of Numb and Numb-Like's ability to repair muscle is unknown. However, our data suggests that Numb-Like is expressed to a greater degree compared to all four isoforms of Numb at two days post-muscle damage (1.05 ± 0.13 and 1.76 ± 0.91 fold changes,

respectively). Thus, Numb-Like may constitute a greater role in muscle repair and satellite cell regulation in response to exercise-induced muscle damage. These data are dissimilar to previous investigations performed in muscle damage in rodents. George et al. (2013) determined that abolition via genetic deletion of Numb, not Numb-Like, resulted in significant decreases in regenerating myofiber size after the induction of muscle damage via barium chloride injections. Similarly, the Numb deficient satellite cells had decrease proliferation capacity, while Numb-Like deficient satellite cells did not results in a decreases in proliferation (108). These data indicated that Numb was the primary regulator of satellite cells and muscle regeneration after injury.

The dissimilarity between results may stem from Numb and Numb-Like having redundant functions, in part due to analogous structure (4). For example, mice that contain knockouts of either *Numb* or *Numb-Like* have less severe impairments of neurogenesis compared to mice that had knockouts of both *Numb* and *Numb-Like*. Therefore, it was concluded that Numb and Numb-Like function in conjunction with one another (110, 112). Interestingly, low levels of Numb have the capacity to salvage Numb and Numb-Like double null mutants during embryogenesis in mice, suggesting small quantities of Numb protein are necessary for function (15). However, it is possible that Numb-Like can salvage the myogenic or embryogenesis pathways in human skeletal muscle in the absence of Numb signaling due to redundancy. Although these homologs have redundant functions, they also have independent functions. During proliferation, satellite cells undergo symmetric or asymmetric division. This balance is critical for satellite cell and skeletal muscle maintenance and homeostasis. During symmetrical division, two identical undifferentiated daughter cells are generated. Throughout asymmetrical division, one daughter cell maintains self-renewal while the other cell progresses through differentiation (25, 26). Numb is asymmetrically

divided to the daughter cells while Numb-Like is symmetrically distributed in *Drosophila* neuronal precursor cells (4). When Numb fails to divide asymmetrically, it produces two daughter cells with the fate determined via Numb-Like asymmetrical division, therefore adding to the redundancy of these two homologs. It should be noted that this was only seen in neocortex of *Drosophila* (4). Evidence has shown that Numb and Numb-Like have distinctive, yet redundant functions and further investigation is necessary to determine the functionality of Numb and Numb-Like.

The differences in the current results compared to previous investigations could be due to utilization of cell cultures compared to muscle homogenate, training status, timing of sample collections and age. Notch, Numb and Numb-Like are expressed across multiple tissues (4, 89, 95, 138). However, in skeletal muscle the primary expression of these proteins and genes are in satellite cells and immature muscle cells (26, 74, 128, 142). To the researcher's knowledge, there is little to no evidence that suggests the expression of Notch, Numb, and Numb-Like in myofibers. This study utilized skeletal muscle homogenate and therefore the changes in expression are most likely due to alterations within the satellite cells, myoblasts, or myocytes. Carey et al. (2007), observed a significant decrease in aging skeletal muscle compared to young muscle and a significant increase in *Notch* and *Numb* mRNA after 12-weeks of resistance training. Resistance training stimulates an increase in the number and activity of satellite cells and myonuclei (143).Therefore, tissue preparation, training status and age may alter satellite cell quantity and activity modifying Notch, Numb and Numb-Like expression (10, 144).

In addition to Notch, Numb and Numb-Like, other satellite cell regulators such as Pax-7, MyoD, FOXO1, Wnt, Myostatin and Myogenin are in flux after a bout of muscle injury (47, 49, 57, 142, 145, 146). These proteins, in addition to others, could regulate satellite cell activity and meditate the Notch, Numb and Numb-Like pathway. Inflammatory markers such as Interleukin-6 and hepatocyte growth factor also induce satellite cell quiescence and reduce proliferation of satellite cells. Therefore, it cannot be excluded that other mechanisms could potentially regulate satellite cell proliferation and differentiation in response to exercise-induced muscle damage (22). Figure 3 represents the hypothesized timeline of gene expression of Numb, Numb-Like, and Notch in conjunction with other known regulators of satellite cells.

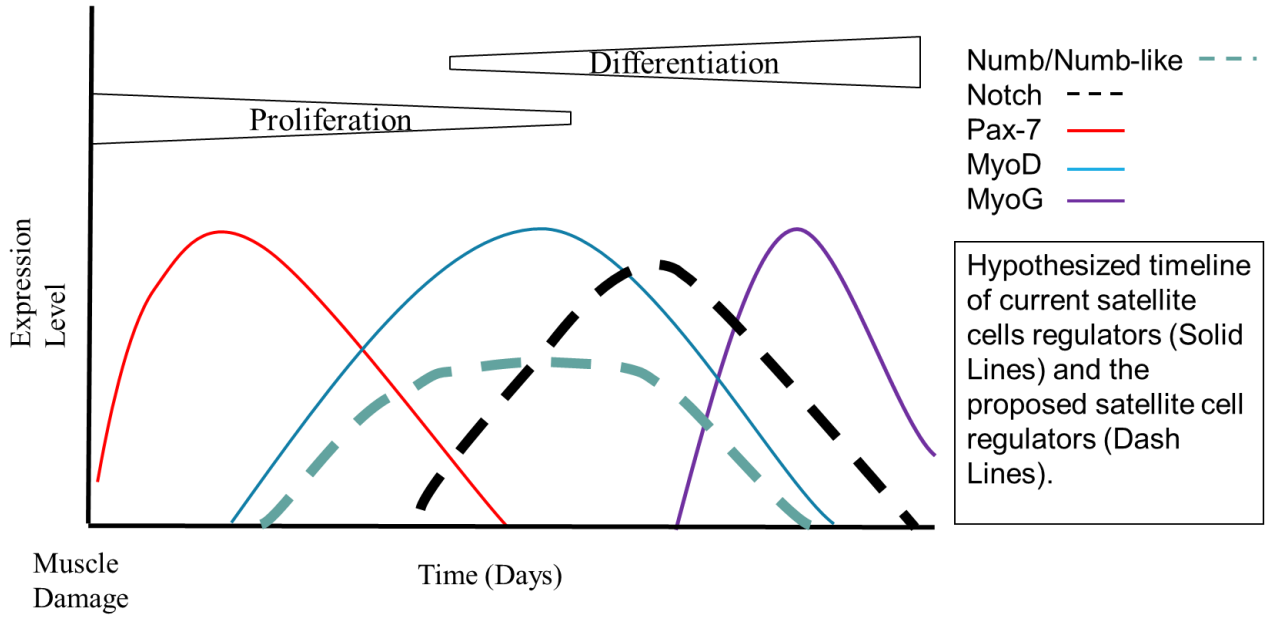

Figure 4: The hypothesized timeline of current satellite cell regulators (solid lines) and the proposed satellite cell regulators (dashed lines). Notch (Black), Numb and Numb-Like (Green), Pax-7(Red), MyoD (Blue) and Myogenin (Purple).

In conclusion, the current data indicates that Numb and Numb-Like expression in humans are unaltered post-muscle damage, while Notch mRNA expression is increased five days after muscle

damage. The temporal regulation and expression of Notch1, at five days post-muscle damage, may reinforce the concept that Notch may regulate satellite cell differentiation in human skeletal muscle after an acute bout of exercise, in addition to proliferation. Fujimaki et al. (2019), indicates that Notch1 and Notch2 are structurally similar and participate symmetrical division. Correspondingly, activation Notch1 Notch2and may prevent differentiation and induce renewal of the satellite cell pool (17). Prior evidence indicates that as early as four days after an acute bout of resistance exercise, satellite cell number increases (37). However, further research is needed to determine if Notch is participating in proliferation or differentiation of satellite cells after exercise. The multiple isoforms of Numb and homogeneity of Numb and Numb-Like add to the potential redundancy of function and difficulty of investigation. However, the results of this study indicate the potential different roles of Numb and Numb-Like. Differences seen among studies can be attributed to timing of muscle sample collections, population utilized, an increase in sample size and greater muscle damage protocols. These variables increase the difficulty of understanding the physiological mechanisms of Notch, Numb and Numb-Like. Further research is needed in order to understand the mechanism of Notch, Numb and Numb-Like in human skeletal muscle post-exercise induced muscle damage as regulators of satellite cell markers of proliferation and differentiation. This could potentially lead to better understanding of satellite cell proliferation and differentiation in skeletal muscle and adaptations to exercise.

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Appendix A: Raw Data

Demographic Data

Subject	Ht (cm)	Wt (kg)	Age (yrs)	Leg Ex Max (kgs)	120% (kgs)	BMI (kg/M^2)
2	171.00	68.42	29			23.40
3	185.00	79.06	20	90.72	108.86	23.10
4	182.50	100.76	21			30.25
5	185.00	82.40	19	108.86	131.54	24.08
6	187.00	87.82	25			25.11
7	169.50	73.38	21	104.33	124.74	25.54
8	178.20	70.42	20			22.18
9	178.00	78.02	19	95.26	113.40	24.62
10	182.90	94.88	19			28.36
11	174.00	63.70	21	90.72	108.86	21.04
12	185.50	85.14	27			24.74
13	181.00	80.22	26	104.33	124.74	24.49
14	185.00	130.42	30	113.40	136.08	38.11
15	192.50	92.86	23	104.33	124.74	25.06
16	179.00	130.54	20	113.40	136.08	40.74
17	177.50	86.86	30	122.47	147.42	27.57
18	183.50	88.22	23	131.54	158.76	26.20
19	170.00	70.48	27	108.86	131.54	24.39
20	181.50	80.84	27	104.33	124.74	24.54
Damage Group (n = 6)						
Damage Mean (n = 6)	178.75	76.13	21	99.04	118.69	23.81
Stdv Damage (n = 6)	6.19	6.79	3	7.81	9.59	1.57
Std Er Damage (n = 6)	2.53	2.77	1	3.19	3.91	0.64
Control Group (n = 6)						
Control Mean (n = 6)	181.18	84.57	24			25.67
Stdv Control (n = 6)	5.83	12.96	4			3.06
Std Er Control (n = 6)	2.38	5.29	2			1.25
Damage Group Without Subject 13 (n = 12)						
Damage Mean	180.04	88.07	23	107.35	128.90	27.08
Stdv Damage	6.76373	21.3358	4	12.13	14.91404	6.01
Std Er Damage	1.95	6.16	1	3.50	4.31	1.73
Control Group Without Subject 12 (n = 5)						

Control Mean	180.32	84.46	23			25.86
Stdv Control	6.07	8.80	4			3.38
Std Er Control	2.71	6.48	2			1.51

RT-qPCR mRNA Expression

Raw Data					
Subject #	**Numb**	**Numb-Like**	**Notch**	**B2M**	**Group**
2 Pre	21.79	22.52	21.22	16.43	Control
2 Day 2	23.72	24.43	23.99	17.57	Control
2 Day 5	25.6	24.15	24.28	17.12	Control
3 Pre	28.01	32.68	30.73	22.56	Damage
3 Day 2	22.22	23.92	22.11	17.35	Damage
3 Day 5	24.19	26.83	24.49	18.28	Damage
4 Pre	21.67	23.93	23.09	16.32	Control
4 Day 2	23.09	24.48	23.54	17.59	Control
24Day 5	25.66	27.61	26.71	20.13	Control
5 Pre	21.81	23.77	21.22	16.63	Damage
5 Day 2	22.16	23.72	21.94	17.14	Damage
5 Day 5	21.78	22.74	20.74	16.48	Damage
6 Pre	23.1	23.88	21.99	17.29	Control
6 Day 2	22.27	24.03	21.22	17.16	Control
6 Day 5	22.17	23.54	23.33	16.46	Control
7 Pre	26.35	27.37	27.4	21.55	Damage
7 Day 2	21.17	22.13	20.9	15.7	Damage
7 Day 5	20.27	21.62	19.28	15.54	Damage
8 Pre	20.28	21.16	19.49	15.02	Control
8 Day 2	21.51	21.45	20.49	15.76	Control
8 Day 5	21.9	22.68	21.98	16.27	Control
9 Pre	21.46	24.26	22.92	17.05	Damage
9 Day 2	27.45	28.99	28.75	22.65	Damage
9 Day 5	22.83	23.59	23.1	17.66	Damage
10 Pre	20.12	21.28	21.02	15.36	Control
10 Day 2	19.57	21.78	21.83	14.83	Control
10 Day 5	20.36	21.54	21.05	14.71	Control
11 Pre	27.66	28.04	28.54	20.81	Damage
11 Day 2	26.75	28.58	29.1	20.11	Damage
11 Day 5	21.45	22.02	22.33	14.8	Damage
12 Pre	24.03	24.55	25.68	18.5	Control
12 Day 2	27.22	29.8	26.99	20.65	Control
12 Day 5	25.65	27.07	25.36	20.44	Control

13 Pre	24.39	26.88		19.69	Damage
13 Day 2	27.18	30.55		20.59	Damage
13 Day 5	25.6	28.79		21.14	Damage
14 Pre	30.06	30.3	30.15	24.14	Damage
14 3-Hour	23.88	25.06	25.19	17.17	Damage
14 Day 2	22.15	24.29	24.17	16.85	Damage
14 Day 5	23.05	24.34	24.05	17.76	Damage
15 Pre	21.79	21.86	23.09	15.97	Damage
15 3-Hour	21.96	22.54	24.02	14.28	Damage
15 Day 2	20.17	20.58	21.43	13.7	Damage
15 Day 5	21.2	23.76	22.75	16.45	Damage
16 Pre	25.83	25.14	26.47	18.93	Damage
16 3-Hour	22.81	23.26	24.76	17.15	Damage
16 Day 2	22.99	24.1	24.36	16.96	Damage
16 Day 5	21.7	22.77	22.6	16.4	Damage
17 Pre	23.34	24.34	24.87	17.19	Damage
17 3-Hour	25.23	29.11	31.08	19.61	Damage
17 Day 2	22.3	22.57	22.5	15.31	Damage
17 Day 5	25.28	30.91	30.6	18.53	Damage
18 Pre	22.15	22.48	21.82	14.56	Damage
18 3-Hour	20.14	21.79	20.13	14.41	Damage
18 Day 2	21.64	22.56	22.25	14.48	Damage
18 Day 5	20.13	21.34	20.69	14.49	Damage
19 Pre	22.16	21.46	22.37	15.01	Damage
19 3-Hour	20.74	22.12	21.37	14.26	Damage
19 Day 2	22.52	22.36	22.72	15.53	Damage
19 Day 5	22.56	23.12	21.68	15.85	Damage
20 Pre	21.43	22.09	22.39	15.94	Damage
20 3-Hour	22.02	21.92	22.89	14.9	Damage
20 Day 2	22.97	22.18	22.05	15.99	Damage
20 Day 5	21.23	21.38	21.53	14.72	Damage

2^-ΔΔCT			
Subject #	**Numb**	**Numb-Like**	**Notch**
2 Pre			
2 Day 2	0.578344092	0.5864175	0.323088208
2 Day 5	0.115023456	0.5212329	0.193445624
3 Pre			
3 Day 2	1.494849249	11.712686	10.62948651
3 Day 5	0.726986259	2.9690471	3.89061979
4 Pre			

4 Day 2	0.901250463	1.647182	1.765405993
4 Day 5	0.882702996	1.0942937	1.140763716
5 Pre			
5 Day 2	1.117287138	1.4742692	0.864537231
5 Day 5	0.920187651	1.8403753	1.257013375
6 Pre			
6 Day 2	1.624504793	0.823591	1.558329159
6 Day 5	1.071773463	0.7120251	0.22221067
7 Pre			
7 Day 2	0.628506687	0.6551967	1.569168196
7 Day 5	1.049716684	0.8350879	4.316912946
8 Pre			
8 Day 2	0.712025098	1.3660403	0.835087919
8 Day 5	0.773782497	0.8293195	0.423372656
9 Pre			
9 Day 2	0.763129604	1.8276629	0.852634892
9 Day 5	0.590496331	2.4283898	1.347233577
10 Pre			
10 Day 2	1.01395948	0.4897101	0.395020656
10 Day 5	0.539614118	0.5321851	0.624165274
11 Pre			
11 Day 2	1.156688184	0.4233727	0.41754396
11 Day 5	1.148698355	1.0069556	1.148698355
12 Pre			
12 Day 2	0.486327474	0.1166291	9.781122222
12 Day 5	1.248330549	0.6689638	4.789914818
13 Pre			
13 Day 2	0.269807059	0.1466044	1.866065983
13 Day 5	1.180992661	0.7269863	2.732080514
14 Pre			
14 3-Hour	0.578344092	0.301452	0.248273124
14 Day 2	1.536875181	0.4117955	0.40332088
14 Day 5	1.547564994	0.7474246	0.823591017
15 Pre			
15 3-Hour	0.275476279	0.1934456	0.162667732
15 Day 2	0.637280314	0.5034778	0.655196702
15 Day 5	2.099433367	0.3737123	1.765405993
16 Pre			
16 3-Hour	2.361985323	1.0717735	0.952637998
16 Day 2	1.8276629	0.5248583	1.101905116
16 Day 5	3.031433133	0.8950251	2.531513188
17 Pre			

17 3-Hour	1.443929196	0.196146	0.072293011
17 Day 2	0.558643569	0.9265881	1.404444876
17 Day 5	0.659753955	0.0266448	0.0476956
18 Pre			
18 3-Hour	3.630076621	1.4539725	2.907945035
18 Day 2	1.347233577	0.8950251	0.702222438
18 Day 5	3.863745316	2.0994334	2.084931522
19 Pre			
19 3-Hour	1.591072968	0.3763117	1.189207115
19 Day 2	1.117287138	0.7684376	1.125058485
19 Day 5	1.356604327	0.5664419	2.887858391
20 Pre			
20 3-Hour	0.323088208	0.5471469	0.343885455
20 Day 2	0.356012549	0.9726549	1.310393404
20 Day 5	0.493116352	0.7022224	0.77916458

Western Blot: Micro BCA Results

Subject	Sample Vol (µl)	Buffer Vol (µl)	Final Vol (µl)	Load (ul)
14 Pre	2.89	8.66	11.54	11.54
14 3-Hour	2.25	6.74	8.99	8.99
14 Day 2	3.78	11.35	15.13	15.13
14 Day 5	2.88	8.64	11.53	11.53
15 Pre	2.84	8.53	11.38	11.38
15 3-Hour	3.07	9.20	12.26	12.26
15 Day 2	2.39	7.18	9.58	9.58
15 Day 5	2.52	7.55	10.06	10.06
16 Pre	2.99	8.97	11.96	11.96
16 3-Hour	2.99	8.98	11.97	11.97
16 Day 2	3.01	9.04	12.05	12.05
16 Day 5	2.80	8.41	11.21	11.21
17 Pre	2.84	8.52	11.35	11.35
17 3-Hour	2.88	8.65	11.54	11.54
17 Day 2	2.59	7.76	10.35	10.35
17 Day 5	2.38	7.14	9.53	9.53
18 Pre	2.86	8.58	11.44	11.44
18 3-Hour	2.96	8.88	11.83	11.83
18 Day 2	3.26	9.77	13.02	13.02
18 Day 5	2.29	6.86	9.15	9.15
19 Pre	2.80	8.39	11.19	11.19
19 3-Hour	2.64	7.91	10.55	10.55
19 Day 2	2.67	8.02	10.69	10.69

19 Day 5	2.46	7.38	9.83	9.83
20 Pre	2.36	7.08	9.43	9.43
20 3-Hour	2.99	8.98	11.97	11.97
20 Day 2	2.78	8.35	11.13	11.13
20 Day 5	2.98	8.95	11.93	11.93
3 Pre	4.78	14.33	19.11	19.11
3 Day 2	1.86	5.59	7.45	7.45
3 Day 5	4.71	14.13	18.84	18.84
5 Pre	3.73	11.19	14.92	14.92
5 Day 2	4.18	12.53	16.71	16.71
5 Day 5	3.54	10.62	14.16	14.16
7 Pre	5.64	16.93	22.58	22.58
7 Day 2	3.91	11.72	15.63	15.63
7 Day 5	4.45	13.36	17.81	17.81
9 Pre	3.44	10.32	13.75	13.75
9 Day 2	4.26	12.79	17.06	17.06
9 Day 5	3.50	10.51	14.01	14.01
11 Pre	3.96	11.87	15.83	15.83
11 Day 2	4.79	14.36	19.14	19.14
11 Day 5	7.01	21.04	28.05	28.05
2 Pre	5.44	16.33	21.78	21.78
2 Day 2	6.89	20.68	27.57	27.57
2 Day 5	4.90	14.69	19.59	19.59
4 Pre	6.80	20.40	27.20	27.20
4 Day 2	5.56	16.67	22.23	22.23
4 Day 5	4.27	12.80	17.06	17.06
6 Pre	6.96	20.87	27.82	27.82
6 Day 2	4.17	12.51	16.69	16.69
6 Day 5	5.56	16.67	22.23	22.23
8 Pre	5.57	16.72	22.30	22.30
8 Day 2	4.76	14.28	19.04	19.04
8 Day 5	5.60	16.79	22.39	22.39
10 Pre	5.29	15.87	21.16	21.16
10 Day 2	6.15	18.44	24.58	24.58
10 Day 5	5.48	16.43	21.91	21.91

Western Blot Poncaeu 3 Trace Average

Notch Poncaeu Average of 3 Tracings					
Subject	**Group**	**Pre**	**3-Hour**	**Day 2**	**Day 5**
2	Control				
3	Damage	12672		25196	22793
4	Control				
5	Damage	17243		28869	25935
6	Control	13132		16129	12747

7	Damage	13910		11743	18181
8	Control	46235		30591	32983
9	Damage	23952		19631	17261
10	Control	16122		22260	23031
11	Damage	26461		23667	2711
14	Damage	140680	166573	175059	141752
15	Damage	180656	177461	148065	171855
16	Damage	157790	173382	150630	162361
17	Damage	10599	11103	11682	10639
18	Damage	36964	41265	30891	39676
19	Damage	46475	47517	37576	51682
20	Damage	52370	55548	533850	60331

Numb Poncaeu Average of 3 Tracings					
Subject	**Group**	**Pre**	**3-Hour**	**Day 2**	**Day 5**
2	Control	49654		49047	66521
3	Damage	100022		57137	117098
4	Control	54999		50625	46617
5	Damage	43175		50551	36339
6	Control	14785		27007	30600
7	Damage	112699		123898	106663
8	Control	110458		111920	93497
9	Damage	64159		46794	51743
10	Control	32193		38237	36686
11	Damage	32124		40026	36284
14	Damage	15396	12992	26905	22232
15	Damage	32752	35532	41576	55646
16	Damage	50793	56745	47306	42869
17	Damage	18171	20486	8482	20936
18	Damage	22033	20693	10894	19594
19	Damage	18715	21852	17080	15982
20	Damage	33154	37133	36526	3012

Numb-Like Poncaeu Average of 3 Tracings					
Subject	**Group**	**Pre**	**3-Hour**	**Day 2**	**Day 5**
2	Control	5756		6259	7461
3	Damage	4970		3628	4260
4	Control	4067		4218	3402
5	Damage	3645		4949	4915
6	Control	5762		7586	12952
7	Damage	19999		24188	15025
8	Control	5471		9776	8928

9	Damage	19065		17719	18747
10	Control	34828		31400	40265
11	Damage	43385		38583	38570
14	Damage	35617	31042	23988	21563
15	Damage	39520	25167	34781	46929
16	Damage	42553	47099	42818	46145
17	Damage	8233	17052	19698	16442
18	Damage	19310	17081	15935	17666
19	Damage	23091	23206	22897	19174
20	Damage	40760	35693	37470	36408

Western Blot Image 3 Trace Average

Notch Average of 3 Tracings					
Subject	**Group**	**Pre**	**3-Hour**	**Day 2**	**Day 5**
2	Control				
3	Damage	5334		21299	5247
4	Control				
5	Damage	1067		3712	11687
6	Control	5823		3980	4144
7	Damage	5654		3935	4429
8	Control	3988		6253	9715
9	Damage	1319		176	1261
10	Control	32818		11704	5169
11	Damage	14806		3433	21442
14	Damage	9815	10379	8196	481
15	Damage	32455	22687	8157	7299
16	Damage	14445	5116	18717	12716
17	Damage	13957	3483	2505	1864
18	Damage	6685	14007	6416	16077
19	Damage	31437	15435	21951	16760
20	Damage	15876	23300	328780	37080

Numb Average of 3 Tracings					
Subject	**Group**	**Pre**	**3-Hour**	**Day 2**	**Day 5**
2	Control	15098		21175	4874
3	Damage	49140		12767	52436
4	Control	35973		17303	16271
5	Damage	23687		49186	54170
6	Control	18769		56594	35105
7	Damage	68195		26346	21806
8	Control	104884		168282	113021
9	Damage	44648		35310	52248

10	Control	71697		41849	36548
11	Damage	43880		27081	23523
14	Damage	9856	6329	14809	6572
15	Damage	10853	12842	36091	2282
16	Damage	16242	12332	19866	1669
17	Damage	17559	44814	33846	31049
18	Damage	13890	17236	4451	31049
19	Damage	22033	19571	22424	18019
20	Damage	38811	89429	26510	29979

Numb-Like Average of 3 Tracings					
Subject	**Group**	**Pre**	**3-Hour**	**Day 2**	**Day 5**
2	Control	13797		10278	27560
3	Damage	5142		8355	15400
4	Control	11021		8813	7536
5	Damage	15838		10088	9068
6	Control	1581		4265	3295
7	Damage	3333		1951	1075
8	Control	3097		2170	851
9	Damage	2900		1609	2295
10	Control	6380		3383	11343
11	Damage	5952		3273	12178
14	Damage	29310	14735	7021	3351
15	Damage	25780	52347	35245	33842
16	Damage	20342	11171	3293	4933
17	Damage	3198	13460	4414	4660
18	Damage	46799	52697	12259	65346
19	Damage	153007	65879	120689	21442
20	Damage	16126	155667	101605	75900

Western Blots Protein/Poncaeu (AU)

Notch/Poncaeu (AU)					
Subject	**Group**	**Pre**	**3-Hour**	**Day 2**	**Day 5**
2	Control				
3	Damage	421		845	230
4	Control				
5	Damage	62		129	451
6	Control	443		247	325
7	Damage	406		335	244
8	Control	86		204	295
9	Damage	55		9	73
10	Control	2036		526	224
11	Damage	560		145	7909

14	Damage	70	62	47	3
15	Damage	180	128	55	42
16	Damage	92	30	124	78
17	Damage	1317	314	214	175
18	Damage	181	339	208	405
19	Damage	676	325	584	324
20	Damage	303	419	616	615

Numb/Poncaeu (AU)					
Subject	**Group**	**Pre**	**3-Hour**	**Day 2**	**Day 5**
2	Control	304		432	73
3	Damage	491		223	448
4	Control	654		342	349
5	Damage	549		973	1491
6	Control	1269		2096	1147
7	Damage	605		213	204
8	Control	950		1504	1209
9	Damage	696		755	1010
10	Control	2227		1094	996
11	Damage	1366		677	648
14	Damage	640	487	550	296
15	Damage	331	361	868	41
16	Damage	320	217	420	39
17	Damage	966	2188	3990	1483
18	Damage	630	833	409	1585
19	Damage	1177	896	1313	1127
20	Damage	1171	2408	726	9953

Numb-Like/Poncaeu (AU)					
Subject	**Group**	**Pre**	**3-Hour**	**Day 2**	**Day 5**
2	Control	2397		1642	3694
3	Damage	1035		2303	3615
4	Control	2710		2089	2215
5	Damage	4345		2038	1845
6	Control	274		562	254
7	Damage	167		81	72
8	Control	566		222	95
9	Damage	152		91	122
10	Control	183		108	282
11	Damage	137		85	316
14	Damage	823	475	293	155

15	Damage	652	2080	1013	721
16	Damage	478	237	77	107
17	Damage	388	789	224	283
18	Damage	2424	3085	769	3699
19	Damage	6626	2839	5271	1118
20	Damage	396	4361	2712	2085