INFLUENCE OF NOTCH INHIBITION ON MTOR AND WNT SIGNALING IN REPAIRING MUSCLE

by

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A thesis submitted to the faculty of
the University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Master of Science in
Kinesiology

Charlotte

2023

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ABSTRACT

JAMES CARL WILLIAMS III. INFLUENCE OF NOTCH INHIBITION ON MTOR AND WNT SIGNALING IN REPAIRING MUSCLE
(UNDER THE DIRECTION OF DR. SUSAN T. ARTHUR)

Protein synthesis and muscle stem cells are crucial components of adequate muscle repair in healthy individuals that are dysregulated in a variety of diseases states. A key regulator of muscle stem cell activation and coordination of downstream muscle repair is the evolutionary conserved Notch signaling pathway. Notch signaling is instrumental in cell fate determination during embryonic myogenesis and mature tissue repair. Furthermore, the mechanistic target of rapamycin (mTOR) and Wingless/Integrated (Wnt) are closely linked to Notch signaling in a variety of other tissues and their interactions are widely studied. However, the exact mechanisms by which they coregulate in skeletal muscle is not fully understood. The convergence of these three signaling networks in skeletal muscle is an area which demands further exploration so effective therapies for the growing global obese and aged population may be developed. Our lab has previously measured the effect Notch1 shRNA knockdown had on muscle protein synthesis (MPS) and found Notch inhibition significantly increased mTOR signaling and MPS. Our attempt to elucidate the interaction of Notch, mTOR, and Wnt utilized Notch1 shRNA lentiviral knockdown and an injurious bout of downhill running. We investigated the effect of these interventions on key downstream regulators of mTOR and Wnt to understand the mechanisms by which Notch1 knockdown increases muscle protein synthesis. We observed no significant differences between empty vector and Notch1 inhibited limbs in P70S6K or 4EBP1, indicating Notch1 shRNA inhibition and DHR was not sufficient to induce changes. However, there were significant time-course changes in GSK3β expression, suggesting a relationship between Notch and Wnt signaling via GSK3β. These findings contribute to the growing understanding of the synergistic roles played by Notch, mTOR, and Wnt signaling in the muscle repair process.

ACKNOWLEDGEMENTS

Dr. Susan Arthur was indispensable in regard to the completion of this work. Her guidance and support helped me overcome the many obstacles that appeared along the way. I also want to thank my committee members Dr. Joseph Marino and Dr. Joshua Huot for their help in answering the plethora of questions I had concerning the laboratory work I accomplished. Lastly, I want to thank my parents, siblings, and close friends for their constant support and love that helped me stick it out through the whole process of earning my degree.

TABLE OF CONTENTS

LIST OF FIGURES

List of Tables

LIST OF ABBREVIATIONS

MuSCs	muscle satellite cells
BMI	body mass index
DALY	daily adjusted life years
HALE	health-adjusted life expectancy
T2DM	type 2 diabetes mellitus
IGF-1	insulin-like growth factor 1
AKT	protein kinase B
ANOVA	analysis of variance
IL-6	interleukin 6
RNA	ribonucleic acid
mRNA	messenger RNA
shRNA	short hair-pin loop RNA
STAT3	signal transducer and activator of transcription 3
LPL	lipoprotein lipase
HFD	high fat diet
ROS	reactive oxygen species
FGF19	fibroblast growth factor 19
Hey1	hairy/enhancer-of-split related with YRPW motif protein 1
Hey2	hairy/enhancer-of-split related with YRPW motif protein 2
Hes1	hairy and enhancer of split-1
MyoD	myoblast determination protein 1
Myf5	myogenic factor 5
MRFs	myogenic regulatory factors
MHC	myosin heavy chain

TGFβ	transforming growth factor beta
GSK3	Glycogen Synthase Kinase 3
P70S6K	S6 kinase 1
Wnt	Wingless/Integrated
eIF4E	eukaryotic translation initiation factor 4E
4EBP1	4E-binding protein 1

CHAPTER 1: INTRODUCTION

Notch signaling is known to coregulate various cell processes throughout the body with mechanistic target of rapamycin (mTOR) and Wingless/Integrated (Wnt) (Bertrand, 2020; Das & Hontiveros, 2020; Hibdon et al., 2019; McCubrey et al., 2014). There is evidence, albeit limited, that Notch, Wnt, and mTOR interact in skeletal muscle to regulate muscle repair (Brack et al., 2008; Gerrard et al., 2021; Huot et al., 2020). The data thus far indicates a multifaceted and complex relationship between these three signaling pathways that depends on combined inhibitory and stimulatory effects of their various factors. For one, the enzyme GSK3β, which is integral to Wnt signaling, has been implicated as a regulator in Notch and mTOR, acting as a nexus between the three signaling networks (Espinosa et al., 2003; Foltz et al., 2002; Inoki et al., 2006; Jin et al., 2009; Stretton et al., 2015). This study examines the effect of Notch signaling knockdown on the expression of the Wnt regulator GSK3β and the downstream translation factors of mTOR 4E-binding protein 1 (4EBP1) and S6 kinase 1.

This study is novel because we examine how Notch knockdown affects mTOR and Wnt after exercise-induced injury. Our lab prefers an exercise-induced muscle injury model because it is a more accurate representation of muscle damage sustained during daily life. In addition, muscle damage because of exercise provides a robust stimulus for all the signaling pathways we examine. The type of inhibition used, short hairpin RNA (shRNA), is praised for its ability to integrate in the genome of test animals and produce a sustained knockdown effect of the target (Moore et al., 2010; Taxman et al., 2006). Other Notch inhibitors such as a γ-secretase inhibitor (GSI) are not specific to just one receptor, whereby an understanding of how each Notch receptor (1-4) is difficult to assess with that type of inhibitor (Huot, 2018). Short interfering RNA (siRNA), while a valuable tool in research, is not as stable as shRNA, can lead to off-target effects, and may fail to induce lasting knockdown of targets

(Chernikov et al., 2019; Lam et al., 2015). We hypothesize that normal Wnt and mTOR signaling will be altered in response to Notch1 knockdown in repairing muscle. The information gathered here will be valuable for determining solutions to dysfunctional muscle repair in diseased populations that display muscle wasting or accelerated muscle loss.

CHAPTER 2: LITERATURE REVIEW

Notch signaling is a key player in the early stages of muscle repair and is shown to be dysfunctional in other tissues (liver, adipose, endothelial cells, myocardium) in obesity, aging, and cancer (Baker et al., 2014; Hasan et al., 2020; Miranda et al., 2018; Richter et al., 2020; Rosati et al., 2009). It has been previously shown that oscillations in Notch activity is necessary for the activation of muscle progenitor stem cells and the subsequent proliferation and differentiation to myoblasts, which give way to fully formed myotubes at the end of muscle repair (Brack et al., 2008; Gerrard et al., 2021; Noguchi et al., 2019).

Notch engages in synergistic regulation of various cell processes with other cell signaling pathways. These other signaling networks that are known to interact with Notch are mechanistic target of rapamycin (mTOR) and Wingless/Integrated (Wnt). The mechanisms of crosstalk between these pathways are not well understood, but there is plenty of evidence of co-regulation in cancer and non-skeletal muscle models (Bertrand, 2020; Collu et al., 2012; Collu et al., 2014; Espinosa et al., 2003; Evangelisti et al., 2020; Hibdon et al., 2019; Inoki et al., 2006; Ma et al., 2010; Vo et al., 2011), but fewer interactions have been described in skeletal muscle (Brack et al., 2008; Huot et al., 2020), and the way they regulate each other has not been clearly described. mTOR and Wnt are also known to be dysregulated in obesity and aging (Abou Ziki & Mani, 2019; Anderson et al., 2008; Beals et al., 2019; Colleluori et al., 2019; Laplante & Sabatini, 2012; Nilsson et al., 2013; Rivas et al., 2016; Tran et al., 2018; Um et al., 2004), so an understanding of how these pathways interact with Notch is helpful in understanding the bigger picture of poor muscle repair in obesity and aging. This review gives context on the relationship between Notch, mTOR, and Wnt to provide better insight into how muscle repair is accomplished by these complex cell signaling networks.

2.1 Role of Notch Signaling in Muscle Repair

Notch is a signaling pathway that exists within many tissue types (liver, adipose, endothelial cells, myocardium, skeletal muscle) and is involved in multiple cell functions such as proliferation and cell fate determination despite its relatively simple signaling pathway. Notch signaling is initiated by cell-to-cell interaction between Notch receptors (Notch-1, Notch-2, Notch-3, Notch-4) and Notch cell-membrane-anchored DSL ligands (Delta/Jagged, Serrate, or Lag2) ligands (Shen et al., 2021; Tsivitse, 2010). Binding of a Notch ligand to Notch-1 receptor induces two rounds of cleavage of the Notch receptor, the first mediated by members of the ADAM-family of metalloproteases and the second by endocytosis mediated γ-secretase (Borggrefe & Oswald, 2009; Chapman et al., 2016). The cleaved portion of the receptor, referred to as the notch intracellular domain (NICD), will translocate to the nucleus to bind on the transcription factor CSL to induce transcription (Shen et al., 2021). Endocytosis of cleaved Notch-1, and possibly the entire ligand-receptor complex, is required for Notch signaling to occur(Chapman et al., 2016; Zheng & Conner, 2018). CSL is coupled with mastermind-like protein (MamL) and together will phosphorylate NICD which leads to ubiquitination and subsequent proteasomal degradation of NICD. The primary gene targets of NICD are Hairy/Enhancer of Split (HES) and Hairy/Enhancer of Split related to YRPW motif (HEY) (Kopan & Ilagan, 2009).

Skeletal muscles are equipped with stem cells called Muscle Satellite Cells (MuSCs), which rest between the sarcolemma and the basal lamina of muscle fibers. These MuSCs are capable of proliferating into myoblasts and differentiating into myotubes to replace damaged muscle tissue. MuSCs are quiescent during adulthood, waiting for activation induced by muscle injury. Several factors are responsible for awakening MuSCs, including leukocytes, stimuli from blood vessels, and signaling pathways, a prominent one being Notch Signaling (54). A variety of myogenic factors interact with Notch to adjust its expression and regulate

myogenesis: myoblast determination protein 1 (MyoD), myogenic factor 5 (Myf5), myogenic regulatory factors (MRFs), myogenin, and myosin heavy chain (MHC) (56).

The strength of Notch signaling is dependent on the amount NICD that accumulates in the nucleus, so proper disposal and supply of NICD is important for efficient cellular function. For example, there are various cancers which result due to flaws on the ubiquitination region of NICD, PEST (proline, glutamic acid, serine, and threonine-enriched) domain (Shen et al., 2021). Researchers determined that 12% of chronic lymphocytic leukemia (CLL) patients possessed a mutation of Notch1 gene that resulted in Notch1 proteins lacking a C-terminal domain containing PEST (Puente et al., 2011). Puente et al. reiterate previous finding that Notch is constantly active in CLL (Rosati et al., 2009) and that the isoform which results from the removal of the PEST containing C-terminal of Notch1 proteins is more stable and active. Therefore, not only is Notch overly expressed in CLL, but the isoforms of Notch1 resulting from mutations related to CLL are even more stable and active. Furthermore, other disease states such as T2DM and obesity are associated with hyperactive Notch in cells (Valenti et al., 2013).

Precise fluctuations of Notch activity are necessary for proper muscle repair response. When Notch activity is high, MuSCs are held in quiescence and their activation is inhibited (Gioftsidi et al., 2022). It was shown that when Notch is inhibited by injury, MuSCs activate and undergo proliferation (Batsis & Villareal, 2018), contributing many MuSCs to differentiate into myoblasts. When MuSCs are activated, levels of MyoD, a regulator of myocyte differentiation, increase (Kim et al., 2014). The increase of MyoD encourages MuSCs to leave G_0 and begin DNA synthesis in preparation to go through several rounds of division (Han & Lean, 2016; Zammit et al., 2004; Zhang et al., 2010). MuSCs are known to exhibit self-renewal by proliferating and saving a number of MuSCs that will become quiescent to maintain dormant SC levels (Abreu & Kowaltowski, 2020). Notch signaling is

then elevated to encourage myoblast proliferation; however, for differentiation to occur, Notch must be downregulated, because Notch activity is known to inhibit differentiation of myoblasts into myotubes (Brack et al., 2008; Noguchi et al., 2019). Noguchi (et al. 2019) utilized transgenic mice lacking gene expression of specific Notch effectors (Hey1 and HeyL) to determine that HeyL and Hes1 together act as inhibitors of myogenic differentiation. Previously, a group inhibited Notch in myoblasts early in the repair process to show there was less Myf5 synthesis, suggesting reduced myoblast proliferation under Notch inhibition (Brack et al., 2007). Furthermore, when Brack inhibited Notch at later stages of muscle repair, repair was not as negatively affected, validating the idea that Notch signaling withdraws during differentiation into myotubes. Finally, myotube fusion is accomplished by suppressing Notch via TGFβ inhibition (Choi et al., 2021).

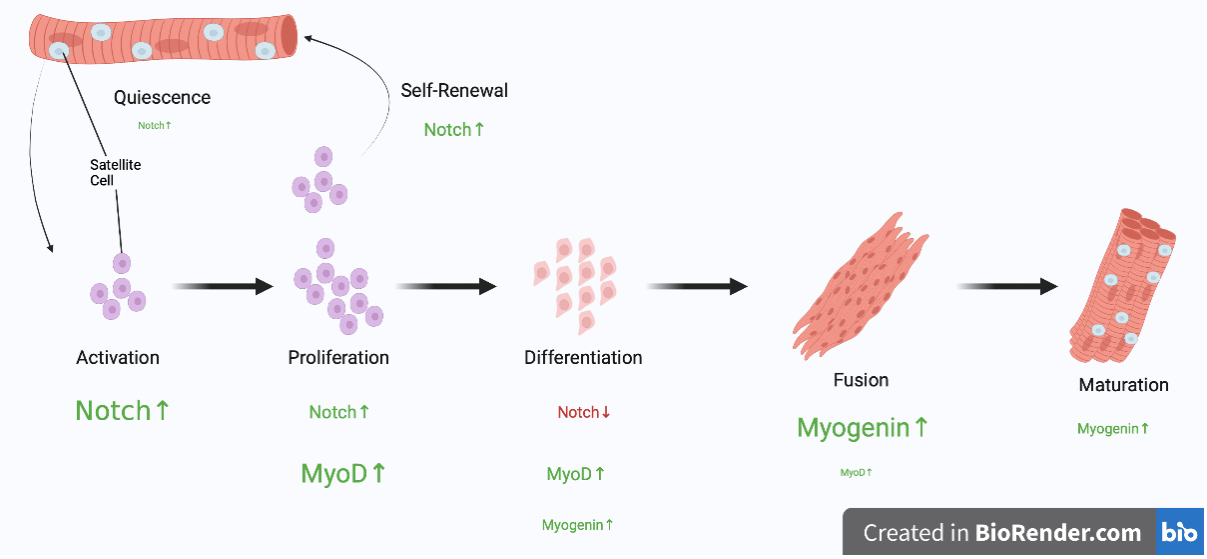


Fig. 1 Diagram of Notch signaling and MyoD/Myogenin activity during muscle repair; Created with BioRender.com

An important protein associated with Notch activity is its downstream regulator, Hes1, which is thought to regulate MuSC proliferation and differentiation (Lahmann et al., 2019). Lahmann (et al. 2019) showed fluctuating expression of Hes1 was likely to act on MyoD and myogenin by inhibiting their expression. When Hes1 inhibits MyoD and myogenin,

proliferation rather than differentiation of MuSCs is occurring. These finding are consistent with previous literature and describe the importance of precise orchestrations of Notch signaling during myogenesis and repair.

Parabiosis experiments have yielded interesting perspectives on how Notch signaling and MuSCs behave in older individuals. When an older mouse's circulatory system is joined to that of a younger mouse, and injury to muscle is induced, the older mouse experiences improved muscle repair compared to two older mice who have been conjoined (Conboy & Rando, 2006). On the other hand, the younger mice's ability to repair muscle was diminished. This reaffirms that elderly stem cell activity is blunted, either due to reduced MuSC number, or impairment in their activity, possibly related to aberrant Notch signaling.

2.2 Role of mTOR signaling in Muscle Repair

mTOR balances anabolic and catabolic activity within cells and it is involved in cell growth and proliferation, suppression of catabolism, cell fate determination, and metabolism. mTOR is extremely sensitive to the presence of nutrients in the environment such as amino acids (AAs) and oxygen and the hormone insulin (Liu & Sabatini, 2020; Sabatini, 2017; Saxton & Sabatini, 2017). There are two distinct complexes of mTOR, mTORC1 and mTORC2, which serve different purposes (Laplante & Sabatini, 2012). One of the major distinctions between the two complexes is that mTORC2 is unresponsive to the inhibitory effects of rapamycin, unlike mTORC1.

mTOR activity is related to several downstream effectors including 4E-binding protein 1 (4EBP1) and S6 kinase 1, otherwise known as P70S6K. 4EBP1 in its inactive form is an inhibitor of translation as it is coupled tightly with eukaryotic translation initiation factor 4E (eIF4E) on the 5' cap of mRNA (Jossé et al., 2016). The mRNAs associated with mTOR regulation are known to possess 5' terminal oligopyrimidine motifs on their 5' caps (Thoreen

et al., 2012). Phosphorylation of 4EBP1 by activated mTORC1 causes it to separate from eIF4E which then disassociates from the 5' cap of mRNA to allow 5' cap dependent translation to begin. Phosphorylation of 4EBP1 (p-4EBP1) is one way that mTOR accomplishes protein synthesis. Phosphorylated P70S6K (p-P70S6K) on T389 may phosphorylate ribosomal protein S6 which may have some dispensable involvement in ribosomal biogenesis (Liu & Sabatini, 2020). P70S6K phosphorylates mTOR directly, and activation of P70S6K is complex and requires phosphorylation of multiple AAs (Pullen et al., 1998). p-P70S6K is also recruited to spliced mRNA by S6K1 Aly/REF-like target (Ahn et al.) to increase translation of post-transcriptionally modified mRNA (Liu & Sabatini, 2020; Ma et al., 2008). Translation regulation is mostly attributed to p-4EB1 rather than p-P70S6K due to studies that examined S6K1 knockout and addition of rapamycin finding a weaker effect of P70S6K inhibition (Mieulet et al., 2007; Pende et al., 2004). On the other hand, mTOR inhibition results in a significant drop in translation of 5' terminal oligopyrimidine capped mRNA carried out by 4-EBP1 (Thoreen et al., 2012).

2.3 mTOR and Notch interactions

mTOR (mechanistic target of rapamycin) and Notch signaling are two important molecular pathways involved in various cellular processes such as cell growth, differentiation, and survival (Bi & Kuang, 2015; Gerrard et al., 2021; Gordon et al., 2013; Laplante & Sabatini, 2012). While these pathways operate independently, recent studies suggest that they can also interact in a complex and context-dependent manner (Baker et al., 2014; Hibdon et al., 2019; Huang et al., 2015; Lee et al., 2012).

Notch signaling can regulate mTOR activity by modulating the expression of upstream mTOR regulators (Hibdon et al., 2019). Notch inhibition (gamma-secretase inhibition) has been shown to affect the phosphatase and tensin homolog (PTEN)/Protein

kinase B (AKT)/mTOR pathway (Huot et al., 2020). GSI treatment of C2C12 cells increased indices of muscle protein synthesis, myosin heavy chain (MHC), myogenin. More importantly, phosphorylation of mTOR at ser2448 and ser2481 as well as 4EBP1 on Thr37/46, which promotes cell growth and protein synthesis, in 96-hour myotubes was increased. Furthermore, AKT and TSC2 (Ser939 and Thr1462) phosphorylation increased and PTEN expression decreased following GSI treatment, suggesting Notch signaling may play an inhibitory role on upstream regulators of mTOR. Another study examining Notch-1 in T-cell acute lymphoblastic leukemia (T-ALL) cells found that PTEN inhibition by Notch-1 could be accomplished by Hes-1 and Myc binding to PTEN (Palomero et al., 2007).

Another observed interaction between the mTOR and Notch pathways can be seen in the development of Alzheimer's Disease (AD). The catalytic subunit of γ-secretase Presenilin-1 (PS1) is known to cleave beta-amyloid precursor protein (APP) (Chyung et al., 2005), as well as Notch1, contributing to the buildup of Abeta42-peptide fragments which form amyloid plaques in neurons (Hardy & Selkoe, 2002). Investigators tested the hypothesis that rapamycin would alleviate plaque buildup by way of reducing PS1 expression (Das & Hontiveros, 2020). They described a decrease in p-mTOR, p-P70S6K1, 4EBP1, PS1, NICD, and Hes1 proteins suggesting mTOR upregulates PS1/γ-secretase activity. Another study examined the regulatory relationship between mTORC1 and Notch3 in human pulmonary arterial smooth muscle cells in the development of Hypoxia-induced pulmonary hypertension (Wang et al., 2014). They came to the conclusion that mTOR acted as an upstream regulator of Notch3 after testing the effect of rapamycin on Notch3 activity. They found that Notch3 activity decreased in response to rapamycin, and to confirm this regulatory effect of mTOR they inhibited Notch3 activity which resulted in no significant changes in mTOR activity.

Overall, the interaction between mTOR and Notch signaling is complex and context dependent. While mTOR can positively regulate Notch signaling, and vice versa, the exact mechanism and outcome of this interaction may vary depending on the cellular context and the specific signaling pathway involved.

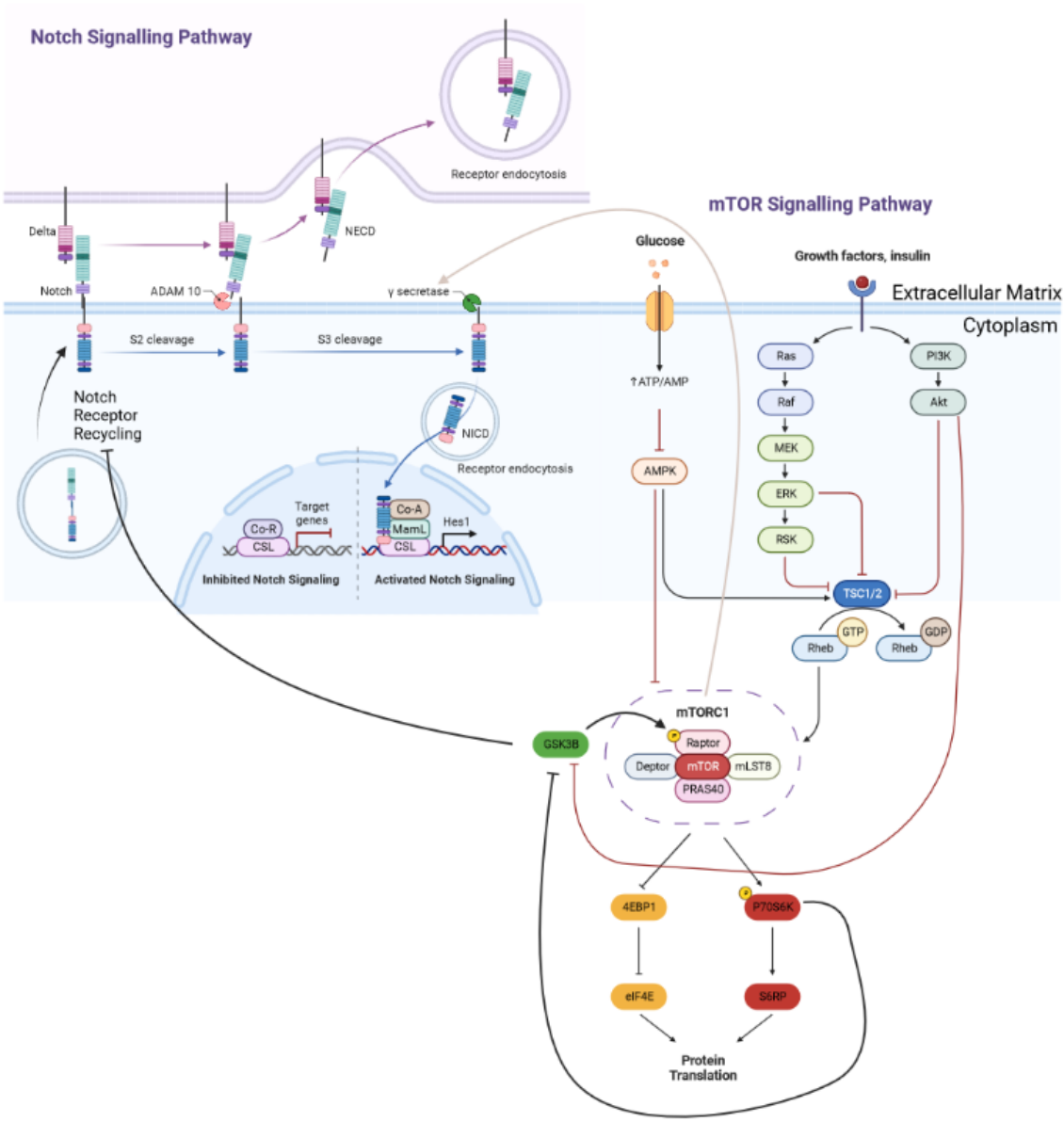


Fig. 2 Possible interactions between Notch, mTOR, and GSK3β; Created with BioRender.com

2.4 Wnt and Muscle Repair

Wnt signaling is a highly conserved molecular pathway that plays a crucial role in embryonic development, tissue homeostasis, and regeneration. In muscle tissue, Wnt signaling has been shown to be involved in the repair and regeneration of damaged muscle fibers (Reggio et al., 2020; Rudnicki & Williams, 2015; Rudolf et al., 2016; Tusavitz et al., 2020; von Maltzahn et al., 2012). Active Wnt signaling promotes the proliferation and differentiation of satellite cells, leading to the generation of new muscle fibers (Fujimaki et al., 2014). Wnt signaling also regulates the expression of myogenic regulatory factors, such as MyoD and myogenin, which are essential for muscle regeneration (Jones et al., 2015; Suzuki et al., 2015).

Canonical Wnt signaling is downregulated during muscle repair via increased levels of one of the homologs of Glycogen Synthase Kinase 3 (GSK3), GSK3β (Amin et al., 2014). Increased Notch signaling at the onset of muscle damage encourages accumulation of GSK3β, specifically the active form GSK3β phosphorylated at tyrosine 216, which acts negatively on β-catenin preventing its effect in the nucleus (Yeh et al., 2023). Furthermore, investigation determined low β-catenin in muscle stem cells delayed differentiation following proliferation due to increased Notch, but constant action of β-catenin in satellite cells resulted in early differentiation (Rudolf et al., 2016). β-catenin phosphorylated by GSK3β that is phosphorylated at Tyr 216 (active GSK3β) is marked for degradation, whereby Wnt signaling is inhibited (Brack et al., 2008; Hughes et al., 1993).

Studies wherein GSK3β was inhibited show a significant increase in muscle regeneration of myogenic cells and expression of the transcription factor myogenin and muscle specific Myosin Heavy Chain (MHC) (Marcella et al., 2023; Polesskaya et al., 2003). In fact, there are many indications that the Wnt signaling regulator GSK3β interacts with and may regulate various points of the PI3K/Akt/ mTOR network. Firstly, Wnt signaling was

shown to stimulate P70S6K via GSK3 activity (Inoki et al., 2006). The study described increased phosphorylation of P70S6K at Thr 389 in response to induction of Wnt-1 and Wnt-3a in multiple cell types. Furthermore, 4EBP1 phosphorylation also increased following Wnt signaling induction. The researchers go on to explain that GSK3β plays an inhibitory role in the mTOR pathway by blocking P70S6K phosphorylation. Prior to this study others had investigated the interaction between GSK3β and tuberous sclerosis complex (Werner et al.) in the degradation complex for β-catenin (Mak et al., 2003). The TSC1/2 (hamartin-tuberin) complex is a negative regulator of mTOR (Huang & Manning, 2008). In the last ten years it was found that GSK3 phosphorylates TSC2 to inhibit mTORC1 in neural cells (Ka et al., 2014).

Alternatively, others have suggested that GSK3β may also phosphorylate Regulatory-associated protein of tor (Raptor) at Ser859, whereby allowing downstream phosphorylation of P70S6K (Stretton et al., 2015). GSK3 inhibition resulted in significantly decreased phosphorylation of P70S6K due to loosening of the bond between Raptor and mTORC1 despite AA availability.

GSK3β is reduced in mice muscle subjected to a bout of injurious downhill running 3 days following muscle damage, but significantly increases beginning on day 4. (Amin et al., 2014). Meanwhile, Amin et al. also described an increase in LEF1 expression which is a downstream Wnt regulator that may induce expression of MRFs (Tsivitse, 2010). LEF1 expression is known to increase when β-catenin enters the nucleus and binds to TCF/LEF transcription factors (Nusse, 2005; Tsivitse, 2010).

2.5 Wnt and Notch interactions

GSK3 homolog GSK3β have been reported to regulate Notch signaling factors such as NICD, Notch surface receptors, and the transcription factor Hes1 (Bertrand, 2020; Gao et

al., 2021; Jin et al., 2009). GSK3β may bind to and phosphorylate active Notch2 surface receptor fragments to modulate active Notch (Espinosa et al., 2003). In addition to the ability of GSK3β to associate with Notch2, it was shown that it also inhibits Hes1 transcription (Li et al., 2012). Furthermore, GSK3β can have a positive effect on Notch by phosphorylating NICD and increasing transcription (Collu et al., 2014). On the other hand, disheveled (Dvl), a positive regulator of β-catenin, was found to bind to CSL and inhibit transcription (Collu et al., 2012). Others have expounded on the negative regulatory effects of Dvl and shown that it may bind to NICD and mark it for degradation, whereby downregulating Notch activity (Muñoz-Descalzo et al., 2010).

Espinosa et al. confirmed this inhibitory effect of GSK3β by using the GSK3β inhibitor lithium chloride and described upregulation of Notch target Hes1. Furthermore, active Wnt signaling inhibited GSK3β and resulted in greater transcription of Hes1, indicating a positive regulatory role of Wnt on Notch signaling. They then tested the effect of GSK3β knockdown on immunolocalization of Notch1 to further understand the regulatory role of GSK3β on Notch1. They determined that in the presence of GSK3β in control cells, Notch1 would localize to a tubulovesicular compartment, but when GSK3β was inhibited or knocked down, Notch1 would be broadly dispersed throughout the cytoplasm or enriched on perinuclear regions. The investigators conclude that GSK3β negatively regulates sorting decisions of Notch1 in the cell.

More recently, research showed that GSK3β also mediates Notch transport throughout the cell (Zheng & Conner, 2018). Before the work of Zheng and Conner, it was discovered that Notch signal transduction requires endocytosis transport initiated by a ligand-receptor interaction, specifically concerning Notch1 (Chapman et al., 2016). Zheng et al.

determined that after inhibiting GSK3β with a small interfering RNA Notch1 signaling increased, revealing a possible negative regulatory function of GSK3β on Notch1.

2.6 Conclusion

Notch, mTOR, and Wnt are inextricably linked and ablation or upregulation of select regulatory factors in one signaling network has been shown to modulate activity in the others (Bertrand, 2020; Collu et al., 2012; Collu et al., 2014; Espinosa et al., 2003; Evangelisti et al., 2020; Hibdon et al., 2019; Inoki et al., 2006; Ma et al., 2010; Vo et al., 2011). GSK3β is a significant coregulator which has positive and negative effects on Notch, mTOR, and Wnt signaling alike acting as a nexus for the three pathways. It is involved in transcriptional, mobility, and stability regulation of various factors in Notch and mTOR (Evangelisti et al., 2020; Foltz et al., 2002; Stretton et al., 2015; Zheng & Conner, 2018). Notch inhibition also affects portions of the mTOR pathway, namely the downstream transcriptional effectors P70S6K and 4EBP1 (Huot et al., 2020). Factors of activated Notch also modify upstream regulators of mTOR such as PTEN and AKT (Palomero et al., 2007).

CHAPTER 3: METHODS

The experimental design for the project studying interactions between Notch, mTOR, and Wnt involved injections of Notch1 shRNA knockdown lentiviral particles in the left gastrocnemius muscle and an empty vector in the right gastrocnemius. The right leg served as a control limb to compare protein expression without Notch1 inhibition to the Notch1 inhibited limb. Mice were randomly allocated to control and DHR groups to further examine the effect of injurious eccentric exercise and induce muscle damage. Mice which ran downhill were euthanized across 96 hours in order to gain insight on the time-course effects of the treatments.

3.1. Animals and Experimental Groups

Young (2-4 months) male C57BL/6 mice (n = 6/group, 30 total) were randomly divided into exercising and non-exercising (control) groups. Mice were group housed in the vivarium at the University of North Carolina at Charlotte in a reverse 12-hour light cycle. Mice were allowed to consume food and water ad libitum.

3.2. Notch1 Knockdown and Control Vector Injection

The mice were injected with 100,000 TUs of shRNA Notch1 inhibitor into the left gastrocnemius (Control vector in right) for five consecutive days prior to DHR. The Notch1 inhibitor and empty vector consisted of lentiviral particles and were purchased from Sigma (Sigma Mission^{TM} shRNA). The inhibitor contained a total of 100,000 transducing units split between 4 different clones of RNA (GCAGATGATCTTCCCGTACTA; GCCCTTTGAGTCTTCATACAT; GCCAGGTTATGAAGGTGTATA; CCCACATTCCAGAGGCATTTA) that targeted Notch1 receptors. 50 uLs were injected in the gastrocnemius muscle along the longitudinal axis of the muscle. All mice groups were

exposed to an injurious bout of DHR save the control group. The control group were sacrificed on the day of DHR, and the remaining mice were sacrificed six at a time at 24hr, 48hr, 72hr, and 96hr so time-course changes could be measured.

3.3. Treadmill Familiarization and Downhill Run Exercise Bout

Animals randomly allocated to the exercise groups underwent five days of treadmill familiarization. A shock grid was used to encourage running and all treadmill exposure occurred in the dark. The grade of the treadmill during familiarization was set to 0% incline. Mice were placed in the lanes of the treadmill for five minutes on day one of treadmill familiarization and not allowed to run. On the second day of familiarization, mice were placed in the lanes and sat for two minutes and ran for 2 minutes at 7 meters/min. On day three of familiarization, mice were placed and rested on the treadmill for 2 minutes and ran for 2 minutes at 7 m/min and another 2 minutes at 9 m/min. The fourth day of familiarization involved two 3-minute bouts of running after resting in the lanes of the treadmill for 2 minutes, the first at 7 m/min and the second 9 m/min. On the final day of familiarization, mice rested for 2 minutes on the treadmill and ran for 3 minutes at 7 m/min for one bout, 3 minutes at 9 m/min for a second bout, and 2 minutes at 11 m/min for the final bout. The control group simulated a sedentary lifestyle (regular cage activity), and the exercise groups engaged in a bout of downhill running (-15% grade 22m/min until exhaustion) to induce muscle injury on the day of the last lentiviral injection. The left and right gastrocnemius muscles were harvested at the time of euthanasia and stored at -80 ºC.

3.4. Tissue Homogenization and Protein Concentration

Lysates were generated using a Benchmark 24 tube Bead blaster. Samples of gastrocnemius muscle were added to 2.0 mL reinforced cryogenic tubes and placed on dry

ice. Tubes and beads were cooled on dry ice, and Radioimmunoprecipitation assay (RIPA) buffer (supplemented with 1% Triton-x, 2% SDS, protease cocktail inhibitor) was chilled in wet ice before bead blast homogenization. 15 volumes of ice-cold RIPA buffer were added to cryogenic tubes with muscle samples and loaded into bead blaster to be shaken at 6 m/s, for two 30 second cycles at 1-minute intervals. Following shaking, the tubes were placed on ice for 5 minutes and then put back into the bead blaster for another round with the same settings as the first. Samples were put on ice for 15 minutes and vortexed every 2-3 minutes. Samples were then centrifuged at 2,000 g for 2 minutes to remove any bubbles, and then transferred to chilled Eppendorf tubes. Finally, the sample tubes were centrifuged for 20 minutes at 20,000g. A Pierce BCA kit was used to determine protein concentration of sample homogenates (23225; ThermoFisher).

3.5 Western Blot

Protein samples were loaded into wells of a 4-12% Bis-Tris gel (3450125; Bio-rad) and run (XT MES running buffer; 1610789; Bio-rad) at 125 V for 2-2 ½ hours in 4 ºC refrigerator. Following electrophoresis, proteins were transferred (Towbin Buffer; 10% methanol) onto a Polyvinylidene difluoride (PVDF) membrane for 1 hour at 100V in 4 ºC refrigerator. Membranes were washed 1x in Tris-buffered saline (TBS) and blocked for 30 minutes in 1:1 Odyssey blocking buffer with TBS. Following blocking, membranes were incubated overnight (14-18 hours) in primary antibodies (p-4EBP1, p-GSK3β, p-P70S6K, Total 4EBP1, Total GSK3β, Total P70S6K) (Dilution Factor of 1:500 for phosphorylated proteins, 1:1000 for total proteins) in 4 ºC refrigerator on a rocker. The following day membranes were washed 5 times x 5 minutes in TBST (TBS: 0.1% Tween 20) and then incubated in secondary antibodies (IRDye 680 RD, Goat anti-Rabbit, #926-68071; IRDye 800 CW, Goat anti-Mouse, #926-32350) directed to primary antibodies (1:10,000 in TBST)

for 1 hour. Following 5 x 5-minute washes in TBST and 1 x 5 min wash in TBS, membranes were imaged to identify mTOR and Wnt proteins. Bands were quantified using the Odyssey® LiCor CLx System. Membranes were stripped between scanning of phosphorylated proteins and incubation of total proteins using NewBlot™ PVDF Stripping Buffer for PVDF Membranes produced by LiCor.

Antibody	Catalog #, Company	Dilution
p-4EBP1 (Thr37/46)	#2855, CS	1:500
p-GSK3α/β (Ser 21/9)	#9331, CS	1:500
p-P70S6K (Thr389)	#9205, CS	1:500
Total 4EBP1	#9644, CS	1:1000
Total GSK3α/β	#5676, CS	1:1000
Total P70S6K	#9202	1:1000

Table 1. Western Blot Antibodies 4EBP1: 4E-binding protein 1; GSK3: Glycogen Synthase Kinase 3; P70S6K: P70 S6 kinase 1; CS: Cell signaling.

3.6 Statistical Analysis

A 2-way ANOVA test was used (Limb (Inhibitor Vs. Vector) x Exercise (DHR vs. No DHR)). Post-hoc comparisons were obtained via a Tukey's test, with statistical significance set at $p \leq 0.05$. All statistical analyses and graphs were made using Graphpad Prism 7.03. All data are presented as means ± SD.

CHAPTER 4: RESULTS

4.1 The Effect of Notch Inhibition on mTOR

Mice were exposed to a Notch1 shRNA inhibitor in the left leg and exercised to induce muscle damage to examine the effects those treatments had on downstream transcription targets of mTOR. Mice which ran downhill were sacrificed daily following DHR (24 hr., 48hr., 72hr., and 96h). The control group that did not engage in DHR were euthanized the same day the exercising mice ran downhill. However, no significant changes in expression of p-4EBP1 or p-P70S6K between control and Notch1 Knockdown were observed (Figure 4)($p>0.05$).

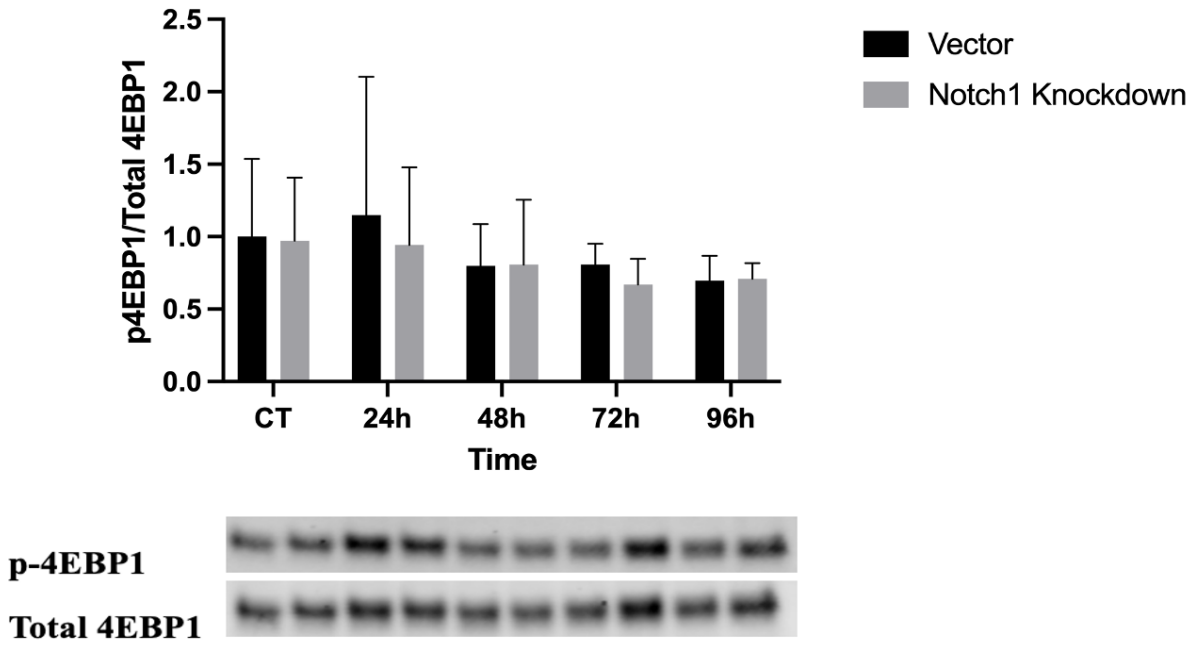


Fig. 3 Expression of p-4EBP1$^{thr37/46}$ investigated with Western blotting. Significance is indicated by "*" ($p < 0.05$).

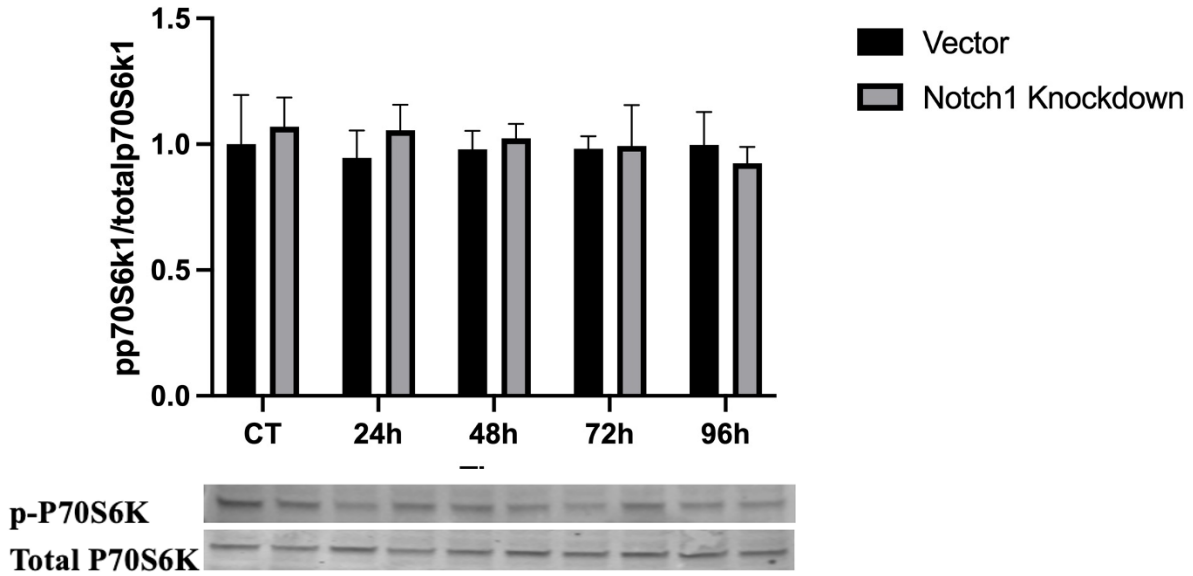


Fig. 4 Expression of p-P70S6K^{thr389} investigated with Western blotting. Significance is indicated by "*" ($p<0.05$).

4.2 The Effect of Notch Inhibition on Wnt target GSK3β

No significant differences were observed between control and Notch1 Knockdown in p-GSK3β^{ser9} expression (Figure 6)($p>0.05$). However, there was a significant decrease in p-GSK3β^{ser9} in CT compared to 48h post-DHR in empty-vector limbs (Figure 6)($p = 0.0444$). In addition, there was a significant difference between CT and 48h post-DHR of p-GSK3β^{ser9} in the Notch inhibited limb (Figure 6)($p = 0.0393$). p-GSK3β^{ser9} was shown to have increased significantly at the 96-hour timepoint normalized to total GSK3β ($p = 0.0135$).

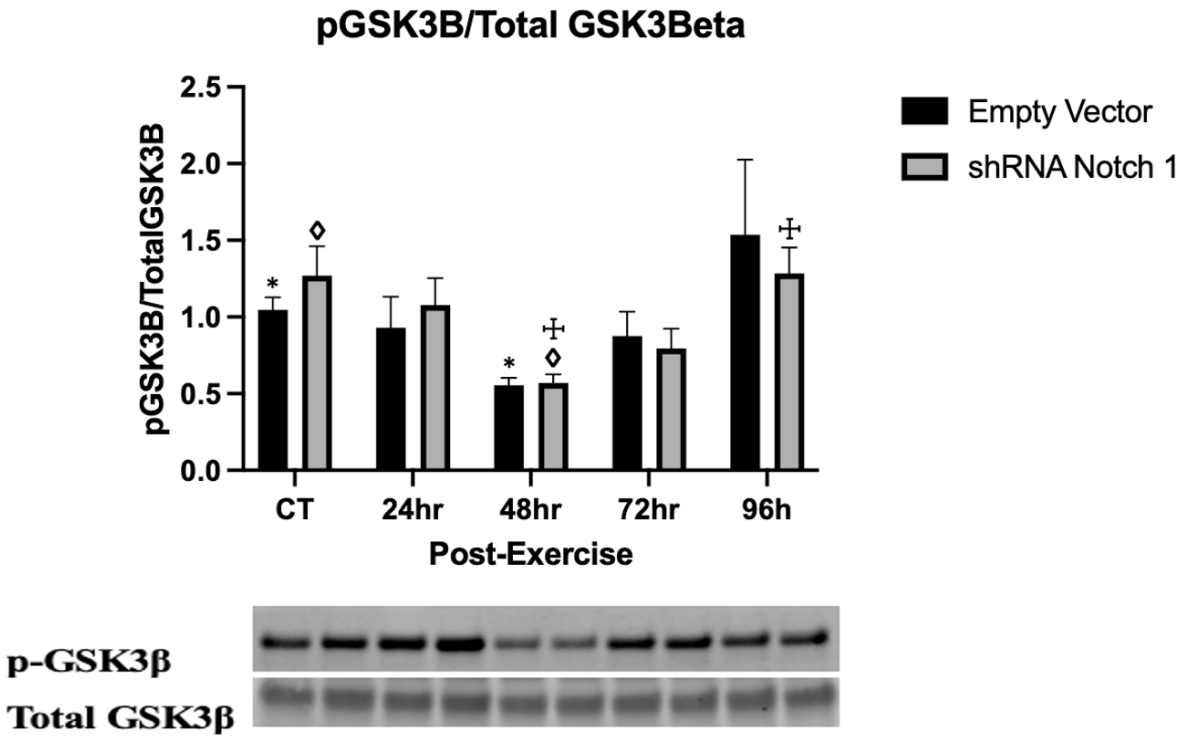


Fig. 5 Expression of p-GSK3β^{ser9} investigated with Western blotting. Significance is indicated by "*" ($p = 0.0444$); "⋄" ($p = 0.0393$); "✢" ($p = 0.0135$). There were significant differences between empty vector limb (right gastrocnemius) between control mice and DHR mice 48hr after exercise, and between shRNA Notch1 limb (left gastrocnemius) between control mice and DHR mice 48hr after exercise, and between DHR mice at 48hr post-DHR and DHR mice 96hr post-DHR.

CHAPTER 5: DISCUSSION

Our study's aim was to describe interactions between Notch, mTOR, and Wnt signaling by inhibiting Notch1 protein synthesis and measuring protein concentrations of specific downstream effectors of mTOR and Wnt during muscle repair induced by a single bout of injurious DHR. Dysregulation of Notch, mTOR, and Wnt signaling are characteristic of obesity, metabolic syndrome, aging, and cancer (Beals et al., 2019; Brack et al., 2007; Collu et al., 2014; Nusse, 2005; Saxton & Sabatini, 2017; Yang & Ming, 2012). The two targets of mTOR investigated were the translational factors P70S6K and 4EBP1.

p-4EBP$^{thr37/46}$ expression was measured to understand the effect Notch inhibition and DHR had on downstream targets of mTOR. The current study did not reveal a significant change in p-4EBP1$^{thr37/46}$ in response to Notch1 inhibition despite a significant increase in mTOR^{ser2448}. Our finding could be explained in part by the form of p-4EBP1 we chose to study (p-4EBP1$^{thr37/46}$) is not the form that would respond to Notch1 inhibition and a singular bout of DHR. The downstream target of mTOR, 4EBP1, negatively regulates translation by binding to eukaryotic translation initiation factor 4E (eIF4E) to prevent its interaction with the 5' cap of mRNA. Activated mTOR phosphorylates 4EBP1, removing it from the complex with eIF4E and allowing translation to commence. Some have described a concurrent increase in p-mTOR^{ser2448} and p-4EBP1 that was phosphorylated at a different site (Murai et al., 2012). So, our lack of an increase in p-4EBP1 could be because another form of p-4EBP1 which was phosphorylated at a site other than thr 37/46 increased but was not measured. Our lab previously described an increase in phosphorylated mTOR^{ser2448} in the mice limbs that were transduced with Notch1 inhibiting shRNA (Huot, 2018). It was our assumption that because mTOR^{ser2448} expression increased in response to Notch1 inhibition, and prior findings in which Notch inhibition led to increased p-4EBP1$^{thr37/46}$ (Huot et al., 2020), that we would see a similar increase in p-4EBP1$^{thr37/46}$. Our assumption was based on

the studies which found mTOR inhibitors decreased p-4EBP1 and active mTOR in response to GSI Notch inhibition increased p-4EBP1 (Huot et al., 2020; Lee et al., 2021; Truitt & Ruggero, 2016). Another study determined that phosphorylation of mTOR on ser2448 had minimal effect *in vitro* on 4EBP1, therefore p-mTOR^{ser2448} may not be an adequate indication of downstream 4EBP1 activity (Mothe-Satney et al., 2004). Furthermore, others have shown that inhibition of mTOR was not always sufficient to reduce p-4EBP1, suggesting activity of a different kinase or kinases on 4EBP1 (Mi et al., 2015; Zhang & Zheng, 2012). A review from 2017 asserted there is more to the mTOR/4EBP1 network than the canonical phosphorylation of 4EBP1 by active mTOR, and that many other kinases may play a role in regulating 4EBP1 (Batool et al., 2017). Prior studies have been met with similar findings in which p-4EBP1 did not increase or decrease with active or inactive mTOR, respectively. Our findings suggest Notch1 inhibition and exercise does not affect phosphorylation of 4EBP1 on thr 37/46, and its activity may be regulated by other factors such as other Notch receptors (2-4), GSK3β, or other kinases independent of or in tandem with p-mTOR^{ser2448}.

We then measured p-P70S6K^{thr389} expression to understand the mechanism by which mTOR^{ser2448} increased in response to Notch1 inhibition. Notch1 shRNA inhibition in tandem with no DHR or DHR did not illicit a significant effect on p-P70S6K^{thr389} expression in Notch1 knockdown limbs compared to empty vector limbs. The absence of a change in p-P70S6K relative to an increase in mTOR^{ser2448} is confounding as the general understanding is that p-P70S6K^{thr389} phosphorylates mTOR^{ser2448} (Glass, 2003)(Figueiredo et al., 2017). Again, our expectation was that if Notch inhibition causes an increase in p-mTOR^{ser2448}, then a parallel increase in p-P70S6K^{thr389} should occur. We believe that we did not see an increase in P70S6K^{thr389} because Notch1 inhibition may have reduced p70S6K activity, and phosphorylation of mTOR^{ser2448} was instead accomplished by AKT. Compensatory phosphorylation of mTOR^{ser2448} by AKT could explain why we witnessed an increased in p-

mTOR^{ser2448} and no relative increase in p-P70S6K^{thr389}.Other studies which examined the relationship between p-mTOR^{ser2448} and p-P70S6K^{thr389} have also described this puzzling concurrent increase in p-mTOR^{ser2448} and stable p-P70S6K^{thr389} expression (Apró & Blomstrand, 2010; Horii et al., 2020). Myotubes treated with GSI were shown to have significantly decreased p-P70S6K^{thr389} despite elevated mTOR^{ser2448}, which is the opposite of our expected outcome (Huot et al., 2020). mTOR^{ser2448} has been shown to be phosphorylated primarily by P70S6K, but to a lesser extent by AKT (Chiang & Abraham, 2005; Navé et al., 1999). Rapamycin was also shown to inhibit P70S6K activation in adipocytes and phosphorylation of mTOR^{ser2448} in adipocytes remained stable despite the inhibition, so it is possible P70S6K is regulated independently of active mTOR (Wang et al., 2006). p-P70S6K expression was decreased following addition of AMPK activators, suggesting AMPK has inhibitory effects on P70S6K that prevent its phosphorylation (Ginion et al., 2011). Our apparent lack of an increase in p-P70S6K^{thr389} despite greater p-mTOR^{ser2448} expression could be the result of Notch1 inhibition reducing P70S6K activity by way of AMPK inhibition or other regulators independent of mTOR.

Finally, we investigated GSK3β^{ser9} expression in response to Notch1 shRNA inhibition to identify cross-over between Notch and Wnt signaling. Based on our data, Notch1 inhibition may have had some effect on GSK3β^{ser9} expression, evidenced by significant time-course changes (Figure 6). This conclusion is in line with prior studies which assessed the effect of Notch3 inhibition via shRNA on p-GSK3β^{ser9} (Giovannini et al., 2013). Giovannini et al. found that Notch3 shRNA inhibition increased p-GSK3β^{ser9} expression in human hepatocarcinoma cell lines. GSK3β may be phosphorylated at multiple sites, one of which is serine 9 (p-GSK3β^{ser9}) and it is the form that we investigated. GSK3β is an inhibitor of β-catenin, preventing its accumulation in the nucleus to induce transcription. However, phosphorylation of GSK3β^{ser9} causes the kinase to become inactive and β-catenin

is no longer under GSK3β inhibition and Wnt signaling increases. p-GSK3β^{ser9} is an inactive form of GSK3β and it follows that GSK3β^{ser9} protein expression would be greater during the phase of muscle repair that Wnt signaling is increased. Notch has also been shown to effect an upstream regulator of GSK3β, AKT (Huot et al., 2020). Some of the variance in p-GSK3β we observed could be the result of an increase in AKT signaling in response to Notch inhibition that in turn inhibited GSK3β. The effect of exercise could also alter Wnt signaling and GSK3β phosphorylation. Exercise has been shown to illicit changes in AKT, GSK3β, and β-catenin (Sakamoto et al., 2004; Sakamoto et al., 2003; Sun et al., 2021). Our data showed an initial decrease in p-GSK3β^{ser9} but a significant increase by the 96h timepoint, which resembles prior studies on the effect of exercise on Wnt signaling and GSK3β.

While shRNA inhibition of Notch1 was able to significantly reduce overall Notch signaling activity, there were drawbacks in terms of how it was applied. DHR was shown to not induce a significant amount of muscle damage in DHR mice compared to control, likely due to damage from needle injection. Transducing units were given through intramuscular injection in the left gastrocnemius muscle of mice, introducing muscle damage and additional inflammation. This additional muscular damage likely skewed the results and made it appear as though DHR mice did not sustain significant damage from DHR. Conditional knockdown of Notch1 could circumvent the issues associated with intramuscular injection injury and would be beneficial to include in futures studies of effect of DHR on muscle regeneration. Furthermore, shRNA can have significant off-target effects that can have unknown effects on other signaling pathways (Konermann et al., 2018). shRNA can bind to and mark off-target proteins for degradation if they have as little as 11 similar nucleotides as the target mRNA (Jackson et al., 2003). Clustered Regularly Interspersed Short Palindromic Repeats/Cas (CRISPR-Cas) and closely related technologies have been shown to effectively alter the genome and generate minimal off-target effects (Gilbert et al., 2014). CRISPR technologies

are not completely error-free and can still lead to considerable off-target effects; however, new breakthroughs continue to improve the fidelity of these gene editing technologies (Shen et al., 2018).

A limitation of the study can be found in its restricted time frame. Muscle was only examined up to 4 days post exercise-induced damage, while typical muscle repair requires about 2 weeks to peak and up to 4 weeks to completely resolve (Ambrosio et al., 2009). Notch signaling has been described to fluctuate throughout the muscle repair process to mediate satellite cell stem activity and muscle repair (Bi et al., 2016). Therefore, the current timeframe studied here, while edifying, is not sufficient to give a full picture of the interplay between Notch, mTOR, and Wnt.

Chronic inflammation is a common characteristic of obesity and aging. It is known that a significant stimulus for Notch activity is signaling from pro-inflammatory cells (Fazio & Ricciardiello, 2016). Furthermore, Notch signaling has been identified as a key regulator of inflammation and therapies which target Notch are likely to become more prevalent in the near future. Notch downregulation in diabetic and liver inflammation models have been shown to reduce inflammation and improve repair of damaged tissue (Gamrekelashvili et al., 2016; Shang et al., 2016). Muscles are often thought of purely as tools for locomotion; however, muscles have complex roles in metabolism, inflammation, and secretion of hormone-like factors (myokines), some of which are either increased in response to exercise or exercise-dependent (Boström et al., 2012; Broholm et al., 2011; Broholm et al., 2008; Gopalan et al., 2021; Huh et al., 2012; Yang et al., 2015). The interaction between Notch signaling and immune cells in the inflammatory response of skeletal would be a fruitful area of study in the future.

Our data contributes to the understanding of the influence Notch signaling on other key signaling pathways involved in skeletal muscle and disease. The intention of the study

was to uncover aspects of the crosstalk between Notch, mTOR, and Wnt in order to aid future efforts to develop therapeutics to counteract muscle wasting in a variety of disease states. The current results indicate crosstalk between Notch and Wnt signaling by way of GSK3β. Prior studies suggest the way Notch inhibition affects GSK3β activity is through AKT, which is a critical upstream factor involved in mTOR signaling.

CHAPTER 6: CONCLUSION

To summarize, our study identified an interaction between Notch and Wnt signaling in skeletal muscle via GSK3β. We observed significant time-course changes in GSK3β in response to Notch1 inhibition, suggesting Notch1 or a downstream factor of Notch signaling exerts some effect on GSK3β. This data confirms previous studies that noted coregulation of GSK3β and Notch.GSK3β is likely to be involved in mTOR regulation, acting as a regulatory nexus between the three signaling pathways. The precise mechanisms of Notch, mTOR, and Wnt coregulation in skeletal muscle repair remain only partially understood and warrants future investigations. In addition, there is a gap in the literature regarding the effect of chronic inflammation on Notch and muscle repair. Future studies would do well to investigate the effect of inflammation and Notch inhibition on key cell signaling networks involved in skeletal muscle repair.

REFERENCES

Abou Ziki, M. D., & Mani, A. (2019). The interplay of canonical and noncanonical Wnt signaling in metabolic syndrome. *Nutr Res*, *70*, 18-25. https://doi.org/10.1016/j.nutres.2018.06.009

Abreu, P., & Kowaltowski, A. J. (2020). Satellite cell self-renewal in endurance exercise is mediated by inhibition of mitochondrial oxygen consumption. *Journal of Cachexia, Sarcopenia and Muscle*, *11*(6), 1661-1676. https://doi.org/https://doi.org/10.1002/jcsm.12601

Ahn, B., Ranjit, R., Premkumar, P., Pharaoh, G., Piekarz, K. M., Matsuzaki, S., Claflin, D. R., Riddle, K., Judge, J., Bhaskaran, S., Satara Natarajan, K., Barboza, E., Wronowski, B., Kinter, M., Humphries, K. M., Griffin, T. M., Freeman, W. M., Richardson, A., Brooks, S. V., & Van Remmen, H. (2019). Mitochondrial oxidative stress impairs contractile function but paradoxically increases muscle mass via fibre branching. *Journal of Cachexia, Sarcopenia and Muscle*, *10*(2), 411-428. https://doi.org/https://doi.org/10.1002/jcsm.12375

Ambrosio, F., Kadi, F., Lexell, J., Fitzgerald, G. K., Boninger, M. L., & Huard, J. (2009). The effect of muscle loading on skeletal muscle regenerative potential: an update of current research findings relating to aging and neuromuscular pathology. *Am J Phys Med Rehabil*, *88*(2), 145-155. https://doi.org/10.1097/PHM.0b013e3181951fc5

Amin, H., Vachris, J., Hamilton, A., Steuerwald, N., Howden, R., & Arthur, S. T. (2014). GSK3β inhibition and LEF1 upregulation in skeletal muscle following a bout of downhill running. *The Journal of Physiological Sciences*, *64*(1), 1-11. https://doi.org/10.1007/s12576-013-0284-5

Anderson, S. R., Gilge, D. A., Steiber, A. L., & Previs, S. F. (2008). Diet-induced obesity alters protein synthesis: tissue-specific effects in fasted versus fed mice. *Metabolism*, *57*(3), 347-354. https://doi.org/10.1016/j.metabol.2007.10.009

Apró, W., & Blomstrand, E. (2010). Influence of supplementation with branched-chain amino acids in combination with resistance exercise on p70S6 kinase phosphorylation in resting and exercising human skeletal muscle. *Acta Physiologica*, *200*(3), 237-248. https://doi.org/https://doi.org/10.1111/j.1748-1708.2010.02151.x

Baker, A. T., Zlobin, A., & Osipo, C. (2014). Notch-EGFR/HER2 Bidirectional Crosstalk in Breast Cancer [Review]. *Frontiers in Oncology*, *4*. https://doi.org/10.3389/fonc.2014.00360

Batool, A., Aashaq, S., & Andrabi, K. I. (2017). Reappraisal to the study of 4E-BP1 as an mTOR substrate – A normative critique. *European Journal of Cell Biology*, *96*(4), 325-336. https://doi.org/https://doi.org/10.1016/j.ejcb.2017.03.013

Batsis, J. A., & Villareal, D. T. (2018). Sarcopenic obesity in older adults: aetiology, epidemiology and treatment strategies. *Nat Rev Endocrinol*, *14*(9), 513-537. https://doi.org/10.1038/s41574-018-0062-9

Beals, J. W., Burd, N. A., Moore, D. R., & van Vliet, S. (2019). Obesity Alters the Muscle Protein Synthetic Response to Nutrition and Exercise. *Front Nutr*, *6*, 87. https://doi.org/10.3389/fnut.2019.00087

Bertrand, F. E. (2020). The cross-talk of NOTCH and GSK-3 signaling in colon and other cancers. *Biochim Biophys Acta Mol Cell Res*, *1867*(9), 118738. https://doi.org/10.1016/j.bbamcr.2020.118738

Bi, P., & Kuang, S. (2015). Notch signaling as a novel regulator of metabolism. *Trends Endocrinol Metab*, *26*(5), 248-255. https://doi.org/10.1016/j.tem.2015.02.006

Bi, P., Yue, F., Sato, Y., Wirbisky, S., Liu, W., Shan, T., Wen, Y., Zhou, D., Freeman, J., & Kuang, S. (2016). Stage-specific effects of Notch activation during skeletal myogenesis. *Elife*, *5*. https://doi.org/10.7554/eLife.17355

Borggrefe, T., & Oswald, F. (2009). The Notch signaling pathway: Transcriptional regulation at Notch target genes. *Cellular and Molecular Life Sciences*, *66*(10), 1631-1646. https://doi.org/10.1007/s00018-009-8668-7

Boström, P., Wu, J., Jedrychowski, M. P., Korde, A., Ye, L., Lo, J. C., Rasbach, K. A., Boström, E. A., Choi, J. H., Long, J. Z., Kajimura, S., Zingaretti, M. C., Vind, B. F., Tu, H., Cinti, S., Højlund, K., Gygi, S. P., & Spiegelman, B. M. (2012). A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature*, *481*(7382), 463-468. https://doi.org/10.1038/nature10777

Brack, A. S., Conboy, I. M., Conboy, M. J., Shen, J., & Rando, T. A. (2008). A Temporal Switch from Notch to Wnt Signaling in Muscle Stem Cells Is Necessary for Normal Adult Myogenesis. *Cell Stem Cell*, *2*(1), 50-59. https://doi.org/10.1016/j.stem.2007.10.006

Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C., & Rando, T. A. (2007). Increased Wnt Signaling During Aging Alters Muscle Stem Cell Fate and Increases Fibrosis. *Science*, *317*(5839), 807-810. https://doi.org/doi:10.1126/science.1144090

Broholm, C., Laye, M. J., Brandt, C., Vadalasetty, R., Pilegaard, H., Pedersen, B. K., & Scheele, C. (2011). LIF is a contraction-induced myokine stimulating human myocyte proliferation. *Journal of Applied Physiology*, *111*(1), 251-259. https://doi.org/10.1152/japplphysiol.01399.2010

Broholm, C., Mortensen, O. H., Nielsen, S., Akerstrom, T., Zankari, A., Dahl, B., & Pedersen, B. K. (2008). Exercise induces expression of leukaemia inhibitory factor in human skeletal muscle. *The Journal of Physiology*, *586*(8), 2195-2201. https://doi.org/https://doi.org/10.1113/jphysiol.2007.149781

Chapman, G., Major, J. A., Iyer, K., James, A. C., Pursglove, S. E., Moreau, J. L. M., & Dunwoodie, S. L. (2016). Notch1 endocytosis is induced by ligand and is required for signal transduction. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1863*(1), 166-177. https://doi.org/https://doi.org/10.1016/j.bbamcr.2015.10.021

Chernikov, I. V., Vlassov, V. V., & Chernolovskaya, E. L. (2019). Current Development of siRNA Bioconjugates: From Research to the Clinic [Review]. *Frontiers in Pharmacology*, *10*. https://doi.org/10.3389/fphar.2019.00444

Chiang, G. G., & Abraham, R. T. (2005). Phosphorylation of Mammalian Target of Rapamycin (mTOR) at Ser-2448 IsMediated by p70S6 Kinase. *Journal of Biological Chemistry*, *280*(27), 25485-25490.

Choi, I. Y., Lim, H. T., Che, Y. H., Lee, G., & Kim, Y. J. (2021). Inhibition of the Combinatorial Signaling of Transforming Growth Factor-Beta and NOTCH Promotes Myotube Formation of Human Pluripotent Stem Cell-Derived Skeletal Muscle Progenitor Cells. *Cells*, *10*(7), 1649. https://www.mdpi.com/2073-4409/10/7/1649

Chyung, J. H., Raper, D. M., & Selkoe, D. J. (2005). γ-Secretase Exists on the Plasma Membrane as an Intact Complex That Accepts Substrates and Effects Intramembrane Cleavage *. *Journal of Biological Chemistry*, *280*(6), 4383-4392. https://doi.org/10.1074/jbc.M409272200

Colleluori, G., Aguirre, L., Phadnis, U., Fowler, K., Armamento-Villareal, R., Sun, Z., Brunetti, L., Hyoung Park, J., Kaipparettu, B. A., Putluri, N., Auetumrongsawat, V., Yarasheski, K., Qualls, C., & Villareal, D. T. (2019). Aerobic Plus Resistance Exercise in Obese Older Adults Improves Muscle Protein Synthesis and Preserves Myocellular Quality Despite Weight Loss. *Cell Metabolism*, *30*(2), 261-273.e266. https://doi.org/https://doi.org/10.1016/j.cmet.2019.06.008

Collu, G. M., Hidalgo-Sastre, A., Acar, A., Bayston, L., Gildea, C., Leverentz, M. K., Mills, C. G., Owens, T. W., Meurette, O., Dorey, K., & Brennan, K. (2012). Dishevelled limits Notch signalling through inhibition of CSL. *Development*, *139*(23), 4405-4415. https://doi.org/10.1242/dev.081885

Collu, G. M., Hidalgo-Sastre, A., & Brennan, K. (2014). Wnt-Notch signalling crosstalk in development and disease. *Cell Mol Life Sci*, *71*(18), 3553-3567. https://doi.org/10.1007/s00018-014-1644-x

Conboy, I. M., & Rando, T. A. (2006). The Regulation of Notch Signaling Controls Satellite Cell Activation and Cell Fate Determination in Postnatal Myogenesis. *Developmental Cell*, *10*(2), 273. https://doi.org/10.1016/j.devcel.2006.01.003

Das, H. K., & Hontiveros, S. S. (2020). Inhibition of p-mTOR represses transcription of PS1 and Notch 1-signaling. *FBL*, *25*(6), 1172-1183. https://doi.org/10.2741/4851

Espinosa, L., Inglés-Esteve, J., Aguilera, C., & Bigas, A. (2003). Phosphorylation by Glycogen Synthase Kinase-3β Down-regulates Notch Activity, a Link for Notch and Wnt Pathways*. *Journal of Biological Chemistry*, *278*(34), 32227-32235. https://doi.org/https://doi.org/10.1074/jbc.M304001200

Evangelisti, C., Chiarini, F., Paganelli, F., Marmiroli, S., & Martelli, A. M. (2020). Crosstalks of GSK3 signaling with the mTOR network and effects on targeted therapy of cancer. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1867*(4), 118635. https://doi.org/https://doi.org/10.1016/j.bbamcr.2019.118635

Fazio, C., & Ricciardiello, L. (2016). Inflammation and Notch signaling: a crosstalk with opposite effects on tumorigenesis. *Cell Death Dis*, *7*(12), e2515. https://doi.org/10.1038/cddis.2016.408

Figueiredo, V. C., Markworth, J. F., & Cameron-Smith, D. (2017). Considerations on mTOR regulation at serine 2448: implications for muscle metabolism studies. *Cellular and Molecular Life Sciences*, *74*(14), 2537-2545. https://doi.org/10.1007/s00018-017-2481-5

Foltz, D. R., Santiago, M. C., Berechid, B. E., & Nye, J. S. (2002). Glycogen Synthase Kinase-3β Modulates Notch Signaling and Stability. *Current Biology*, *12*(12), 1006-1011. https://doi.org/10.1016/S0960-9822(02)00888-6

Fujimaki, S., Hidaka, R., Asashima, M., Takemasa, T., & Kuwabara, T. (2014). Wnt protein-mediated satellite cell conversion in adult and aged mice following voluntary wheel running. *The Journal of biological chemistry*, *289*(11), 7399-7412. https://doi.org/10.1074/jbc.M113.539247

Gamrekelashvili, J., Giagnorio, R., Jussofie, J., Soehnlein, O., Duchene, J., Briseño, C. G., Ramasamy, S. K., Krishnasamy, K., Limbourg, A., Häger, C., Kapanadze, T., Ishifune, C., Hinkel, R., Radtke, F., Strobl, L. J., Zimber-Strobl, U., Napp, L. C., Bauersachs, J., Haller, H., . . . Limbourg, F. P. (2016). Regulation of monocyte cell fate by blood vessels mediated by Notch signalling. *Nature Communications*, *7*(1), 12597. https://doi.org/10.1038/ncomms12597

Gao, J., Fan, L., Zhao, L., & Su, Y. (2021). The interaction of Notch and Wnt signaling pathways in vertebrate regeneration. *Cell Regeneration*, *10*(1), 11. https://doi.org/10.1186/s13619-020-00072-2

Gerrard, J. C., Hay, J. P., Adams, R. N., Williams, J. C., Huot, J. R., Weathers, K. M., Marino, J. S., & Arthur, S. T. (2021). Current Thoughts of Notch’s Role in Myoblast Regulation and Muscle-Associated Disease. *International Journal of Environmental Research and Public Health*, *18*(23), 12558. https://www.mdpi.com/1660-4601/18/23/12558

Gilbert, L. A., Horlbeck, M. A., Adamson, B., Villalta, J. E., Chen, Y., Whitehead, E. H., Guimaraes, C., Panning, B., Ploegh, H. L., & Bassik, M. C. (2014). Genome-scale CRISPR-mediated control of gene repression and activation. *Cell*, *159*(3), 647-661.

Ginion, A., Auquier, J., Benton, C. R., Mouton, C., Vanoverschelde, J. L., Hue, L., Horman, S., Beauloye, C., & Bertrand, L. (2011). Inhibition of the mTOR/p70S6K pathway is not involved in the insulin-sensitizing effect of AMPK on cardiac glucose uptake. *Am J Physiol Heart Circ Physiol*, *301*(2), H469-477. https://doi.org/10.1152/ajpheart.00986.2010

Gioftsidi, S., Relaix, F., & Mourikis, P. (2022). The Notch signaling network in muscle stem cells during development, homeostasis, and disease. *Skeletal Muscle*, *12*(1), 9. https://doi.org/10.1186/s13395-022-00293-w

Giovannini, C., Baglioni, M., Baron Toaldo, M., Ventrucci, C., D'Adamo, S., Cipone, M., Chieco, P., Gramantieri, L., & Bolondi, L. (2013). Notch3 inhibition enhances sorafenib cytotoxic efficacy by promoting GSK3b phosphorylation and p21 down-regulation in hepatocellular carcinoma. *Oncotarget*, *4*(10), 1618-1631. https://doi.org/10.18632/oncotarget.1221

Glass, D. J. (2003). Molecular mechanisms modulating muscle mass. *Trends in molecular medicine*, *9*(8), 344-350. https://doi.org/10.1016/s1471-4914(03)00138-2

Gopalan, V., Yaligar, J., Michael, N., Kaur, K., Anantharaj, R., Verma, S. K., Sadananthan, S. A., Le, G. T. T., Goh, J., & Velan, S. S. (2021). A 12-week aerobic exercise intervention results in improved metabolic function and lower adipose tissue and ectopic fat in high-fat diet fed rats. *Biosci Rep*, *41*(1). https://doi.org/10.1042/bsr20201707

Gordon, B. S., Kelleher, A. R., & Kimball, S. R. (2013). Regulation of muscle protein synthesis and the effects of catabolic states. *The International Journal of Biochemistry & Cell Biology*, *45*(10), 2147-2157. https://doi.org/https://doi.org/10.1016/j.biocel.2013.05.039

Han, T. S., & Lean, M. E. (2016). A clinical perspective of obesity, metabolic syndrome and cardiovascular disease. *JRSM Cardiovascular Disease*, *5*, 2048004016633371. https://doi.org/10.1177/2048004016633371

Hardy, J., & Selkoe, D. J. (2002). The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. *Science*, *297*(5580), 353-356. https://doi.org/doi:10.1126/science.1072994

Hasan, S. S., Jabs, M., Taylor, J., Wiedmann, L., Leibing, T., Nordström, V., Federico, G., Roma, L. P., Carlein, C., Wolff, G., Ekim-Üstünel, B., Brune, M., Moll, I., Tetzlaff, F., Gröne, H.-J., Fleming, T., Géraud, C., Herzig, S., Nawroth, P. P., & Fischer, A. (2020). Endothelial Notch signaling controls insulin transport in muscle. *EMBO Molecular Medicine*, *12*(4), e09271. https://doi.org/https://doi.org/10.15252/emmm.201809271

Hibdon, E. S., Razumilava, N., Keeley, T. M., Wong, G., Solanki, S., Shah, Y. M., & Samuelson, L. C. (2019). Notch and mTOR Signaling Pathways Promote Human Gastric Cancer Cell Proliferation. *Neoplasia (New York, N.Y.)*, *21*(7), 702-712. https://doi.org/10.1016/j.neo.2019.05.002

Horii, N., Hasegawa, N., Fujie, S., Uchida, M., & Iemitsu, M. (2020). Resistance exercise-induced increase in muscle 5α-dihydrotestosterone contributes to the activation of muscle Akt/mTOR/p70S6K- and Akt/AS160/GLUT4-signaling pathways in type 2 diabetic rats. *The FASEB Journal*, *34*(8), 11047-11057. https://doi.org/https://doi.org/10.1096/fj.201903223RR

Huang, B., Wang, Y., Wang, W., Chen, J., Lai, P., Liu, Z., Yan, B., Xu, S., Zhang, Z., Zeng, C., Rong, L., Liu, B., Cai, D., Jin, D., & Bai, X. (2015). mTORC1 Prevents

Preosteoblast Differentiation through the Notch Signaling Pathway. *PLOS Genetics*, *11*(8), e1005426. https://doi.org/10.1371/journal.pgen.1005426

Huang, J., & Manning, B. D. (2008). The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. *The Biochemical journal*, *412*(2), 179-190. https://doi.org/10.1042/BJ20080281

Hughes, K., Nikolakaki, E., Plyte, S. E., Totty, N. F., & Woodgett, J. R. (1993). Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. *Embo j*, *12*(2), 803-808. https://doi.org/10.1002/j.1460-2075.1993.tb05715.x

Huh, J. Y., Panagiotou, G., Mougios, V., Brinkoetter, M., Vamvini, M. T., Schneider, B. E., & Mantzoros, C. S. (2012). FNDC5 and irisin in humans: I. Predictors of circulating concentrations in serum and plasma and II. mRNA expression and circulating concentrations in response to weight loss and exercise. *Metabolism*, *61*(12), 1725-1738. https://doi.org/10.1016/j.metabol.2012.09.002

Huot, J. R. (2018). *Notch Serves as a Molecular Brake on Myogenesis, Protein Synthesis, and Growth in Skeletal Muscle* The University of North Carolina at Charlotte].

Huot, J. R., Marino, J. S., Turner, M. J., & Arthur, S. T. (2020). Notch Inhibition via GSI Treatment Elevates Protein Synthesis in C2C12 Myotubes. *Biology*, *9*(6), 115. https://doi.org/10.3390/biology9060115

Inoki, K., Ouyang, H., Zhu, T., Lindvall, C., Wang, Y., Zhang, X., Yang, Q., Bennett, C., Harada, Y., Stankunas, K., Wang, C.-y., He, X., MacDougald, O. A., You, M., Williams, B. O., & Guan, K.-L. (2006). TSC2 Integrates Wnt and Energy Signals via a Coordinated Phosphorylation by AMPK and GSK3 to Regulate Cell Growth. *Cell*, *126*(5), 955-968. https://doi.org/https://doi.org/10.1016/j.cell.2006.06.055

Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G., & Linsley, P. S. (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nature Biotechnology*, *21*(6), 635-637. https://doi.org/10.1038/nbt831

Jin, Y. H., Kim, H., Oh, M., Ki, H., & Kim, K. (2009). Regulation of Notch1/NICD and Hes1 expressions by GSK-3alpha/beta. *Mol Cells*, *27*(1), 15-19. https://doi.org/10.1007/s10059-009-0001-7

Jones, A. E., Price, F. D., Le Grand, F., Soleimani, V. D., Dick, S. A., Megeney, L. A., & Rudnicki, M. A. (2015). Wnt/β-catenin controls follistatin signalling to regulate satellite cell myogenic potential. *Skeletal Muscle*, *5*(1), 14. https://doi.org/10.1186/s13395-015-0038-6

Jossé, L., Xie, J., Proud, C. G., & Smales, C. M. (2016). mTORC1 signalling and eIF4E/4E-BP1 translation initiation factor stoichiometry influence recombinant protein productivity from GS-CHOK1 cells. *Biochem J*, *473*(24), 4651-4664. https://doi.org/10.1042/bcj20160845

Ka, M., Condorelli, G., Woodgett, J. R., & Kim, W.-Y. (2014). mTOR regulates brain morphogenesis by mediating GSK3 signaling. *Development*, *141*(21), 4076-4086. https://doi.org/10.1242/dev.108282

Kim, T. N., Park, M. S., Ryu, J. Y., Choi, H. Y., Hong, H. C., Yoo, H. J., Kang, H. J., Song, W., Park, S. W., Baik, S. H., Newman, A. B., & Choi, K. M. (2014). Impact of Visceral Fat on Skeletal Muscle Mass and Vice Versa in a Prospective Cohort Study: The Korean Sarcopenic Obesity Study (KSOS). *PLoS ONE*, *9*(12), e115407. https://doi.org/10.1371/journal.pone.0115407

Konermann, S., Lotfy, P., Brideau, N. J., Oki, J., Shokhirev, M. N., & Hsu, P. D. (2018). Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors. *Cell*, *173*(3), 665-676.e614. https://doi.org/https://doi.org/10.1016/j.cell.2018.02.033

Kopan, R., & Ilagan, M. X. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*, *137*(2), 216-233. https://doi.org/10.1016/j.cell.2009.03.045

Lahmann, I., Bröhl, D., Zyrianova, T., Isomura, A., Czajkowski, M. T., Kapoor, V., Griger, J., Ruffault, P.-L., Mademtzoglou, D., Zammit, P. S., Wunderlich, T., Spuler, S., Kühn, R., Preibisch, S., Wolf, J., Kageyama, R., & Birchmeier, C. (2019). Oscillations of MyoD and Hes1 proteins regulate the maintenance of activated muscle stem cells. *Genes & Development*, *33*(9-10), 524-535. https://doi.org/10.1101/gad.322818.118

Lam, J. K. W., Chow, M. Y. T., Zhang, Y., & Leung, S. W. S. (2015). siRNA Versus miRNA as Therapeutics for Gene Silencing. *Molecular Therapy - Nucleic Acids*, *4*, e252. https://doi.org/https://doi.org/10.1038/mtna.2015.23

Laplante, M., & Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell*, *149*(2), 274-293. https://doi.org/10.1016/j.cell.2012.03.017

Lee, B. J., Boyer, J. A., Burnett, G. L., Thottumkara, A. P., Tibrewal, N., Wilson, S. L., Hsieh, T., Marquez, A., Lorenzana, E. G., Evans, J. W., Hulea, L., Kiss, G., Liu, H., Lee, D., Larsson, O., McLaughlan, S., Topisirovic, I., Wang, Z., Wang, Z., . . . Rosen, N. (2021). Selective inhibitors of mTORC1 activate 4EBP1 and suppress tumor growth. *Nat Chem Biol*, *17*(10), 1065-1074. https://doi.org/10.1038/s41589-021-00813-7

Lee, K., Nam, K. T., Cho, S. H., Gudapati, P., Hwang, Y., Park, D.-S., Potter, R., Chen, J., Volanakis, E., & Boothby, M. (2012). Vital roles of mTOR complex 2 in Notch-driven thymocyte differentiation and leukemia. *Journal of Experimental Medicine*, *209*(4), 713-728. https://doi.org/10.1084/jem.20111470

Li, B., Jia, Z., Wang, T., Wang, W., Zhang, C., Chen, P., Ma, K., & Zhou, C. (2012). Interaction of Wnt/β-catenin and notch signaling in the early stage of cardiac differentiation of P19CL6 cells. *J Cell Biochem*, *113*(2), 629-639. https://doi.org/10.1002/jcb.23390

Liu, G. Y., & Sabatini, D. M. (2020). mTOR at the nexus of nutrition, growth, ageing and disease. *Nature reviews Molecular cell biology*, *21*(4), 183-203.

Ma, J., Meng, Y., Kwiatkowski, D. J., Chen, X., Peng, H., Sun, Q., Zha, X., Wang, F., Wang, Y., Jing, Y., Zhang, S., Chen, R., Wang, L., Wu, E., Cai, G., Malinowska-Kolodziej, I., Liao, Q., Liu, Y., Zhao, Y., . . . Zhang, H. (2010). Mammalian target of rapamycin regulates murine and human cell differentiation through STAT3/p63/Jagged/Notch cascade. *The Journal of Clinical Investigation*, *120*(1), 103-114. https://doi.org/10.1172/JCI37964

Ma, X. M., Yoon, S. O., Richardson, C. J., Jülich, K., & Blenis, J. (2008). SKAR links pre-mRNA splicing to mTOR/S6K1-mediated enhanced translation efficiency of spliced mRNAs. *Cell*, *133*(2), 303-313. https://doi.org/10.1016/j.cell.2008.02.031

Mak, B. C., Takemaru, K.-I., Kenerson, H. L., Moon, R. T., & Yeung, R. S. (2003). The Tuberin-Hamartin Complex Negatively Regulates β-Catenin Signaling Activity*. *Journal of Biological Chemistry*, *278*(8), 5947-5951. https://doi.org/https://doi.org/10.1074/jbc.C200473200

Marcella, B. M., Copeland, E. N., Hamstra, S. I., Hockey, B. L., Braun, J. L., Geromella, M. S., Whitley, K. C., Watson, C. J. F., Baranowski, B. J., Maddalena, L. A., Mohammad, A., Silvera, S., Baranowski, R. W., Ochoa, E. C., Wasilewicz, L., Cleverdon, R. E. G., Beaudette, S., Vandenboom, R., Roy, B. D., . . . Fajardo, V. A. (2023). Treating muscle and brain alike: benefits of GSK3 inhibition in mdx mice. *bioRxiv*, 2022.2002.2016.480726. https://doi.org/10.1101/2022.02.16.480726

McCubrey, J. A., Steelman, L. S., Bertrand, F. E., Davis, N. M., Abrams, S. L., Montalto, G., D'Assoro, A. B., Libra, M., Nicoletti, F., Maestro, R., Basecke, J., Cocco, L., Cervello, M., & Martelli, A. M. (2014). Multifaceted roles of GSK-3 and Wnt/β-catenin in hematopoiesis and leukemogenesis: opportunities for therapeutic intervention. *Leukemia*, *28*(1), 15-33. https://doi.org/10.1038/leu.2013.184

Mi, W., Ye, Q., Liu, S., & She, Q. B. (2015). AKT inhibition overcomes rapamycin resistance by enhancing the repressive function of PRAS40 on mTORC1/4E-BP1 axis. *Oncotarget*, *6*(16), 13962-13977. https://doi.org/10.18632/oncotarget.3920

Mieulet, V., Roceri, M., Espeillac, C., Sotiropoulos, A., Ohanna, M., Oorschot, V., Klumperman, J., Sandri, M., & Pende, M. (2007). S6 kinase inactivation impairs growth and translational target phosphorylation in muscle cells maintaining proper regulation of protein turnover. *Am J Physiol Cell Physiol*, *293*(2), C712-722. https://doi.org/10.1152/ajpcell.00499.2006

Miranda, K., Yang, X., Bam, M., Murphy, E. A., Nagarkatti, P. S., & Nagarkatti, M. (2018). MicroRNA-30 modulates metabolic inflammation by regulating Notch signaling in adipose tissue macrophages. *International Journal of Obesity*, *42*(6), 1140-1150. https://doi.org/10.1038/s41366-018-0114-1

Moore, C. B., Guthrie, E. H., Huang, M. T., & Taxman, D. J. (2010). Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown. *Methods Mol Biol*, *629*, 141-158. https://doi.org/10.1007/978-1-60761-657-3_10

Mothe-Satney, I., Gautier, N., Hinault, C., Lawrence, J. C., Jr., & Van Obberghen, E. (2004). In rat hepatocytes glucagon increases mammalian target of rapamycin phosphorylation on serine 2448 but antagonizes the phosphorylation of its downstream targets induced by insulin and amino acids. *The Journal of biological chemistry*, *279*(41), 42628-42637. https://doi.org/10.1074/jbc.M405173200

Muñoz-Descalzo, S., Sanders, P. G., Montagne, C., Johnson, R. I., Balayo, T., & Arias, A. M. (2010). Wingless modulates the ligand independent traffic of Notch through Dishevelled. *Fly (Austin)*, *4*(3), 182-193. https://doi.org/10.4161/fly.4.3.11998

Murai, A., Asa, S. A., Kodama, A., Hirata, A., Yanai, T., & Sakai, H. (2012). Constitutive phosphorylation of the mTORC2/Akt/4E-BP1 pathway in newly derived canine hemangiosarcoma cell lines. *BMC Veterinary Research*, *8*(1), 128. https://doi.org/10.1186/1746-6148-8-128

Navé, B. T., Ouwens, M., Withers, D. J., Alessi, D. R., & Shepherd, P. R. (1999). Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J*, *344 Pt 2*(Pt 2), 427-431.

Nilsson, M. I., Dobson, J. P., Greene, N. P., Wiggs, M. P., Shimkus, K. L., Wudeck, E. V., Davis, A. R., Laureano, M. L., & Fluckey, J. D. (2013). Abnormal protein turnover and anabolic resistance to exercise in sarcopenic obesity. *The FASEB Journal*, *27*(10), 3905-3916. https://doi.org/https://doi.org/10.1096/fj.12-224006

Noguchi, Y.-t., Nakamura, M., Hino, N., Nogami, J., Tsuji, S., Sato, T., Zhang, L., Tsujikawa, K., Tanaka, T., Izawa, K., Okada, Y., Doi, T., Kokubo, H., Harada, A., Uezumi, A., Gessler, M., Ohkawa, Y., & Fukada, S.-i. (2019). Cell-autonomous and redundant roles of Hey1 and HeyL in muscle stem cells: HeyL requires Hes1 to bind diverse DNA sites. *Development*, *146*(4). https://doi.org/10.1242/dev.163618

Nusse, R. (2005). Wnt signaling in disease and in development. *Cell Research*, *15*(1), 28-32. https://doi.org/10.1038/sj.cr.7290260

Palomero, T., Sulis, M. L., Cortina, M., Real, P. J., Barnes, K., Ciofani, M., Caparros, E., Buteau, J., Brown, K., Perkins, S. L., Bhagat, G., Agarwal, A. M., Basso, G., Castillo, M., Nagase, S., Cordon-Cardo, C., Parsons, R., Zúñiga-Pflücker, J. C., Dominguez,

M., & Ferrando, A. A. (2007). Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nature Medicine*, *13*(10), 1203-1210. https://doi.org/10.1038/nm1636

Pende, M., Um, S. H., Mieulet, V., Sticker, M., Goss, V. L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S. C., & Thomas, G. (2004). S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol Cell Biol*, *24*(8), 3112-3124. https://doi.org/10.1128/mcb.24.8.3112-3124.2004

Polesskaya, A., Seale, P., & Rudnicki, M. A. (2003). Wnt Signaling Induces the Myogenic Specification of Resident CD45⁺ Adult Stem Cells during Muscle Regeneration. *Cell*, *113*(7), 841-852. https://doi.org/10.1016/S0092-8674(03)00437-9

Puente, X. S., Pinyol, M., Quesada, V., Conde, L., Ordóñez, G. R., Villamor, N., Escaramis, G., Jares, P., Beà, S., & González-Díaz, M. (2011). Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. *Nature*, *475*(7354), 101-105.

Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., & Thomas, G. (1998). Phosphorylation and Activation of p70^{s6k} by PDK1. *Science*, *279*(5351), 707-710. https://doi.org/doi:10.1126/science.279.5351.707

Reggio, A., Rosina, M., Palma, A., Cerquone Perpetuini, A., Petrilli, L. L., Gargioli, C., Fuoco, C., Micarelli, E., Giuliani, G., Cerretani, M., Bresciani, A., Sacco, F., Castagnoli, L., & Cesareni, G. (2020). Adipogenesis of skeletal muscle fibro/adipogenic progenitors is affected by the WNT5a/GSK3/β-catenin axis. *Cell Death Differ*, *27*(10), 2921-2941. https://doi.org/10.1038/s41418-020-0551-y

Richter, L. R., Wan, Q., Wen, D., Zhang, Y., Yu, J., Kang, J. k., Zhu, C., McKinnon, E. L., Gu, Z., Qiang, L., & Pajvani, U. B. (2020). Targeted Delivery of Notch Inhibitor Attenuates Obesity-Induced Glucose Intolerance and Liver Fibrosis. *ACS Nano*, *14*(6), 6878-6886. https://doi.org/10.1021/acsnano.0c01007

Rivas, D. A., McDonald, D. J., Rice, N. P., Haran, P. H., Dolnikowski, G. G., & Fielding, R. A. (2016). Diminished anabolic signaling response to insulin induced by intramuscular lipid accumulation is associated with inflammation in aging but not obesity. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *310*(7), R561-R569. https://doi.org/10.1152/ajpregu.00198.2015

Rosati, E., Sabatini, R., Rampino, G., Tabilio, A., Di Ianni, M., Fettucciari, K., Bartoli, A., Coaccioli, S., Screpanti, I., & Marconi, P. (2009). Constitutively activated Notch signaling is involved in survival and apoptosis resistance of B-CLL cells. *Blood, The Journal of the American Society of Hematology*, *113*(4), 856-865.

Rudnicki, M. A., & Williams, B. O. (2015). Wnt signaling in bone and muscle. *Bone*, *80*, 60-66. https://doi.org/10.1016/j.bone.2015.02.009

Rudolf, A., Schirwis, E., Giordani, L., Parisi, A., Lepper, C., Taketo, Makoto M., & Le Grand, F. (2016). β-Catenin Activation in Muscle Progenitor Cells Regulates Tissue Repair. *Cell Reports*, *15*(6), 1277-1290. https://doi.org/https://doi.org/10.1016/j.celrep.2016.04.022

Sabatini, D. M. (2017). Twenty-five years of mTOR: Uncovering the link from nutrients to growth. *Proceedings of the National Academy of Sciences*, *114*(45), 11818-11825. https://doi.org/doi:10.1073/pnas.1716173114

Sakamoto, K., Arnolds, D. E., Ekberg, I., Thorell, A., & Goodyear, L. J. (2004). Exercise regulates Akt and glycogen synthase kinase-3 activities in human skeletal muscle.

Biochem Biophys Res Commun, *319*(2), 419-425. https://doi.org/10.1016/j.bbrc.2004.05.020

Sakamoto, K., Aschenbach, W. G., Hirshman, M. F., & Goodyear, L. J. (2003). Akt signaling in skeletal muscle: regulation by exercise and passive stretch. *American Journal of Physiology-Endocrinology and Metabolism*, *285*(5), E1081-E1088. https://doi.org/10.1152/ajpendo.00228.2003

Saxton, R. A., & Sabatini, D. M. (2017). mTOR Signaling in Growth, Metabolism, and Disease. *Cell*, *168*(6), 960-976. https://doi.org/10.1016/j.cell.2017.02.004

Shang, Y., Smith, S., & Hu, X. (2016). Role of Notch signaling in regulating innate immunity and inflammation in health and disease. *Protein & Cell*, *7*(3), 159-174. https://doi.org/10.1007/s13238-016-0250-0

Shen, C.-C., Hsu, M.-N., Chang, C.-W., Lin, M.-W., Hwu, J.-R., Tu, Y., & Hu, Y.-C. (2018). Synthetic switch to minimize CRISPR off-target effects by self-restricting Cas9 transcription and translation. *Nucleic Acids Research*, *47*(3), e13-e13. https://doi.org/10.1093/nar/gky1165

Shen, W., Huang, J., & Wang, Y. (2021). Biological Significance of NOTCH Signaling Strength [Review]. *Frontiers in Cell and Developmental Biology*, *9*. https://doi.org/10.3389/fcell.2021.652273

Stretton, C., Hoffmann, T. M., Munson, M. J., Prescott, A., Taylor, P. M., Ganley, I. G., & Hundal, H. S. (2015). GSK3-mediated raptor phosphorylation supports amino-acid-dependent mTORC1-directed signalling. *Biochem J*, *470*(2), 207-221. https://doi.org/10.1042/bj20150404

Sun, L., Wang, G., Wu, Z., Xie, Y., Zhou, L., Xiao, L., & Wang, H. (2021). Swimming exercise reduces the vulnerability to stress and contributes to the AKT/GSK3β/CRMP2 pathway and microtubule dynamics mediated protective effects on neuroplasticity in male C57BL/6 mice. *Pharmacology Biochemistry and Behavior*, *211*, 173285. https://doi.org/https://doi.org/10.1016/j.pbb.2021.173285

Suzuki, A., Pelikan, R. C., & Iwata, J. (2015). WNT/β-Catenin Signaling Regulates Multiple Steps of Myogenesis by Regulating Step-Specific Targets. *Molecular and Cellular Biology*, *35*(10), 1763-1776. https://doi.org/doi:10.1128/MCB.01180-14

Taxman, D. J., Livingstone, L. R., Zhang, J., Conti, B. J., Iocca, H. A., Williams, K. L., Lich, J. D., Ting, J. P., & Reed, W. (2006). Criteria for effective design, construction, and gene knockdown by shRNA vectors. *BMC Biotechnol*, *6*, 7. https://doi.org/10.1186/1472-6750-6-7

Thoreen, C. C., Chantranupong, L., Keys, H. R., Wang, T., Gray, N. S., & Sabatini, D. M. (2012). A unifying model for mTORC1-mediated regulation of mRNA translation. *Nature*, *485*(7396), 109-113. https://doi.org/10.1038/nature11083

Tran, L., Kras, K. A., Hoffman, N., Ravichandran, J., Dickinson, J. M., D'Lugos, A., Carroll, C. C., Patel, S. H., Mandarino, L. J., Roust, L., & Katsanos, C. S. (2018). Lower Fasted-State but Greater Increase in Muscle Protein Synthesis in Response to Elevated Plasma Amino Acids in Obesity. *Obesity*, *26*(7), 1179-1187. https://doi.org/https://doi.org/10.1002/oby.22213

Truitt, M. L., & Ruggero, D. (2016). New frontiers in translational control of the cancer genome. *Nature Reviews Cancer*, *16*(5), 288-304. https://doi.org/10.1038/nrc.2016.27

Tsivitse, S. (2010). Notch and Wnt Signaling, Physiological Stimuli and Postnatal Myogenesis [Review]. *International Journal of Biological Sciences*, *6*(3), 268-281. https://doi.org/10.7150/ijbs.6.268

Tusavitz, S., Keoonela, S., Kalkstein, M., McCormick, S., Gasser, B., Arrigale, M., Rafferty, P., & Carpenter, A. C. (2020). Macrophage-derived Wnt signaling increases

endothelial permeability during skeletal muscle injury. *Inflamm Res*, *69*(12), 1235-1244. https://doi.org/10.1007/s00011-020-01397-z

Um, S. H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P. R., Kozma, S. C., Auwerx, J., & Thomas, G. (2004). Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature*, *431*(7005), 200-205. https://doi.org/10.1038/nature02866

Valenti, L., Mendoza, R. M., Rametta, R., Maggioni, M., Kitajewski, C., Shawber, C. J., & Pajvani, U. B. (2013). Hepatic Notch Signaling Correlates With Insulin Resistance and Nonalcoholic Fatty Liver Disease. *Diabetes*, *62*(12), 4052-4062. https://doi.org/10.2337/db13-0769

Vo, K., Amarasinghe, B., Washington, K., Gonzalez, A., Berlin, J., & Dang, T. P. (2011). Targeting notch pathway enhances rapamycin antitumor activity in pancreas cancers through PTEN phosphorylation. *Molecular Cancer*, *10*(1), 138. https://doi.org/10.1186/1476-4598-10-138

von Maltzahn, J., Chang, N. C., Bentzinger, C. F., & Rudnicki, M. A. (2012). Wnt signaling in myogenesis. *Trends Cell Biol*, *22*(11), 602-609. https://doi.org/10.1016/j.tcb.2012.07.008

Wang, L., Rhodes, C. J., & Lawrence, J. C. (2006). Activation of Mammalian Target of Rapamycin (mTOR) by Insulin Is Associated with Stimulation of 4EBP1 Binding to Dimeric mTOR Complex 1*. *Journal of Biological Chemistry*, *281*(34), 24293-24303. https://doi.org/https://doi.org/10.1074/jbc.M603566200

Wang, W., Liu, J., Ma, A., Miao, R., Jin, Y., Zhang, H., Xu, K., Wang, C., & Wang, J. (2014). mTORC1 Is Involved in Hypoxia-Induced Pulmonary Hypertension Through the Activation of Notch3. *Journal of Cellular Physiology*, *229*(12), 2117-2125. https://doi.org/https://doi.org/10.1002/jcp.24670

Werner, J. U., Tödter, K., Xu, P., Lockhart, L., Jähnert, M., Gottmann, P., Schürmann, A., Scheja, L., Wabitsch, M., & Knippschild, U. (2018). Comparison of Fatty Acid and Gene Profiles in Skeletal Muscle in Normal and Obese C57BL/6J Mice before and after Blunt Muscle Injury. *Front Physiol*, *9*, 19. https://doi.org/10.3389/fphys.2018.00019

Yang, H. T., Luo, L. J., Chen, W. J., Zhao, L., Tang, C. S., Qi, Y. F., & Zhang, J. (2015). IL-15 expression increased in response to treadmill running and inhibited endoplasmic reticulum stress in skeletal muscle in rats. *Endocrine*, *48*(1), 152-163. https://doi.org/10.1007/s12020-014-0233-y

Yang, Z., & Ming, X.-F. (2012). mTOR signalling: the molecular interface connecting metabolic stress, aging and cardiovascular diseases. *Obesity Reviews*, *13*(S2), 58-68. https://doi.org/https://doi.org/10.1111/j.1467-789X.2012.01038.x

Yeh, C.-J., Sattler, K. M., & Lepper, C. (2023). Molecular regulation of satellite cells via intercellular signaling. *Gene*, *858*, 147172. https://doi.org/https://doi.org/10.1016/j.gene.2023.147172

Zammit , P. S., Golding , J. P., Nagata , Y., Hudon , V. r., Partridge , T. A., & Beauchamp , J. R. (2004). Muscle satellite cells adopt divergent fates : a mechanism for self-renewal? *Journal of Cell Biology*, *166*(3), 347-357. https://doi.org/10.1083/jcb.200312007

Zhang, K., Sha, J., & Harter, M. L. (2010). Activation of Cdc6 by MyoD is associated with the expansion of quiescent myogenic satellite cells. *Journal of Cell Biology*, *188*(1), 39-48. https://doi.org/10.1083/jcb.200904144

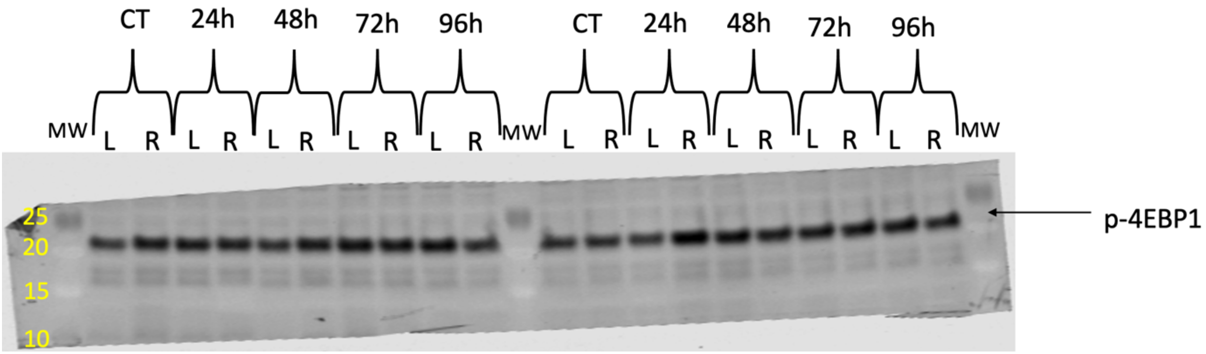
Zhang, Y., & Zheng, X. S. (2012). mTOR-independent 4E-BP1 phosphorylation is associated with cancer resistance to mTOR kinase inhibitors. *Cell cycle*, *11*(3), 594-603.

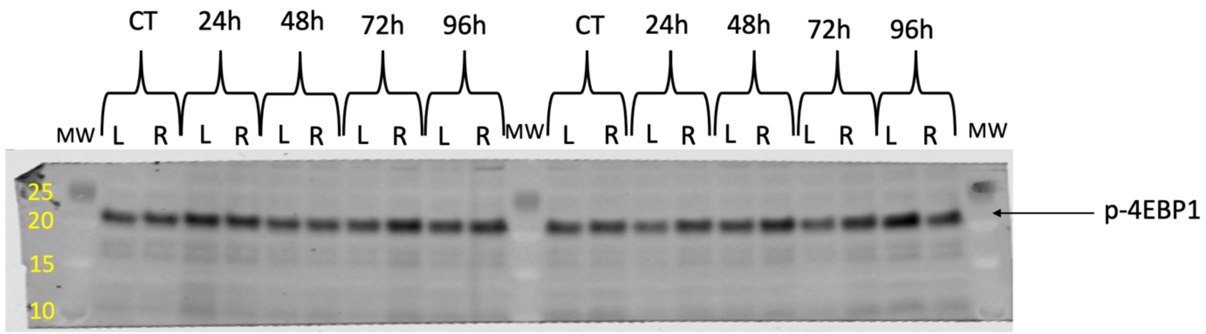
Zheng, L., & Conner, S. D. (2018). Glycogen synthase kinase 3β inhibition enhances Notch1 recycling. *Mol Biol Cell*, *29*(4), 389-395. https://doi.org/10.1091/mbc.E17-07-0474

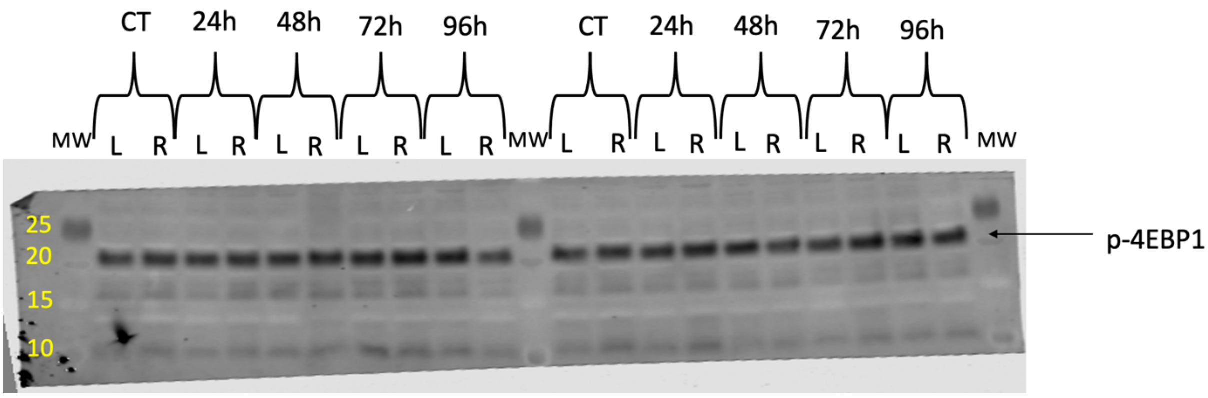
APPENDIX A: WHOLE WESTERN BLOT IMAGES

Samples were loaded in repeated sequence of left gastrocnemius (L; shRNA Notch1 inhibition) and right gastrocnemius (R; empty vector) beginning with control group to 96h post DHR. Molecular weights are labeled in yellow text. Membranes were cut at ~37 kD to allow for more efficient and convenient application of primary antibodies.

p-4EBP1$^{thr37/46}$







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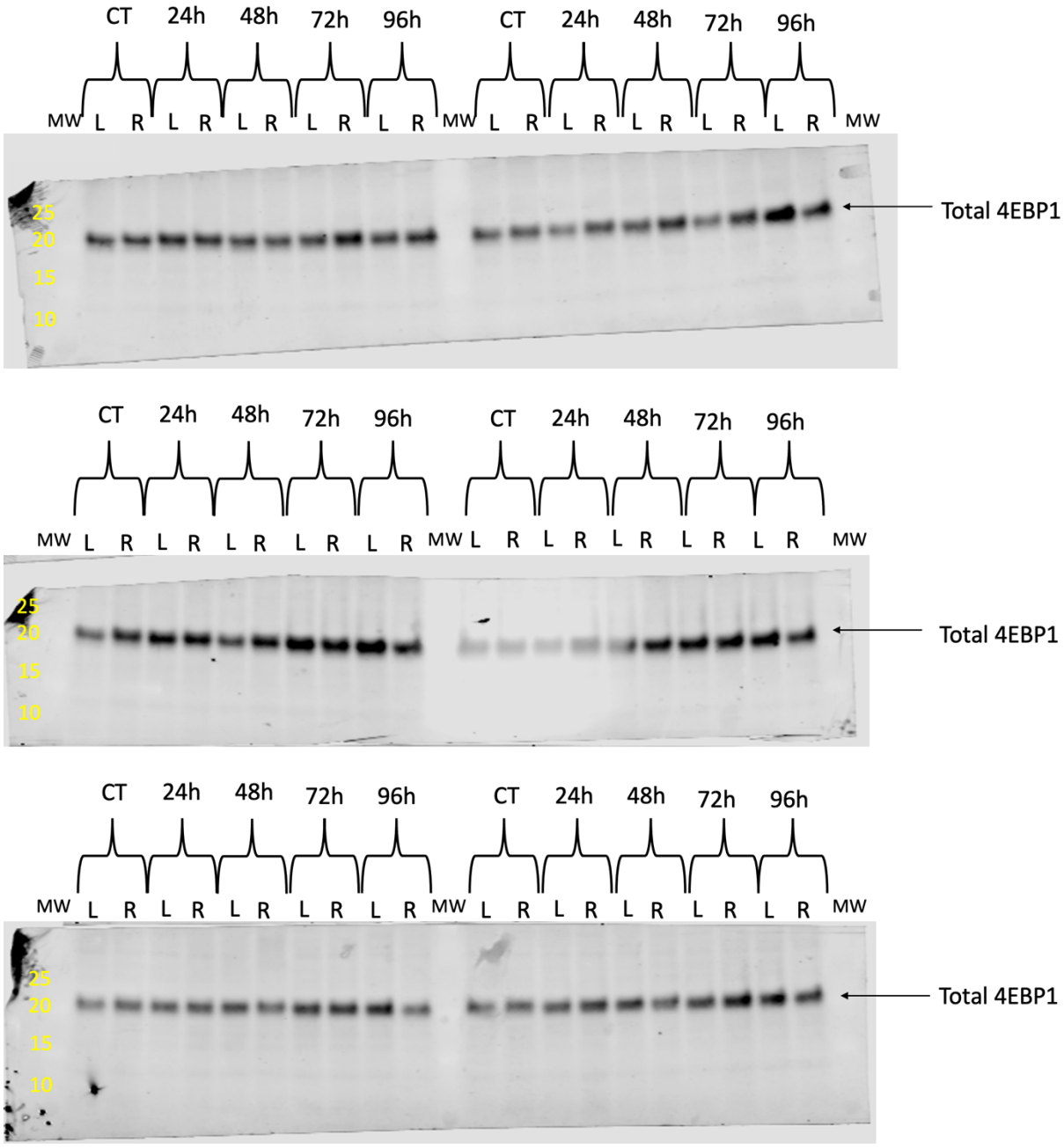
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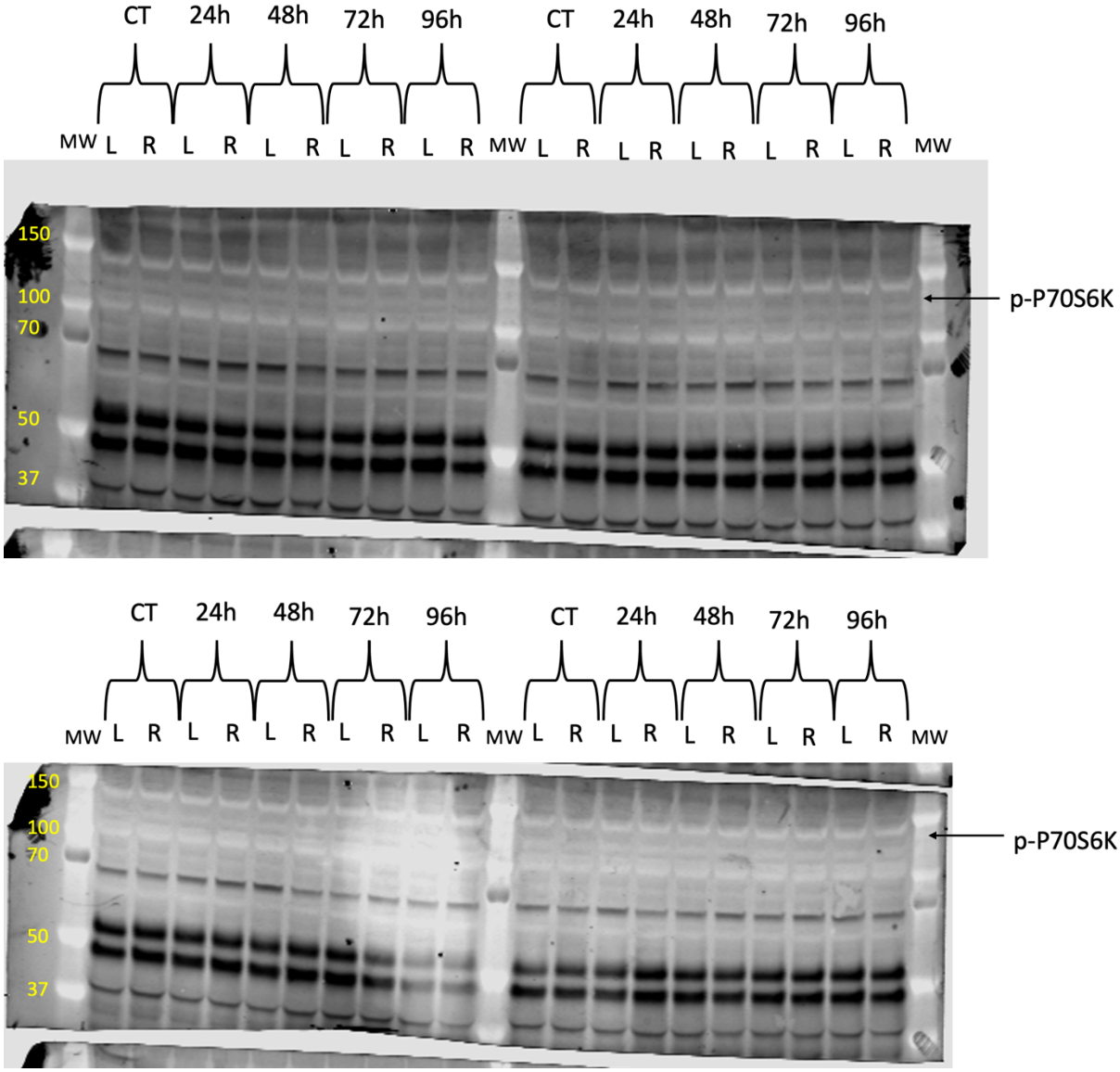
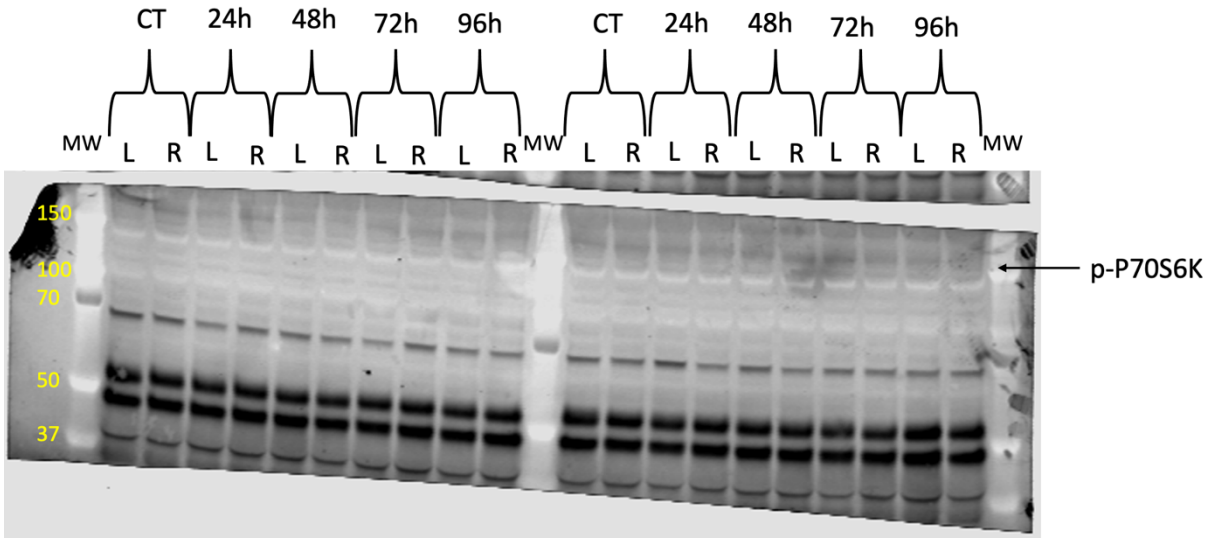
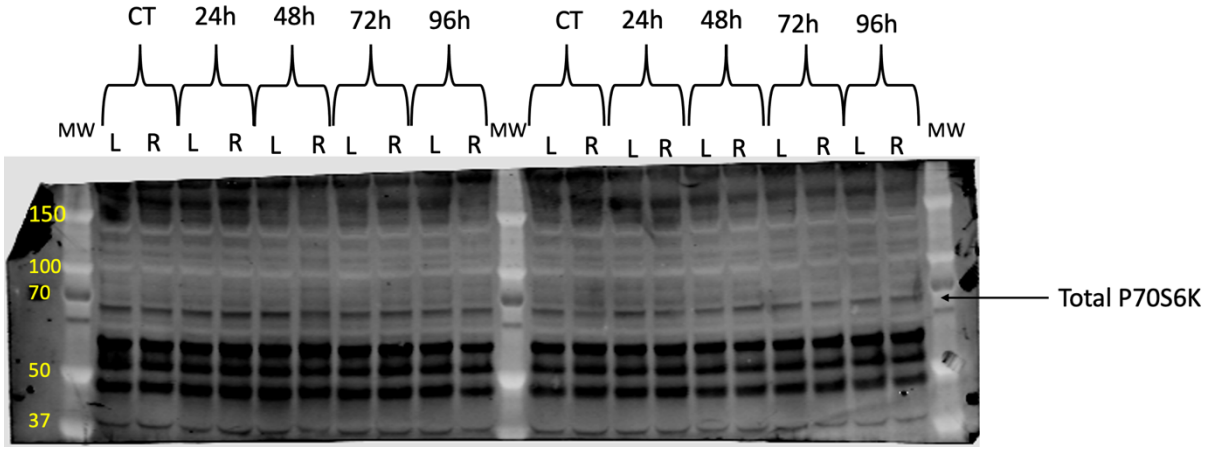
25
20
15
10
Total 4EBP1
CT 24h 48h 72h 96h CT 24h 48h 72h 96h
MW L R L R L R L R L R MW L R L R L R L R L R MW
25
20
15
10
Total 4EBP1
CT 24h 48h 72h 96h CT 24h 48h 72h 96h
MW L R L R L R L R L R MW L R L R L R L R L R MW
25
20
15
10
Total 4EBP1

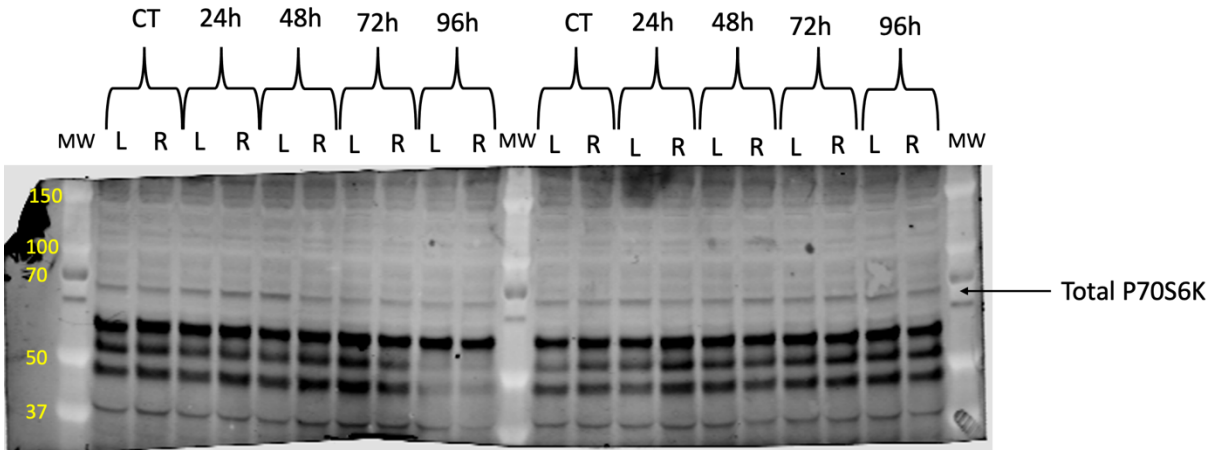
p-P70S6K^{thr389}

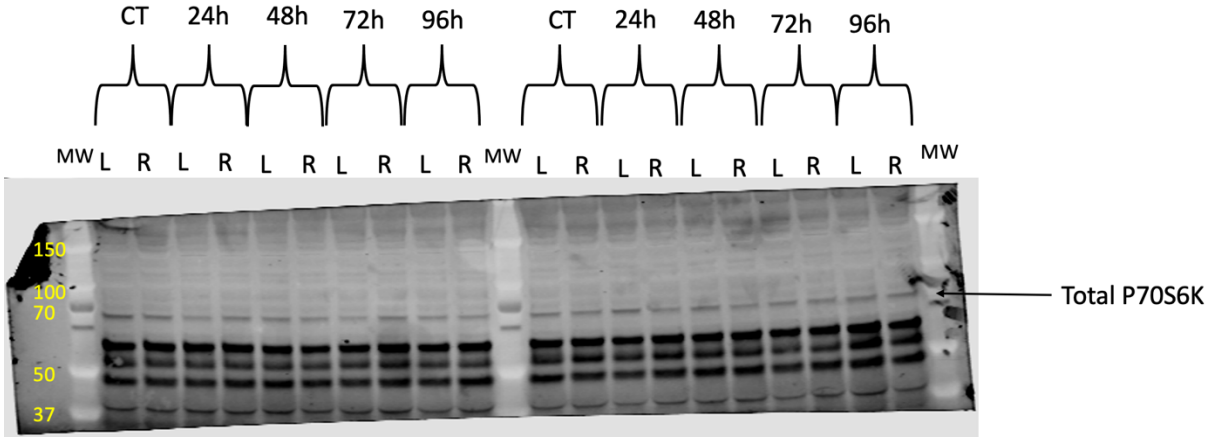




Total P70S6K



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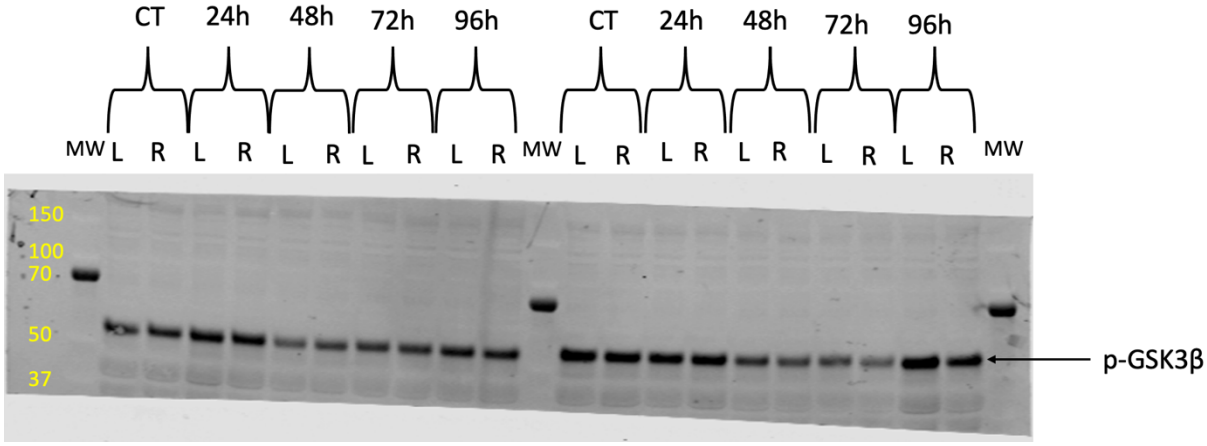
150
100
70
50
37
Total P70S6K

p-GSK3β^{ser9}


CT 24h 48h 72h 96h CT 24h 48h 72h 96h
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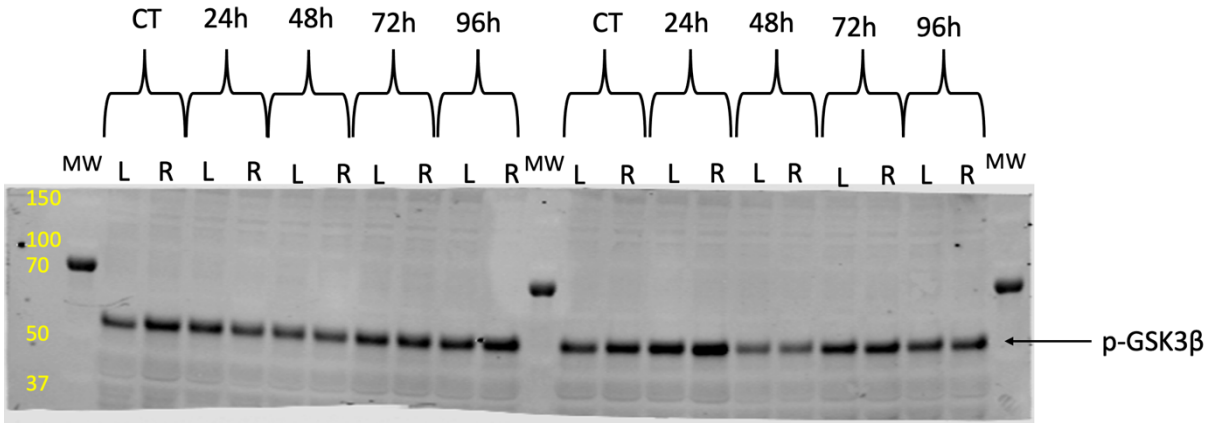
150
100
70
50
37
p-GSK3β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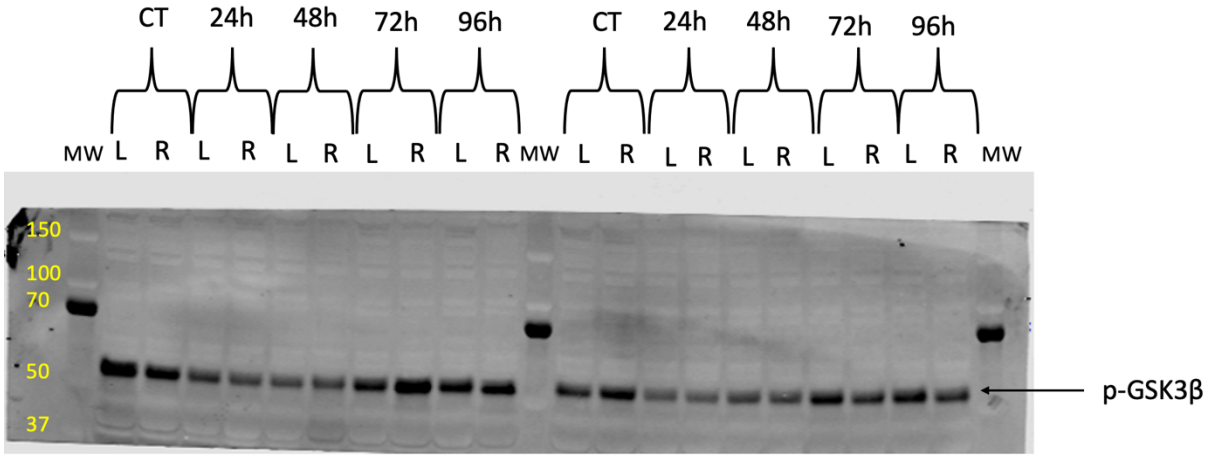
150
100
70
50
37
p-GSK3β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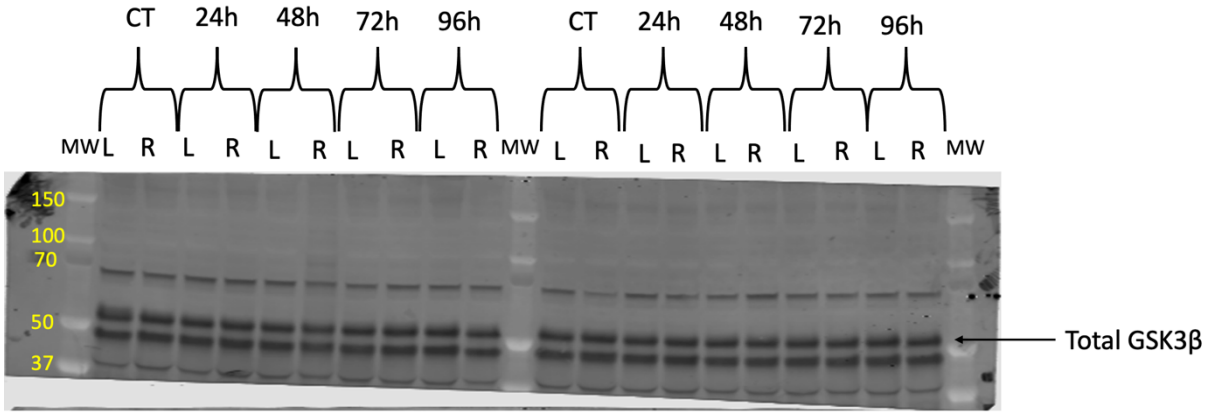
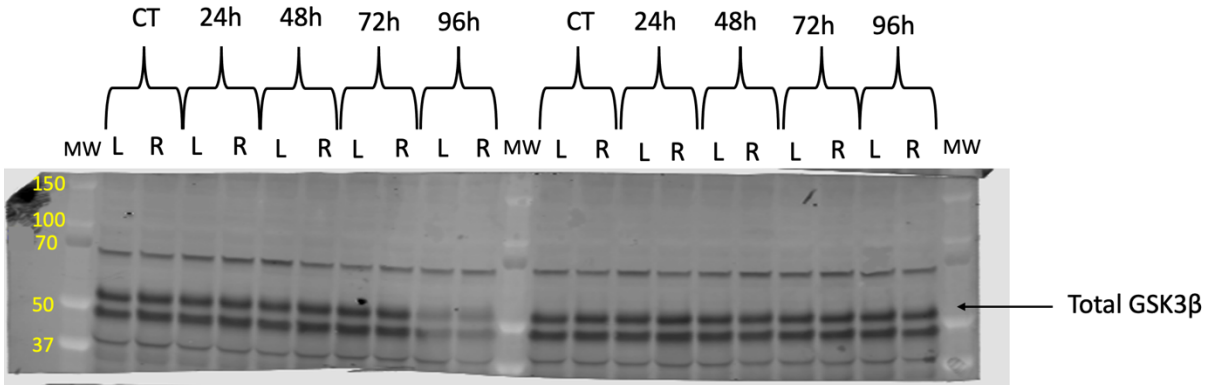
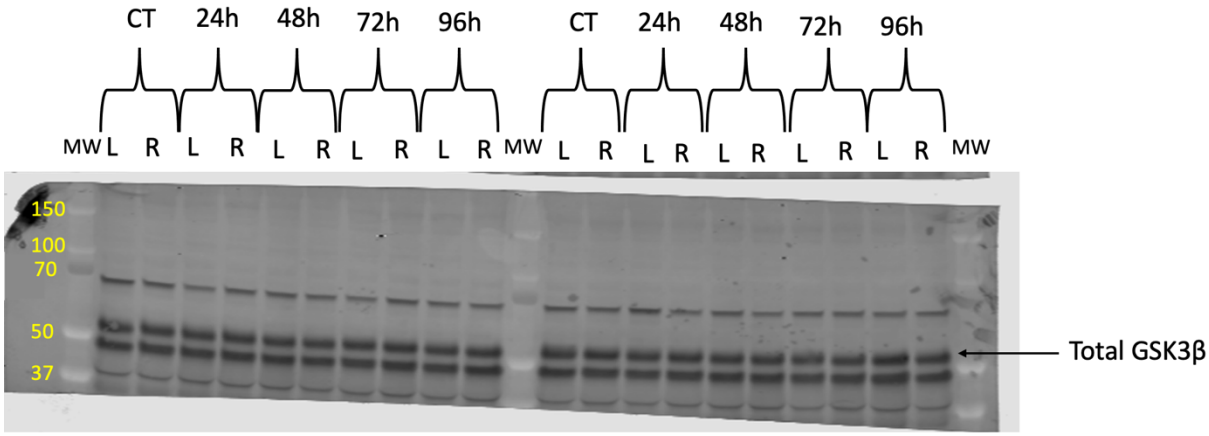
MW L R L R L R L R L R MW L R L R L R L R L R MW
150
100
70
50
37
p-GSK3β

Total GSK3β


CT 24h 48h 72h 96h CT 24h 48h 72h 96h
MW L R L R L R L R L R MW L R L R L R L R L R MW
150
100
70
50
37
Total GSK3β


CT 24h 48h 72h 96h CT 24h 48h 72h 96h
MW L R L R L R L R L R MW L R L R L R L R L R MW
150
100
70
50
37
Total GSK3β


CT
24h
48h
72h
96h
CT
24h
48h
72h
96h
MW L R L R L R L R L R MW L R L R L R L R L R MW
150
100
70
50
37
Total GSK3β