Studying Novel Muscle Atrophy Factors Using Adenoviral Gene Delivery Vectors in Mice

by

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Studying Novel Muscle Atrophy Factors Using Adenoviral Gene Delivery Vectors in Mice

Koç University

Graduate School of Sciences and Engineering

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and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
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ABSTRACT

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Cachexia is a wasting syndrome correlated with high basal energy expenditure and loss of adipose and skeletal muscle tissues. It is associated with many chronic disorders like renal failure and cancer and is an important risk factor for mortality. Currently, there is no successful therapy present to slow down or stop cachexia. We have identified several novel cachexia-related factors and studied their involvement in muscle tissue wasting. Here, we especially focused on a secreted factor, Ectodysplasin A2 (EDA2), which promotes cellular atrophy in cultured myotubes. We developed adenoviral vectors which overexpress these proteins in cultured muscle cells and muscle tissue when injected to animals. We first tested viral vectors on cultured primary muscle cells to validate overexpression. We then purified and injected adenovirus vectors into the tibialis anterior muscles of mice. In this project, we have found that EDA-A2 is a novel cachexia factor that significantly increases the expression of both muscle atrophy and NFκB2 signaling pathway-related genes in primary myotubes and tibialis anterior muscles of mice. EDA-A2-driven muscle atrophy is accompanied by activation of the NFκB2 signaling pathway shown by the increased processing of NFκB2 protein. Our findings suggest that targeting of EDA-A2 could be used in fighting muscle wasting associated with cancer and other chronic diseases.

ÖZETÇE

Yeni Kas Atrofisi Faktörlerinin Adenoviral Gen Aktarımı Vektörleri Kullanılarak Farelerde İncelenmesi

Batu Toledo

Moleküler Biyoloji ve Genetik, Yüksek Lisans

10 Ağustos 2020

Kaşeksi, yüksek bazal enerji harcaması ve yağ ile iskelet kası dokularının kaybıyla ilişkili bir yıkım sendromudur. Böbrek yetmezliği ve kanser gibi birçok kronik bozukluk ile ilişkilidir ve mortalite için önemli bir risk faktörüdür. Şu anda kaşeksiyi yavaşlatmak veya durdurmak için başarılı bir tedavi mevcut değildir. Kaşeksi ile ilgili birkaç yeni faktör belirledik ve kas dokusu kaybına katılımlarını inceledik. Burada özellikle kültür edilmiş miyotüplerde hücresel atrofiyi destekleyen Ectodysplasin A2 (EDA-A2) olmak üzere salgılanan faktörlere odaklandık. Hayvanlara enjekte edildiğinde kültürlenmiş kas hücrelerinde ve kas dokusunda bu proteinleri fazladan ifade edecek adenoviral vektörler geliştirdik. İlk önce ifade seviyesini doğrulamak için kültürlenmiş birincil kas hücreleri üzerinde viral vektörleri test ettik. Daha sonra saflaştırılmış adenovirüs vektörlerini farelerin tibialis anterior kaslarına enjekte ettik. Bu projede, EDA-A2'nin birincil miyotüplerde ve farelerin tibialis anterior kaslarında hem kas atrofisi hem de NFκB2 sinyal yoluna bağlı genlerin ifadesini önemli ölçüde artıran yeni bir kaşeksi faktörü olduğunu bulduk. EDA-A2 ile uyarılan kas atrofisi, NFκB2 sinyal yolunun aktivasyonu ve artan NFκB2 proteininin işlenmesi dolayısıyla olmaktadır. Bulgularımız, EDA-A2'nin tedaviye yönelik hedeflenmesinin, kanser ve diğer kronik hastalıklarla ilişkili kas kaybıyla mücadelede kullanılabileceğini göstermektedir.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

EDA-A2	Ectodysplasin A2
XEDAR	Ectodysplasin A2 receptor
EDA-A1	Ectodysplasin A1
EDAR	Ectodysplasin A1 receptor
LacZ	Beta-galactosidase
NIK	Nuclear Factor Kappa B Inducing Kinase
NIK mut	Nuclear Factor Kappa B Inducing Kinase mutant
NFkB2	Nuclear Factor Kappa B
NFkBia	Nuclear Factor Kappa B Inhibitor Alpha
NFkBie	Nuclear Factor Kappa B Inhibitor Epsilon
Ad-EDA-A2	Ectodysplasin A2 adenovirus
Ad-EDA-A1	Ectodysplasin A1 adenovirus
Ad-LacZ	Beta-galactosidase adenovirus
Ad-NIK	Nuclear Factor Kappa B Inducing Kinase adenovirus
Ad-NIK mut	Nuclear Factor Kappa B Inducing Kinase mutant adenovirus
BAT	Brown Adipose Tissue
WAT	White Adipose Tissue
TNFα	Tumor Necrosis Factor Alpha
TNFR	Tumor Necrosis Factor Receptor
IL-6	Interleukin 1
IL-1	Interleukin 6
MAPK	Mitogen Activated Protein Kinase
XHLED	X-linked hypohidrotic ectodermal dysplasia
IKK1 / IKKα	Inhibitor of Nuclear Factor Kappa-B Kinase Subunit Alpha / I-Kappa-B Kinase 1
IKK2 / IKKβ	Inhibitor of Nuclear Factor Kappa-B Kinase Subunit Beta / I-Kappa-B Kinase 2
IKKγ / NEMO	Inhibitor of Nuclear Factor Kappa-B Kinase Subunit Gamma / Nuclear Factor Kappa-B essential modulator
JNK	c-Jun N-Terminal Kinase

DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
NaCl	Sodium chloride
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
TEMED	Tetramethylethylenediamine
APS	Ammonium Persulfate
HCl	Hydrochloric Acid
TBS	Tris-buffered Saline
ddH_2O	Double-distilled Water
RPM	Revolutions per Minute
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
PBS	Phosphate-Buffered Saline
dNTP	Deoxynucleoside Triphosphate
LB	Lysogeny Broth / Luria-Bertani Medium
PCR	Polymerase Chain Reaction
TE	Tris-EDTA
ITR	Inverted Terminal Repeat

Chapter 1:
INTRODUCTION

1.1 Cancer Cachexia

Etymologically, cachexia is a phrase derived from the Greek words, *kakos* and *hexis*, which means 'bad condition'. There are several different descriptions of cachexia in the literature however, a confident agreement implies that each patient contains two common elements: weight loss which is primarily from skeletal muscle and body fat, and inflammation. Therefore, the major signs that are correlated with cancer cachexia patients are associated with these indications and also include anorexia, fatigue, and anemia, which entirely, reduces life quality progressively in time. (Fearon et al., 2012) Cancer cachexia is a multifactorial syndrome causing fluctuations in numerous metabolic pathways, in many different tissue types and organs. It is indirectly responsible for the death of at least 20% of all cancer patients. The occurrence of the syndrome amongst cancer patients is very high. Although it changes with the type of tumor; in patients with gastric or pancreatic cancer, the occurrence is higher than 80%, where almost 50% of patients with lung, prostate or colon cancer are suffering from cancer cachexia, and about 40% of patients with breast cancer or leukemia mature the syndrome (Fearon et al., 2011). Skeletal muscle contributes to more than 40% of body weight, thus it is the central tissue participating in the wasting that occurs throughout the cancer cachexia. The latest findings propose that tissues and organs such as adipose tissue (both BAT and WAT), heart, gut, liver, and brain are completely affected in the cancer cachexia syndrome and can be associated with skeletal muscle wasting. For example, anomalies in heart function, variations in liver protein synthesis, differences in hypothalamic mediators, and initiation of brown adipose tissue wasting are seen in the cachectic patients. Therefore, cachexia can be named as a multi-organ syndrome. Some of these systemic effects of cachexia can be seen in Table 1.1 (Argilés et al., 2014).

The uncontrolled weight loss ends up with weakness in patients, limits their physical movement, and thus decreases their life quality. Also, anti-cancer therapy is restricted with cachexia too. It often postpones the initiation and accomplishment of

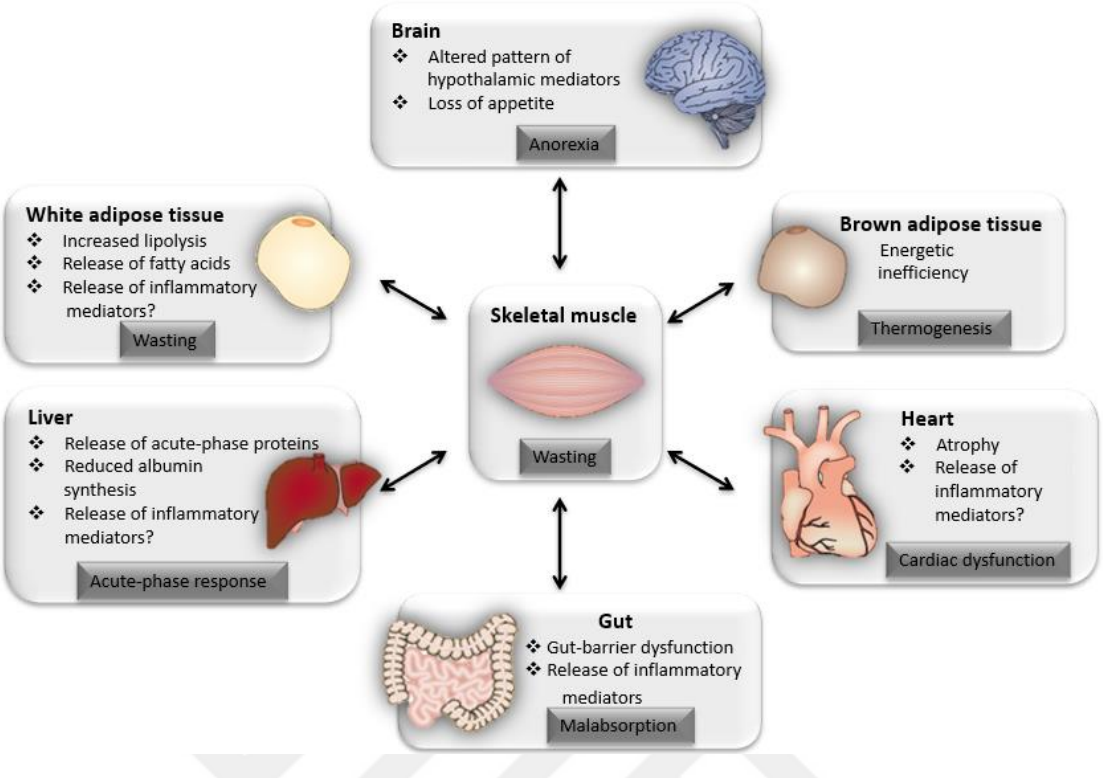

Table 1.1: Consequences of cachexia in different organs.

aggressive anti-cancer therapies and impedes to answers to treatment. Therefore, anti-cachexia treatments can provide effective help to a huge number of cancer patients. Cancer cachexia is often thought of as an inopportune result of cancer and frequently ignored. However, in order to have synergistic impacts on improving response rates and life quality, it becomes more appreciated that cancer management should include mutually anti-tumor and anti-cachexia therapies. Unfortunately, at this time there is no successful therapy against cancer cachexia, and the fundamental molecular mechanisms are even now weakly understood (Kir & Spiegelman, 2016).

Skeletal muscle wasting is one of the most crucial phenotypic aspects of cancer cachexia and it is one of the important sources of functional deficiencies, respiratory obstacles, and fatigue (Mueller et al., 2016). A new research on cachectic patients with cancer of the gastrointestinal tract revealed that, in addition to the muscle mass loss, significant loss of both muscle strength and mechanical quality are seen in the syndrome. While these patients show starvation, the non-muscle protein partition of the body stays fairly unaffected, and the liver mass is even expanded, indicating a tumor-related metabolic disruption that particularly targets skeletal muscle and adipose tissue. Although pathophysiological processes of the diminution of skeletal muscle tissue through cancer

cachexia have been deeply examined in recent years, the discovery of the vital processes and therapeutic targets has been delayed because of the presence of a high number of mediators and signaling pathways. Moreover, there is proof of complicated tissue interactions in this systemic syndrome, which are mediated by cytokines, tumor-derived factors, hormones, and neuropeptides (Mueller et al., 2016).

1.2 Skeletal Muscle Tissue

Skeletal muscle is one of the most active and flexible tissue types in the human body. In humans, skeletal muscle embraces around 40 % of total body mass, encompasses 50–75% of all body proteins, and corresponds to 30–50% of whole-body protein turnover. Skeletal muscle tissue is primarily made up of water (around 75%), protein (around 20%), and other materials such as inorganic salts, fat, minerals, and carbohydrates (5%) (Frontera & Ochala, 2015). Skeletal muscle mass generally depends on the equilibrium between protein synthesis and degradation. These processes are sensitive to environmental factors such as nutritional status, hormonal balance, physical activity/exercise, and injury or disease. The numerous protein sections (structural, contractile, and regulatory) have collected substantial scientific consideration because of their important support to the movement, physical activity and capability, functioning, and health. Skeletal muscle supports substantially to various body events (Frontera & Ochala, 2015). From a mechanical point of view, the principal purposes of skeletal muscle are to translate chemical energy into mechanical energy to produce power and force, provide strength for posture, and create movement. With these helps, it has an influence on activity, allows for the contribution to social and professional situations, preserves or augments health, and play a role in functional independence. From a metabolic view, the skeletal muscle has many roles such as involvement in basal energy metabolism. It functions as storage for vital substrates like amino acids or carbohydrates, and produces heat to preserve body temperature and the exploitation of the mainstream of oxygen and fuel consumed throughout the physical exercise and action. Also, skeletal muscle serves as a reservoir of amino acids that are necessary for other tissues like skin, heart, or brain for the production of organ-specific proteins. In the meantime, amino acid released from skeletal muscle participates in the maintenance of blood glucose levels during the

starvation. A decrease in skeletal muscle mass weakens the body's capability to react to stress and chronic diseases. (Wolfe, 2006)

The two most abundant myofilaments (proteins) found in skeletal muscle are actin and myosin covering around 70–80% of the total protein mass of an individual myofiber. Myosin is the major molecular engine and a total of 11 sarcomeric myosin genes with their consequent protein artifacts have been discovered in mammals (Frontera & Ochala, 2015).

Skeletal muscle tissue is made up of cells known as myocytes that create thread-like fibers; that are bundled into fascicles which are assembled all together to establish the skeletal muscles. Myocytes are the bundles of protein filaments in which myofibrils are located, and they all arrange into replicating components recognized as sarcomeres. The striated shape of skeletal muscle is the consequence of the frequent arrangement of sarcomeres in the myocytes (Argilés et al., 2014). A skeletal muscle fiber (myocyte) is encircled by a plasma membrane recognized as the sarcolemma having the sarcoplasm which is the cytoplasm of skeletal muscle cells. The dark sections that resemble to where thick filaments exist are named as A bands, in the meantime, I bands are light zones which include just thin filaments. The Z disc is in the I band and attaches the thin filaments and unites contiguous myofibrils, whereas the H zone is positioned in the center of every A band. The M line is composed of protein fibers which link adjacent thick filaments. The sarcomere is the area of the myofibril in the middle of two Z discs. When a sarcomere contracts, the Z discs shift and become closer, then I band turns out to be smaller. The A band preserves the equal thickness. At full contraction, both the thin and thick filaments intersect (Figure 1.1) (Argilés et al., 2014).

Skeletal muscle is one of the major parts of the human body, whose act is vital for a sort of biological activities, from movement to respiration. A stringent equilibrium between protein synthesis and degradation is necessary to conserve homeostasis. In the meantime, a drop in the synthesis or an extreme degradation of skeletal muscle results in wasting. The complicated hormonal web of anabolic and catabolic elements typically disrupted during tumor progression (Porporato, 2016).

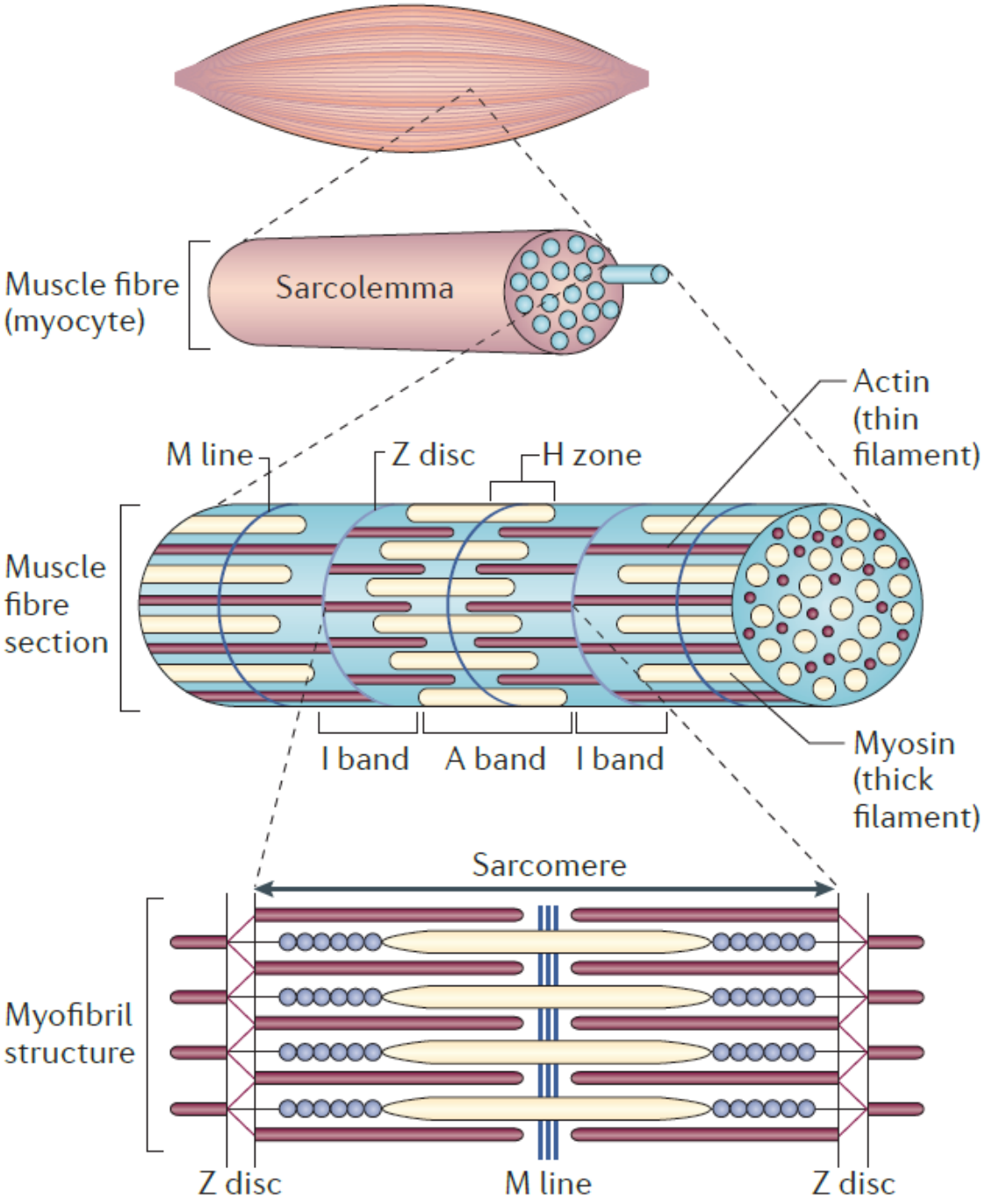

Figure 1.1: Structure of muscle tissue and cell. Adapted from Argilés et al., 2014.

1.3 Muscle Atrophy and Cancer Cachexia

For the past decades, researchers have investigated the obscure mediators of cancer cachexia intending to establish original therapeutics that either slow down or stop the tumor-induced adipose and skeletal muscle loss. It is assumed that these mediators may arise from immune cells, tumor cells, or the targeted mesenchymal tissues going through wasting. Chronic inflammation is the major condition in carcinogenesis, as cancer cells depend on the production of proinflammatory mediators. These mediators provide cancer cells protection from apoptosis, growth, and support for angiogenesis/metastasis. Proinflammatory cytokine "Cachectin" was found at the top of the mediator list. Cachectin was found primarily in association with the wasting in rabbits and later it was known as the TNFα. This cytokine was consequently demonstrated in mice to stimulate cachexia (Beutler and Cerami, 1986). Another candidate is IL-6 which may cooperate with TNFα or function alone as a regulator of systemic inflammation in cancer cachexia. A variety of cancer forms produce IL-6 and this can be amplified by host-derived proinflammatory cytokines (e.g., IL-1). Contrary to TNFα, the circulation of IL-6 has been demonstrated to relate to weight loss in cancer patients, and crucially, IL-6 concentrations correlate with diminished survival. Also, gain- and loss-of-function experiments in tumor-bearing mice verify the necessity of IL-6 in the regulation of cancer cachexia (Moses et al., 2009). Although pro-inflammatory cytokines, such as TNFα, IL-1, and IL-6, have long been clustered as regulators of cancer cachexia, their duties in muscle atrophy are still questionable and further research on this area is needed. (Cohen et al., 2014)

Several intracellular elements participate in protein turnover and thus, in the muscle wasting process. Therefore, these inflammatory cytokines which are secreted by either immune cells or tumors directly stimulate signaling pathways that upregulate enzymes inducing skeletal muscle protein turnover. Pro-inflammatory and pro-cachectic cytokines such as TNFα or IL-1 are implicated in two well-known signaling pathways, the nuclear factor-κB (NF-κB) and the p38 MAPK pathways. These mediators stimulate upregulation of the expression of the key E3 ubiquitin ligases which are muscle RING finger-containing protein 1 (MURF1; also known as TRIM63) and muscle atrophy F-box protein (ATROGIN1; also known as MAFBX and FBXO32) that facilitate structural muscle protein breakdown and inhibition of protein synthesis. MURF1 is facilitating the

ubiquitylation of the thick filament of the sarcomere, as well as other thick filament components. (Glass, 2010). ATROGIN1 also is a high-fidelity indicator of acute muscle atrophy, which is upregulated in various situations of cachexia. ATROGIN1 upregulation arises with the p38 stimulation and by the induction of the C/EBPb transcription factor, that is triggered through p38 phosphorylation. MAFbx performs the ubiquitination of an eIF3f that is an element of the protein translation machinery (Fearon et al., 2012).

There are three pathways which are in charge of the intracellular protein breakdown in skeletal muscle: the lysosomal pathway, the calcium-dependent pathway, and the ubiquitin-proteasome pathway (UPP). Lysosomal and calcium-dependent proteases have been presented to participate in 15% – 20% of total protein breakdown in muscle. On the other hand, the UPP is thought to perform the main role in the skeletal muscle loss in experimental and human models of cancer cachexia (Biolo et al., 2000). UPP initiates with the early transmission of E1, the Ub-activating enzyme, to the Ub conjugating enzyme, E2, by the way of ATP-dependent establishment of a thiol-ester bond. This process is repeated to create a polyubiquitin chain that is transesterified to E3, the Ub ligase. The E3 conjugates the polyubiquitin complex to a lysine residue on the target protein N-terminus. Only, selected proteins are recognized by E3 and targeted for degradation in the 26S proteasome, a multi-subunit catalytic enzyme complex that is located in the nucleus and cytosol. *Atrogin-1* and *Murf-1* conducts this phenomena and the ubiquitinated proteins are then inserted into the core (20s) proteasome for proteolysis into smaller peptides in skeletal muscle cells (Esper & Harb, 2005).

1.4 Ectodysplasin A, Edar and Xedar

The human *EDA* gene was initially discovered by positional cloning in XLHED patients and is placed on the long arm of the X chromosome. EDA is a 391 amino acid residues long membrane protein with a transmembrane domain, a short intracellular domain, a short positively-charged sequence essential for relations with heparan-sulfate proteoglycans, a consensus furin cleavage sequence responsible for proteolytic processing of EDA, a bi-partite collagen-like domain, a stalk region of an unidentified role, and a 150 amino acid residues-long C-terminal TNF homology domain (THD) responsible for receptor binding (Kowalczyk-Quintas & Schneider, 2014). The *Eda*

transcript is well-known to go through complex alternative splicing generating various ectodysplasin transcripts. The original cDNA defined in 1996 found out to code for a truncated molecule lack of the TNF domain and with the cloning of the murine homolog EDA was accepted as a part of the TNF superfamily. Six different human transcripts deficient of the TNF domain have been described, two of them were also been present in mice. Many of the alternative transcripts are differentially expressed in numerous human tissues. However, whether they are biologically significant (for example, used to control the quantity of the operational protein) or simply correspond to atypical splicing outcomes is unknown. However, the two longest isoforms of EDA, EDA-A1, and EDA-A2, are the operational types of the molecule. EDA-A1 (391 aa) and EDA-A2 (389 aa) differ only with an addition of two amino acids in the TNF domain. EDA-A1 and EDA-A2 were found to associate with two different receptors; *Edar* is the specific receptor for EDA-A1, on the other hand, another related but distinct TNFR X-linked Eda-A2 receptor (*Eda2r*; *Xedar*) binds EDA-A2, but not EDA-A1 (Yan et al., 2000).

Positional cloning of the murine *downless* gene guides the invention of Edar which is a TNF receptor molecule. There were phenotypic similarities between *Tabby* and *downless* mice thus, it was directly supposed that *Edar* was the receptor of EDA. Supplementary research carried by numerous groups has confirmed that EDA-A1 and *Edar* were physically interacting. EDAR is a 448 amino acid type I transmembrane protein where the mature extracellular domain (159 aa) consists of one broad and two incomplete cysteine-rich domains tracked by a single transmembrane region and a C-terminal intracellular domain (237 aa) (Headon and Overbeek, 1999). On the other hand, XEDAR was discovered as a unique TNFR family member molecule in a database examination based on sequence similarity. Two alternatively spliced isoforms of XEDAR have been described that diverge in their membrane-proximal section of the cytoplasmic domain. XEDAR is a 297/318 amino acid, type III transmembrane protein (deficient of NH2-terminal signal peptide) that includes an extracellular section with three cysteine-rich domains, an intracellular region without significant homology to other TNFRs and a single transmembrane region. The ligand-binding domain of XEDAR is only about 32% matching with EDAR. From the seventeen TNF ligands tested, XEDAR bound, particularly to EDA-A2. Fundamentally, there was no cross-reactivity with EDA-A1 (or EDA-A2 for EDAR), although high concentrations of the ligand applied (Yan et al., 2000).

Signaling by TNFRs ends up with pleiotropic reactions dependent on the cell type and the other signals that the cell takes the most usual result being the stimulation of the transcription factor NF-κB. Transfection of mammalian cells with *Edar* ends up with a dose-dependent activation of NF-κB. The NF-κB response can be efficiently inhibited by dominant-negative mutants of IKK1, and IKK2 (Kumar et al., 2001). *Edar* is not able to bind any of the Trafs precisely, as an alternative it utilizes a distinctive adapter molecule EDARADD (encoded by the *crinkled* gene in mice) for signal transduction. EDARADD is a 208 amino acid protein involving an NH_2-terminal Traf-binding consensus sequence and a COOH-terminal death domain (Yan et al., 2002).

The intracellular domain of XEDAR is much smaller (140 aa) than that of EDAR. The intracellular part of XEDAR interacts with Traf 1,3 and 6 but not with 2. Traf 6 is expected to be the key connector molecule in *Xedar* signaling because a point mutation in *Xedar* eliminating Traf 6 association ends up with a substantial loss of NF-κB activation though the only insignificant loss was found when the consensus sequence for Traf 3 was mutated. Similar to many other TNFRs, *Xedar* also triggers the JNK pathway. *Xedar* that contains a point mutation in the Traf 6 binding site was incapable of JNK activation, on the other hand, a mutation in the Traf 3 consensus site had only a negligible outcome. Also, XEDAR (but not EDAR) stimulated the MAPK pathway when assessed by an Elk1 transcriptional assay (Mikkola & Thesleff, 2003).

1.5 NF-κB, Canonical and Non-canonical Pathway

Nuclear Factor kappa B (NF-κB) was discovered by David Baltimore et al. in 1986 as a factor in the nucleus of B cells that binds to the enhancer of the kappa light chain of immunoglobulin. It has been demonstrated to be present in the cytoplasm of every cell in its inactive state and is conserved through the animals, starting from Drosophila to humans. It is translocated to the nucleus only when it is activated, where it controls the expression of more than 300 immune, growth, and inflammation-related genes. Five different NF-κB family members have been found which are NF-κB 1 (p105/p50), NF-κB 2 (p100/p52), RelA (p65), RelB, and c-Rel. Two separate NF-κB initiation pathways have been discovered which are canonical pathway and non-canonical (alternative) pathway (Aggarwal, 2003).

In mammals, the transcription factor of the NF-κB family consists of two clusters of proteins which are different in their composition, act, and the way of synthesis. The first cluster contains p105 and p100 which are produced as huge polypeptides and as to create limited proteolysis short active molecules that are p50 (NF-κB 1) and p52 (NF-κB 2). While p50 is created by constitutive p105 processing, p100 proteolysis is firmly controlled through its inducible phosphorylation by NIK (NF-κB inducing kinase) and its polyubiquitination. The second NF-κB subfamily contains p65 (Rel-A), Rel-B, and c-Rel proteins that do not come from precursors and have a C-terminal transcriptional activation domain (Ghosh et al., 1998).

There are two different signaling pathways present controlling the activation of NF-κB which are known as the canonical pathway (classical) and the non-canonical pathway (alternative pathway). The joint directing step in both pathways is the activation of an IκB kinase (IKK) that consists of catalytic kinase subunits (IKKα/IKKβ) and the directing non-enzymatic scaffold protein NEMO (NF-kappa B essential modulator also known as IKKγ). Activation of NF-κB is due to IKK-mediated phosphorylation induced proteasomal degradation of the IκB inhibitor allowing the active NF-κB transcription leads to the expression of the IκBα gene which accordingly sequesters NF-κB subunits and finished transcriptional activity unless a continual activation signal present. In the canonical NF-κB signaling pathway, binding of the ligand to a cell surface receptor such as a member of the Toll-like Receptor super-family starts the recruitment of adaptors (such as TRAF) to the cytoplasmic domain of the receptor. These adaptors recruit the IKK complex that causes the phosphorylation and then degradation of the IκB inhibitor. This pathway is vital for the stimulation of innate immunity and inflammation. The non-canonical pathway is necessary for the stimulation of p100/Rel-B complexes and arises through the growth of lymphoid organs that are responsible for the production of B and T lymphocytes and only a B and B cell-activating factor (BAFF). This pathway uses an IKK complex that consists of two IKKα subunits but not NEMO. In the non-canonical (alternative) pathway, ligand stimulated initiation ends up with the activation of NF-κB inducing kinase (NIK) that phosphorylates and activates the IKKα complex which phosphorylates p100 ending up with the processing and release of the p52/Rel-B active heterodimers (Strickland and Ghosh, 2006).

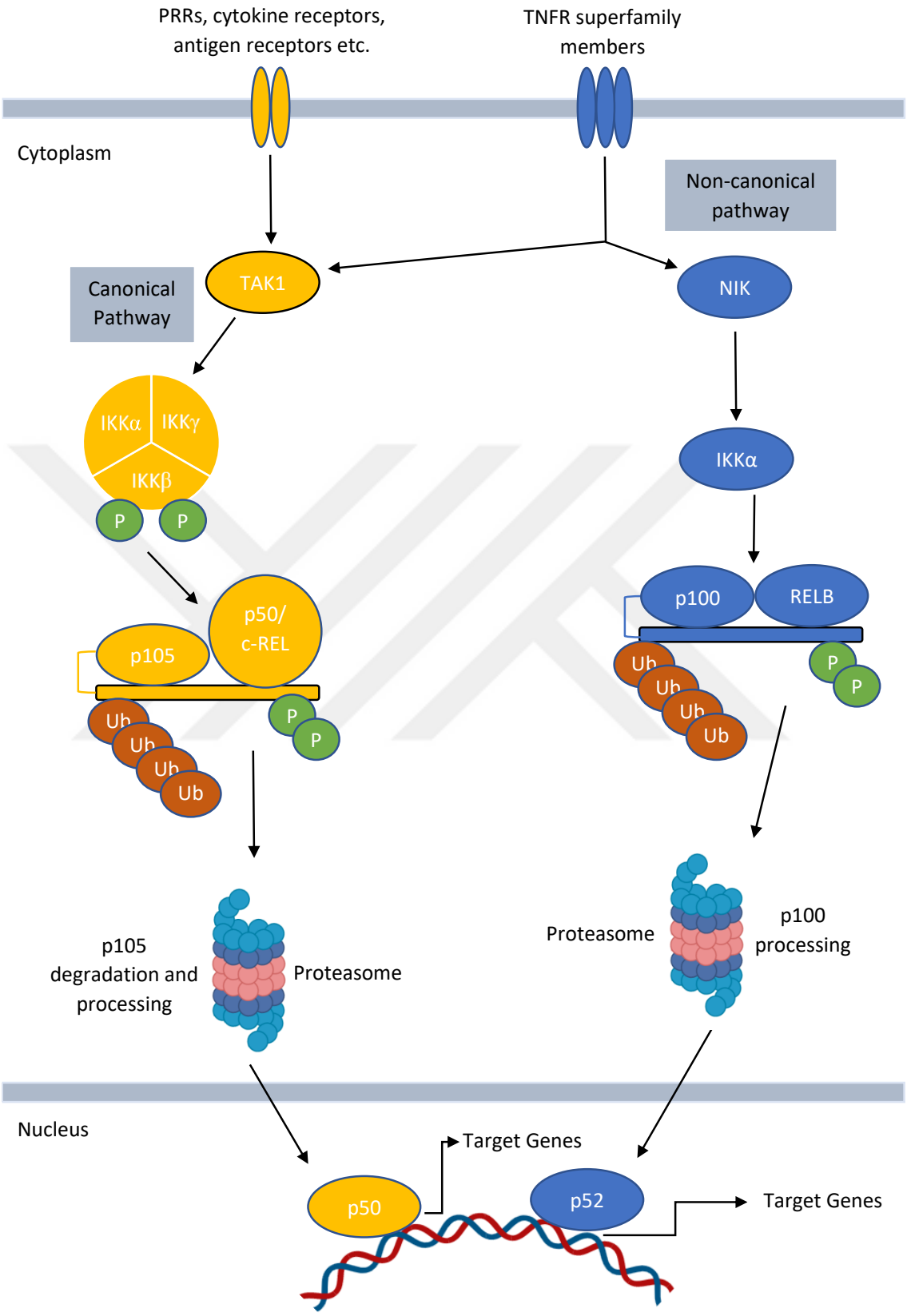

Figure 1.2: Canonical and Non-Canonical NFkB Signaling Pathways

NF-κB is a transcription factor which operates as a principal regulator for turning on certain immune and inflammatory responses. NF-κB adjusts cell behavior in many different ways; it prevents apoptosis (programmed cell death), enhances cell proliferation, and rises inflammatory and immune reactions. Stimulation of NF-κB supports the growth of several types of human cancer tumors that contain cancers of the blood and some breast cancer types (Serasanambati & Chilakapati, 2016).

1.6 Adenoviruses

Adenoviruses are DNA viruses and they have 36 kb of double-stranded DNA. Following the entry into the nucleus, genes from the early region 1 (E1a and E1b) are immediately transcribed. Through the initial stage of viral replication, four non-contiguous segments of the adenovirus genome are expressed (E1 to E4). They act as the principal transcriptional controllers that initiate the process of viral gene expression which ends up with the adenoviral genome replication. Following the commencement of DNA replication, the main late promoter leads the viral transcription (Volpers & Kochanek, 2004). Adenoviral vectors permit for transmission of their genetic content to the host genome but do not incorporate them into the host DNA. Thus, there is a very minimal possibility of interruption of essential cellular genes or activities. On the other hand, the adenoviral-vector methodology restricts gene therapy to medication approaches where there is transient protein expression is required. Since the viral DNA ultimately vanishes, therapies for chronic disorders would need to be repeated routinely. In preclinical experiments, it has been demonstrated that adenoviral vector DNA is expressed in the liver, skeletal muscle, heart, brain, lung, pancreas, and tumor tissue. When adenoviral vectors are given intravenously, most of the virus accumulates in the liver (Schiedner et al., 1998).

The adenoviruses have numerous marks that make them appropriate to use in gene therapy applications. Firstly, they are omnipresent, they have been isolated from many distinct organisms and more than 100 separate serotypes have been found where forty-three was in humans. Most of the adults in the world have been exposed to the adenoviruses that are most frequently used in gene therapy (serotypes 2 and 5). Secondly, adenoviral vectors quickly affect a large number of human cells and have a tendency to

produce high levels of gene transfer with respect to amounts reached with some of the other vectors. Thirdly, adenoviral vectors have a low level of pathogenicity in humans. They trigger rare and only benign symptoms related to the widespread cold. Fourthly, adenoviral vectors can hold comparatively significant sections of DNA (up to 7.5 kilobase pairs [kb]) and transduce these transgenes in nonproliferating cells. Also, the adenoviral genome does not go through readjustment frequently. Finally, adenoviral vectors are comparatively straightforward to manipulate using recombinant DNA techniques (Vorburger & Hunt, 2002).

Chapter 2:
METHODS

2.1 Cell Culture

Primary myoblasts were isolated from male mice previously in our laboratory and an established primary myoblast cell line was used in the experiments. Firstly, primary myoblasts were expanded in DMEM containing 20% FBS (Sigma) supplemented with basic fibroblast growth factor (bFGF) (Promega), 1% penicillin-streptomycin (Gibco) for forty-eight hours. Myoblasts were then passaged into 12 well plates and differentiated in DMEM with 5% (for RNA extraction) or 2% (for protein isolation) horse serum and 1% penicillin-streptomycin (Gibco) were used until mature myotube formation seen (~24 hours) and then treated with adenovirus. After myotube formation, the medium (DMEM with 5% (for RNA extraction) or 2% (for protein isolation) horse serum and 1% penicillin-streptomycin (Gibco)) were renewed in every 24 hours. Throughout the culturing, collagen-I coated plates were used (Corning).

Human embryonic kidney (HEK) 293A cells were cultured in DMEM containing 10% FBS (Sigma) supplemented with 1% penicillin-streptomycin (Gibco) for forty-eight hours.

2.2 Animal Experimentations

C57BL/6 mice were used in all animal experiments that were approved by the Institutional Animal Care and Use Committee of Koç University. Mice (Mus musculus) were kept in 12 h light-dark cycles (06.00-18.00) at 22 ^{o}C and fed with a standard irradiated rodent chow diet. Eight-to-ten-week-old male mice were used in all animal experiments. The sample size, which was concluded empirically by performing preliminary experiments, was decided to be five. Mice were separated into treatment groups arbitrarily while fulfilling the average body weight equivalence in each group criteria. There was no blinding where the treatment groups were visibly marked to avoid mistakes. $5x10^{8}$ adenoviral particles were injected into the tibialis anterior muscles of the

animals intramuscularly. One leg received Ad-LacZ as control whereas the contralateral leg received the test group adenovirus. All mice were killed in the late light cycle (13.00-16.00). Mice were kept independently in all adenovirus injection experiments. Tibialis anterior muscles were collected into sterile 1.5 ml microcentrifuge tubes and frozen in liquid nitrogen immediately for storage.

2.3 Protein Isolation and Western Blotting

Cells were homogenized in a 150µl lysis buffer that is composed of 50 mM Tris HCl (pH 7.5), 500 mM NaCl, 5 mM EDTA, 10% Triton X and 1 mM phenylmethyl-sulphonyl fluoride (PMSF) complemented with Pierce Protease and Phosphatase Inhibitor Mini Tablets (Thermo). Muscle tissues were homogenized in 1 ml of the tissue lysis buffer that is composed of 50 mM tris HCl (pH:7.5), 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% NP40 and 1 mM phenylmethyl-sulphonyl fluoride (PMSF) complemented with Pierce Protease and Phosphatase Inhibitor Mini Tablets (Thermo)by using Kinematica Polytron PT 1200E Handheld Homogenizer. The homogenates were centrifuged at 17,000 rpm for 10 min and the supernatants were used as whole-cell lysates.

Protein concentration was measured with Bio-Rad Protein Assay according to manufacturer's manual and 30 mg protein lysate was differentiated in separate SDS–PAGE lanes at 120V for 2.5 hours. SDS-PAGE 8% separation (resolving) gels were poured as; 2.3 ml ddH_2O, 1.3 ml 30% Acrylamide, 1.3 ml 1.5 M Tris-HCL (pH:8.8), 50µl 10% SDS, 3µl TEMED and 50µl 10% APS. SDS-PAGE 5% stacking gels were poured as; 1.1 ml ddH_2O, 335µl 30% Acrylamide, 0.5 ml 0.5 M Tris-HCL (pH:8.8), 20µl 10% SDS, 2µl TEMED and 20µl 10% APS. Then, the proteins were transferred to a nitrocellulose membrane at 120V for 1 hour and were blotted with primary antibodies that are in TBS containing 0.1% Tween (TBS-T) and 5% Bovine Serum Albumin overnight at 4 ^oC after blocking with 5% milk in TBS-T and washing steps with TBS-T (3 times / 10 minutes). As secondary antibody incubation, HRP conjugated or IRDye800CW antibodies in TBS-T containing 5% milk at room temperature for 1 hour were applied after washing steps with TBS-T (3 times / 10 minutes). Enhanced

chemiluminescence (ECL) western blotting substrates (Advansta) or Near-Infrared Western Blot Detection (Licor Biosciences) were used for visualization of the results.

2.4 RNA Isolation and qRT-PCR

RNA was isolated from cultured cells or frozen tissue samples using TRIzol. Briefly, for the cells, TRIzol was added to the plates and the solution was taken to tubes after 5 minutes of incubation. On the other hand, tissues were homogenized with QIAGEN TissueLyser LT Tissue Homogenizer and 1 ml of TRIzol for 15 minutes. RNA isolation was done with the phenol/chloroform RNA extraction technique. Briefly, 100µl (200µl for tissue) of chloroform was added and vortexed for 20 seconds three times. The solutions were centrifuged at 17000 rpm for 15 minutes at 4 ^{o}C and the aqueous phase was taken to a new tube. 250µl (500µl for tissue) isopropanol was added and precipitated at -80 ^{o}C for one hour. Then, solutions were dissolved at room temperature and centrifuged at 17000 rpm for 20 minutes at 4 ^{o}C. Supernatants were discarded and the pellets were washed with 0.5 ml (1 ml for tissue) 70% ethanol which was previously cooled at -20 ^{o}C. The solutions were centrifuged at 17000 rpm for 5 minutes at 4 ^{o}C and the supernatants were discarded. The pellets were airdried and dissolved with 50µl ddH_2O. RNA concentrations were measured with NanoDrop One. Reverse transcription of RNAs were done with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's manual. 1 ug RNA was added to reverse transcription reactions.

The consequential cDNA was examined with qRT-PCR. 25 ng cDNA and 150 nmol of each primer were combined with SYBR GreenER qPCR Super Mix (Invitrogen). qRT-PCR reactions were done in the 96-well format using a CFX Connect Real-Time PCR Detection System (BIORAD). Relative mRNA levels were calculated by using the comparative CT method and normalized to cyclophilin mRNA. The subsequent primers were used: *Cyclophilin* F: 5'-GGAGATGGCACAGGAGGAA-3', R: 5'-GCCCGTAGT GCTTCAGCTT-3'; *Atrogin-1 (Fbxo32)* F: 5'-TCAGAGAGGCAGATTCGCAA-3', R: 5'-GGGTGACCCCATACTGCTCT; *Murf-1 (Trim63)* F: 5'-TCCTGATGGAAACGCT ATGGAG-3', R: 5'-ATTCGCAGCCTGGAAGATGT-3'; *EDA1 & EDA2* F:5'-AACTC GGGAAAATCAGCCAGC-3', R: 5'-TTGAGCACTCCACCTGAAAGAT-3'; *NIK* F:

5’-CGAGCTACTTCAACGGGGTC-3’, R:5’-GGCAATGTCTCCCACCTTGA-3’; *NFkB2* F: 5’-CCTTCGTAGTTACAAGCTGGC-3’, R: 5’-GGCACTGTCTTCTTTCAC CT-3’; *NFkBia* F: 5’-TAGCAGTCTTGACGCAGACC-3’, R: 5’-CGTGTGGCCATTGT AGTTGG-3’; *NFkBie* F: 5’-GACATTGATGTACAGGAGGGCA-3’, R: 5’-GGTG TGC ACCCGTTAAGCAT-3’.

2.5 Molecular Cloning and Adenovirus Production

Briefly; LacZ, EDA-A2, EDA-A1, NIK, and NIK mut genes were cloned into pENTR-TOPO by classical double digestion cloning, NotI, and AscI restriction endonuclease enzymes and T4 DNA ligase were used. pENTR-TOPO vectors that contain our genes, were inserted into pAd/CMV/DEST (destination) vector with LR recombination (Figure 2.1). The destination vector was digested with PacI and HEK293A cells were transfected with PacI digested destination vectors. After the cytopathic effect has seen, cells were harvested. This was the crude lysate. Adenoviruses were amplified by infecting HEK293A cells with crude lysate. Amplified adenoviruses were harvested and used in in-vitro experiments. Purification and titering were done to amplified adenoviruses to use them in in-vivo experiments.

Firstly, all genes were amplified with PCR. In PCR reaction, 1X Standard Taq Reaction Buffer, 200 µM dNTP Mix, 0.2 µM forward and reverse primers, <1,000 ng Template DNA, 1.25 units Taq DNA Polymerase (NEB) and ddH_2O up to 50µl were used. PCR reaction conditions were: 95 ^oC for 30 seconds as initial denaturation; 30 cycles of, 95 ^oC for 30 seconds as denaturation, 55-65 ^oC for 30 seconds as annealing, 68 ^oC for 60 seconds per kb as extension; 68 ^oC for 5 minutes as final extension. After the PCR reaction, products were gel purified by using Macherey-Nagel Nucleospin Gel and PCR Clean-up Kit according to the manufacturer’s instructions. Purified PCR products and acceptor plasmid pENTR/TOPO digested with restriction endonuclease enzymes NotI and AscI. Restriction digest was set as; 1X CutSmart Buffer, 1 µg DNA, 1-unit restriction enzymes, and ddH_2O up to 50µl; at 37 ^oC for 3 hours. Inserts were PCR purified whereas plasmids were gel purified by using Macherey-Nagel Nucleospin Gel and PCR Clean-up Kit according to the manufacturer’s instructions. Acceptor plasmids were also treated with; 1 unit of Alkaline-Calf Intestinal Phosphatase (CIP) with 1X

CutSmart Buffer and ddH_2O up to 20µl, at 37 ^oC for 30 minutes and 70 ^oC heat was applied to inactivate the enzyme. The reaction products were purified with Macherey-Nagel Nucleospin Gel and PCR Clean-up Kit according to the manufacturer's instructions. Then, ligation reaction was set as; 1X Ligase Buffer, 100 ng vector DNA, 17 ng insert DNA, 1-unit T4 Ligase, and ddH_2O up to 10µl, at 15 ^oC for 18 hours. After ligation, half of the reaction product was transformed into competent bacteria. Briefly, ligation products were added to 100µl competent bacteria and incubated on ice for 30 minutes. Then, the mixture was heat-shocked at 42 ^oC for 1 minute and put back on the ice for 5 minutes. One ml of LB (without antibiotics) was added to the solution and incubated in 37 ^oC shaker for 1 hour. Then, the bacteria were concentrated and plated on LB agar plates with kanamycin. Plates were incubated overnight at 37 ^oC incubator. Single grown colonies were grown in 5 ml of LB with kanamycin and their ingredients were confirmed with colony PCR. Basically, the same PCR reaction was set with the DNA obtained from cultures. The validated candidates were isolated with Thermo Fisher Scientific GeneJET Plasmid Miniprep Kit according to the manufacturer's manual and sent to sequencing to confirm their ingredients. Then, the LR recombination reaction was set as; 50-150 ng of entry clone, 300 ng destination vector, 2µl LR Clonase II enzyme mix, and TE buffer up to 10µl, at 25 ^oC, for 3 hours. Then, 1µl Proteinase K was added to the reaction and incubated at 37 ^oC for 10 minutes. LR recombination reaction products were transformed into competent bacteria and plated on LB agar plates with ampicillin. Single colonies were grown and colony PCR was done for validation as explained before. The validated candidates were isolated with Thermo Fisher Scientific GeneJET Plasmid Miniprep Kit according to the manufacturer's manual. Then, the PacI digestion reaction was set to open the ITRs as; 5 ug purified DNA, 1X CutSmart Buffer, 1-unit PacI, and up to 50µl ddH_2O, at 37 ^oC for 3 hours. PacI digested destination plasmids that contain genes of interest were purified with Thermo Fisher Scientific GeneJET Plasmid Miniprep Kit according to the manufacturer's manual. Purified constructs transfected to HEK293A cells by using Lipofectamine 2000. Basically, 2 ug of DNA incubated in DMEM was mixed with Lipofectamine 2000 incubated with DMEM and after 30 minutes of incubation, the mixture was added to HEK293A cells by dropwise. Then, these HEK293A cells were grown according to Virapower Adenoviral System instructions and were harvested to obtain crude lysate of adenovirus after the cytopathic effect. Briefly, the harvest was done as, triple freeze (at -80 ^oC) – thaw (at 37 ^oC) cycles before cell debris

spin down to take supernatant that contains the viral particles. Adenovirus crude lysate then was used for amplification of the adenoviral particles by reinfection of HEK293As with 100-200µl crude lysate. Cells were harvested as explained before, after the proper

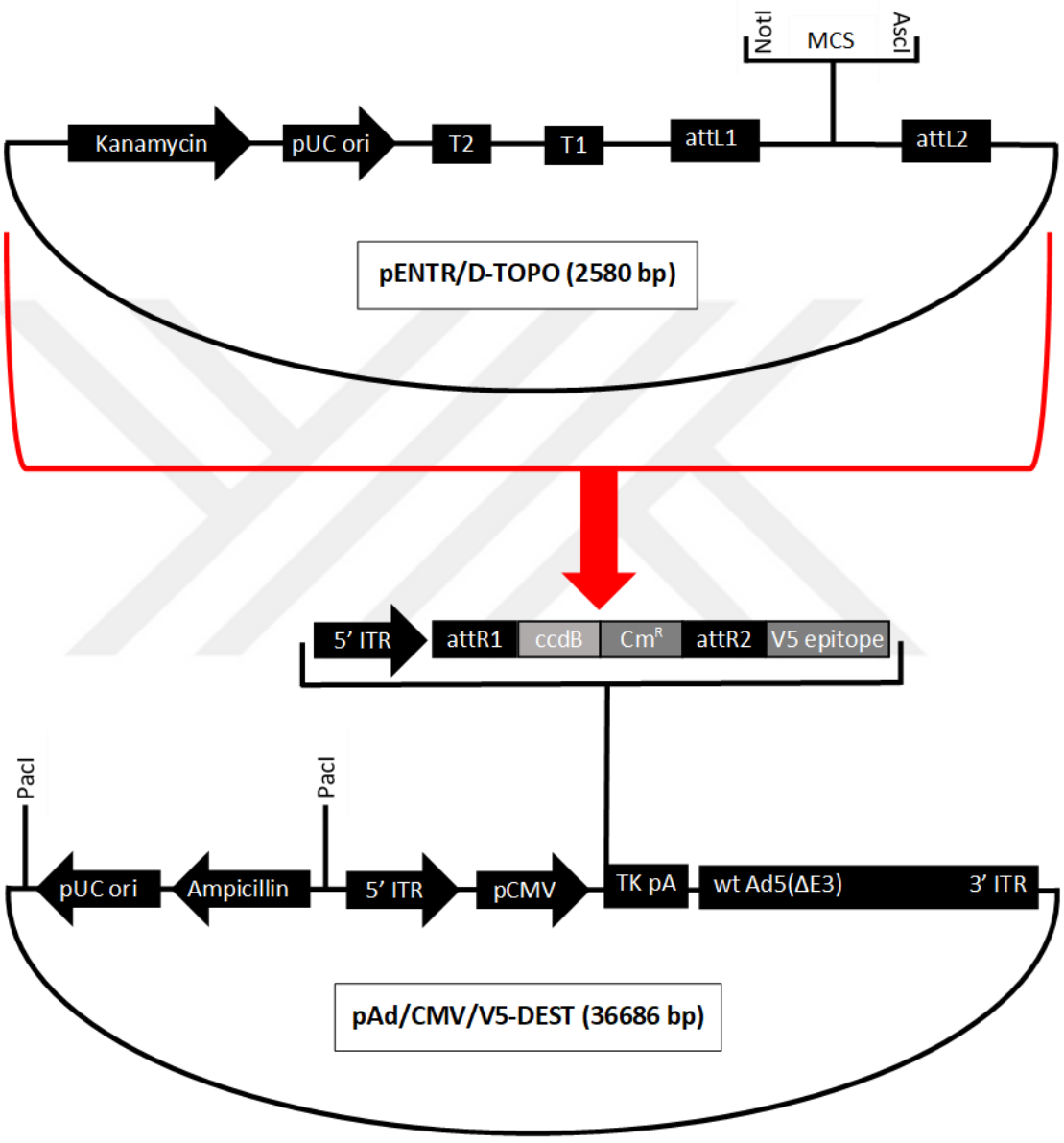

Figure 2.1: Cloning Strategy for Adenovirus Production (Vector Maps)

level of the cytopathic effect seen on cells to obtain the amplified adenovirus. Amplified adenoviruses were used to reinfect HEK293As again to obtain purified adenovirus which was used in animal experiments. After the cytopathic effect, cells harvested as explained before. Then, purification was done by using the Clontech Adeno-X Maxi Purification

Kit according to the manufacturer's manual. Adenovirus titer was found out with Clontech Adeno-X Rapid Titer Kit according to the manufacturer's manual. Purified and tittered adenoviruses were used in in-vivo experiments.

Chapter 3:
RESULTS

3.1 EDA-A2 Overexpression in Primary Myotubes Induces Expression of Muscle Atrophy and NFκB Signaling Pathway Related Genes

Adenoviruses have been used for overexpression of the candidate genes in mammalian cells for many years. To study the effect of EDA-A2 on muscle cells, primary myotubes were cultured and treated with proper adenoviruses for 24 hours. Firstly, gene expression changes were examined with qRT-PCR. Muscle atrophy related genes *Atrogin-1* and *Murf-1* were upregulated when EDA-A2 overexpressed in primary myotubes which suggests that muscle wasting is induced. In the same samples, expression of NFκB signaling pathway-related genes; *Nik, NFκB2, NFκBia,* and *NFκBie* were also upregulated. From these elements, *Nik* and *NFκB2* are the main elements of the alternative pathway which indicates that EDA-A2 may signal through the alternative NFκB signaling pathway *in vitro*. LacZ and EDA-A1 adenovirus treatment did not show any effect and confirmed that EDA-A1 and EDA-A2 have different effects on muscle tissue (Figure 3.1). These findings argue that EDA-A2 is a potent inducer of muscle atrophy that may signal through the alternative NFκB pathway.

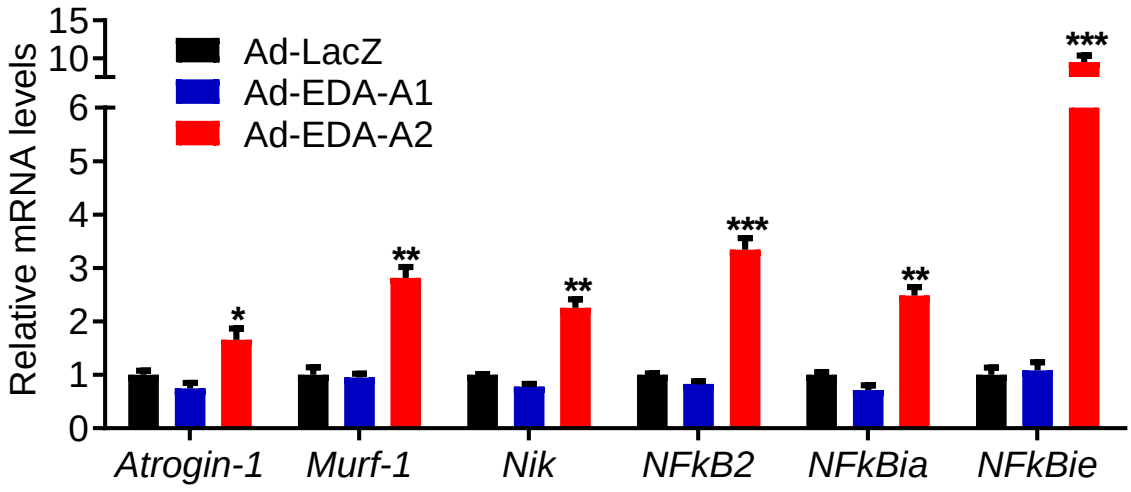

*Figure 3.1: Primary myotubes were treated with 20µl of LacZ, EDA-A1 and EDA-A2 adenoviruses for 24 hours and gene expression changes were determined with qRT-PCR. The values are mean ± SEM. *p < 0.05, **p < 0.005, ***p < 0.0005.*

To test the effects of EDA-A2 on muscle cells at the protein expression we treated primary myotubes with EDA-A2 and EDA-A1 adenoviruses for 48 hours and harvested the cell lysates. NIK protein level was upregulated only when the cells were overexpressing EDA-A2. NFκB2 signaling pathway was also activated which can be seen with the decrease in p100 and increase in p52 protein levels, in the EDA-A2 overexpression condition that shows enhanced NFκB2 processing (Figure 3.2). None of these effects were present when LacZ or EDA-A1 were overexpressed that shows the solid effect of EDA-A2. The canonical NFκB signaling pathway was not activated also because the p50 protein expression level was the same in all conditions (Figure 3.2).

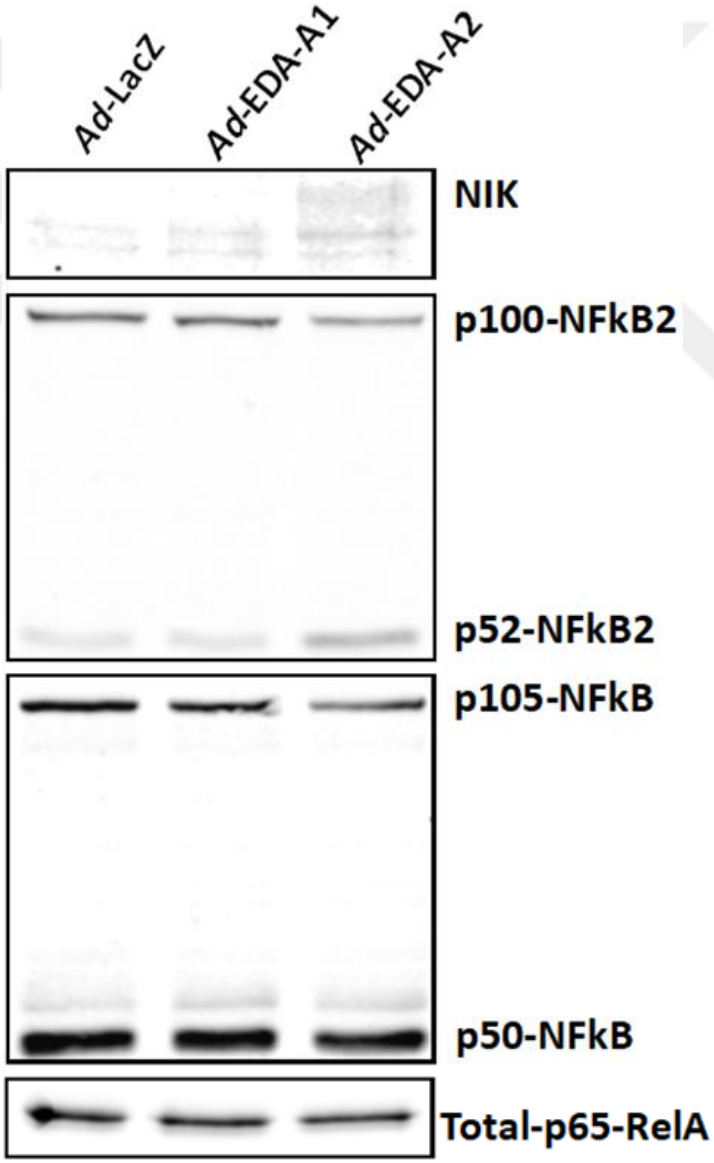

Figure 3.2: NIK, NFkB2-p100, NFkB2-p52, NFkB-p105 and NFkB-p50 protein levels in primary myotubes with 48 hours of LacZ, EDA-A1 and EDA-A2 adenovirus treatments.

These findings one more time indicate that EDA-A2 signaling may be working through the alternative NFκB signaling pathway rather than the canonical NFκB pathway.

3.2 EDA-A2 Overexpression in Mouse Tibialis Anterior Muscle Induces Expression of Muscle Atrophy and NFκB Signaling Pathway Related Genes

After the validation of the effects and signaling pathway of EDA-A2 on primary myotubes in-vitro, the effect of this factor in real life should be examined. A similar approach was applied for in-vivo experiments. Purified adenoviral particles used to overexpress the genes of interest in mice tibialis anterior muscle by injecting and changes in RNA and protein levels inspected.

Firstly, the effects on the gene expression profile examined with qRT-PCR. Proper amounts of EDA-A2 adenovirus injected into mouse tibialis anterior muscles and incubated for 7 days. Then, animals were sacrificed, and tibialis anterior and gastrocnemius muscles were harvested. The muscle tissues were homogenized, and total RNA isolation was performed. After the cDNA production, qRT-PCR performed and according to gene expression profiles; muscle atrophy related genes *Atrogin-1* and *Murf-1*, and NFκB signaling pathway-related genes; *Nik*, *NFκB2*, *NFκBia*, and *NFκBie* expression were upregulated with respect to their basal level that can be seen in Ad-LacZ treatments. Among these, *Nik* and *NFκB2* are the main elements of the alternative NFκB signaling pathway (Figure 3.3). This again demonstrates that EDA-A2 causes muscle

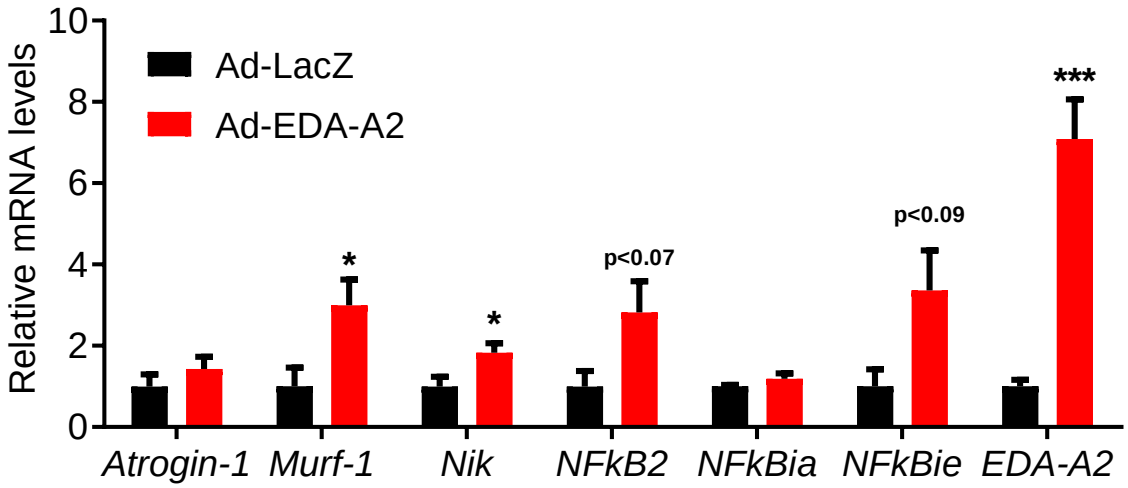

*Figure 3.3: Mice tibialis anterior muscles were injected with 5x10^8 pfu LacZ and EDA-A2 adenoviruses and were incubated for 7 days (n=5). Gene expression changes were determined with qRT-PCR. The values are mean ± SEM. *p < 0.05, **p < 0.005, ***p < 0.0005.*

atrophy and it signals through the alternative NFκB signaling pathway *in-vivo* as well. The EDA-A2 expression level was also examined which was significantly higher than the basal level.

The presence of muscle atrophy was also examined as a change in muscle weight in the same experiment. Mice tibialis anterior and gastrocnemius muscle weights were measured and there was a significant decrease in EDA-A2 overexpressing muscle (tibialis anterior) weight whereas there was no significant change in gastrocnemius that did not get any treatment (Figure 3.4). This decrease in muscle weight hinted at the existence of muscle atrophy in the presence of EDA-A2.

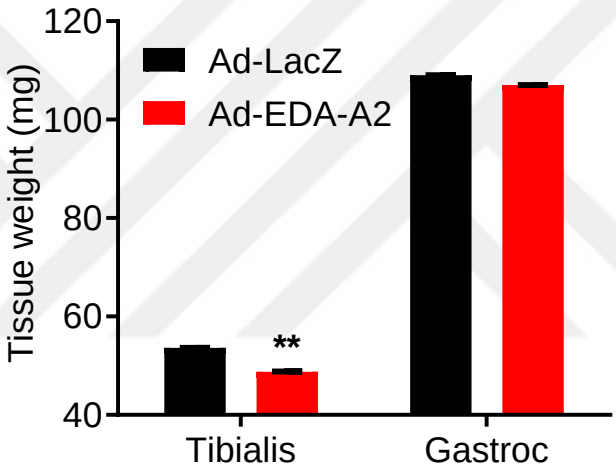

*Figure 3.4: Mice tibialis anterior muscles were injected with 5x10^8 pfu LacZ and EDA-A2 adenoviruses and were incubated for 7 days (n=5). Tibialis anterior and gastrocnemius muscle weight measured. *p < 0.05, **p < 0.005, ***p < 0.0005.*

The effect of EDA-A2 on protein expression levels were also investigated *in-vivo*. To this end, protein levels were determined with western blot, and again p52 protein level was increased in EDA-A2 overexpressing mice tibialis anterior muscle tissues that demonstrate the alternative NFκB signaling pathway activity whereas neither p105 nor p50 protein levels changed (Figure 3.5). This again indicates that EDA-A2 signals through the alternative NFκB signaling pathway and it does not induce canonical NFκB signaling pathway *in vivo* too.

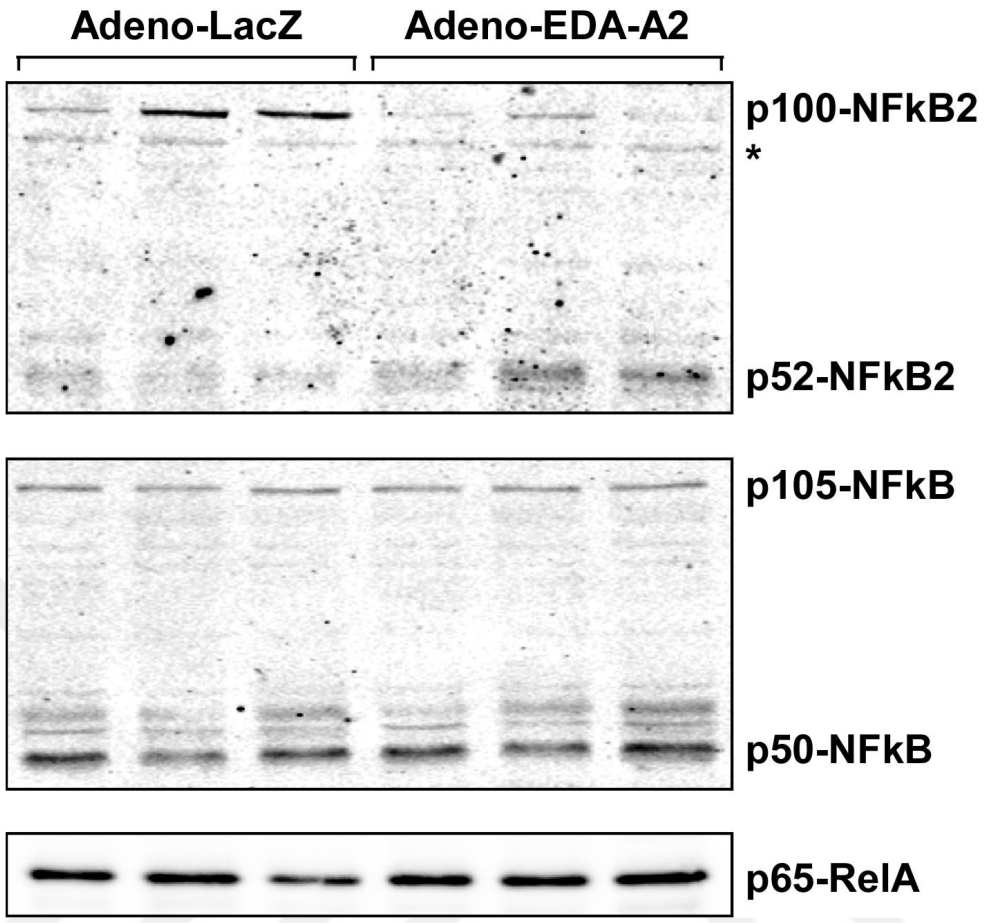

Figure 3.5: Mice tibialis anterior muscles were injected with 5x10^8 pfu LacZ and EDA-A2 adenoviruses and were incubated for 7 days (n=5). NFkB2-p100, NFkB2-p52, NFkB-p105 and NFkB-p50 protein levels measured with western blotting.

3.3 NIK Overexpression in Primary Myotubes Induces Expression of Muscle Atrophy and NFκB Signaling Pathway Related Genes

To study the downstream signaling pathway of EDA-A2 induced muscle atrophy further, NIK adenovirus was produced. To confirm the effect of NIK on the protein level, primary myotubes were treated with NIK adenovirus for 48 hours and protein expression levels were examined with western blotting. The alternative NFκB signaling pathway was again activated with the NIK overexpression which can be seen with the decrease in p100 and increase in p52 protein levels (Figure 3.6). These results supported our previous findings and demonstrated that the alternative NFκB pathway is inducing the atrophy in muscle cells.

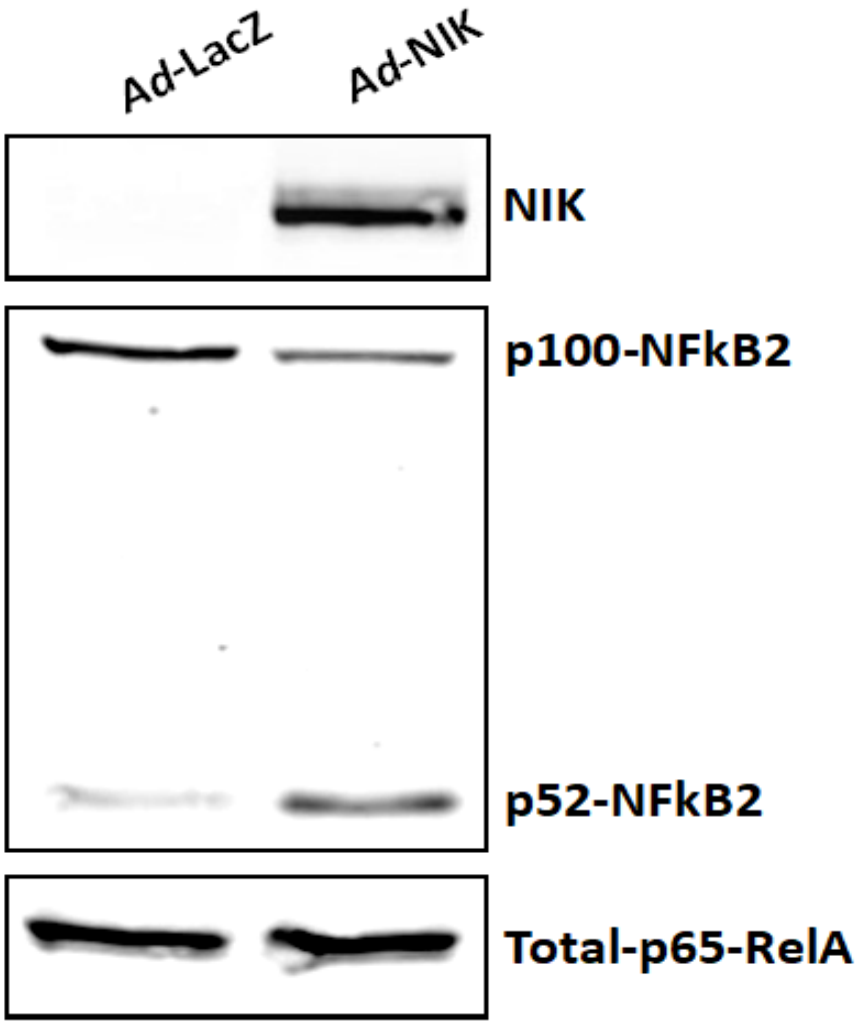

Figure 3.6: NIK, NFkB2-p100 and NFkB2-p52 protein levels in primary myotubes with 48 hours of LacZ and NIK adenovirus treatments.

Then, primary muscle cells treated with NIK adenovirus again and gene expression changes were examined with qRT-PCR. The expression of muscle atrophy related genes *Atrogin-1* and *Murf-1*, and NFκB signaling pathway-related genes *Nik, NFκB2, NFκBia,* and *NFκBie* were upregulated where *Nik* and *NFκB2* are the main elements of alternative NFκB signaling pathway (Figure 3.7). These findings support our previous results and suggest that the alternative NFκB signaling pathway is inducing muscle atrophy in muscle cells.

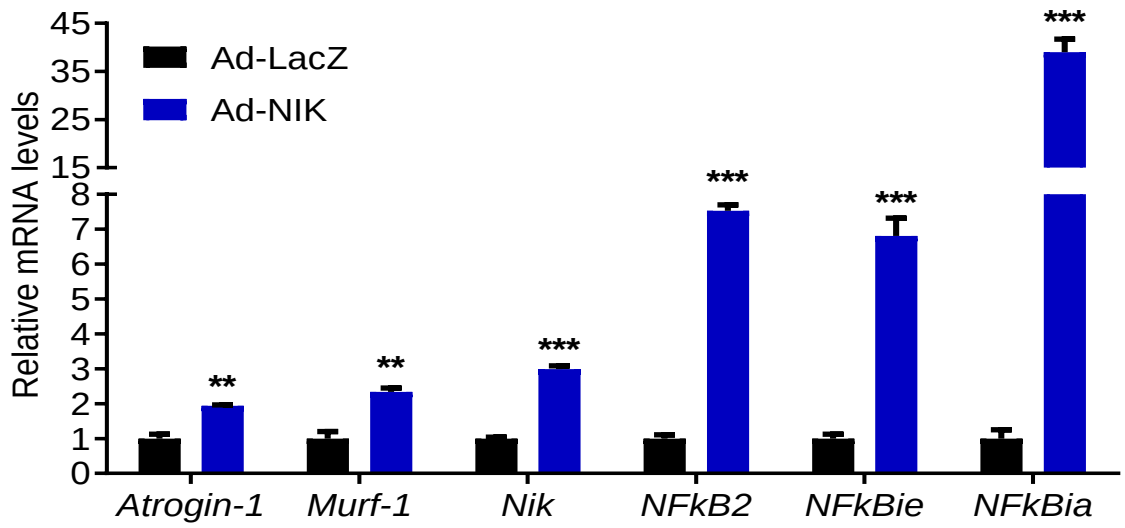

*Figure 3.7: Primary myotubes were treated with 20µl of LacZ and NIK adenoviruses for 24 hours and gene expression changes were determined with qRT-PCR. The values are mean ± SEM. *p < 0.05, **p < 0.005, ***p < 0.0005.*

As the next step and to validate the previous outcomes, NIK overexpression was desired to be chemically inhibited. To this end, NIK overexpression in cultured primary myotubes combined with the NIK inhibitor B022 treatment to confirm the successful inhibition of the NIK by B022. NIK overexpression and B022 treatment were examined first at the protein level. NIK adenovirus was given to cultured primary myotubes with increasing concentrations of B022 for 48 hours and harvested for protein. According to western blot results p52 protein level increased with NIK overexpression and it decreased with B022 treatment. Also, the increasing level of B022 treatment impairs p52 protein processing in a dose-dependent manner (Figure 3.8). These findings indicate that B022 is successfully inhibiting the protein expression outcomes of NIK overexpression.

Relative mRNA levels were also examined with qRT-PCR in the NIK adenovirus-B022 treatment condition. Muscle atrophy related gene *Murf-1* and NFκB signaling pathway-related genes *Nik*, *NFκB2*, *NFκBia*, and *NFκBie* expressions increased with NIK overexpression. However, when NIK inhibitor treatment applied, the expression levels of the atrophy related and NFκB signaling pathway-related genes decreased with respect to increasing dose of the B022 κB (Figure 3.9). This indicates that B022 is successfully inhibiting the gene expression outcomes of NIK overexpression too.

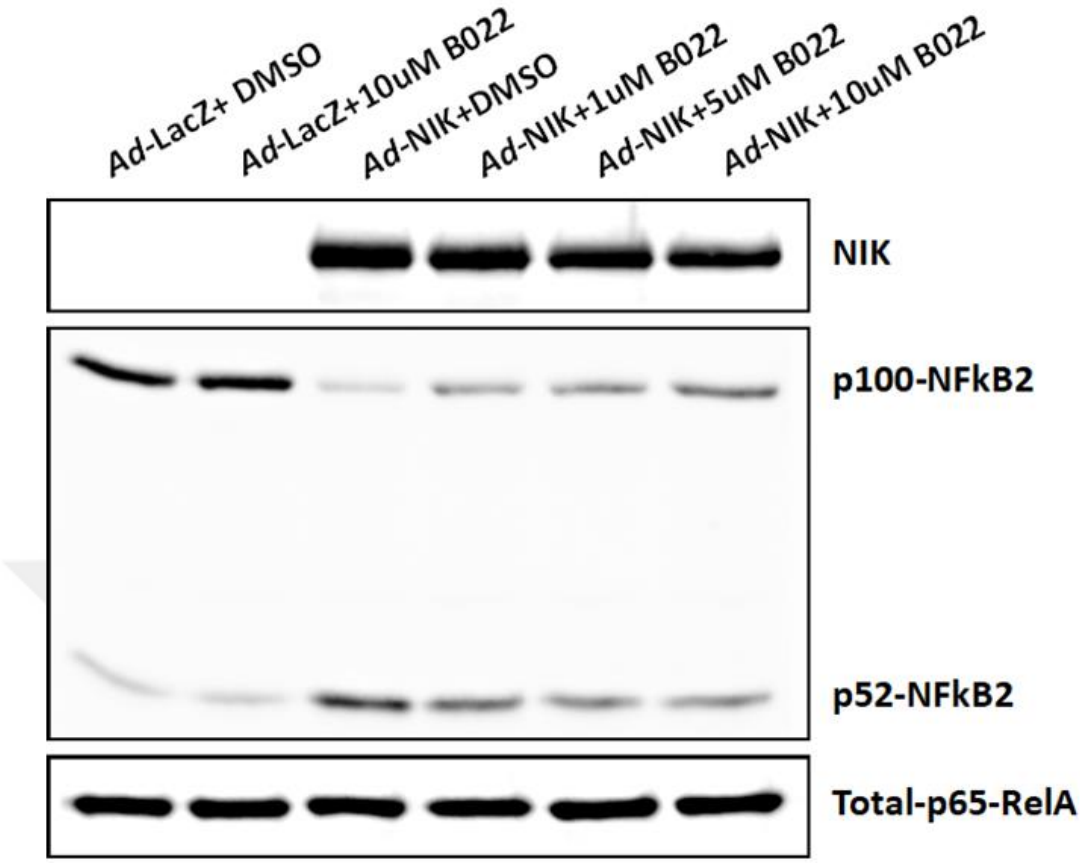

Figure 3.8: NIK, NFkB2-p100, and NFkB2-p52 protein levels in primary myotubes with 48 hours of LacZ, NIK adenovirus and B022 treatments.

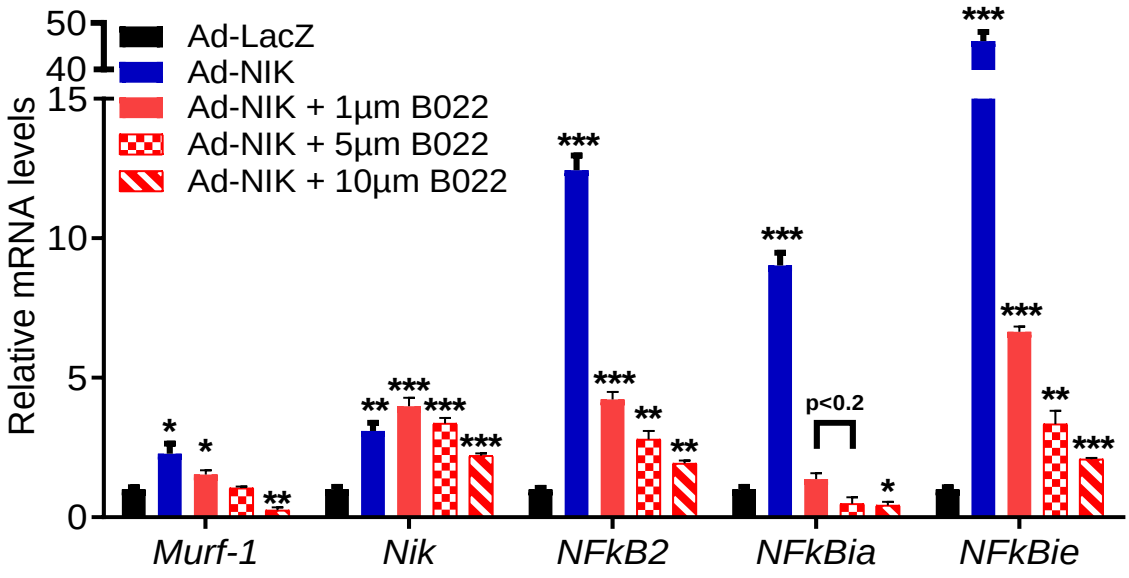

*Figure 3.9: Primary myotubes were treated with 20µl of LacZ, NIK adenoviruses and NIK Inhibitor B022 for 24 hours and gene expression changes were determined with qRT-PCR. The values are mean ± SEM. *p < 0.05, **p < 0.005, ***p < 0.0005.*

After the validation of successful inhibition of NIK by B022, we further wanted to test the contribution of NIK activity to EDA-A2 driven muscle atrophy. First, protein expression profile was investigated with western blotting Cultured primary myotubes treated with EDA-A2 adenovirus and NIK inhibitor B022 for 48 hours and harvested for protein. EDA-A2 overexpression caused an activation in the alternative NFκB signaling pathway which can be seen with the increase in p52 protein level that shows the NFκB2 processing. This activation was suppressed when the primary myotubes treated with 10 μM B022 in combination with EDA-A2 adenovirus (Figure 3.10). This further shows that EDA-A2 signals through the alternative NFκB signaling pathway.

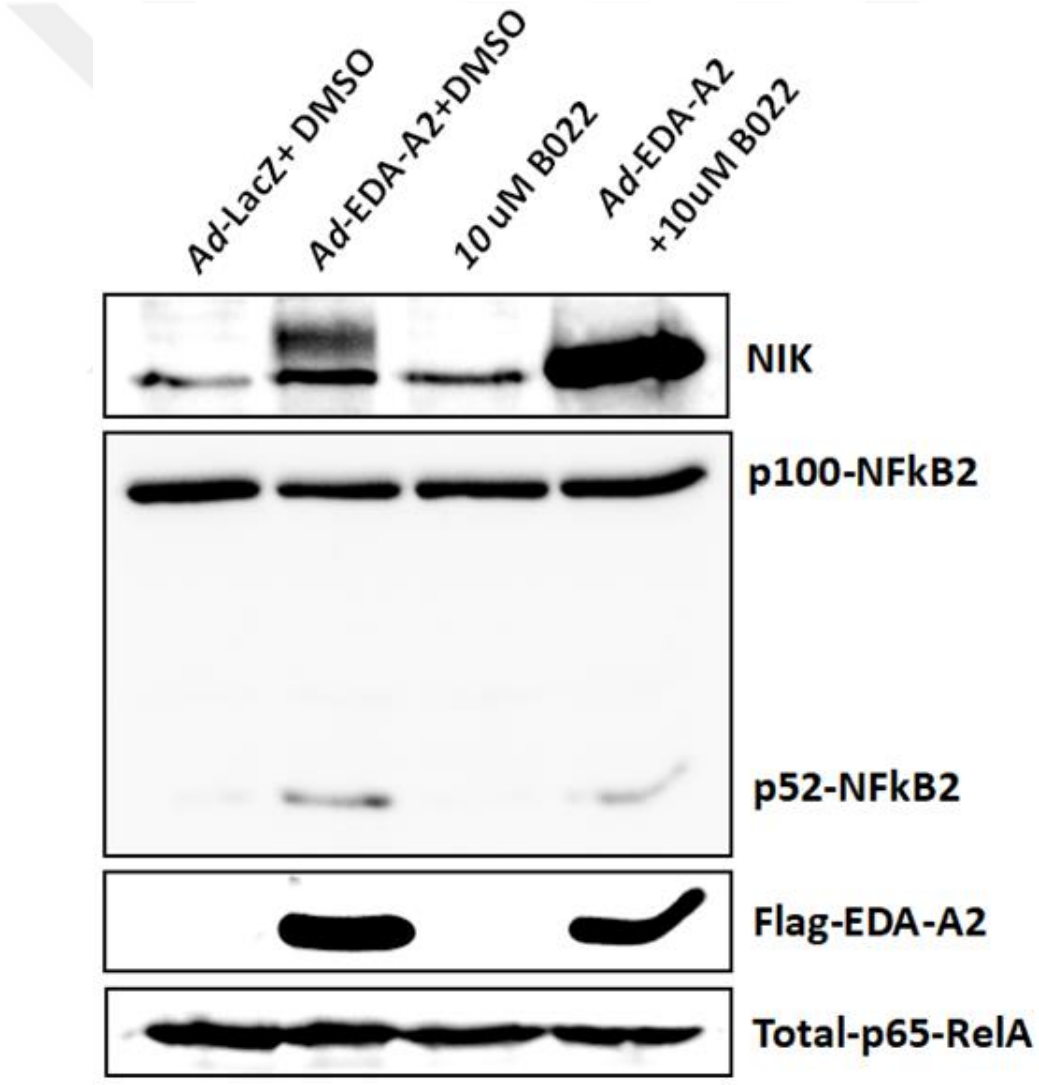

Figure 3.10: NIK, NFkB2-p100, NFkB2-p52 and EDA-A2 protein levels in primary myotubes with 48 hours of LacZ, EDA-A2 adenovirus and 10 uM B022 treatments.

Then, the gene expression profile was examined with the EDA-A2 adenovirus-B022 co-treatment. Muscle atrophy related gene *Murf-1*'s expression level significantly increased with EDA-A2 overexpression with respect to its basal level. However, this effect was suppressed with the B022 treatment and, an increase in the inhibitor concentration provided better suppression. NFκB signaling pathway-related genes *Nik, NFκB2, NFκBia,* and *NFκBie* expressions also decreased, where *Nik* and *NFκB2* are the main elements of alternative NFκB signaling pathway, with the B022 treatment with respect to the increasing level of inhibitor although they were upregulated with EDA-A2 overexpression (Figure 3.11). These findings one more time demonstrated that EDA-A2 is a muscle atrophy factor and suggest that EDA-A2 induced muscle atrophy requires NIK activity.

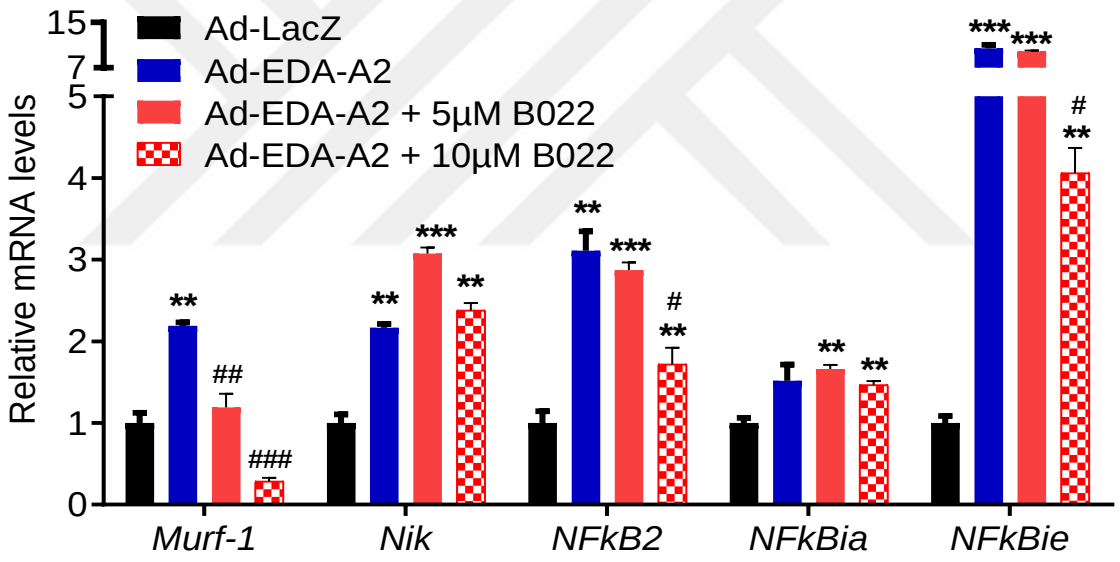

*Figure 3.11: Primary myotubes were treated with 20µl of LacZ, EDA-A2 adenoviruses and NIK inhibitor B022 for 24 hours and gene expression changes were determined with qRT-PCR. The values are mean ± SEM. *p < 0.05, **p < 0.005, ***p < 0.0005 with respect to Ad-LacZ. #p < 0.05, ##p < 0.005, ###p < 0.0005 with respect to Ad-EDA-A2.*

NIK and NIK mutant overexpression were combined in one experiment to further study the signal transduction where NIK mutant was used by Holtmann et al. as a dominant-negative in the literature (1999). To validate this dominant-negative effect, cultured primary myotubes treated with NIK and NIK mutant adenoviruses for 48 hours and harvested for protein. Western blot results showed that, in the case of NIK overexpression, p52 protein levels increased which demonstrates that the alternative

NFκB signaling pathway is activated. However, when both NIK and NIK mutant adenoviruses were given to primary myotubes, the phenotype was repressed and the p52 protein level decreased. (Figure 3.12). Then, the results that were obtained with NIK and NIK mutant overexpression were also examined in the gene expression level. Cultured primary myotubes were treated with NIK and NIK mutant adenoviruses for 24 hours and the cells harvested for RNA. Gene expression changes were examined with qRT-PCR. NIK overexpression in cultured myotubes caused an increase in muscle atrophy related genes *Atrogin-1* and *Murf-1*, and the alternative NFκB signaling pathway-related genes *Nik, NFκB2, NFκBia,* and *NFκBie* expressions, where *Nik* and *NFκB2* are the main elements of alternative NFκB signaling pathway. However, when both NIK and NIK mutant adenoviruses were given to primary myotubes, the phenotype was repressed and the respective gene expression levels decreased (Figure 3.13). These findings further support the idea that the alternative NFκB signaling pathway is the axis in the muscle atrophy.

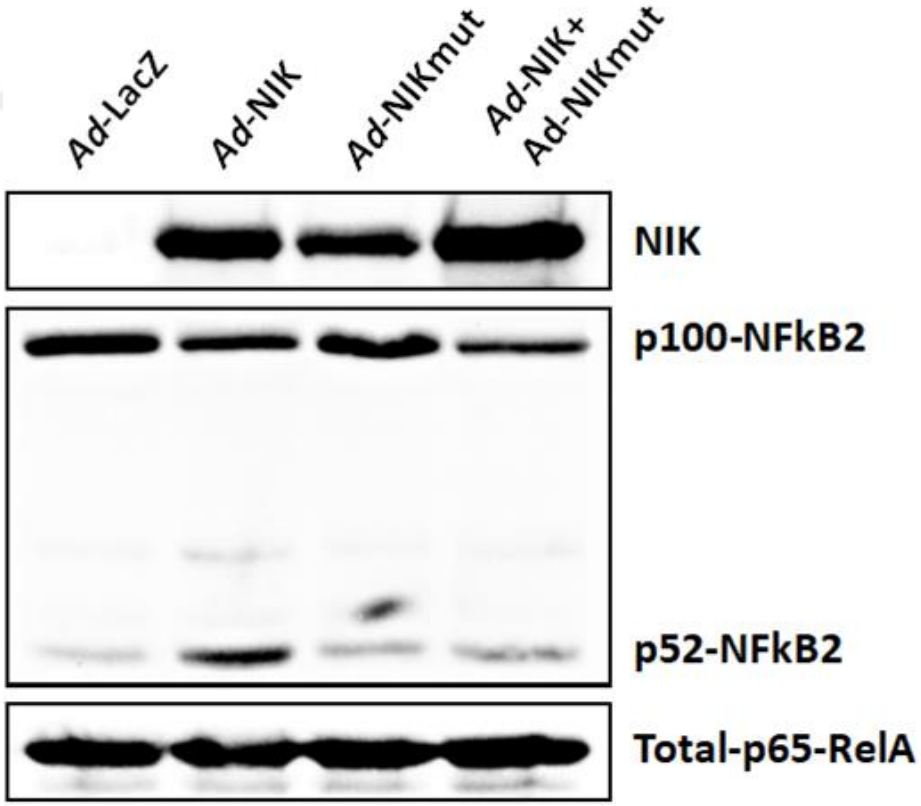

Figure 3.12: NIK, NFkB2-p100 and NFkB2-p52 protein levels in primary myotubes with 48 hours of LacZ, NIK and NIK mutant adenovirus treatments.

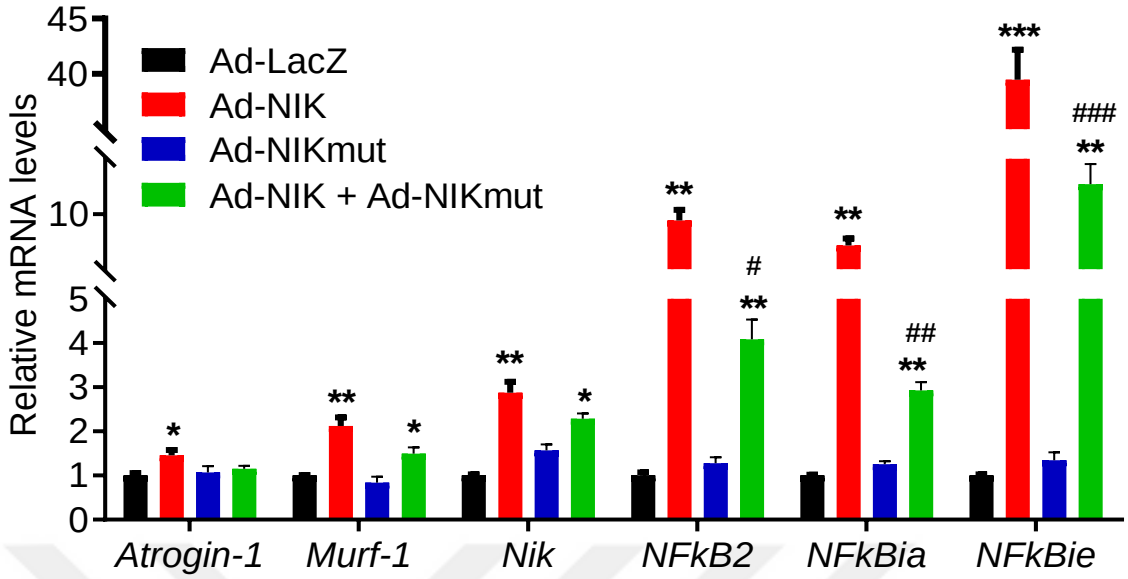

*Figure 3.13: Primary myotubes were treated with 20µl of NIK and NIK mutant adenoviruses for 24 hours and gene expression changes were determined with qRT-PCR. The values are mean ± SEM. *p < 0.05, **p < 0.005, ***p < 0.0005 with respect to Ad-LacZ. #p < 0.05, ##p < 0.005, ###p < 0.0005 with respect to Ad-NIK.*

As the final approach, cultured primary myotubes treated with EDA-A2 and NIK mutant adenoviruses for 48 hours and the effects on protein level examined with western blotting. After EDA-A2 overexpression, the p52 protein level increased which shows the alternative NFκB signaling pathway activity. However, when both EDA-A2 and NIK mutant overexpressed at the same time, the phenotype was repressed, and the p52 protein level decreased (Figure 3.14). This result again demonstrates that EDA-A2 signals through the alternative NFκB signaling pathway.

Gene expression profile of EDA-A2-NIK mutant overexpression at the same time was also investigated. Cultured primary myotubes treated with the respective adenoviruses for 24 hours and the effects on gene expression level investigated with qRT-PCR. EDA-A2 overexpression increased the muscle atrophy related gene *Murf-1's* and NFκB signaling pathway-related genes *Nik*, *NFκB2*, *NFκBia*, and *NFκBie*, where *Nik* and *NFκB2* are the main elements of the alternative NFκB signaling pathway. However, these genes' expressions repressed with EDA-A2-NIK mutant overexpression at the same time (Figure 3.15). This result again suggests that EDA-A2 is a novel atrophy factor which requires NIK kinase activity and signals through the alternative NFκB signaling pathway in the muscle cells.

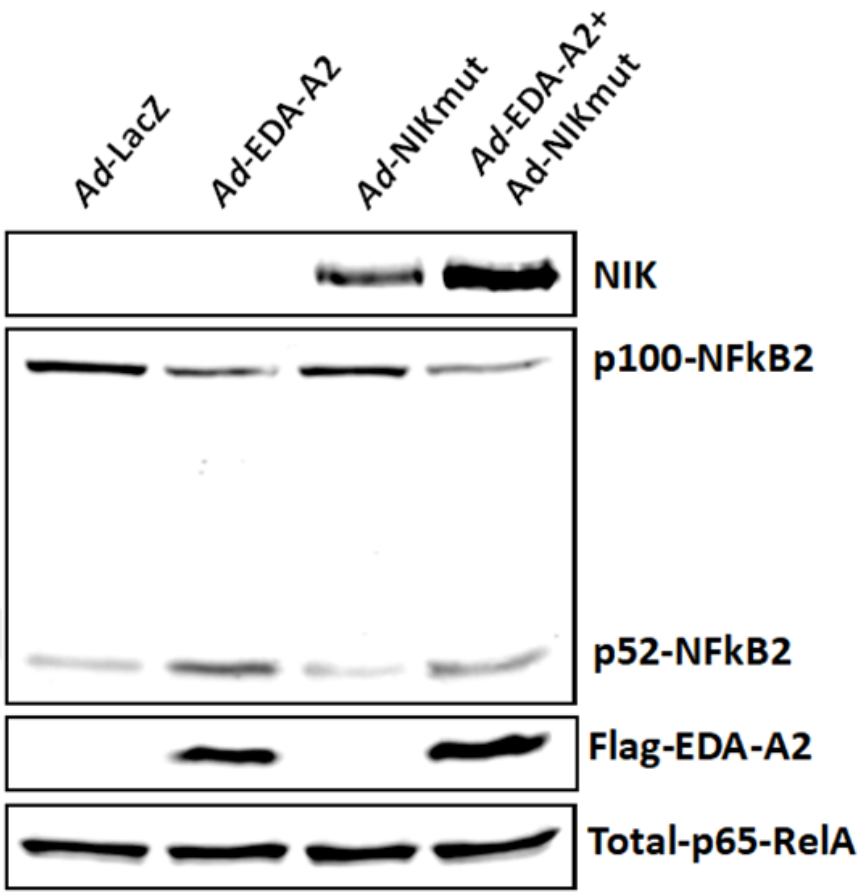

Figure 3.14: NIK, NFkB2-p100, NFkB2-p52 and EDA-A2 protein levels in primary myotubes with 48 hours of LacZ, EDA-A2 and NIK mutant adenovirus treatments.

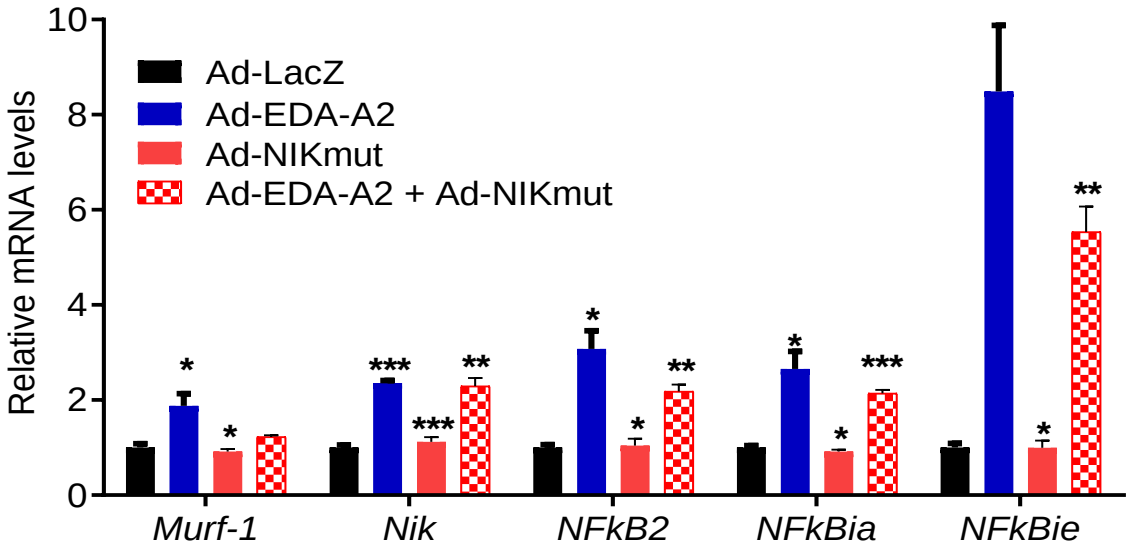

*Figure 3.15: Primary myotubes were treated with 20µl of EDA-A2 and NIK mutant adenoviruses for 24 hours and gene expression changes were determined with qRT-PCR. The values are mean ± SEM. *p < 0.05, **p < 0.005, ***p < 0.0005*

Chapter 4:
CONCLUSIONS/DISCUSSION

Cancer cachexia is a serious disease that negatively affects cancer patients and anti-cancer therapy. Also, there is no successful cure to slow down or stop the syndrome. In this project, we aimed to elucidate the working principles of novel cancer cachexia factors that take -part, especially in muscle tissue wasting. We desired to enlighten the effects of novel factors on skeletal muscle tissue and aimed to solve how these factors signal in the cells to function. To solve these questions, we developed adenoviral expression vectors that overexpress our genes of interests, EDA-A2 and NIK, and used them in muscle tissues both in-vitro and in-vivo. We also, coupled with the adenoviral overexpression with inhibitors and dominant-negatives to exactly solve the signaling in the cell.

According to our results, it can solidly be stated that novel cachexia factor EDA-A2 is causing muscle wasting because every time we overexpressed it in muscle tissue (both in-vitro and in-vivo), it upregulated the expression of the muscle atrophy related genes *Atrogin-1* and *Murf-1*. We also overexpressed NIK by using adenoviral overexpression vectors too which gave the same result that was the upregulation of the muscle atrophy related genes *Atrogin-1* and *Murf-1*. In the meantime, NIK mutant overexpression and NIK inhibitor treatment decreased the expression level of muscle atrophy related genes, *Atrogin-1* and *Murf-1* that is another clue that supports our findings. Also, EDA-A2 overexpression caused a significant decrease in muscle mass of mice in only 7 days of incubation. Thus, EDA-A2 can be named as a novel factor that causes skeletal muscle atrophy in cancer cachexia patients.

Some supportive experiments to our main findings such as muscle fiber diameter measurements can also be performed both in-vitro and in-vivo as the next step of this project. Cultured primary muscle cells can be infected with EDA-A2 or NIK adenovirus in combination with the GFP adenovirus to measure the decrease in the fiber diameters. EDA-A2 or NIK adenovirus injected mice muscles can be stained with hematoxylin and eosin to again measure the decrease in the fiber diameter. Because of the time limitations, these experiments could not be conducted but they would be good supports to our main findings to demonstrate the presence of muscle atrophy.

Our results also showed that EDA-A2 signal through the alternative NFκB (non-canonical) signaling pathway. Every time we overexpress EDA-A2 in muscle tissue (both in-vitro and in-vivo) NFκB signaling pathway elements *Nik, NFKB2, NFκBie,* and *NFκBia* expression increased. From these elements, *Nik* and *NFκB2* are the main elements of the alternative pathway which indicates that EDA-A2 signals through the alternative NFκB signaling pathway. In every experiment, we observed NFκB2 processing which can be seen with the increase in p52 protein levels that is the processed version of the NFκB2-p100. As a downstream signaling component, NIK overexpression gave the same results with the EDA-A2 overexpression case which is the increase of the alternative NFκB (non-canonical) signaling pathway elements *NFKB2, NFκBie,* and *NFκBia* expressions and the increase in the level of NFκB2 processing. Whereas NIK mutant overexpression and NIK inhibitor treatment caused a decline in the alternative NFκB (non-canonical) signaling pathway elements *NFKB2, NFκBie,* and *NFκBia* expression and stopped NFκB2 processing. These results also demonstrated that EDA-A2 signals in the cell through NIK as the regulator and through the alternative NFκB (non-canonical) signaling pathway. The proposed model can be seen in figure 4.1.

Signaling of EDA-A2 through the alternative NFκB (non-canonical) signaling pathway was one of the most novel findings of this study because the first time in the literature it is shown that a muscle atrophy related factor signals through the alternative NFκB (non-canonical) signaling pathway. The factors that were found before like TNFα were generally working through the canonical NFκB signaling pathway and the first time in the literature such a factor was utilizing the NFκB2 (non-canonical) pathway to signal in the cell.

These results proposed that EDA-A2 should be studied more deeply as a potential target for anti-cachectic drugs. Its novel effects on muscle wasting in cachexia syndrome make it a good candidate to prevent or slow down the muscle wasting seen in cachexia patients. Novel therapies against its own or its downstream signaling elements could be a good way to prevent muscle wasting seen in cancer cachexia patients. For example, research to find out novel strategies like the development of an EDA-A2 neutralizing antibody or EDA2R antagonist to stop EDA-A2 activity in cancer cachexia patients or gene therapy strategies that will prevent EDA-A2 expression could be highly beneficial to prevent the muscle wasting associated with cancer cachexia that will increase the efficiency of the anti-cancer therapies and extend the survival rate. Because EDA-A2 is

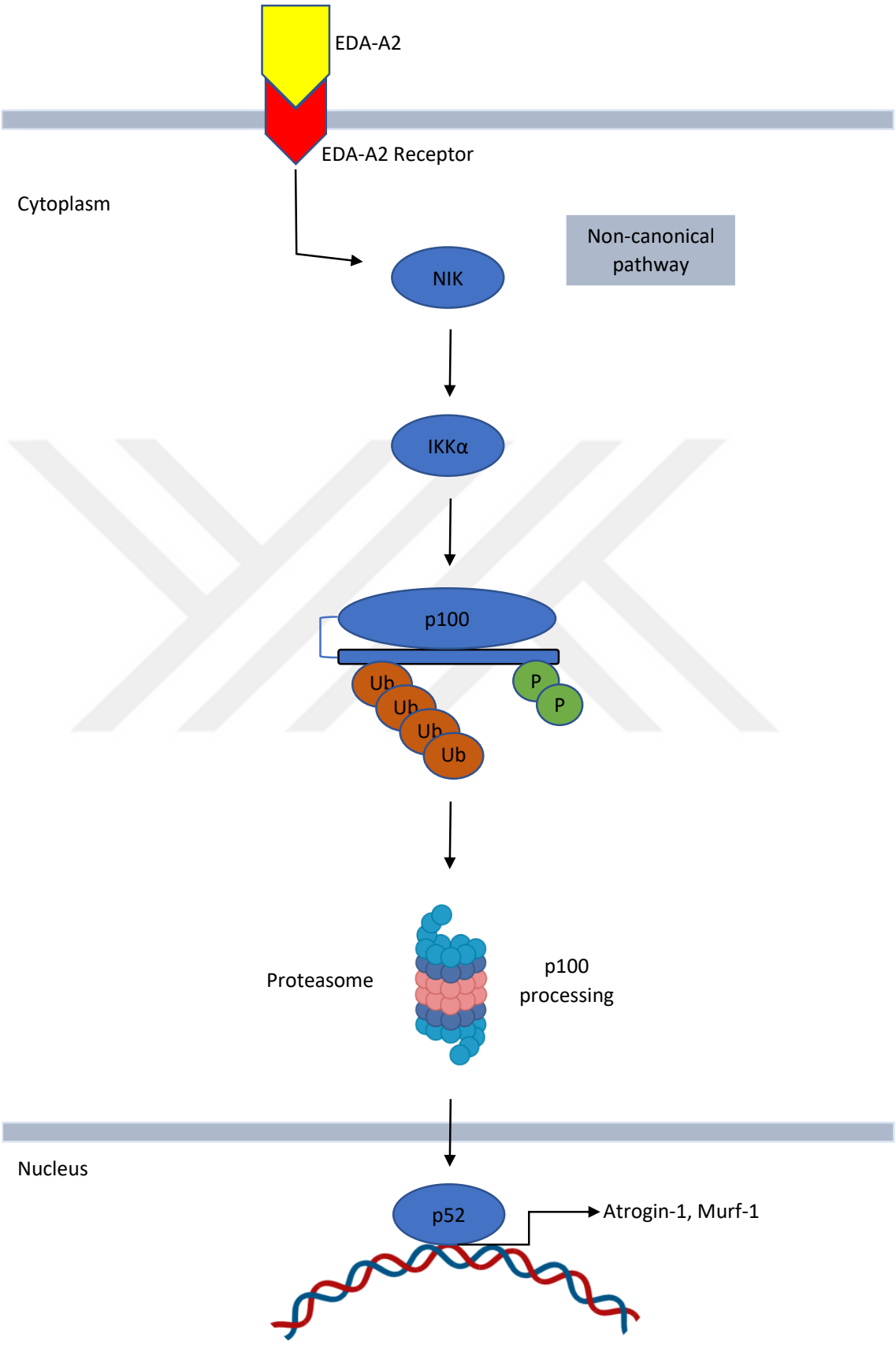

Figure 4.1: Intracellular Signaling Model of EDA-A2

a secreted factor, its elimination through the muscle tissues would be easier than intracellular factors and several different approaches like intravenous or intramuscular injections can be tried. Some structural or proteomic approaches for EDA-A2 or EDA2R would be highly beneficial to develop an antibody or antagonist against them and these studies could guide the researchers about the sides of the protein that could be targeted or these studies could give information about the post-translational modifications of EDA-A2. Interaction partners of EDA-A2 in or out of the cell could be found, if there is any, with BioID or co-immunoprecipitation studies that could provide more solid information about the protein's working principles. If the effect of EDA-A2 against cancer patient muscle tissues could be prevented, this can save many lives by increasing the anti-cancer therapy efficiency and the life quality of the cancer patients.

In this study, we also find out that NIK can be a good candidate target for anti-cachexia therapies too. It is working in downstream of EDA-A2 and it is as efficient as EDA-A2 in preventing muscle atrophy. We speculate that studies to find out efficient enough inhibitors for NIK could be the next step of this research. However, it should be known that NIK is an intracellular molecule and its inhibition will be highly challenging. Also, NIK has other signaling duties in the cells too which brings the idea that its inhibition could end up with systemic side effects that could negatively affect the patients. However, its pivotal role in muscle atrophy makes it a promising candidate to conduct further research on it. Same as EDA-A2, research to find out an inhibitor against NIK that does not have severe systemic side effects could be performed after deep structural and proteomic approaches. Its successful inhibition in cancer cachexia patient muscles could stop or slow down the syndrome and can increase the efficiency of anti-cancer therapies and patient lifetime and quality.

To sum up, EDA-A2 is a novel muscle atrophy and cancer cachexia related factor and it is signaling through NIK and alternative NFκB (non-canonical) signaling pathway. EDA-A2 and NIK could be good candidates to perform further research on them to cure cancer cachexia.

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