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Ankrd2 modulates NF-kB mediated inflammatory responses during muscle differentiation

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CONTENTS

Abstract (English)

Adaptive responses of skeletal muscle regulate the nuclear shuttling of the sarcomeric protein Ankrd2 that can transduce different stimuli into specific adaptations by interacting with both structural and regulatory proteins. In a genome-wide expression study on Ankrd2 knockout or overexpressing primary proliferating or differentiating myoblasts, we found an inverse correlation between Ankrd2 levels and the expression of proinflammatory genes and identified Ankrd2 as a potent repressor of inflammatory responses through direct interaction with the NF-kB repressor subunit p50. In particular, we identified Gsk3ß as a novel direct target of the p50:Ankrd2 repressosome dimer and found that the recruitment of p50 by Ankrd2 is dependent on Akt2-mediated phosphorylation of Ankrd2 upon oxidative stress during myogenic differentiation. Surprisingly, the absence of Ankrd2 in slow muscle negatively affected the expression of cytokines and key calcineurin-dependent genes associated with the slow-twitch muscle program. Thus, our findings support a model in which alterations in Ankrd2 protein and phosphorylation levels modulate the balance between physiological and pathological inflammatory responses in muscle.

Keywords: Gsk3ß, inflammation, NF-kB, oxidative stress, skeletal muscle

Abstract (Italiano)

La proteina Ankrd2 (Ankyrin repeat domain 2) può interagire sia con proteine del sarcomero sia con proteine nucleari che regolano l'espressione genica e quindi è in grado di trasdurre stimoli di natura diversa in specifiche risposte adattative del muscolo scheletrico. In un'analisi trascrittomica condotta su mioblasti primari (proliferanti o in differenziamento) dove Ankrd2 è stata silenziata o sovra-espressa, abbiamo: a) trovato una correlazione inversa tra i livelli di Ankrd2 e l'espressione di geni pro-infiammatori; b) dimostrato che Ankrd2 agisce da potente repressore della risposta infiammatoria tramite interazione diretta con la subunità p50 del fattore di trascrizione NF-kB. In particolare, abbiamo dimostrato che la chinasi Gsk3ß è il bersaglio privilegiato del dimero di repressione p50:Ankrd2; inoltre, durante il differenziamento miogenico il reclutamento di p50 da parte di Ankrd2 dipende dalla fosforilazione di Ankrd2 mediata dalla chinasi Akt2 in condizioni di stress ossidativo. Stranamente, l'assenza di Ankrd2 influenza in maniera negativa l'espressione di citochine e di geni chiave calcineurina-dipendenti associati al programma di contrazione lenta del muscolo scheletrico. I nostri risultati supportano quindi un modello nel quale alterazioni della proteina Ankrd2 o dei suoi livelli di fosforilazione modulano l'equilibrio tra la risposta infiammatoria fisiologica e patologica nel muscolo scheletrico.

Parole chiave: Gsk3ß, infiammazione, NF-kB, stress ossidativo, muscolo scheletrico.

Ankyrin repeat domain protein 2 (Ankrd2/Arpp) belongs to the Muscle Ankyrin Repeat Protein (MARP) family, whose members are involved in muscle stress response pathways (Miller et.al, 2003). MARPs bind to the elastic I-band region of the giant protein titin and are thought to translocate to the nucleus in response to stretch to transduce stress-induced signaling pathways into transcriptional adaptations (Baumeister et.al, 1997; Kuo et.al, 1999; Aihara et.al, 2006). In particular, MARPs have been characterized as transcriptional co-inhibitors, repressing the expression of cardiac and skeletal muscle (Jeyaseelan et.al, 1997; Zou et.al, 1997; Bean et.al, 2008).

In addition to a well documented role of Ankrd2 in mechano transduction (Tsukamoto et.al, 2002; Barash et.al, 2002; McKoy et.al, 2005; Hentzen et.al, 2006; Lehti et.al, 2007). We recently demonstrated an important role of Ankrd2 in myogenic differentiation, coordinating proliferation and apoptosis (Bean et.al, 2008). More specifically, we found that muscle cells stably overexpressing Ankrd2 protein were unable to acquire the apoptosis-resistance phenotype upon differentiation and thus to fully differentiate. Subsequent studies, showed that the effect of Ankrd2 on muscle differentiation is dependent on Akt2-mediated phosphorylation at Ser-99, which is induced in response to oxidative stress following hydrogen peroxide stimulation (Cenni et.al, 2011). Although previous studies of triple knockout (KO) mice for the three MARP family members Ankrd1/CARP, Ankrd2/Arpp, and DARP/Ankrd23, revealed only a relatively mild skeletal muscle phenotype (Barash et.al, 2007), the binding of Ankrd2 to numerous nuclear and cytoplasmic proteins (Kojic et.al, 2004) suggest its involvement in multiple signaling pathways.

In the present study, based on Ankrd2 ablation and overexpression in differentiating primary mouse skeletal muscle and C2C12 cells, we demonstrate that Ankrd2 plays an important role in modulating NF-kB transcriptional activity through

direct interaction with the NF-kB repressor p50. NF-kB directly affects the expression of more than 150 genes and plays a key role in regulating multiple signaling pathways involved in inflammatory and cell survival responses by inhibiting the apoptotic machinery (Pahl et.al, 1999). Tight regulation of the expression of NF-kB target genes, such as cytokines, chemokines, cell adhesion molecules, and proteolytic enzymes, is essential for ensuring the appropriate induction and subsequent resolution of inflammation. Importantly, fluctuations in the concentrations of cytokines establish whether the effects of inflammatory signaling pathways are beneficial or detrimental (Pedersen et.al, 2008). NF-kB is composed of the Rel family proteins RelA (p65), c-Rel, RelB, p50, and p52, are able to dimerize with one another to form multiple homo- and heterodimers that are associated with various cellular processes. Before activation, most of the NF-kB dimers are retained in the cytoplasm as inactive complexes by binding to the classical inhibitors of the kB (IkB) family members IkBα, IkBβ, and IkBε. Upon stimulation, IkB proteins are rapidly degraded by the proteasome, allowing the NF-kB dimer to translocate to the nucleus, where it activates the transcription of target genes. The most rapid and direct way to ensure resolution of inflammation is based on the transcription of IkBα by p65:p50 through a negative feedback mechanism (Hoffmann et.al, 2002).

However, given the physiological potency of the genes regulated by NF-kB, several additional regulatory checkpoints regulating its activity exist, including the formation of specific NF-kB complexes. Interestingly, it has recently emerged that the predominant role of p50 may be related to the resolution phase of inflammation during the later stages of NF-kB activation (Elsharkawy et.al, 2010). As p50 lacks a transactivation domain, its influence on transcription is dependent either on

dimerization with other members of the NF-kB family or on different co-regulators (Elsharkawy et.al, 2010; Bours et.al, 1993; Cao et.al, 2006).

Here we show that Ankrd2 recruits p50, dominating over p65:p50 dimers, and orchestrates the repression of inflammation-related genes during muscle differentiation. In particular, we show that the inhibition of NF-kB activity is modulated by Akt2-mediated phosphorylation of Ankrd2 in response to oxidative stress. Pro-inflammatory cytokines stimulate the production of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide that have thus been proposed as universal second messengers of the NF-kB activation pathway (Li et.al, 1998). ROS, in turn, activate NF-kB and enhance the expression of pro-inflammatory genes, such as TNFα, IL6, and C-reactive protein, which are involved in the pathogenesis of inflammation (Wei et.al, 2008). Cells possess several defense systems to protect against the harmful effects of ROS. Here we propose a novel molecular mechanism by which activation of Akt2 leads to the resolution of oxidative stress and inflammation via Ankrd2-dependent NF-kB inhibition. Furthermore, we identify Gsk3β as a direct target of the novel NF-kB dimer p50:Ankrd2. GSK3β is an emerging player in inflammatory signaling, serving as a nodal point for both the generation and resolution of inflammatory response (Hu et.al, 2006; Gong et.al, 2008). Since Gsk3β can promote the transcription of only a subset of NF-kB-induced genes, it appears to have inhibitory or stimulatory effects dependent on context and cell type (Steinbrecher et.al, 2005). Thus, taken together our *in vitro* results indicate a role of Ankrd2 in the resolution of inflammatory responses through interplay with NF-kB, Akt and Gsk3β pathways. Unexpectedly, *in vivo,* the absence of Ankrd2 in slow muscle was associated with decreased expression levels of cytokines and key calcineurin-dependent genes, involved in the maintenance of the slow-twitch muscle program. Thus, since muscle produce cytokines both constitutively and in response to

various inflammatory stimuli (De Rossi et.al, 2000, Nagaraju et.al, 1998). Our findings suggest a multifaceted role of Ankrd2 in inflammatory signaling pathways.

2.1. Skeletal muscle

2.1.1. Types of muscle

There are three types of muscle found in human body skeletal, cardiac and smooth. ***Skeletal muscle*** is the most common of the three types of the muscle in the body. Skeletal muscles cover the bone and confer a shape to the body. They make approximately 40% of our total body weight. They provide heat, movement and the blood flow, thus maintains homeostasis in the body (Sievanen et.al, 2005). Tendons (a fibrous connective tissue), usually connects muscles to the bone. Cytoskeleton is organized into multinucleated myofibres. Skeletal muscles are under voluntary control; unlike smooth and cardiac muscle. They are also known as striped, striated, somatic, or voluntary muscles. These muscles are most abundant, exhibit cross-striations under microscope (Carole et.al, 2001), therefore called as striped muscles and are recognized to be the best differentiated form of muscle.

Smooth muscle is an involuntary non-striated, are found in the walls of hollow organs like intestine and stomach, oesophageous, and most of the internal organs. However, cytoskeleton of smooth muscle is not organized in to myofibril (Small JV and Gimona M, 1998).

Cardiac muscle is an involuntary muscle, exist only in the heart. Unlike other muscle they are self-contracting, work autonomically and must continue to contract in rhythmic fashion for blood pumping. The arrangement of actin and myosin is similar to skeletal striated muscle (Figure 2.1).

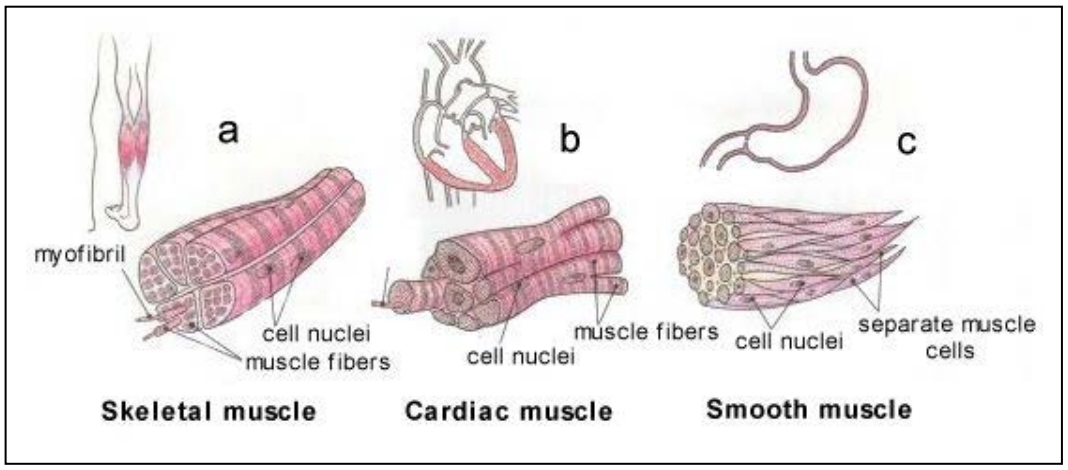

Figure 2.1 Types of muscle cells and their appearance Skeletal, Cardiac and smooth. Figure adapted from (human body anatomy cea1.com).

2.1.2. Skeletal myogenesis

Myogenesis is the formation of muscular tissue which is a complex and tightly controlled process, results in the formation of a multinucleated myofibre. They are multinucleate and formed by the fusion of myoblasts. The fusion of myoblasts is a major step in muscular differentiation, generating multinucleated muscle fibers from mononucleated myoblasts. This phenomenon was first reported by (Holtzer et.al, 1958). Myogenic regulatory factors (MRFs) act as master regulator of skeletal myogenesis (Figure 2.2), the highly conserved MyoD, Myf5, Myogenin, and MRF4 genes are collectively expressed in the skeletal muscle lineage and therefore known as myogenic regulatory factors (MRFs) (Weintraub et.al, 1991; Rudnicki et.al, 1995). The basic domain of the MRFs mediates DNA binding, whereas the helix–loop–helix motif is involved in the heterodimerization with E proteins and in the recognition of genomic E-boxes, a motif found in the promoters of many muscle-specific genes (Massari and Murre, 2000).

Myogenesis involves multiple steps in which a specialized population of myogenic cells termed multi-potential mesodermal precursor cells (also called as “descendents of satellite cells”) give rise to a muscle cells lineage as myoblasts. These myoblasts are committed to proliferate until they encounter an environment lacking mitogen in serum such as growth factors, at which point they exit from the cells cycle and differentiate. This process is followed by fusion of mononucleate myoblasts to form multinucleate muscle fibers, transcriptional activation of muscle-specific genes and repression of genes associated with cell proliferation (Sabourin et.al, 2000).

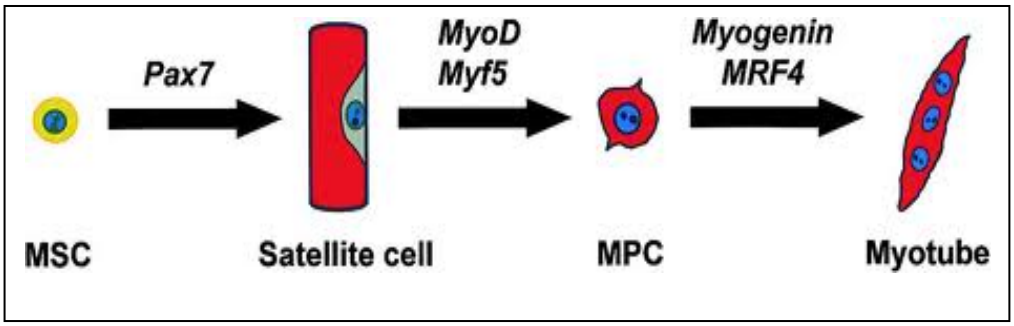

Figure 2.2 Specification of the satellite cells. Satellite cells are subsequently activated with response to physiological stimuli to generate daughter myogenic precursor cells (MPC) before terminal differentiation into new or previously existing fibers (Seale et.al, 2000).

Distinct population of mesodermal precursor cells give rise to the all three muscle cell lineages, skeletal, cardiac, and smooth, during embryogenesis. These different muscle cell types express many of the same muscle-specific genes, which shows that they might share a common myogenic regulatory programme. However, each muscle cell type is different with respect to the types of muscle genes they express, morphology, ability to divide, and contractile properties. Therefore, if they do share any common myogenic programme it should be modified by additional regulatory factors which could generate the specific diversity within muscle cell types.

2.1.3. Skeletal muscle differentiation

Skeletal muscle differentiation is a highly orchestrated programme of gene expression. Embryonic muscle grows by the proliferation of myogenic cells whereas postnatal muscle grows/repair mainly by muscle remodeling (Brown et.al, 2011). Recently, a complex view of the myogenic transcription factors and kinases that control vertebrate muscle development by myoblast proliferation, migration, fusion and differentiation has been documented (Figure 2.3).

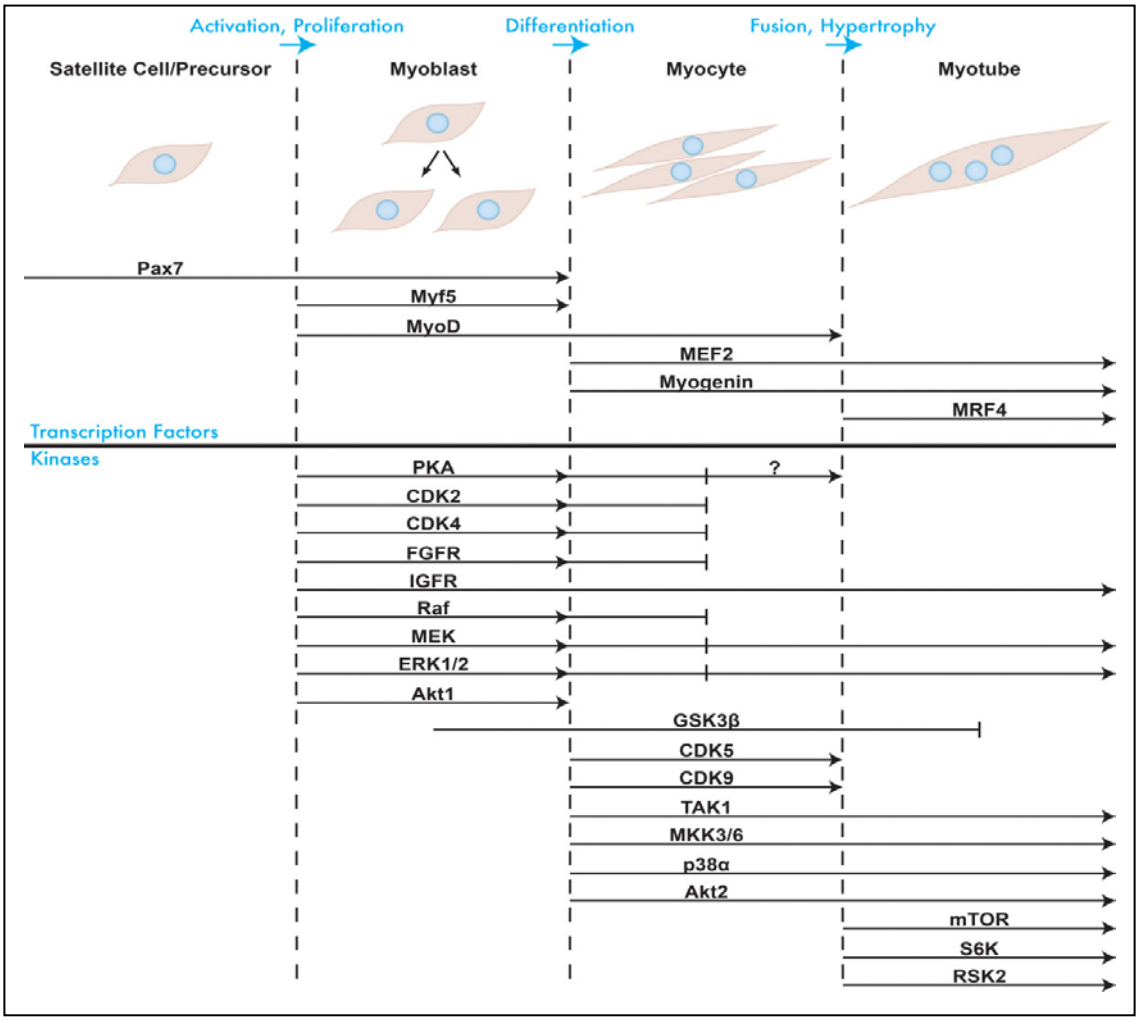

Figure 2.3 Hierarchy of transcription factors and kinases regulates the myotube progression via myogenic lineage. Figure adapted from (Knight et.al, 2011).

Myoblasts fusion and differentiation is a tightly interlocked regulatory process, it is a key step of muscular differentiation, give rise to elongated multinucleated muscle fibre. Differentiation involves the activation of the muscle specific transcription factors such as MYOD, myogenin (Wright et.al, 1989), MRF4/ Myf-6 (Braun et.al, 1990) and MEF2C. Therefore, myogenic potential of the Satellite cell mostly depends on the expression of Pax genes and myogenic regulatory factors (MRFs: MyoD, Myf5, myogenin, and MRF4). It seems that sequential activation and repression of Pax3/7 and MRFs is required for the progression of skeletal myoblasts through myogenesis. MyoD and Myf5 act early and especially determine the differentiation potential of activated myoblasts and later, they works in concert with myogenin and MEF2 family to drive the differentiation (Arnold et.al, 1998).

2.1.4. Structure of skeletal muscle

Myoblasts fused together and form a cylindrical multinucleated structure known as myofibre. The sarcolemma, or plasma membrane of the muscle cell, is highly invaginated by transverse tubules (T tubes) that permeate the cell. The sarcoplasm or cytoplasm of the muscle cell, contains calcium-storing sarcoplasmic reticulum, the specialized endoplasmic reticulum of a muscle cell. Striated muscle cells are multinucleated; the nuclei lie along the periphery of the cell, forming swellings visible through the sarcolemma. The myofibrils consist of protein chains called myofilaments, myofilaments have a symmetrical, alternating pattern of thick (comprised of myosin) and thin (comprised of actin) elements. Thick and thin filaments slide, past each other and contractile force is generated which produces movement (Kay et.al, 2006). These myotubes gain their own basal lamina (endomysium) and led to the formation of

independent adult muscle fibers. All the neighboring muscle fibers aggregate to form muscle bundles or fascicles, each bundle encased in a connective tissue called perimysium (Figure 2.4).

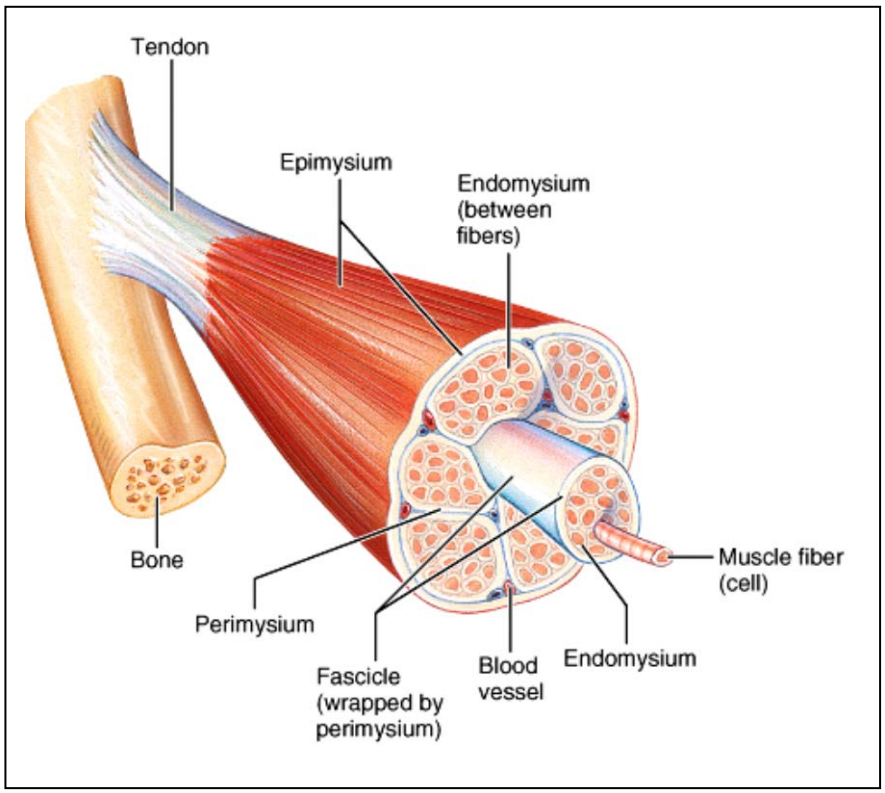

Figure 2.4 Anatomy of skeletal muscle. The complexity and arrangement of the muscle. Figure adapted from (Benjamin Cummings 2001).

2.2. Sarcomere formation and its structure

All types of muscle cells use actin and myosin for voluntary and involuntary contraction however, these proteins are organized in to sarcomeric units only in skeletal and cardiac muscle. Process by which sarcomere formed termed as myofibrillogenesis. The sarcomere is highly complex, ordered multiprotein molecular machine. Sarcomere shows three unique properties that are crucial for its function; 1) Ability to shorten rapidly as well as efficiently 2) Ability to contract's in milliseconds 3) its precise self

assembly and structural regularity. Myofibrillar proteins assemble to form the highly ordered repetitive contractile structural unit known as a sarcomere. Myofibrils are known to assemble through a three-step model: premyofibrils to nascent myofibrils to mature myofibrils (Sanger et.al, 2002).

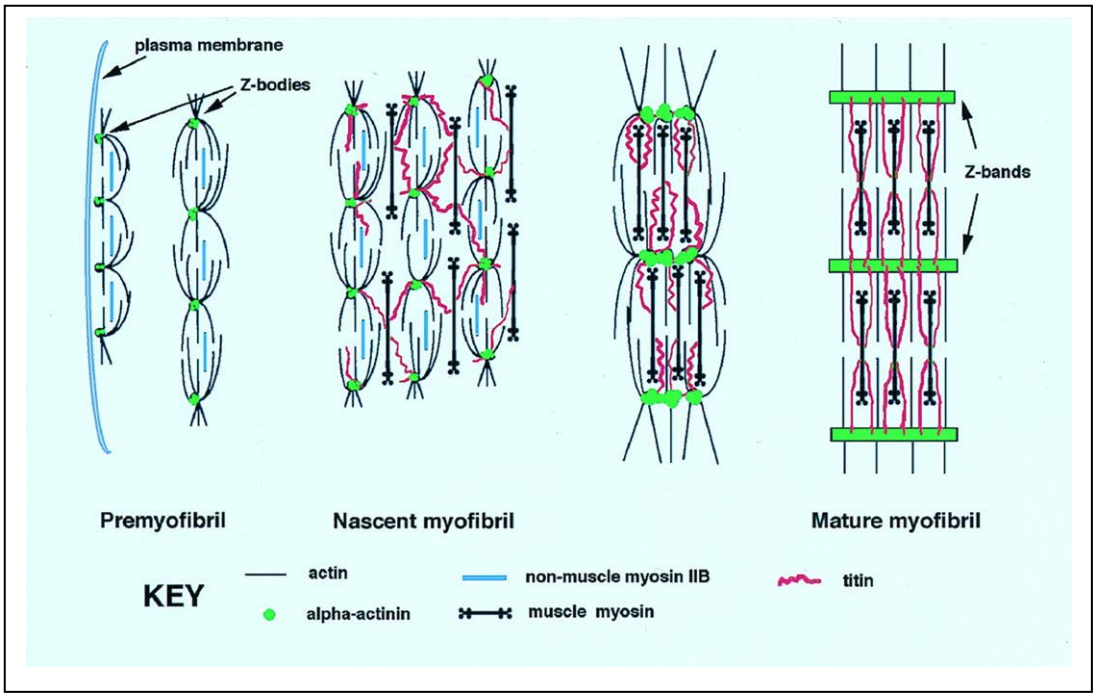

Figure 2.5 Model of myofibrillogenesis. Formation of mature myofibrils is preceded by two intermediary structures: premyofibrils and nascent myofibrils. Picture adapted from (Dabiri et.al, 1997).

The premyofibril model proposes that the formation of mature myofibrils is preceded by two intermediary structures: premyofibrils and nascent myofibrils as shown in (Figure 2.5). Premyofibrils are characterized by presence of minisarcomeric arrangements that lay the initial stages for building sarcomeres (Rhee et.al, 1994). As the formation of Z disc from Z bodies persist it requires addition as well as organization of Z disc proteins to form stable contractile unit.

During this transition from Z bodies to Z disc many other proteins recruited those are necessary for Z disc organization and stability (Sanger et.al, 2000). The boundaries of the minisarcomere consist of Z-bodies containing sarcomeric alpha-actinin. In between the Z bodies mini-A-bands is present which is composed of non-muscle myosin II. Actin filaments overlap in these minisarcomeres, give rise to a continuous actin pattern after staining with the phalloidin (fluorescent F-actin staining reagent). In the second proposed step of myofibrillogenesis, titin and overlapping muscle myosin II filaments are recruited to premyofibrils, called as nascent myofibrils. Titin is the only protein that extends entire half of the sarcomere (Granzier et.al, 2006), therefore, act as scaffold thereby interacting with various sarcomeric proteins. Mature myofibrils formed in the absence of nonmuscle myosin II, the alignment of muscle myosin II filaments to form A-bands, and the Z-bodies fuses to form Z-bands (Sanger et.al, 2000).

Sarcomere contains more than 28 different proteins but both contractile proteins actin and myosin accounts more than 70% of myofibrilar protein. These two proteins are responsible for the transduction of chemical energy into mechanical work. The actin-myosin complex has been shown to generate energy needed for contraction, by hydrolyzing ATP. In addition to the myosin, vertebrate striated muscle also contains significant amount of nonmyosin proteins like myosin binding proteins, which are present in thick filaments and myomesin, and creatine kinase in the M line. These proteins are thought to play primarily a structural role in the formation of Z line. In terms of contraction, the most important players are the troponin and tropomyosin, which are known to form a complex (tropomyosin-troponin), involved in regulation of contraction in response to calcium (Figure 2.6).

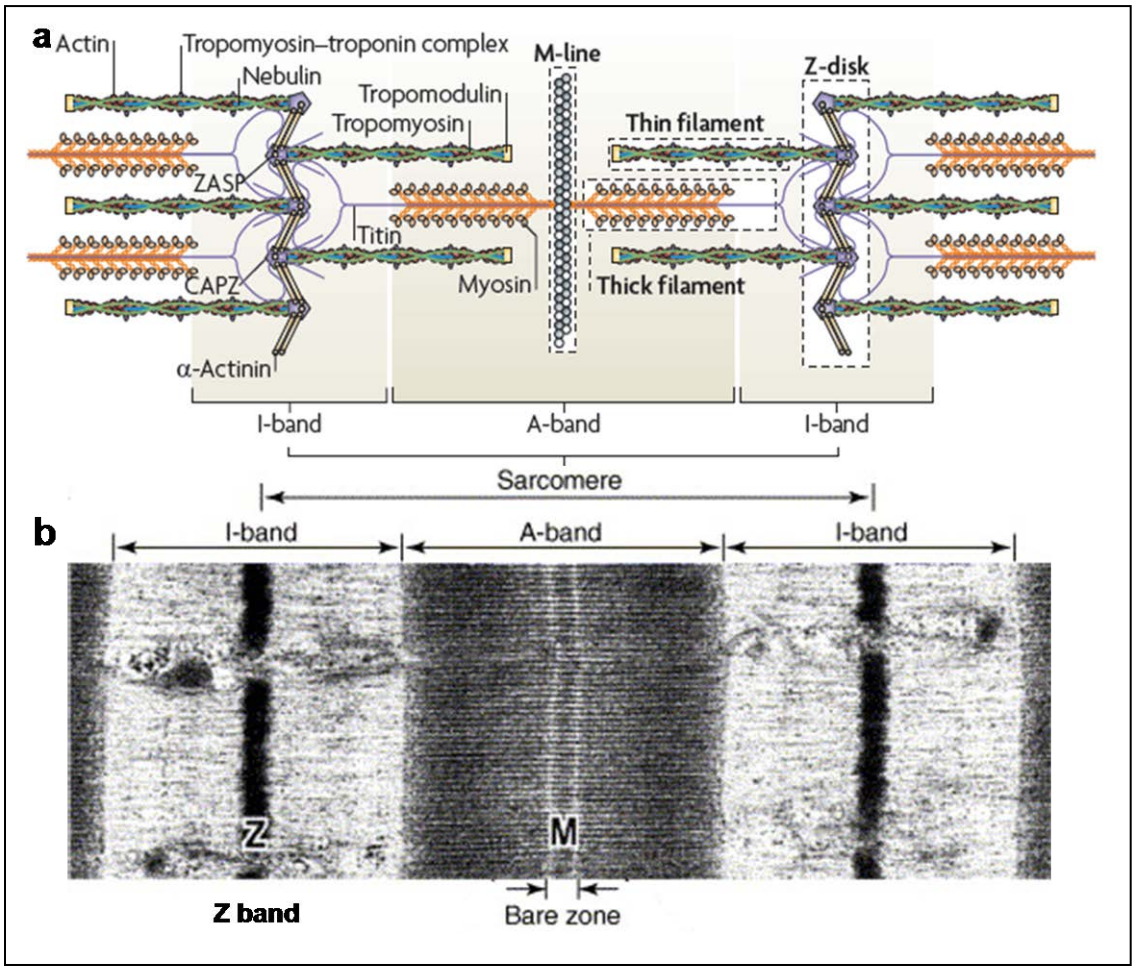

Figure 2.6 Molecular organization of sarcomere the basic contractile unit of muscle. (a) Schematic diagram of sarcomere summarizing organizations and locations of major sarcomeric components **(b)** Corresponding electron micrograph of a skeletal sarcomere. Figure modified from (Sparrow J.C & Schock F, 2009).

Overview of skeletal muscle from its cytology to a brief summary of the major proteins composing its highly specialized cytoskeleton has been described above (Figure 2.6). Apart from its role as a mechanosensor, we aimed to explore the Ankrd2 mediated signaling pathways, involved in skeletal muscle remodeling. Interestingly, many of the Z disc associated proteins those have dynamic distribution within the cells, are known to shuttle in between Z disc and nucleus. Therefore, it is important to give a short description of the MARP family proteins that represents the subject of this study.

2.3. MARP Family proteins

Beyond a well-defined structural role, in recent years it is emerging out that the Z-disc is directly involved in the perception and transmission of muscular stress signals. Muscle Ankyrin repeat protein (MARP) family have been found to be highly responsive to the muscle mechanical status (Kemp et.al, 2000). MARP family members are found in the interface between Z disc and I band, involved in the regulation of gene expression in response to muscle stress. MARP family comprises three structurally similar proteins, Ankyrin repeat domain 2 (Ankrd2/Arpp), Cardiac Ankyrin repeat protein (CARP/Ankrd1) and Diabetes associated Ankyrin repeat protein (DARP/Ankrd23) (Miller et.al, 2003). MARPs act as early responding genes and their expression got upregulated upon any kind of muscle stress such as denervation/ eccentric contraction or muscle injury in case of CARP (Aihara et.al, 2000; Kuo H et.al, 1999); Ankrd2 (Kemp et.al, 2000) and DARP (Ikeda et.al, 2003).

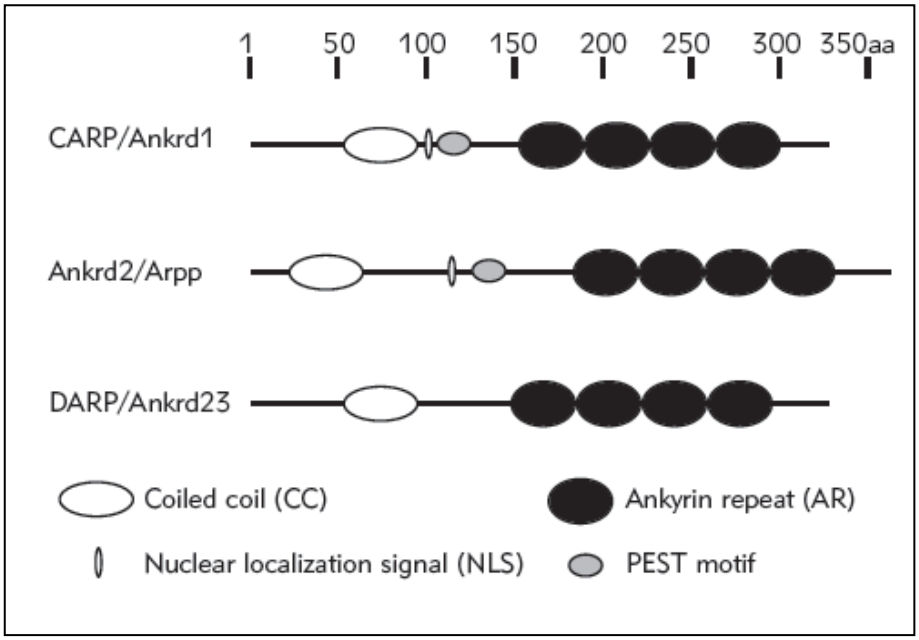

Figure 2.7 Schematic representation and domain organization of MARP family member proteins. Figure adapted from (Kojic et.al, 2010).

They share four conserved copies of 33-residue ankyrin repeats and contain a nuclear localization signal, allowing the sorting of MARPs to the nucleus (Figure 2.7). They are found both in the nucleus and in the cytoplasm of skeletal and cardiac muscle cells, suggesting that MARPs shuttle within the cell and thus play a role in signal transduction in striated muscle. Aim of this report was to study the role of the best characterized protein of MARPs family (Ankrd2), in skeletal muscle remodeling.

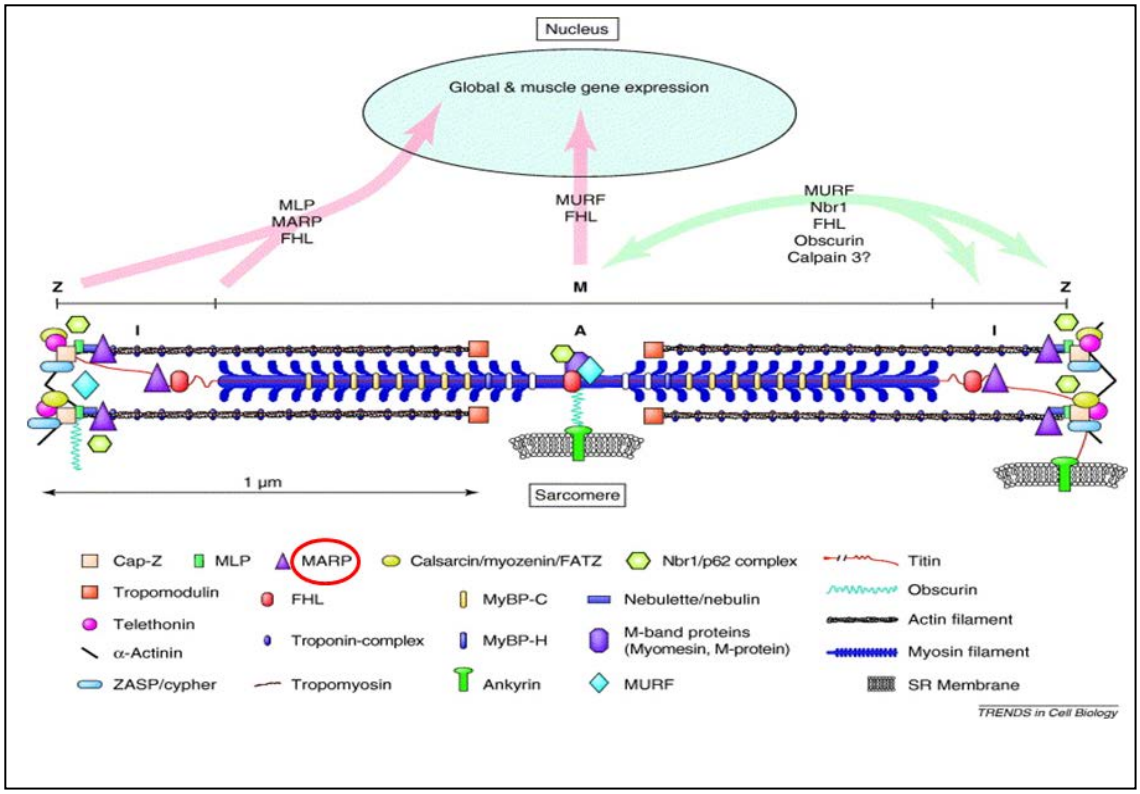

Figure 2.8 Illustrates the localization of MARP family proteins the key sensors of the skeletal muscle. Picture adapted from (Lange et.al, 2006).

2.3.1. Ankrd2 structure and functions

Ankrd2 was first identified as a protein got upregulated during mechanical stress response both *in-vivo* and *in-vitro* (Kemp et.al, 2000, Miller et.al, 2003). Ankyrin repeat-containing protein 2 (Ankrd2) also known as ARPP (Moriyama et.al, 2001).

The amino acid sequence of Ankrd2 is highly homologus with CARP (52%) and DARP (32%) (Moriyama et.al, 2001, Ikeda et.al, 2003). The Ankrd2 gene contains 9 exons and spans about 12 kb region. Ankrd2 is comprises of 360 amino acids. Ankrd2 contains a coiled-coil domain that contributes to its self-dimerization, a nuclear localization signal (NLS) allowing its sorting to the nucleus, a PEST protein degradation sequence and numerous potential modification sites mainly for phosphorylation (Moriyama et.al, 2001), (Figure 2.7). The 280 bp long region upstream of the transcription initiation site of the human Ankrd2 gene is sufficient to confer spatial and temporal expression specificity and contains mainly cis-elements specific for the muscle-specific transcription factor MyoD and for NF-kB (Pallavicini et.al, 2001). It is mainly expressed in skeletal muscle, preferentially in type 1 fibers (Tsukamoto et.al, 2002). Interestingly, denervation of slow muscle (soleus) decreases the level of Ankrd2 to below the detection limit in 4 weeks (Mckoy et.al, 2005), whereas denervation of fast muscle (gastrocnemius) increases its expression (Tsukamoto et.al, 2002). Further, immuno-histochemical studies revealed that Ankrd2 colocalizes in the I band of striated muscle (Tsukamoto et.al, 2002). Ankrd2 accumulates in the nuclei of damaged myofibres after muscle injury especially in euchromatin region where genes are transcriptionally active (Tsukamoto et.al, 2008), suggesting its role in signal transduction pathways.

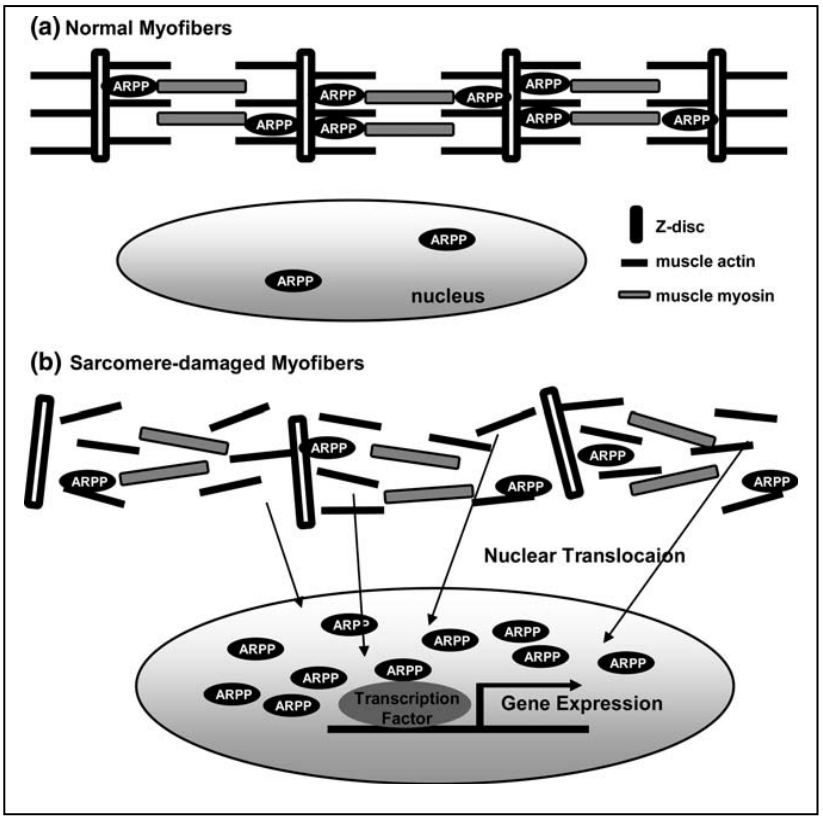

Figure 2.9 Nuclear translocation of Ankrd2/Arpp in sarcomere-damaged myofibers. (a) Normal myofibres arrangement **(b)** During the stretch/muscle injury Ankrd2/Arpp may translocate to the nucleus and bind to some transcription factors (Tsukamoto et.al, 2002).

To investigate the function of the MARP proteins in skeletal muscle Barash and colleagues developed mice with either single, double or triple knockouts of these MARP family members. However, these animals showed only minor differences in fiber size and type compared to wild type mice, with a trend towards a slower fiber-type distribution. In triple MARP knockout mice, after eccentric contractions, slight differences in mechanical behaviour were observed, and both MyoD and muscle LIM protein were up-regulated (Barash et.al, 2003). Although MARP knockout mice showed a relatively mild phenotype, the MARP proteins are important for normal function of striated muscle. It seems that MARPs have influence on the gene expression program of skeletal muscle cells (Barash et.al, 2003).

2.3.2. Role of Ankrd2 in skeletal myogenesis

Ankrd2 is thought to play dual, structural and signaling roles in skeletal muscle. Ankrd2 interacts with several structural and regulatory proteins (transcription factor's), and could link the elastic I-band region as a stress sensor for transcriptional control in the nucleus, likely sending information to the nucleus concerning the changes in the structure or function of the contractile machinery. During the myogenic differentiation Ankrd2 is expressed in both cytoplasm and nucleus however, as the differentiation into multinucleated myotubes progresses, Ankrd2 seems to change localization and to accumulate mainly in the cytoplasm (Pallavicini et.al, 2001). Mainly Ankrd2 protein has a major role in skeletal muscle formation, our previous study revealed that as a myogenic regulator its over-expression in C2C12 myoblasts significantly affects and down-regulates MyoD, myogenin and their target gene Myh1 (Bean et.al, 2005). Furthermore, we documented that Ankrd2 regulates skeletal muscle differentiation via p53 network (Bean et.al, 2008). Recently, it has been shown that Ankrd2 is involved in the regulation of multiple signaling pathways depending upon the stretch condition (Mohamed et.al, 2011). Intriguingly, Ankrd2 gene promoter contains evolutionary conserved functional putative binding motifs especially for NF-kB and AP-1 (Mohamed et.al, 2011). Therefore, Ankrd2 interact with many structural and regulatory proteins (transcription factors), thus regulates the expression of several muscle specific genes involved in skeletal myogenesis.

2.3.3. Ankrd2 Interactions

Ankrd2 is known to interact with several structural and regulatory proteins. Ankrd2 is found in the central I-band of the sarcomere, where it binds to the N2A region of titin,

forming a mechanosensing complex along with p94/Calpain-3 and anchoring protein myopalladin (Miller et.al, 2003), interacts with telethonin/T-cap (Kojic et.al, 2004), and with many other regulatory proteins (transcription factors) such as promyelocytic leukemia protein (PML),YB-1, p53 (Kojic et.al, 2004), Akt2 and zonula occludens 1 (ZO1) (Belgrano et.al, 2011).

There are substantial evidences documenting its crucial role in skeletal myogenesis. Ankrd2 acts as a molecular messenger by shuttling in between sarcomere to the nucleus, in order to protect muscle from the injury. Myogenesis is an intricate phenomenon that co-ordinately involves expression of multiple intracellular signaling cascades critical for proper cell proliferation, fusion and differentiation. The striking similarities between Ankrd2 and both NF-kB and its inhibitor I-kB, (Pallavicini et.al, 2001), strengthen the fact that probably there might be a cross talk between Ankrd2 and NF-kB and several other effectors such as Akt/Gsk3b, that contributes to skeletal muscle remodeling. The classical pathway of NF-kB regulates skeletal myogenesis therefore, we sought to explore the interplay between Ankrd2 and NF-kB/Gsk3b/Akt in context to skeletal myogenesis. To understand the nature of the cross talk between Ankrd2 and NF-kB/Gsk3b/Akt signaling pathways is indeed a major hurdle to understand the molecular basis of all cellular processes. Proper regulation of these pathways in context to the complex signaling network, with in the cell is strongly dependent on the other signaling molecules (kinases, adopter proteins) those results in either synergistic or antagonistic relationship.

2.4. NF-Kb signaling

First reported as a transcription factor crucial for the activation of kappa (κ) light chain genes in B cells, NF-kB is now recognized as a ubiquitously expressed factor involved in regulation of a wide array of pathways such as cell survival, proliferation and differentiation (Sen et.al; 1986). NF-kB family composed of several members and can be activated by variety of signals, shows the complexity of its regulation. NF-kB factor was found bounded to the κ light-chain enhancer (specific binding sequence GGGACTTTCC) of B cells, therefore called as NF-kB. Sen and Baltimore presented the first evidence that DNA binding activity of NF-kB is inducible and that its expression is not restricted to the B cell lineage (Sen et.al; 1986). Fact that NF-kB is crucial for κ chain transcription came from a breakthrough experiment (Xu et.al; 1996) in mouse. Later, NF-kB has emerged as the central orchestrator of inflammation and immune responses and has been shown to have a decisive role in homeostasis of cells of the immune system thereby, regulating the expression of prosurvival genes. During the past years scientists have shown an increase interest in the role of NF-kB in the regulation of skeletal muscle differentiation, but much more remains elusive. No doubt further research will identify additional regulatory modules that either activate or repress NF-kB related gene expression. This literature aimed to uncover role of NF-kB in skeletal muscle differentiation.

Being a powerful transcription factor NF-kB affects large number of genes involved in different biological functions of the cell and resulted in huge number of publication (almost 35000) and many more to follow. NF-kB likely plays a critical role in the development and function of the immune system and it is thus not surprisingly, when deregulated, led to the pathophysiology of inflammatory disease.

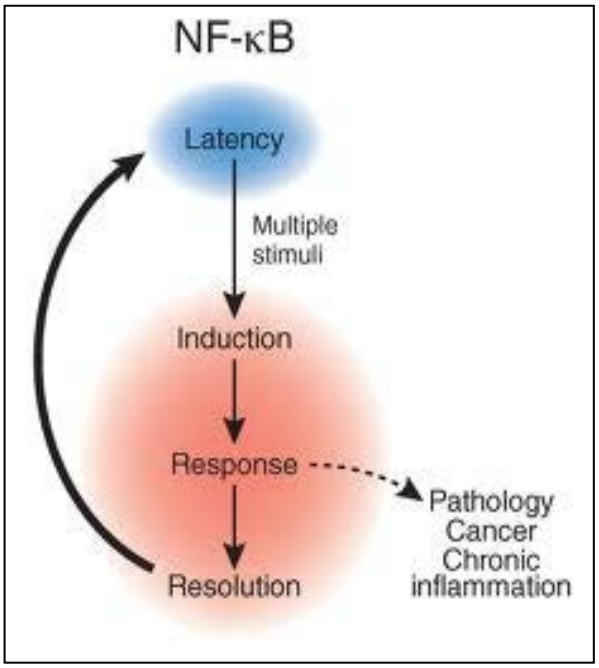

Figure 2.10 Aspects of NF-kB biology. Formulation of NF-kB mediated inflammatory response. Figure adapted from (Baltimore 2011, with permission from Nature Publishing Group).

2.4.1. NF-kB and IkB family members

NF-kB family comprised of five related Rel-family proteins RELA/p65, NF-kB1/p50 (and its precursor p105), NF-kB2/p52 (and its precursor p100), RelC, RelB. Each member of the NF-kB transcription factor family, except RelB, can form homodimers as well as heterodimers with one another (Li et.al, 2002). These structurally related proteins share a highly conserved 300 amino acid N-terminal domain, called Rel homology domain (RHD), that mediates binding to promoters and enhancer regions containing kB sites (Hayden et.al, 2004), subunit dimerization, and interaction with inhibitory proteins, known as IkBs (Hacker et.al, 2006). Among the Rel/NF-kB family, only p65, c-Rel and RelB contain a C-terminal transcriptional activation domain (TAD) and therefore are able to directly activate the transcription. The other two members, p50

and p52, lacs transcriptional activation domain (TAD), are synthesized as large precursors called p105 and p100, respectively. Irregardless, they can positively regulate transcription via heterodimerization with TAD-containing NF-kB subunits.

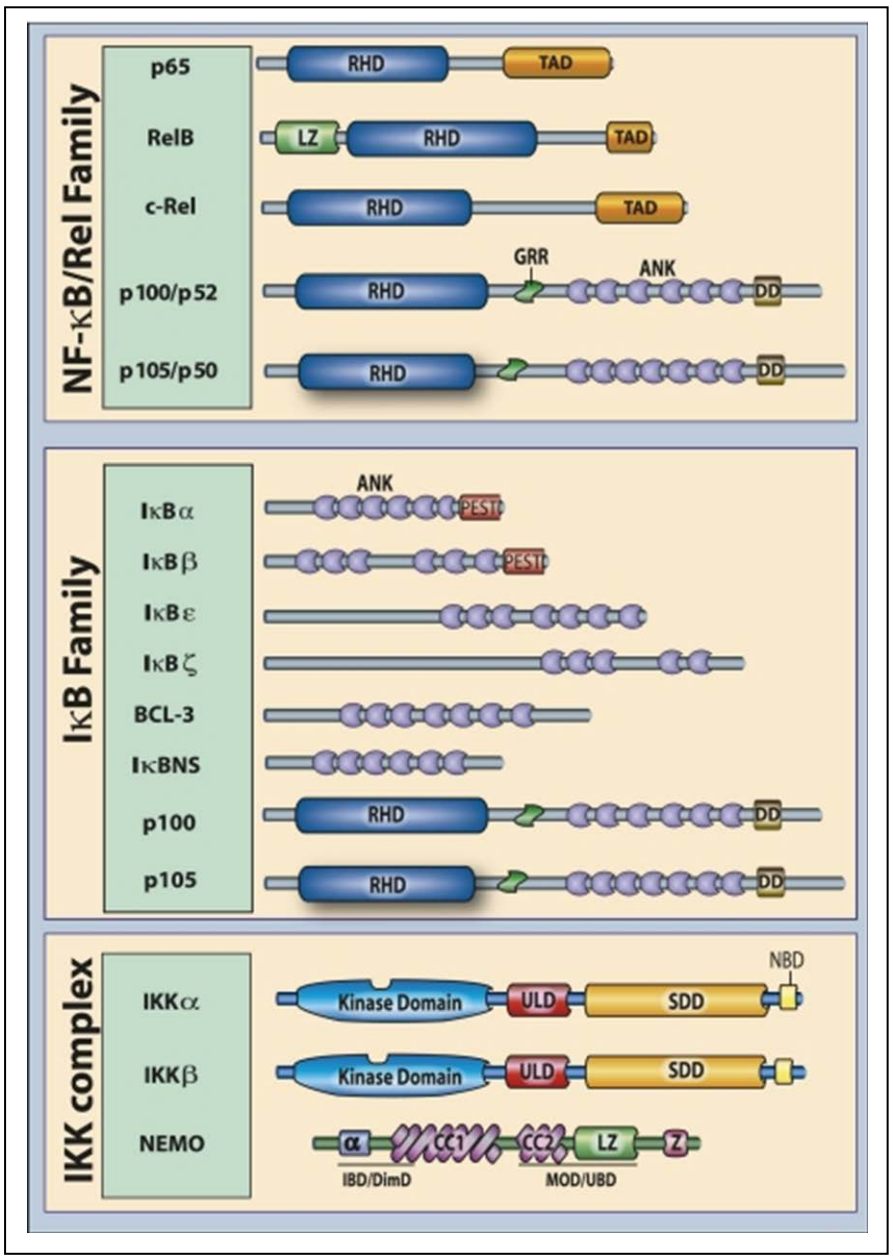

Figure 2.11 Members of the Rel/NF-kB and IkB families of proteins showing structural similarities between NF-kB family members and its inhibitors protein. Picture adopted from (Hayden et.al, 2004 with permission from Nature Publishing Group).

Alternatively p50 and p52 homodimers may negatively regulate transcription by competing with TAD domain containing heterodimers for binding to kB site (Mathew et.al, 2012). In most cells, NF-kB homodimers and heterodimers are maintained latent in the cytoplasm, by binding to IkB prevents the NF-kB:IkB complex from translocating to the nucleus, thereby maintaining NF-kB in an inactive state. The interaction with IkBs masks the nuclear localization sequence in the NF-kB complex, thus preventing nuclear translocation and maintaining NF-kB in an inactive state in the cytoplasmic compartment. NF-kB complexes act either as inducers or repressors of gene expression. The main transcriptional activated forms of NF-kB are the heterodimers p65/p50 or p65/p52 (Hayden et.al, 2004), while homodimers such as p50/50 or p52/p52 are transcriptionally repressive (Li et.al, 2002; Schmitz et.al, 1991).

2.4.2. IKK Complex: The central regulator of NF-kB activation

The IKK complex is the core element of the NF-kB cascade, also known as the IKK signalosome. It is essentially made up of two kinases IKKα/IKK1/CHUK and IKKβ/IKK2, as well as several copies of a regulatory protein, IKKγ/NEMO. IKKα and IKKβ share a strong sequence homology especially in their catalytic region (65%) and also contain helix-loop-helix domains (HLH) which is well known for protein interaction. However IKKγ is smaller and defined by coiled-coil, leucine zipper, and Zn finger like domains. (Zandi et.al, 1997) The IkappaB kinase complex (IKK) contains two kinase subunits, IKK alpha and IKK beta, necessary for IkappaB phosphorylation and NF-kappaB activation (Zandi et.al, 1997). In addition, IKKα shows a putative nuclear localization signal (NLS) (Sil et.al, 2004), these inhibitory kinases dimerizes through their Leucine zipper (LZ) domain either homodimerization or

heterodimerization, generally heterodimerization is preferred which is catalytically more efficient. (Mercurio et.al, 1999) IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation (Mercurio et.al, 1997).

The exact mechanism by which the kinase subunits become activated remains elusive. However, for the activation they need to be phosphorylated on two serine residues (Ser 176 and 180 for IKKα and Ser 177 and 181 for IKKβ) located in an activation loop, similar to a large number of other kinases (Mercurio et.al, 1997; Delhase et.al, 1999). The IkB family members, which have common ankyrin-like repeat domains, regulate the DNA binding and subcellular localization of NF-kB/Rel proteins by masking a nuclear localization signal located near the C terminus of the Rel homology domain (Beg A and Ruben S, 1992). Phosphorylation led to the conformational changes and kinase activation.

Although, phosphorylation of IKKβ is required for activation of the canonical pathway, phosphorylation of IKKα is not, though it is required for activation of the alternative pathway. The role of phosphatases that deactivate the kinases in order to shutdown the signalling is very important. PP2A has been shown to dephosphorylate IKKβ in vitro (Didonato et.al, 1997), However PP2Cβ seems to be associated with IKKβ when overexpressed (Prajapati et.al, 2004). As they shares several structural similarities, and found within the same complex strengthen the fact that they would probably have overlapping function. Canonical pathway of NF-kB is turned on by proinflammatory stimuli, such as TNF, IL1, or TLR ligands such as LPS (Israel et.al, 2010).

2.4.3. NF-kB signaling pathways

The nuclear translocation of NF-kB is regulated by two main pathways: the classical and the alternative NF-kB pathways. The NF-kB complex is activated in response to a variety of stimuli, including viral and bacterial infection, exposure to pro-inflammatory cytokines, mitogens, growth factors, and oxidative and biomechanical stresses (Kumar et.al, 2004). The classical NF-kB pathway activates the IKK complex that controls the inducible degradation of most IkB family members. The alternative NF-kB pathway induces p100 processing and p52 generation through the activation of at least two kinases, which are NIK and IKKalpha (Figure 2.12).

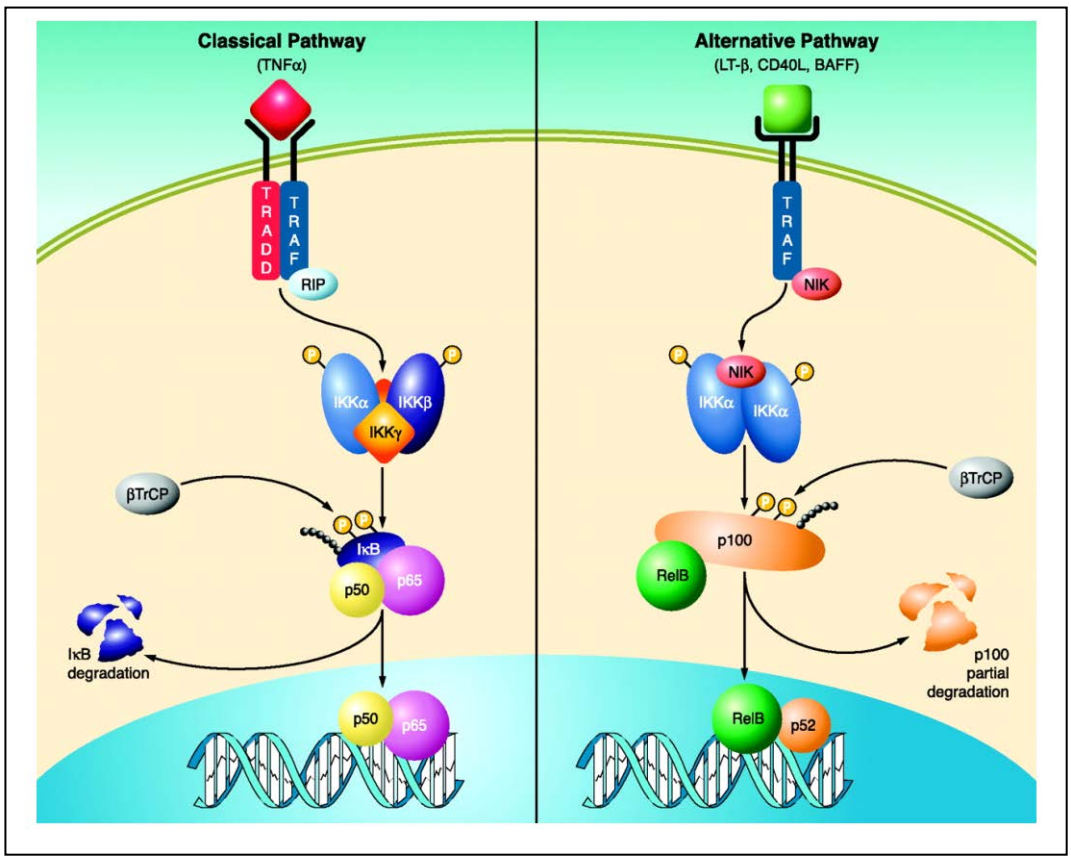

Figure 2.12 Showing activation of classical and alternate NF-kB signaling pathways. Figure adapted from (Bakkar et.al, 2010).

2.4.4. Precursors of IkBs

The Nfkb1 and Nfkb2 proteins, p105 and p100, have dual functions in the NF-kB signaling cascade. They can dimerize with another NF-kB molecule via their Rel homology domain (RHDs), in contrast their carboxy-terminal ankyrin repeats serve the function of IkB proteins. (Rice et.al, 1992; Naumann et.al, 1993; Solan et.al, 2002) They are best known as precursors of p50 and p52. The rate of processing of the p105 and p100 regulates the availability of NF-kB dimers whose activity is regulated by IkB proteins. Thus, proper physiological balance between these precursors is required. The absence of p100 results in perinatal lethality (Ishikawa et.al, 1996), and p105 deficiency results in an inflammatory phenotype and increased susceptibility to opportunistic infections (Ishikawa et.al, 1998). NF-kB activity is regulated by IkB proteins and generation of p50 and p52 from their respective precursors occurs through distinct processing mechanisms.

2.4.5. Upstream activators of NF-kB

NF-kB is triggered by a variety of signals such as bacterial product (LPS), pro-inflammatory cytokines, stress signals and mitogens. These signals are recognized by the specific pattern recognition receptors (PRRs) and then channelled through intracellular adaptor proteins to induce the signaling cascades. This induction results into the activation of inhibitory protein kinases (IKK) and thus there is release of active heterodimer complex which will translocates to the nucleus and thus affects the gene expression.

The TNF receptor associated factors (TRAF) are a group of adapter proteins which mediates the signals and have a crucial role in activation of NF-kB. They are defined by a conserved C-terminal TRAF domain, which is critical for homo and

hetero-dimerization of the TRAF proteins, as well as for their direct and indirect interactions with cognate surface receptors (Kaufman et.al, 1999). This family consist of 6 members and all the TRAF family members except TRAF1 contain N-terminal RING finger domains and thus they function as a E3 ubiquitin ligases (Hsu et.al, 1996). In case of TNF alpha signalling TRAF2 get recruited via receptors upon ligand induced oligomerization through interaction with TRADD, while MYD88 and IRAK kinase recruits TRAF6 which might relay the signals to activate NF-kB.

Receptor-interacting protein (RIP) belongs to threonine/serine protein kinases possess conserved kinase domain that can recruit the IKK complex through binding to IKKγ. RIP1 is essential for NF-kB activation via TNFα and TLR3 and TLR4 (Meylan et.al, 2005).

2.4.6. NF-kB in skeletal muscle differentiation

NF-kB plays a critical role in skeletal myogenesis which is highly sequential and regulated process that involves the determination of multipotential mesodermal cells to give rise to the myoblasts. These myoblasts exit from the cell cycle and differentiates into muscle fibre (Charge et.al, 2004). Thus myogenesis is necessary not only for growth but also for maintenance and repair of myofibres. NF-kB is essential for myoblast proliferation and maintenance in undifferentiated state. Furthermore, it has been shown that in C2C12 myoblast cell line, NF-kB binds on kB sites of the cyclin D1 promoter, regulates its transcription and leads to progression into the S phase of cell cycle. The binding activity of NF-kB on cyclin D1 is reduced during myogenesis, suggesting that NF-kB plays an important role for transition from the proliferation to the differentiation stage (Guttridge et.al, 1999), presumably confirming that NFkB inhibits myogenesis.

The notion that NF-kB acts as a negative regulator of late-stage muscle differentiation was supported by recent findings that the p65/p50 heterodimer complex binds to the transcriptional repressor YinYang1 (YY1) and results in inhibition of skeletal myogenesis (Wang H et.al, 2007). NF-kB also maintains myoblasts in undifferentiated state by suppressing muscle specific gene expression probably by limiting the level of MyoD (Dogra et.al, 2006, Langen et.al, 2004). Interestingly, transcriptional regulation of NF-kB depends on the muscle's state, during atrophy conditions, NF-kB binds on the promoter of an E3 ubiquitin ligase murine ring finger-1 (MuRF1), suppose to be involved in multiple models of skeletal muscle atrophy.

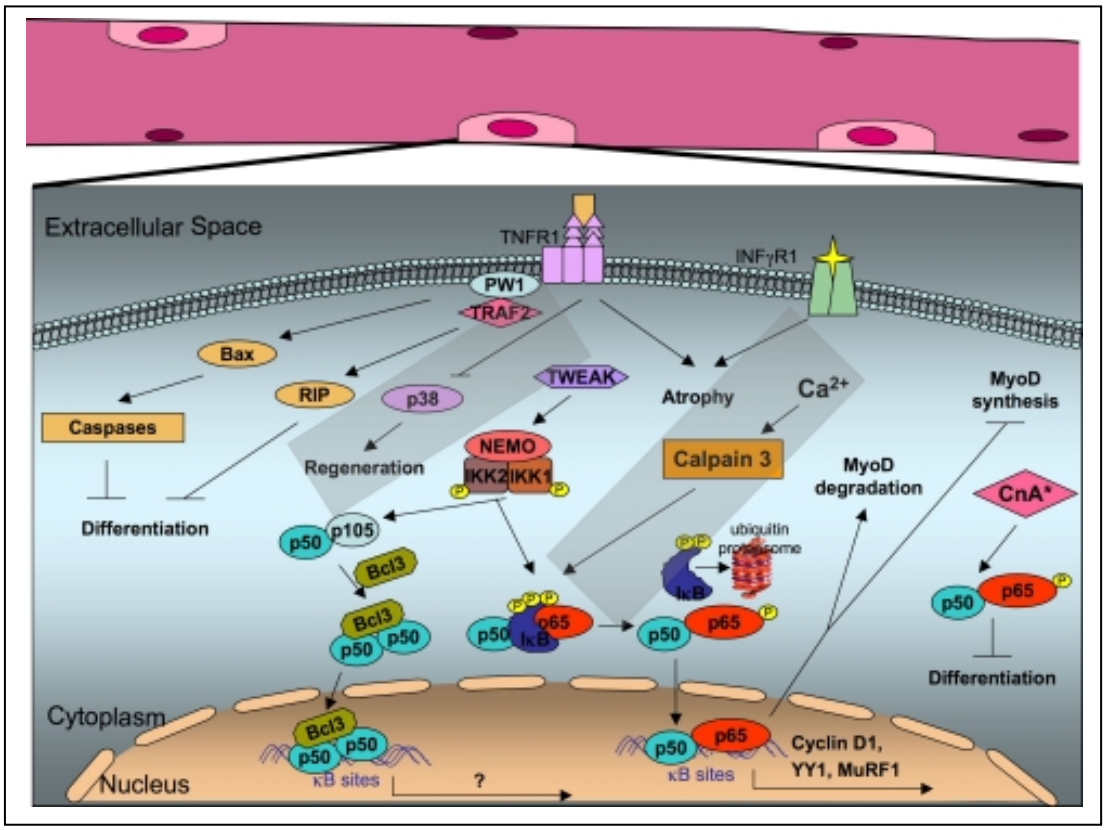

Figure 2.13 An outline of NFkB pathways in skeletal muscle. NF-kB binds on kb sites of the cyclin D1 promoter and regulates its transcription. Figure adapted from (Mourkioti et.al, 2008).

2.4.7. Role of NF-kB in inflammation and skeletal muscle regeneration

Inflammation is the protective response of tissue to the irritation, infection, injury. Common symptoms of inflammation include redness and swelling. It has been reported that acute inflammation is a part of the defense response, while chronic inflammation can lead to cancer, diabetes, cardiovascular, pulmonary, and neurological diseases. It is the basic cause of skeletal muscle wasting and fatigue that led to the aging. Pro-inflammatory cytokines such as TNF-α, IL1β, IFN-γ and TWEAK are important mediators of skeletal muscle wasting in chronic disorders (Spate et.al, 2004, Dogra et.al, 2007). NF-kB is thought to be one of the most important regulators of pro-inflammatory gene expression and synthesis of cytokines, such as TNF-α, IL-1β, IL-6, and IL-8, is mediated by NF-kB. Thus it is intuitive that NF-kB could be a target for new anti-inflammatory treatments.

Muscle regeneration followed by injury is characterized by myonecrosis, accompanied by inflammation, activation of satellite cells, and repair of injured fibers. Muscle regeneration is a process which depends on stem cell recruitment, activation and thus myogenesis (Charge and Rudnicki, 2004). The resolution of the inflammatory response is necessary to proceed toward muscle repair (Pleosi et.al, 2007). There are substantial evidences showing that NF-kB negatively regulates skeletal myogenesis. From past decades NF-KB emerged as important regulator of cell proliferation, differentiation, and regeneration. The classical signaling pathway of NF-kB regulates early muscle development whereas alternative signaling pathway involve in the maintenance of myofibre and mitochondrial biogenesis (Bakkar et.al, 2010). Myogenic differentiation is hampered in response to exogenous tumor necrosis factor-α (TNFα) and this pathway involves the activation of caspases as well as NF-kB (Coletti et.al, 2002, Guttridge et.al 2000, Ladner et.al, 2003).

Intriguingly, NF-kB could transcriptionally regulates the levels of some cytokines such as (IL-6) and inhibitors of muscle differenatiation TNF-α. Taken together, it seems that NF-kB have a great impact on skeletal muscle development and inflammation. NF-kB negatively regulates myogenesis however they may activate inflammation, and led to the acquisition and maintenance of skeletal muscle mass.

2.5. Gsk3β signaling and muscle differentiation

The name Gsk3β was given because of its ability to phosphorylate Glycogen synthase a key enzyme involved in the regulation of glycogen biosynthesis. There are two highly homologous forms of mammalian GSK3, GSK3ά and GSK3β (Woodgett et.al, 1990). GSK3 ά has a mass of 51 kDa, whereas GSK3 β is 47 kDa (Woodgett et.al, 1990, Hansen et.al, 1997). There is slight differences in size is due to glycine rich extension at amino terminus of Gsk3β. Both Gsk3α and Gsk3β shares 97% identity in their catalytic domain, but outside of the catalytic domain they are only 37% identical (Woodgett et.al, 1990). Although they have very high identity in catalytic domain but their functions are distinct. GSK3β was originally isolated from skeletal muscle (Embi et.al, 1980, Rylatt et.al, 1980) the enzyme is widely expressed in all tissues, with particularly abundant levels in brain (Woodgett et.al, 1990). Gsk3β acts as a central regulator and regulates multiple signaling pathways and many other important transcription factors which in turn can affect expression of numerous genes. GSK3β blocks protein translation initiated by the eIF2B protein (Hardt & Sadoshima, 2002). GSK3β inhibition may induce hypertrophy by stimulating protein synthesis independent of the mTOR pathway (Glass et.al, 2005).

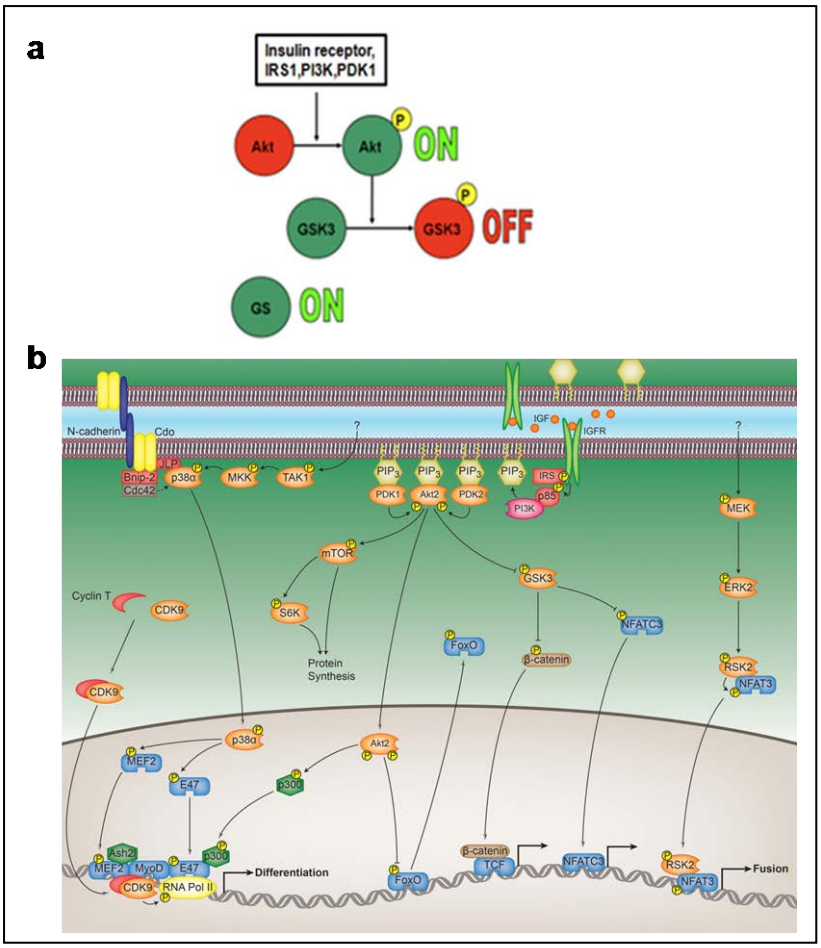

Figure 2.14 Kinases Akt/Gsk3β coordinates skeletal myogenesis. (a) Phosphorylation-dependent inhibition of GSK3β, phosphorylation of Ser-9 of GSK3β inhibits its activity.
(b) The IGF-1/PI3K/Akt pathways induces hypertrophy and blocks atrophy pathways in muscle. GSK3β inhibits differentiation and hypertrophy through phosphorylation and cytoplasmic sequestration of NFATC3 (Knight et.al, 2011).

2.5.1. Gsk3β in inflammation and its bona fide substrates

Muscle inflammation is a condition in which muscle become stiff, swollen and led to the muscle hypertrophy. There are several evidences showing that GSK3β is required for the production of proinflammatory cytokines including interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor (TNF) (Beurel and Jope, 2008, Martin et.al, 2005). Conversely, GSK3β inhibits the production of anti-inflammatory cytokine IL-10. Consequently, pharmacological inhibitors of GSK3 were found to have strong

anti-inflammatory effects (Martin et.al, 2005). Inhibition of Gsk3β led to the ablation of TLR Induced production of cytokines. Gsk3β regulates diverse functions including gene transcription regulation, inflammatory responses, development, cell division cycle, DNA damage responses, and cell survival (Meijer et.al, 2004). Thus, Gsk3β act as a central regulator of many signal transducing signaling pathways and emerged as an important positive regulator of inflammatory responses (Figure 2.14).

Metabolic and signaling proteins	**structural proteins**	**Transcription factor**
AcetylCoA carboxylase	Dynamin-like protein	AP-1 (Jun family)
Amyloid precursor protein	MAP1B	β-Catenin
ATP-citrate lyase	MAP2	C/EBP
Cyclic AMP-dependent Ninein receptor (rat) protein kinase	Neural cell-adhesion	CREB
Cyclin D1	Neurofilaments	Glucocorticoid receptor
eIF2B	Tau	HSF-1
Glycogen synthase Insulin receptor substrate-1		Myc
Pyruvate dehydrogenase		NFAT
Protein phosphatase 1		NFkB

Table 2.1 I Bona fide substrate of Gsk3b. Represents some mammalian proteins that are targeted by Gsk3β (Jope and Johnson, 2004).

2.5.2. Regulation of Gsk3β

Glycogen synthase kinase 3 (GSK3) is a multifunctional Ser/Thr kinase found in eukaryotes. This enzyme phosphorylates and regulates the function of more than 50

substrates (Table 2.1 I). It belongs to one of the few kinases that prefer prior phosphorylation of its substrate before it can further phosphorylate the substrate. This conserve phosphorylation sequence recognized by GSK3β (SXXXS) contains two Ser residues separated by three residues. Multiple copies of this motif can be present in the substrate (Terhaar et.al, 2001). The protein contains an N-terminal domain, a kinase domain and a C-terminal domain (Figure 2.15). Phosphorylation of Tyrosine 216 located in the T-loop (activation site) facilitates substrate phosphorylation by GSK3β but is not strictly required for its kinase activity. Phosphorylation of GSK3β at Ser9 in N-terminal region leads to inhibition of its kinase activity. Many of the protein kinases are capable of phosphorylating GSK3β at this residue, such as Akt, ILK, PKA, and p90Rsk (Delcommenne et.al, 1998, Fang et.al, 2000). GSK3β is regulated by serine (inhibitory) and tyrosine (activating) phosphorylation.

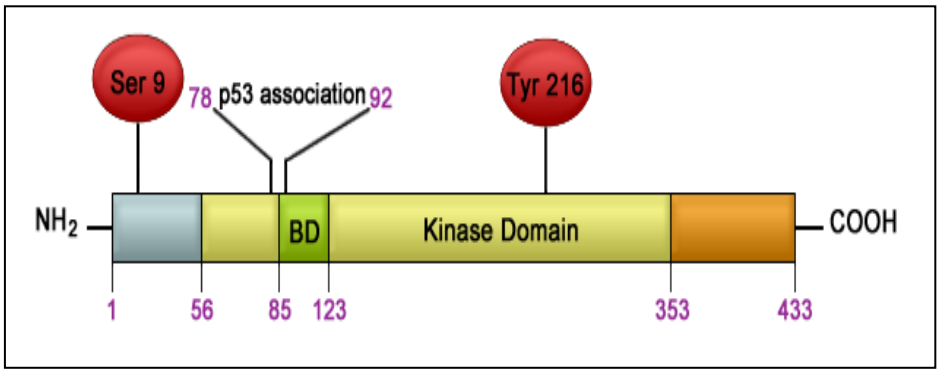

Figure 2.15 Gsk3β structure showing putative modification sites and other regulatory domains Figure adapted from (atlasgeneticsoncology.org/Genes).

2.6. Akt, ROS signaling in skeletal myogenesis

Phosphoinositide 3-Kinases (PI3K)s are lipid kinases with central roles in proliferation, differentiation, cell migration, glucose homeostasis, cell survival and growth (Figure 2.14). One of the most established roles of PI3K is observed in insulin signaling and

Akt/protein kinase B (PKB) is a central component of this cascade. Structural homologs of Akt are conserved throughout vertebrates and invertebrates (Franke et.al, 2003). Three widely expressed isoforms of Akt in mammals are Akt1, Akt2 and Akt3, each of which contain an N-terminal PH domain, a central kinase domain and a C-terminal hydrophobic domain.

Akt controls both protein synthesis, via the kinases mammalian target of rapamycin (mTOR) and glycogen synthase kinase 3β (GSK3β), and protein degradation, via the transcription factors of the Foxo family (Schiaffino et.al, 2011, Knight et.al, 2011). Subsequent phosphorylation of Akt at serine 308 by PDK1, leads to the activation of Akt. Activated Akt is dephosphorylated and deactivated by the phosphatase PP2A, GSK3β, and PARP (Sussman et.al, 2011). Knockdown of the Akt1 gene causes growth retardation and apoptosis (Cho H et.al, 2001), whereas deletion of Akt2 causes defects in glucose metabolism but not altered growth (Cho H et.al, 2001). When both Akt1 and Akt2 genes were disrupted, skeletal muscle atrophy at embryonic day 18.5 was observed, alongwith dwarfism, impaired skin, bone development, and reduced adipogenesis ((Peng XD et.al, 2003). Akt activation affect muscle gene activation and the effect of Akt on muscle mass regulation can be mediated by several other downstream effectors, such as GSK3β, mTOR and FoxO (Figure 2.14b).

Initially ROS were considered as merely toxic species to the skeletal muscle tissue. There are substantial evidences documenting that ROS are useful signaling molecules and regulates many physiological events. ROS could have multiple effects on myogenic differentiation mostly affects cell proliferation and differentiation. In particular, oxidative stress is known to play a detrimental role in a variety of muscular pathologies characterized by imbalance between proliferation and differentiation such

as Duchene muscular dystrophy **(**Terill et.al, 2013), sarcopenia and cachexia. Intracellular accumulation of ROS led to the NF-kB activation, which contributing to the lower expression of MyoD and thus impaired myogenesis (Ardite et.al; 1998). The cells exposed to H_2O_2, although exhibiting delay in recovery of protein synthesis, viability, were unable to continue and execute the differentiation. These cells also displayed a strong and long-lasting reduction of the mRNA levels of MyoD, which is involved in early stem cell commitment, and of myogenin and MRF4, both recruited at later phases of differentiation (S. Dedieu et.al, 2002, Ishibashi et.al, 2005). ROS generated by the inflammatory cytokine TNFα are known to inhibit myogenesis, and this effect is widely attributed to oxidative activation of NF-kB and subsequent gene expression (Thaloor et.al, 1999, Coletti et.al, 2005).

ROS negatively or positively regulate muscle differentiation via signaling pathways involving NF-kB activation. Now it became apparent that ROS plays decisive role in signal transduction of several signaling pathways including pivotal cell survival kinase Akt/PKB. As Akt/PKB phosphorylation is mediated by H_2O_2, our studies show a previously unappreciated role for this pivotal kinase having intrinsic crosstalk with other parallel running signaling network and ensuing its response in muscle development and growth.

Skeletal muscles are plastic tissue. In striated muscle, focal points for mechano-transduction are found at the Z-disc, the Z-disc/I-band interface and the M-band. Among the most intriguing candidates, for a role in the adaptive response of skeletal muscle, are mechanically sensitive proteins displaying dynamic distributions within the cells. We focused on the sarcomeric protein Ankrd2 that is highly responsive to muscle plasticity by shuttling in between sarcomere and the nucleus. It has been demonstrated that Ankrd2 binds to numerous regulatory and structural proteins, affects the transcription of several muscle specific genes. This mechanism is closely coordinated with many other signaling pathways. Therefore, we sought to explore the signaling pathways to determine the functional significance of the intrinsic crosstalk between Ankrd2 and the activation of transcriptional programs that regulate muscle remodeling. We study the specific contribution of Ankrd2 in skeletal muscle by investigating the gene networks that are disturbed in Ankrd2 knockout (KO) or overexpressing primary muscle cells during differentiation. This study reveals the novel molecular link between Ankrd2 and NFkB/Gsk3b/Akt and uncover the underlying molecular mechanism regulates skeletal muscle remodeling.

4.1. Generation of Ankrd2 KO mice

Genomic DNA was isolated from a mouse 129-SVJ genomic DNA library (Stratagene, La Jolla, CA), using full-length Ankrd2 cDNA as a probe. The first 2 exons and half of exon 3 were replaced by cDNA encoding lacZ and a pGK neo cassette, thereby bringing the β-galactosidase cDNA under the control of the endogenous promoter, while ablating the endogenous Ankrd2 gene (Figure 4.1a). The targeting construct was verified by sequencing and linearized before electroporation into 129-SVJ-derived ES cells at the Transgenic Core Facility at the University of California, San Diego. ES clones were screened for homologous recombination by EcoRI digestion of ES cell DNA and Southern blot analysis (Figure 4.1b) with the probe shown in (Figure 4.1a). Cells from two independent targeted ES cell clones were microinjected into C57BL/B6 blastocysts and transferred into pseudopregnant mice. Male chimeras resulting from the microinjections were mated to Black Swiss mice and gave rise to germline transmitted heterozygous mice, which were subsequently interbreed to generate homozygous mice. Offspring from intercrosses were genotyped by PCR analysis using mouse tail DNA and WT and KO allele specific primers giving rise to a 228 bp band from the WT allele and a 770 bp band from the KO allele.

The following primers were used.

WT	Forward 5-‘AACTTCGAAGATCCGCTCCTGG-3’
	Reverse 5’-CATCAATGATCTCACGTCGCAG-3’
KO	Forward 5’-CACACTGGACAGGCCTCTTTCC-3’
	Reverse 5’-AGATGAAACGCCGAGTTAACGC-3’

Successful ablation of the Ankrd2 gene was confirmed by Northern (Figure 4.1c) and Western blot analyses (Figure 4.1d) using polyclonal antibodies against Ankrd2 (Miller

et.al, 2003). All animal procedures were in full compliance with the guidelines approved by the University of California San Diego Animal Care and Use Committee and the Italian Ministry of Health.

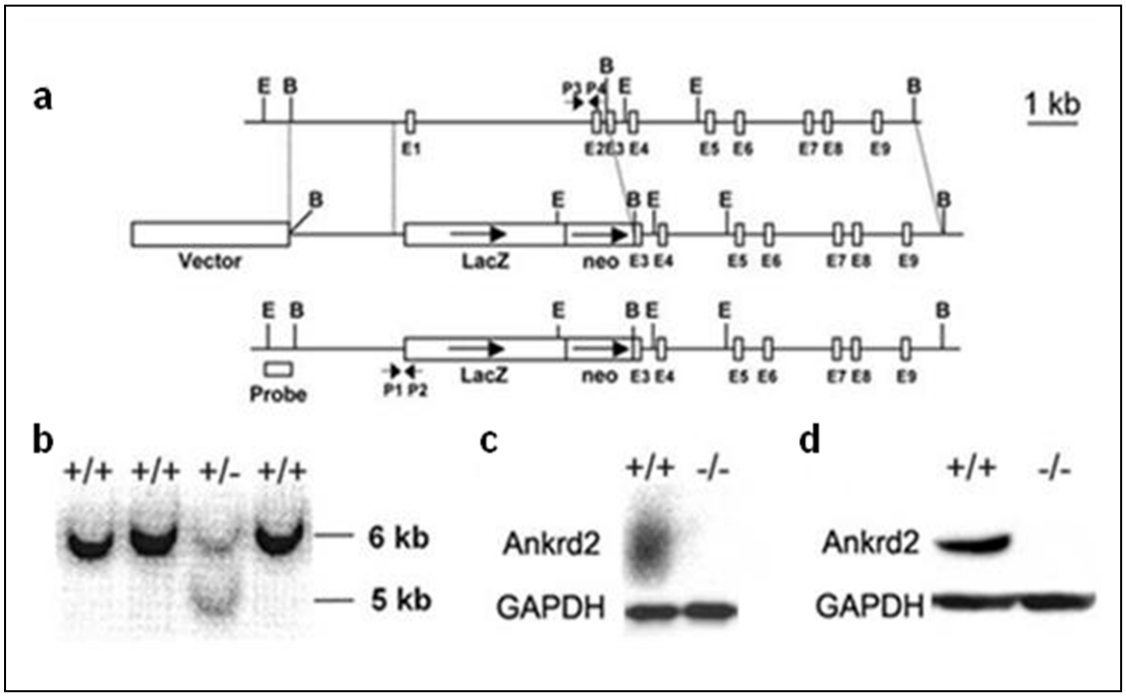

Figure 4.1 Targeting of the *Ankrd2* gene. Targeting strategy for generation of Ankrd2 KO mice **(a)** A restriction map of the relevant genomic region of Ankrd2 is shown on the top, the targeting construct is shown in the center, and the mutated locus after recombination is shown on the bottom. B, BamHI; E, EcoRI; neo, neomycin resistance gene; E, Exon **(b)** Detection of WT and targeted alleles by Southern blot analysis after digestion with EcoRI using the probe indicated in a **(c)** Northern blot analysis for detection of Ankrd2 RNA. Aliquots of 10 μg of total RNA isolated from skeletal muscle of WT and Ankrd2 KO mice were analyzed using a cDNA probe spanning the entire coding region of Ankrd2. A GAPDH probe was used as a loading control **(d)** Western blot analysis on skeletal muscle lysate from WT and Ankrd2 KO mice using anti-Ankrd2 antibodies (Miller et.al, 2003). GAPDH was used as a loading control.

4.2. Adenovirus production

The adenovirus expressing Ankrd2-HA was created using the AdEasy strategy (Agilent Technologies). The HA-tagged mouse Ankrd2 cDNA was cloned into the pAdTrack

cytomegalovirus vector (Agilent Technologies). Subsequent steps were performed according to the manufacturer's instructions. Briefly, the shuttle vector containing the Ankrd2 cDNA was linearized and cotransformed into the *E. coli* DH10B stain with the adenoviral backbone plasmid pAdEasy-1. The resulting recombinant adenoviral DNA with Ankrd2 cDNA was transfected into a packaging cell line (293A cells) to produce the recombinant adenoviral vectors. The adenoviral vectors contain two distinct promoters that independently drive the expression of the gene of interest and GFP. The mock plasmid expresses only GFP.

4.3. Plasmid constructs

Full-length mouse p50 and p65 cDNA, amplified by PCR from mouse skeletal muscle cDNA using primers with flanking sequences for EcoRI and SacII restriction enzymes as mentioned below.

Primer sequences used to generate expression vectors

P50	Forward 5'-CG**GAATTC**CGGCATGGCAGACGATGATCCCTAC-3'
	Reverse 5'- ATCG**CCGCGG**CGCTAATGGGTGACCCCTGCGTT-3'
P65	Forward 5'- CG**GAATTC**CGGCATGGGACGATCTGTTTCCCCT-3'
	Reverse 5'- ATCG**CCGCGG**CGCTATTAGGAGCTGATCTGAC-3'

Bold face; E.CoRI, and SacII

The HA-tag was inserted by site-directed mutagenesis into the Ankrd2-pcDNA3.0 expression vector (Bean et.al, 2008) both in the N- (HA-Ankrd2- pcDNA3.0) and C-terminal (Ankrd2-HA-pcDNA3.0) position. 18 rounds of extension using prmers below, were performed with Pfu polymerase, followed by digestion with DpnI (New England Biolabs) before transformation into the *E. coli* DH10B strain.

Ankrd2	Forward 5'-CAGCCTATACCAGCCCAGTACCCCTACGATGTGCCTGACTACGCTTCCCTGTAGCCGCGGATACCTACCCGGAGCTGG-3'
	5'-AGTGTGCTGGAATTCATGGCCTACCCCTACGATGTGCCTGACTACGCTTCCCTGCCG CGGGAGGGTACCATGGAGGGG-3'

WT and Ser99Ala mutant FLAG-tagged human Ankrd2 expression vectors have been described previously (Cenni et.al, 2011). The 3xNF-kBluc reporter vector, containing a triplicate NF-kB response element, was generated by subcloning from a 3xNF-kB-βgal reporter vector (kindly supplied by Prof. Stefano Schiaffino, University of Padova, Italy) into the pGL3basic reporter vector (Promega). Briefly, the 230bp DNA sequence containing the triplicate NF-kB response element was KpnI/HindIII excised from the 3xNF-kBβgal plasmid and cloned into the same restriction sites of the pGL3basic reporter vector. Two luciferase reporter constructs were produced: shortPROwtluc and longPROwtluc containing 317 and 663 nucleotides upstream of the Ankrd2 ATG start codon, respectively.

Ankrd2	Forward 5'-GGG**AAGCTT**CTGTTTCTGCAAGCCACAGGGC-3'
	5'-CCG**CTCGAG**AGCCAGTTCCCAGCACTGAGGACAC-3'
	5'CCG**CTCGAG**CCTGAGCTCTTAAAACAACACTAG-3'

Bold face; Hind III, and XhoI

Above mentioned primers were used for PCR amplification with Ankrd2 mouse genomic DNA as template. The PCR products cloned into the pGL3-basic vector (Promega). Site-directed mutagenesis to remove the MyoD or the NF-kB binding sequence was performed to generate three mutant reporters: shortPRO-MyoD-luc, short-PRO-NF-kB-luc, and long-PRO-NF-kB-luc. Oligonucleotide primers containing the desired mutations using following primers.

ΔMyoD	5'-TGTCCGAGGTGAAGGAGTTGAGCTAGTAAGCTC-3'
Δ NF-kB	5'- TGGCTTCCCATGCTCCCTGAGGTGAAGGTGACAG-3'

The PCR products were treated with DpnI before transformation into the *E. coli* DH10B strain. For generation of the luciferase reporter construct GSK3β PRO-luc, containing 765 nucleotides upstream of the ATG of the Gsk3β gene driving the firefly luciferase gene, PCR was performed on mouse genomic DNA using the following primers. The PCR product was cloned into the pGL3-basic vector (Promega). All plasmids were confirmed by sequencing.

Gsk3β	Forward 5'- CCG**CTCGAG**CGGTTGCCTGGTTCCCATCATG -3'
	Reverse 5'- CCG**CTCGAG**CGGTTGCCTGGTTCCCATCATG -3'

Bold face; Hind III, and XhoI

4.4. RNA extraction

4.4.1. RNA extraction from primary muscle cells

Samples from proliferating, fusing and differentiated myoblasts were collected in 1 ml TRIzol reagent (Invitrogen) by maintaining the samples on ice. Then 200ul choloroform was added, in order to allow complete dissociation of the nucleoprotein complex and samples were mixed rigorously and incubated at room temperature (RT) for 15 minutes. Furthermore, Samples were centrifuged at 12,000 x g for 20 min at 4°C. After the centrifugation, samples were separated into two phases a lower phenol-chloroform phase, containing the protein fraction, an interphase, with the DNA, and a colorless upper aqueous phase containing RNA. RNA was precipitated by adding an

equal volume of isopropyl alcohol, incubating samples at -20°C for 30 min., and centrifuging at 12,000 x g for 20 min. at 4°C.

RNA pellet was washed twice with 1 ml of 75% ethanol vortexing, and centrifuging at 7,500 x g for 10 min. at 4°C. Finally, RNA pellet was air dried at RT for 10 minutes and then resuspended in 20 µl of RNase free water (Promega).

4.4.2. RNA quantification and quality control

Total RNA extracted from samples was quantified by spectrophotometer, using the NanoDrop ND1000 (Celbio). RNA quality was determined with the RNA 6000 Pico/Nano LabChip on a 2100 Bioanalyzer (Agilent technology) was used for RNA quality control in conjunction with an Agilent Bioanalyzer 2001. All chips were prepared according to the manufacturer instruction's using 200 ng of RNA. RNA samples were separated electrophoretically; then, the bio analyzer software generated an electropherogram and gel-like image. For the experiment comparison and repeatability, the program provided the RNA Integrity Number (RIN), only samples with an RNA Integrity Number (RIN) > 9 were included in the study.

4.4.3. RNA amplification and labelling

50 ng of RNA sample were amplified and labelled using one color Microarray based gene expression analysis (Low Input Quick Amp WT Labelling) protocol (Agilent Technology), according to the manufacturer's instructions. Agilent one-color spike mix was added to samples. Then, cyanine labelled cRNA (complimentary RNA) was

generated using linear amplification protocol. Further, T7 RNA Polymerase were added in the reaction mixture which simultaneously amplifies target material and incorporates Cy3-labelled CTP and incubated at 40°C for 2 h. The labelled cRNAs were purified by RNAeasy Mini Kit (Qiagen), following the manufacturer instructions.

4.4.4. cRNA Quantification

The concentration and specific activity of the labelled cRNAs (picomole of Cy3/ microgram of cRNAs) were measured by Nanodrop ND1000. The specific activity was determined by the formula as below.

Pmol Cy3 per µg cRNA = Concentration of Cy3/Concentration of cRNA x1000

For cRNA yield was about 10 µg and the specific activity was around 30 pmol Cy3 per µg cRNA.

4.5. Microarray experiment

The Agilent SurePrint G3 Mouse GE 8x60K Microarrays include 39430 Entrez Gene RNAs, 16251 large intergenic non-coding RNAs, 339 x 10 replicates of biological probes, and 128 x 10 positive controls. Probe design is sourced using RefSeq, Ensembl, RIKEN, GenBank, and UniGene database. Each slide composed of 8 arrays printed using Agilent's 60-mer SurePrint technology. This technology uses a proprietary non contact industrial inkjet printing process, in which oligo monomers are deposited uniformly onto specially prepared glass slides. This in situ synthesis process prints 60-mer length oligonucleotide probes, base-by-base, from digital sequence files.

600 ng labelled cRNAs was mixed with 5 µl of 10X Blocking Agent, 1 µl of 25X Fragmentation Buffer, and water to a final volume of 25 µl. The reaction was incubated at 60°C for 30 min. to fragment cRNA. Then, 25 µl of 2X GEx Hybridization Buffer HI-RPM was added to obtain the hybridization mix. 40 µl of Hybridization mix was putted into one of the 8 arrays of SurePrint G3 Mouse GE 8 × 60K Microarrays. The slides were incubated into the Agilent SureHyb chamber at 65°C for 17 hours and rotation was set at 10 rpm. Finally, microarray chambers were disassembled into GE Wash Buffer 1 at room temperature. Then microarray slides were washed into GE Wash Buffer 1 at room temperature for 1 min and final wash for 1 minute in GE Wash Buffer 2 at elevated temperature.

4.5.1. Data analysis

Microarray data is captured using a scanner for fluorescent signal detection via a confocal detector or a charge coupled device (CCD) camera. The microarray scanner excites the fluorophores adhered to the spots on the array and acquire data about the intensities of the light emissions from the field of the microarray. The hybridized arrays were scanned using GenePix 4000B scanner (Agilent Technologies) at 3µm resolution. Next, Scanned images were analyzed with Feature Extraction Software (Agilent Technologies), which is an automatic process for the commercial arrays that use platform specific software with defined spot finding algorithms, background subtraction methods and selection of poor quality spots (Elvidge et.al, 2006). Only arrays within a range of least 8/9 quality control metrics were used for the data analysis. Then, intensity measurements were adjusted to minimize several experimental variables, such as differences in labeling, hybridization and detection. This adjustment

is called as normalization (Chen et.al, 2007). Feature Extraction Software performed intra-array normalization. Inter-arrays quantile normalization was performed with the Expander software (Sharan et.al, 2003), using the quantile normalization. This method aims at making the distribution of probe intensities for each array in a set of arrays the same by taking the mean quantile and substituting it as the value of the data item in the original dataset (Bolstad et.al, 2003). After normalization, the intensity from multiple probes that measure the same gene was combined to generate a single expression level for the gene. Then, normalized data were Log2 transformed, in order to reduce the value range. Next, data were filtered by removing probes with at least 5 not available (NA) values.

4.5.2. Cluster analysis

Clustering analysis allows us to sort the data and group genes or samples together on the basis of their separation in expression space. Hierarchical cluster analysis was performed by MultiExperiment Viewer (MeV, v4.6.2), software included in TM4 Microarray Software Suite (Saeed et.al, 2006), using Euclidean Correlation.

4.5.3. Identification of differentially expressed (DE) genes

Significance Analysis of Microarrays (SAM), a non-parametric statistical test, identified differentially expressed genes (Tusher et.al, 2001). SAM uses an adjusted t-test, with permutations of repeated measurements to calculate the False Discovery Rate (FDR) value, defined as the percentage of genes falsely identified as differentially expressed. For the sample analysis, paired two-class SAM analysis was performed to

find differentially expressed (DE) genes. FDR values minor of 5% are commonly considered as highly significant. SAM analyses were performed by MeV.

4.5.4. Gene functional enrichment analysis

To explore the biological mechanism of differentially expressed genes or cluster of genes is necessary to perform a GO analysis, which assigns genes to one or more molecular functions, biological processes and cellular components. To identify the biological system/pathways where the genes differentially expressed and acts, is necessary to analyze the microarray data in pathways. Gene enrichment in pathways was performed at the DAVID web server, using KEGG database. In all the analysis entire mouse genome was used as background.

4.5.5. Web based software

Gene Ontology enrichment was performed with Functional Annotation Clustering of the Database for Annotation, Visualization and Integrated Discovery (DAVID; Huang et.al, 2009; http://david.abcc. ncifcrf.gov/). Enrichment of Transcription Factors in the promoter of DE genes was accomplished by using the oPOSSUM tool (http://opossum.cisreg.ca; Kwon et.al, 2012). To identify the occurrence and frequency of transcription factor binding sequences in the Gsk3β promoter, Genomatix software (http://www.genomatix.de/matinspector.html) and Patch tool (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi) were used.

4.6. Cell culture, DNA transfection and adenovirus infection

Mouse C2C12 myoblast cells (American Type Culture Collection) were grown in growth medium containing DMEM - high glucose, supplemented with 10% heat inactivated fetal bovine serum (FBS) and at around 90% confluency switched to differentiation medium containing DMEM – high glucose, supplemented with 2% heat inactivated horse serum. For infection, C2C12 cells were incubated with adenovirus at a multiplicity of infection (MOI) of 250 in growth or differentiation medium. The human embryonic kidney cell line (HEK293A) was cultured in DMEM supplemented with 10% heat inactivated FBS. Cells were transected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Primary myoblasts were isolated from the limbs of 3-day-old neonatal WT and Ankrd2 KO mice using a protocol modified from (Brodie and Sampson, 1990). Briefly, skinned mouse limbs were collected from neonatal pups, minced, and subjected to serial trypsinization (successive 25 min periods until all tissue was digested) in 0.05% trypsin solution B (Biological Industries) in PBS by continuous stirring at 37°C. Cells were collected by centrifugation at 1000g for 5 min and resuspended in DMEM containing 20% FBS. After 30 min preplating to remove fibroblasts, the supernatant was filtered through a nylon mesh and 1.0 x 10^6 cells / well were plated on collagen-coated 6-well plates. Cells were proliferated in DMEM–high glucose containing 20% FBS and at a confluency of 80-95%, cell differentiation was induced by reducing serum levels to 2% horse serum. For adenoviral infection, cells were incubated with AdGFP or AdAnkrd2 at an MOI of 500 for 4 hours in serum free medium and collected 20 hours after the initiation of infection. Adenovirus treatment was performed at three different stages: 1) proliferating myocytes at about 50% confluency (proliferation stage); 2) proliferating

myocytes at about 90% confluency prior to induction of differentiation (fusion stage); and 3) differentiating cells 3 days after the induction of differentiation (differentiated stage).

4.7. Co-Immunoprecipitation

Subconfluent 293A cells were transfected with FLAG-p50 together with Ankrd2-HA-pcDNA3.0 or HA-Ankrd2-pcDNA3.0. Cells were lysed 24 hours after transfection in a buffer containing 150 mM NaCl, 50 mM Tris-HCl, 0.05% Triton X-100, and protease inhibitors (Sigma) and immunoprecipitations were performed using the ProFound HA Tag IP/Co-IP Kit (Pierce, Rockford IL) according to the manufacturer's instructions. Briefly, protein extracts were incubated overnight with agarose beads conjugated with monoclonal anti-HA tag antibody (H3663, Sigma). The immunoprecipitates were subjected to Western blot analysis using monoclonal anti-HA and anti-FLAG (F3165, Sigma) antibodies. Protein extracts from C2C12 cells were transfected with FLAG-tagged WT or Ser99Ala mutant human Ankrd2 and incubated overnight at 4°C with anti-FLAG and resin provided by the IP/Co-IP Kit (Pierce, Rockford IL). Subsequently, immunoprecipitates were subjected to Western blot analysis using anti-FLAG and anti–p50 (sc-114, Santa Cruz) antibodies.

4.8. Immunofluorescence microscopy

Proliferating (myoblasts) and differentiating (myotubes) C2C12 cells were grown on collagen-coated coverslips, fixed with 4% paraformaldehyde for 10 min and subsequently processed for co-immunofluorescence staining using rabbit anti-p50

polyclonal antibody (sc-114, Santa Cruz) and a mouse monoclonal antibody specific for the Ankrd2 N-terminus (Pallavicini et.al, 2001) as previously described (Bean et.al, 2008). Briefly, following permeabilization and blocking in PBS with 1% BSA, coverslips were incubated with primary antibodies in PBS with 0.05% Tween 20 for 2 h at 37°C (primary antibodies) and subsequently with Rhodamine (TRITC)-conjugated anti-mouse (Dako) and FITC-conjugated anti-rabbit (Sigma) secondary antibodies for 1 h at room temperature. The immunostained samples were observed with a Leica5000 DFC300FX microscope at 40x and 100x magnification.

4.9. Luciferase reporter assay

Luciferase reporter constructs were cotransfected with a modified pGL4.74 control reporter vector (Promega) in mouse C2C12 cells. The pGL4.74 vector was mutated by deletion of the thymidine kinase promoter to provide basal levels of Renilla luciferase expression in cotransfected cells. Six hours after transfection, the medium was replaced with growth or differentiation-promoting medium and at 24 h, 48 h, and 144 h after transfection, cell lysates were prepared from proliferating and differentiating myoblasts. Luciferase and Renilla activity were measured on a TD-20/20 Single-Tube Luminometer (Turner BioSystems) using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was normalized to Renilla activity to account for variations in transfection efficiency. Where indicated, transfected cells were treated with 250 μM H_2O_2 for 2, 4, and 6 hours before collection. In some experiments, 2 μg/ml of LPS (Sigma) was added to the cells 24 h or 48 h after transfection, whereafter cell lysates were prepared 24 hours later.

4.10. Chromatin immunoprecipitation assay

ChIP assays were carried out using the ChIP assay kit (Millipore) according to the manufacturer's instructions.

Gene	Forward	Reverse
Proximal NF-kB box	5'-CACACTGGACAGGCCTCTTT-3'	5'-CACACTGGACAGGCCTCTTT-3'
Distal NF-kB box	5'-AGCATAGCCGTGTTTCCCTA-3'	5'-GGCTCGTTTCTCCATCTGTC-3'
Gsk3βpromoter primer0	5'-TCCTCATTGGTTATCCAGGTC-3'	5'-CTAGCCCTTCCCCACTCC-3'
Gsk3βpromoter primer1	5'-GCGGAGGACGAGTAGGAAG-3'	5'-GGCTGCTCGGGAAGTGTC-3'
Gsk3βpromoter primer2	5'-CCGAGTGACAAAGGAAGGAA-3'	5'-GAGGCAGCTCCCTTCAGAC-3'
Gsk3βpromoter primer3	5'-CGTATGGGGAGCAGTCAGG-3'	5'-AGGAGATGGCTCGGAGATG-3'
Gsk3βpromoter primer4	5'-CATCTCCGAGCCATCTCCT-3'	5'-AAGGGTGGAGTGGAATCCTT-3'
Gsk3βpromoter primer5	5'-TGAAAAGCCAAGAGAACGAA-3'	5'-CAAAAGCTGAAGGCTGCTG-3'

Table 4.1 Oligonucleotide primers used for amplification of NF-kB binding sites on the Ankrd2 gene promoter

C2C12 myoblasts and myotubes at the fourth day of differentiation were fixed by adding formaldehyde solution into the culture medium to a final concentration of 1%, rinsed twice with ice-cold PBS, and lysed according to the manufacturer's specifications. Sonication was performed on ice using a Sonic Dismembrator Model 300 (Fisher Scientific) at a power of 50% for 3 cycles of sonication, each cycle for 30 s followed by a 10 s break on ice. Chromatin complexes were immunoprecipitated with

anti-Ankrd2, anti-p50, or anti-p65 antibodies and used as template for qRT-PCR as described above.

4.11. Real-time PCR

qRT-PCR based on SYBR Green chemistry (Finnzymes) was carried out using muscle samples from Ankrd2 KO and WT mice. Total RNA from Soleus, EDL muscle was extracted using TRIzol reagent (Invitrogen) as described above. 1 μg of RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's directions (listed in table 4.2).

Gene	Forward	Reverse
Ankrd2	5'-GAGAGCCACAGAGCTCATCG-3'	5'-GCTCTTGGCCCTTAACCTTT-3'
Ankrd1	5'-CGGACCTCAAGGTCAAGAC-3'	5'-GCTCTTCTGTTGGGAAATGC-3'
Ankrd23	5'-TGCCTAGAGCACCTTATCGAG-3'	5'-TCTGGGAAGCCACATTCTTC-3'
Il-6	5'-CCACTTCACAAGTCGGAGGCTTA-3'	5'-GCAAGTGCATCATCGTTGTTCATAC-3'
Tnfα	5'- CACAAGATGCTGGGACAGTGA-3'	5'-TCCTTGATGGTGGTGCATGA-3'
IkBα	5'-GCTACTCCCCCTACCAGCTT-3'	5'-TAGGGCAGCTCATCCTCTGT-3'
Glut4	5'-TGTCGCTGGTTTCTCCAACTG-3'	5'-CCATACGATCCGCAACATACTG-3'
Rcan1	5'-GTGTGGCAAACGATGATGTC-3'	5'-AGGAACTCGGTCTTGTGCAG-3'
B2m	5'-CCGTCTACTGGGATCGAGAC-3'	5'-GCTATTTCTTTCTGCGTGCAT-3'

Table 4.2 Primer sequences used for qRT-PCR analysis

Gene specific primers were selected with Primer 3 software, in order to amplify fragments of 150–250 bp in length, close to the 3'end of the transcript (table 4.2). Experiments were performed in a 7500 Real-Time PCR System (Applied Biosystems), using GoTaq qPCR Master Mix (Promega), according to the manufacturer's instructions. Relative expression levels were quantified by constructing a standard

curve with dilution of the purified PCR product generated for each specific primer pair. Values were normalized to the expression of reference genes beta 2 microglobulin (b2m), with invariant expression level in all experimental conditions.

4.12. Protein extraction and western blot analysis

Total protein lysate from C2C12 cells was extracted by solubilization in RIPA lysis buffer (50 mM Tris pH 7.4, 1% NP40, 150 mM NaCl, 2 mM EDTA, 0.1% SDS and protease inhibitor cocktail (Sigma-Aldrich). Further Proteins were quantified by using Bradford reagent. 20 μg of cleared protein lysates were separated by SDS-PAGE and electroblotted on nitrocellulose membranes (Hybond), and proteins were visualized with the appropriate primary and secondary antibodies and ECL (Pierce) on superRX films. Depending on the species origin of antibodies, immunoblots were either probed sequentially or on multiple membranes. Corel Draw has been used to excise the relevant portion of the immunoblots from the original scans of X-ray films exposed to chemoluminescence visualization of specific proteins. Proteins were visualized by using antibodies against Ankrd2 (Pallavicini et.al, 2001), phospho-Ankrd2 (Cenni et.al, 2011), Gsk3β, phospho-Gsk3β (Ser-9), Akt, phospho-Akt (Ser-473) (all from Cell Signaling Technology) and β-actin (Sigma).

5.1. Deletion of Ankrd2 induces the up regulation of genes involved in inflammatory pathways

To study the role of Ankrd2 in the pathways controlling myogenic differentiation, we took advantage of the Ankrd2 KO mouse model (Figure 4.1) and performed global transcriptome analysis on proliferating, fusing, and differentiated primary myoblasts derived from wild type (WT) and Ankrd2 KO mice infected with adenovirus expressing HA-tagged Ankrd2 (AdAnkrd2) or control GFP (AdGFP) 20 hours prior to the collection of cells. In particular, since Ankrd2 is known to be strongly upregulated in response to various stress stimuli, the use of AdAnkrd2 allowed us to study the effect of acute overexpression of Ankrd2. The gene expression analysis was performed using the Whole Mouse Genome Oligo Microarray system from Agilent Technologies and our experimental design is outlined in (Figure 5.1). For each condition, three biological replicates were performed, producing data sets from a total of 36 microarray experiments.

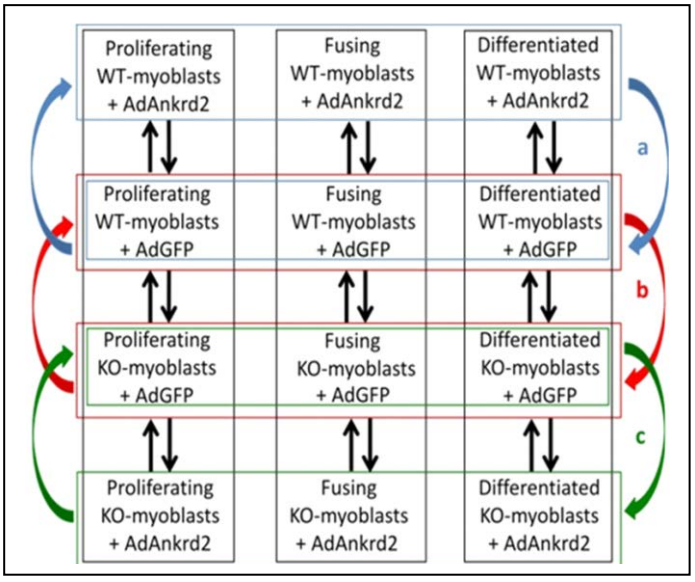

Figure 5.1 Schematic summary of comparisons between gene signatures obtained from proliferating, fusing, and differentiated WT and Ankrd2 KO primary myoblasts infected with AdAnkrd2 or AdGFP.

To evaluate the efficiency of the adenoviral expression, Ankrd2 mRNA and protein levels in AdAnkrd2- and AdGFP-infected WT and KO myoblasts were determined by quantitative real time PCR (qRT-PCR) and Western blot analyses as shown in (Figure5.2a and 5.2b).

a

Sample comparison	Stage 1		Stage 2		Stage 3	
	logFC	SD	logFC	SD	logFC	SD
Ankrd2-WT vs GFP-WT	2.55	0.23	2.87	0.27	3.07	0.18
Ankrd2-KO vs GFP-WT	1.98	0.1	1.54	0.53	1.9	0.38

b

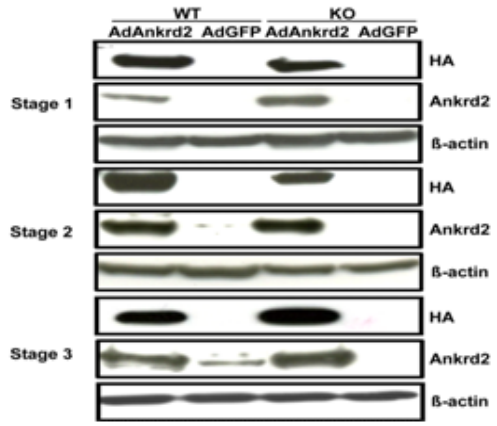

Figure 5.2 Gene expression analyses on AdAnkrd2 and AdGFP-infected primary myoblasts and quantification of Ankrd2 mRNA and protein levels. (a) Relative quantification of Ankrd2 mRNA levels in proliferating (stage 1), fusing (stage 2) and differentiated (stage 3) WT and Ankrd2 KO myoblasts infected with AdAnkrd2 or AdGFP as determined by qRT-PCR analysis (n = 3). Standard deviations (SD) were estimated using the error propagation theory. LogFC, fold change of logarithmic values **(b)** Representative Western blots showing Ankrd2 levels in AdAnkrd2-infected proliferating and differentiating myoblasts as determined using anti-Ankrd2 and anti-HA-tag antibodies.

These results confirmed the absence of Ankrd2 from KO cells and showed highly efficient gene transfer of Ankrd2 with an up till (3.07 in logarithmic scale) overexpression of Ankrd2 transcript levels in WT cells. At the protein level, a 5 to 7 fold increase in the Ankrd2 expression level was observed in AdAnkrd2-infected cells compared to AdGFP-infected WT myotubes. Furthermore, in agreement with our

previous observations, endogenous Ankrd2 expression increased during differentiation (Bean et.al, 2008).

We initially compared gene expression signatures of AdGFP-infected WT and Ankrd2 KO myoblasts (Figure 5.1, comparison b). Unpaired two-class Significance Analysis of Microarray (SAM) analysis was performed using MultiExperiment Viewer (MeV) v4.8 software (Saeed et.al, 2003) with a False Discovery Rate (FDR) of 0%. Not surprisingly, since Ankrd2 is expressed at increasing levels during differentiation, the major transcriptional changes were found at the fully differentiated myotube stage. At this stage, a total of 2,291 differentially expressed (DE) genes were identified (1,381 upregulated and 910 downregulated) in Ankrd2 KO versus WT myotubes. To obtain an overall view of the effect of Ankrd2 deletion in myotubes, KEGG pathway analysis was performed on DE genes.

Ankrd KO vs. WT	Pathway	P-value	DE genes
Downregulated	PPAR signaling pathway (mmu03320)	1.47E-09	18
	Fatty acid metabolism (mmu00071)	2.30E-05	10
	Propanoate metabolism (mmu00640)	3.81E-03	6
	Adipocytokine signaling pathway (mmu04920)	9.48E-03	8
	Insulin signaling pathway (mmu04910)	5.79E-02	10
	Cytokine-cytokine receptor interaction (mmu04060)	9.60E-02	14
Upregulated	Chemokine signaling pathway (mmu04062)	1.09E-05	30
	NOD-like receptor signaling pathway (mmu0462)	4.95E-05	15
	Leukocyte transendothelial migration (mmu04670)	1.22E-04	21
	Natural killer cell mediated cytotoxicity (mmu04650)	1.74E-04	21
	Cytokine-cytokine receptor interaction (mmu04060)	4.46E-04	32
	Fc gamma R-mediated phagocytosis (mmu04666)	8.04E-04	17
	Toll-like receptor signaling pathway (mmu04620)	2.51E-03	16
	Jak-STAT signaling pathway (mmu04630)	3.00E-03	21
	Cytosolic DNA-sensing pathway (mmu04623)	1.08E-02	10
	Regulation of actin cytoskeleton (mmu04810)	1.13E-02	25
	MAPK signaling pathway (mmu04010)	1.19E-02	29
	Cell adhesion molecules (CAMs) (mmu04514)	1.58E-02	19
	ECM-receptor interaction (mmu04512)	2.35E-02	12
	T cell receptor signaling pathway (mmu04660)	2.77E-02	15
	Apoptosis (mmu04210)	3.20E-02	12

Table 5.1 I. KEGG pathway enrichment analysis for up- and down-regulated genes in differentiated myoblasts (stage 3) from AdGFP-infected Ankrd2 KO cells compared to WT.

As shown in Table 5.1 I, many of the affected functional pathways are known to be involved in distinct inflammatory response phases (i.e. cytokine, chemokine, extracellular matrix (ECM), apoptosis, and MAPK signaling (Loza et.al, 2007). Among the genes upregulated in Ankrd2 KO cells were tumor necrosis factor alpha (Tnfα), interleukin 6 (Il-6), and chemokine (C-C motif) ligand 2 (Ccl2), important inflammatory mediators activated by NF-kB (Guha M, 2001). Furthermore, genes belonging to the peroxisome proliferator-activated receptor (PPAR) signaling pathway that can exert anti-inflammatory activity in several cell types, including skeletal muscle cells (Remels et.al, 2009), were found to be downregulated in Ankrd2 KO cells and accordingly many well-known PPAR-connected pathways (i.e. lipid homeostasis, insulin pathway, and fatty acid metabolism) were downregulated as well. Based on these results, Ankrd2 emerges for the first time as a repressor of immune and inflammatory responses.

5.2. Overexpression of Ankrd2 negatively regulates pro-inflammatory genes

To explore the effect of Ankrd2 rescue/overexpression in Ankrd2 KO cells, we next compared gene expression profiles of AdAnkrd2- and control AdGFP-infected Ankrd2 KO myoblasts during proliferation, fusion, and terminal differentiation (Figure 5.1, comparison c). Using MeV software, three sets of unpaired two-class SAM analyses were carried out in order to identify alterations in gene expression induced by Ankrd2 at each specific stage. Using KEGG pathway analysis, the most significantly affected network is represented by genes associated with inflammatory and immune responses (Table 5.1 II), similar to the results obtained from the comparison between AdGFP-infected Ankrd2 KO (Table 5.1 I).

Stage	Pathway	P-value	DE genes	Upregulated	Downregulated
1 (Proliferating)	Cytokine-cytokine receptor interaction (mmu04060)	1.26E-02	4	1	3
	Apoptosis (mmu04210)	1.36E-02	3	0	3
	Chemokine signaling pathway (mmu04062)	5.36E-02	3	1	2
2 (Fusing)	NOD-like receptor signaling pathway (mmu0462)	9.65E-10	15	0	15
	Cytokine-cytokine receptor interaction (mmu04060)	5.39E-08	25	0	25
	Cytosolic DNA-sensing pathway (mmu04623)	2.25E-06	11	0	11
	Chemokine signaling pathway (mmu04062)	2.67E-06	19	0	19
	Toll-like receptor signaling pathway (mmu04620)	3.15E-06	14	0	14
	Cell adhesion molecules (CAMs) (mmu04514)	2.85E-02	10	0	10
	Proteasome (mmu03050)	4.18E-02	5	0	5
	Jak-STAT signaling pathway (mmu04630)	6.31E-02	9	0	9
	Apoptosis (mmu04210)	9.66E-02	6	0	6
3 (Differentiated)	NOD-like receptor signaling pathway (mmu0462)	6.61E-11	15	0	15
	Cytokine-cytokine receptor interaction (mmu04060)	1.23E-07	22	3	19
	Chemokine signaling pathway (mmu04062)	2.54E-04	14	2	12
	Apoptosis (mmu04210)	8.05E-04	9	0	9
	Glutathione metabolism (mmu00480)	1.13E-03	7	0	7
	Toll-like receptor signaling pathway (mmu04620)	1.87E-03	9	0	9
	MAPK signaling pathway (mmu04010)	7.59E-03	14	1	13
	Cytosolic DNA-sensing pathway (mmu04623)	3.70E-02	5	0	5

Table 5.1 II. KEGG pathway enrichment analysis for up- and down-regulated genes in AdAnkrd2- compared to control AdGFP-infected Ankrd2 KO cells at different stages.

To identify genes that are differentially expressed independent from the differentiation stage, we performed an additional SAM analysis (0% FDR), as shown in (Table 5.1 III).

Pathway	P-value	DE genes	Upregulated	Downregulated
NOD-like receptor signaling pathway (mmu0462)	1.90E-11	11	0	11
Chemokine signaling pathway (mmu04062)	7.83E-07	11	0	11
Cytokine-cytokine receptor interaction (mmu04060)	1.11E-05	11	0	11
Apoptosis (mmu04210)	4.22E-03	5	0	5
Toll-like receptor signaling pathway (mmu04620)	6.67E-03	5	0	5
Adipocytokine signaling pathway (mmu04920)	1.43E-02	4	0	4
Cytosolic DNA-sensing pathway (mmu04623)	6.61E-02	3	0	3

Table 5.1 III. KEGG pathway enrichment analysis for up- and down-regulated genes in AdAnkrd2- compared to control AdGFP-infected Ankrd2 KO cells at all stages.

The majority of these genes belong to pathways related to inflammation, having NF-kB as a central node (Figure 5.3).

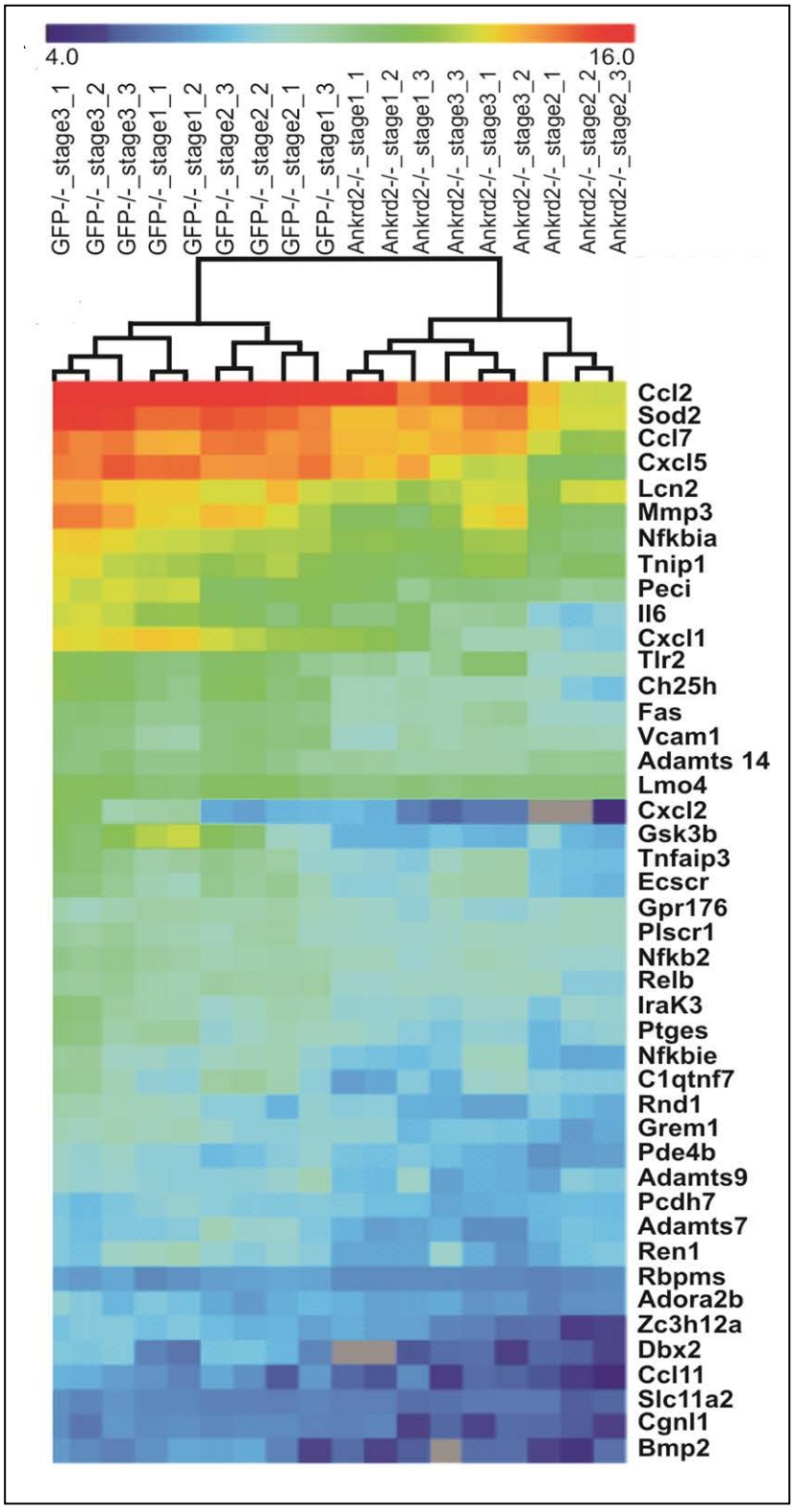

Figure 5.3 Ankrd2 overexpression affects the expression of NF-kB related genes. Gene expression heat map for the functional annotated transcripts that are differentially expressed (0% FDR) at all differentiation stages in response to acute overexpression of Ankrd2 in Ankrd2 KO cells.

Heat map shows that over expression of Ankrd2 in all the stages (Proliferating, fusing and differentiating primary cells) led to the down regulation of well-known NF-kB targets, including IL-6 genes, a known effector of the classical inflammatory response pathway (Guha & Mackman; 2001). Consistent with a role of Ankrd2 as a negative regulator of NF-kB directed inflammation.

Further we selected these down regulated genes (as shown in heat map) and then try to make a search for the enrichment of transcription factor bindings sites (TFBSs), a specific enrichment of NF-kB binding motifs was found using the oPOSSUM tool (Figure 5.4), supporting our notion that Ankrd2 as a negative regulator of NFkB induced Inflammation.

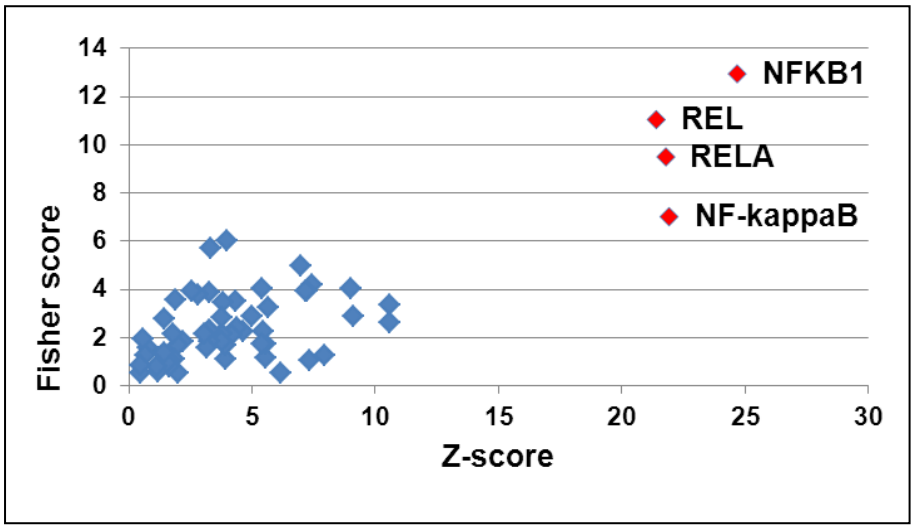

Figure 5.4 Identification of transcription factor binding sites (TFBSs) present in the DE genes by oPOSSUM, showing a significant enrichment for NF-kB binding sites.

5.3. Ankrd2 regulates NF-kB activity

5.3.1. Ankrd2 inhibits NF-kB transcriptional activity

To test the hypothesis that the anti-inflammatory effect of Ankrd2 is achieved through inhibition of NF-kB, a NF-kB-specific reporter assay was performed in the myogenic C2C12 cell line by transfection of a NF-kB reporter vector containing a triplicate NF-kB responsive element driving a firefly luciferase reporter (3xNF-kB-luc) prior to induction of overexpression of HA-tagged Ankrd2 by AdAnkrd2 infection. Cells infected with AdGFP served as controls. To adjust for differences in transfection efficiency, cells were cotransfected with a Renilla luciferase expression vector (pGL4.74) as internal control. NF-kB activity was measured in proliferating and differentiating cells after 1, 3, and 6 days of differentiation. For each stage, cells were infected with AdAnkrd2 or control AdGFP 20 hours before harvesting. As shown in (Figure 6.5a), values represent the firefly/Renilla luciferase ratio in proliferating and differentiating cells the results, expressed as firefly/Renilla luciferase ratio, confirmed previous data showing that the activation of NF-kB increases during myogenic differentiation (Baeza-Raja and Munoz-Canoves, 2004; De Alvaro et.al, 2008). The major finding is that Ankrd2 represses the transactivation of NF-kB, thus identifying Ankrd2 as a negative regulator of the NF-kB signaling pathway. Recently it has been demonstrated that under H_2O_2-induced oxidative stress triggering the production of ROS, Akt2 phosphorylates Ankrd2 at Ser-99, inducing its nuclear translocation and inhibition of muscle differentiation (Cenni et.al, 2011).

ROS are known to interact with the NF-kB signaling pathway in a variety of different ways dependent on context and cell type (Morgan and Liu, 2011), i.e. ROS can both activate and inhibit NF-kB and conversely NF-kB activity can influence ROS levels. Thus, to determine whether Akt2-dependent Ankrd2 Ser-99 phosphorylation

mediates NF-kB regulation, the 3xNF-kB-luc reporter vector was cotransfected into C2C12 myoblasts together with FLAG-tagged WT or Ser99Ala mutant human Ankrd2. As shown in (Figure 5.5b), overexpression of WT Ankrd2 inhibited NF-kB activity, while overexpression of unphosphorylatable Ser99Ala-Ankrd2 did not affect NF-kB activity neither in proliferating or differentiating cells after 1 and 6 days of differentiation.

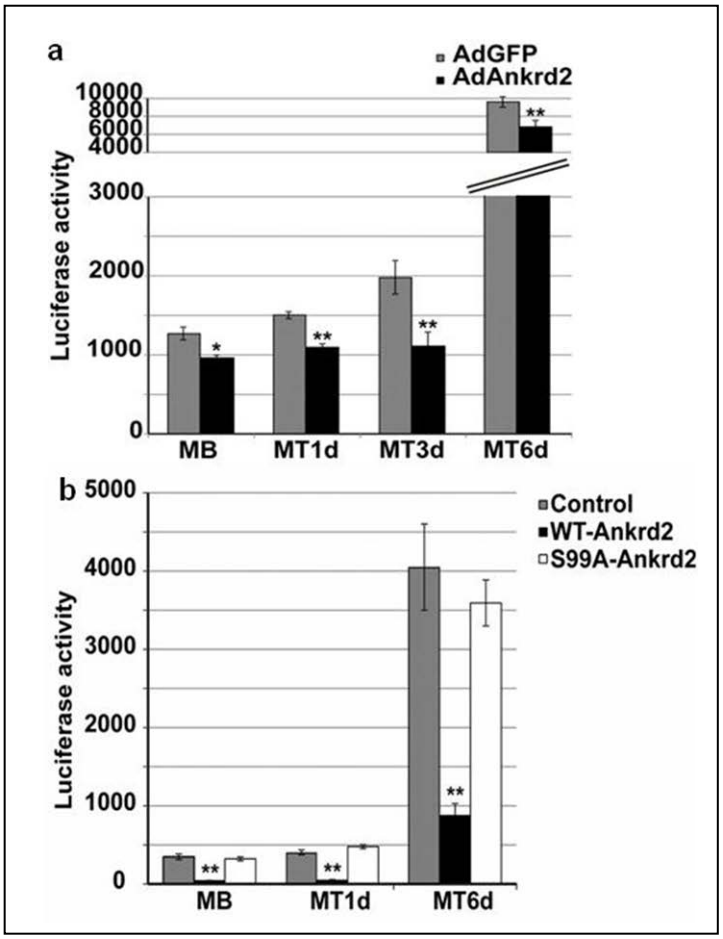

Figure 5.5 Ankrd2 represses the transcriptional activity of NF-kB. (a) C2C12 myoblasts were transfected with the 3xNF-kB-luc vector and pGL4.74 control vector before infection with AdAnkrd2 or AdGFP. The values represent the firefly/Renilla luciferase ratio in proliferating and differentiating cells. (**b**) Luciferase activity assay in C2C12 cells following cotransfection of 3xNF-kB-luc vector with FLAG-tagged WT or Ser99Ala mutant (S99A) Ankrd2.

Thus, the Akt signaling axis inhibits NF-kB-regulated inflammatory genes via phosphorylation of Ankrd2 at Ser-99 and Ankrd2 may play a role as a sensor of the

oxidative state through impairment of NF-kB. To assess whether Akt exerts an inhibitory effect on NF-kB in a ROS and Ankrd2 dependent way during muscle differentiation, we treated C2C12 cells with H_2O_2 and measured NF-kB, Akt, and Ankrd2 activities.

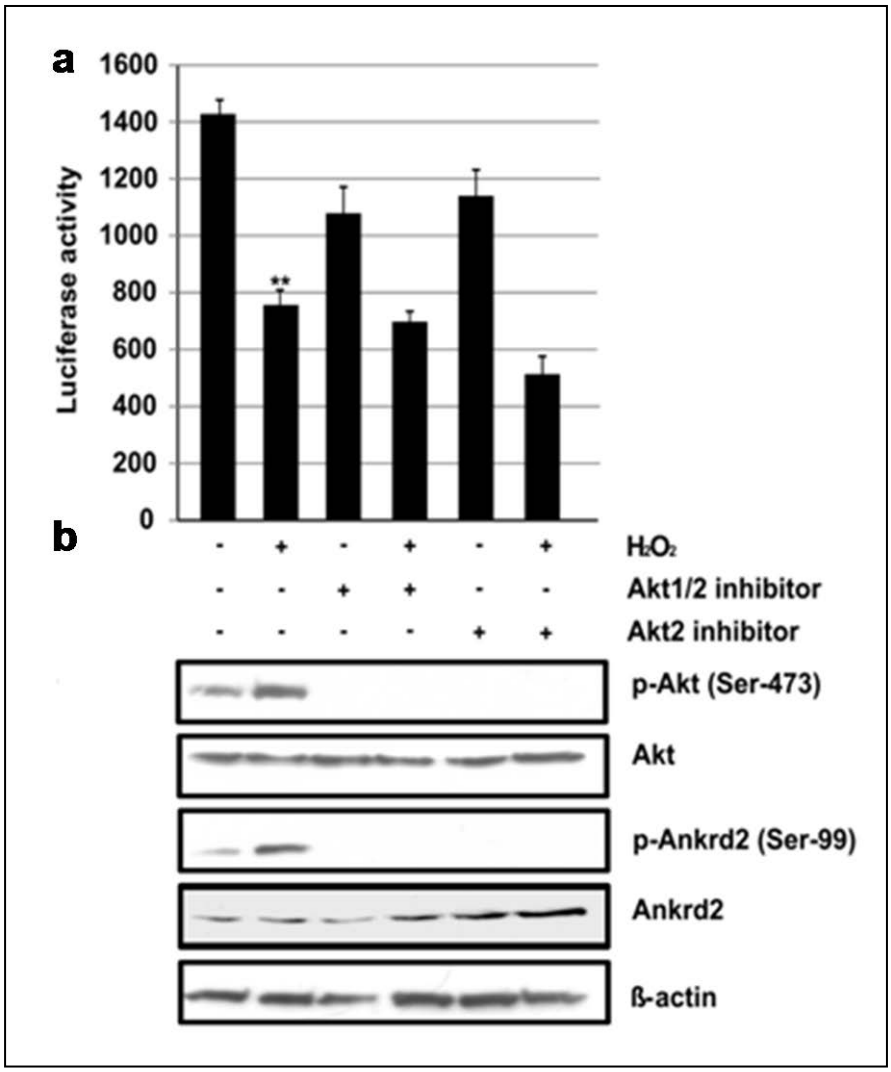

Figure 5.6 Ankrd2 represses the transcriptional activity of NF-kB during oxidative stress. **(a)** Effect of 4 hours treatment with hydrogen peroxide on NF-kB transcriptional activity in C2C12 myoblasts one day after induction of differentiation. Where indicated, cells were pretreated with Akt1/2- and Akt-specific inhibitors, as previously described (Cenni et.al; 2011). (**b**) Western blot analyses on the same samples as in (a), showing increased phosphorylation levels of Akt and Ankrd2. MB: myoblast; MT: differentiating myotubes 1, 3, and 6 days (d) after induction. Data are represented as mean ± SD (n=3). *P <0.05 and **P < 0.01.

As shown in (Figure 5.6a), a decrease in NF-kB luciferase activity was reflected by increased levels of phosphorylated Akt and Ankrd2 (Figure5.6b), resulting in downregulation of NF-kB. This result suggests a novel link between NF-kB and oxidative stress, establishing Ankrd2 as an important direct regulator of NF-kB during oxidative stress.

5.3.2. Ankrd2 interacts and colocalizes with the NF-kB member p50

Based on the striking similarity between Ankrd2 and the NF-kB inhibitor I-kB (Pallavicini et.al, 2001), we hypothesized that Ankrd2 may modulate NF-kB activity through direct interaction. To test the ability of Ankrd2 to bind to the two main subunits of NF-KB, p65 or p50, we transfected C2C12 cells with FLAG-tagged human WT Ankrd2 and performed immunoprecipitation (IP) using anti-FLAG-tag antibody, followed by Western blot analysis for p50 and p65.

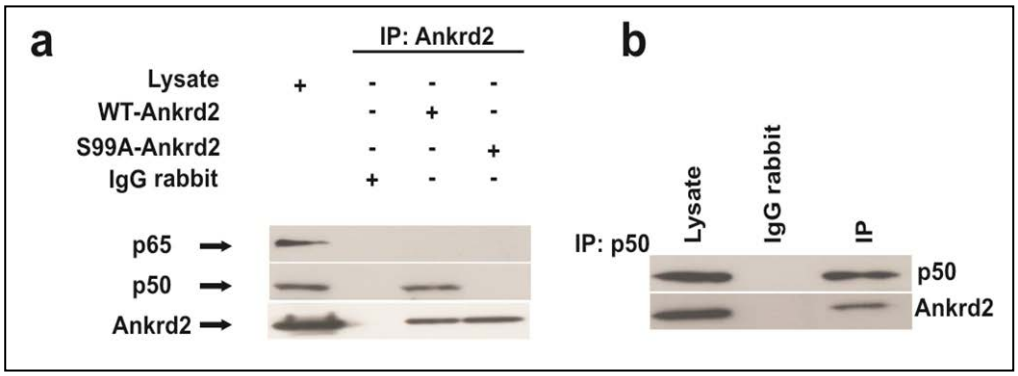

Figure 5.7 Ankrd2 interacts with p50. (a) IP of Ankrd2 following transfection of C2C12 cells with FLAG-tagged WT or Ser99Ala mutant Ankrd2. Ankrd2 is able to interact with p50, but not with p65. Moreover, Ankrd2 Ser99Ala does not bind to p50, demonstrating that Ankrd2 phosphorylation is essential for p50 binding. **(b)** Reverse experiment confirming the interaction between Ankrd2 and p50: IP of p50 following transfection of C2C12 cells with FLAG-tagged p50.

This revealed the binding of Ankrd2 to p50, but not p65 (Figure 5.7a), which was confirmed by a reverse experiment, showing the ability of p50 to immunoprecipitate Ankrd2 (Figure 5.7b). FLAG-tagged Ser99Ala mutant Ankrd2 failed to co-immunoprecipitate p50, demonstrating that phosphorylation of Ser-99 is critical for the Ankrd2-p50 interaction.

To identify the cellular compartment where p50 interacts with Ankrd2, we investigated the localization of the endogenous proteins in both proliferating and differentiating C2C12 myoblasts (1, 3, 6 days after induction) using antibodies against p50 and the Ankrd2 N-terminus. In undifferentiated and early differentiating cells, perfect colocalization of Ankrd2 and p50 in structures resembling promyelocytic leukemia (PML) nuclear bodies (NBs) (Zhong et.al, 2000) was found (Figure 5.8, panels I and II). However, upon advancing differentiation, Ankrd2 moved to the cytoplasm where Ankrd2 and p50 do not colocalize, except in a few cells where Ankrd2 was still present in the NBs (Figure 5.8, panels III and IV).

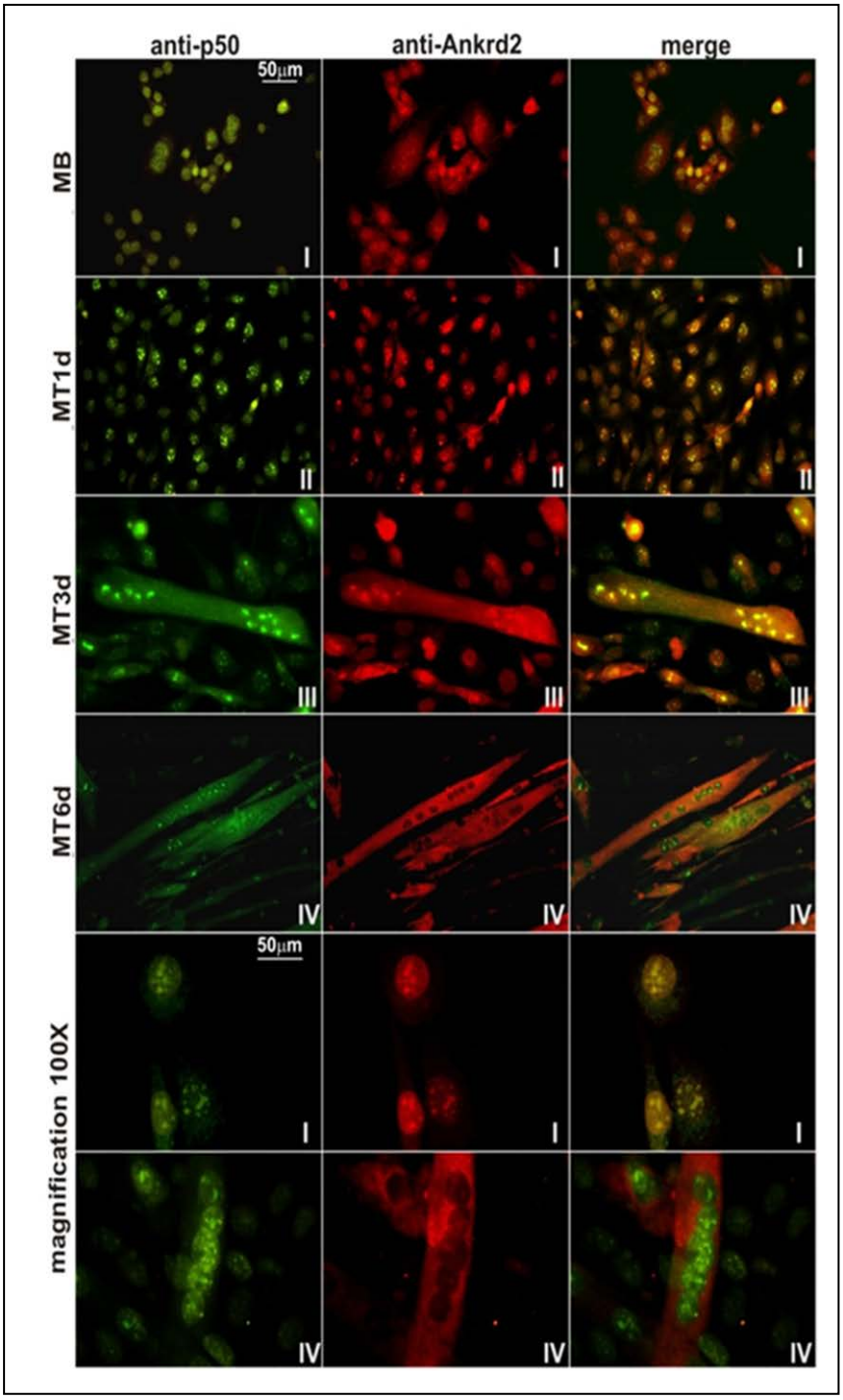

Figure 5.8 Ankrd2 interacts and colocalizes with p50. Immunostainings of proliferating and differentiating C2C12 cells for Ankrd2 and p50. Both proteins were visible in a speckled pattern in the nuclei of proliferating and early differentiating myoblasts (panels I at two different magnifications and II). With progressive differentiation, Ankrd2 became almost completely cytoplasmic and did not colocalize with p50 (panels III and IV at two different magnifications). MB: proliferating myoblasts, MT: differentiating myotubes 1, 3, and 6 days (d) after induction.

5.3.3. Overexpression of Ankrd2 by Ad-Ankrd2 colocalizes with the p50

Since we observed repression of NF-kB activity by exogenous Ankrd2 protein in terminally differentiated myotubes in which endogenous Ankrd2 and p50 do not colocalize, we performed immunostainings of AdAnkrd2-infected C2C12 myotubes using an anti-HA antibody for detecting exogenous Ankrd2.

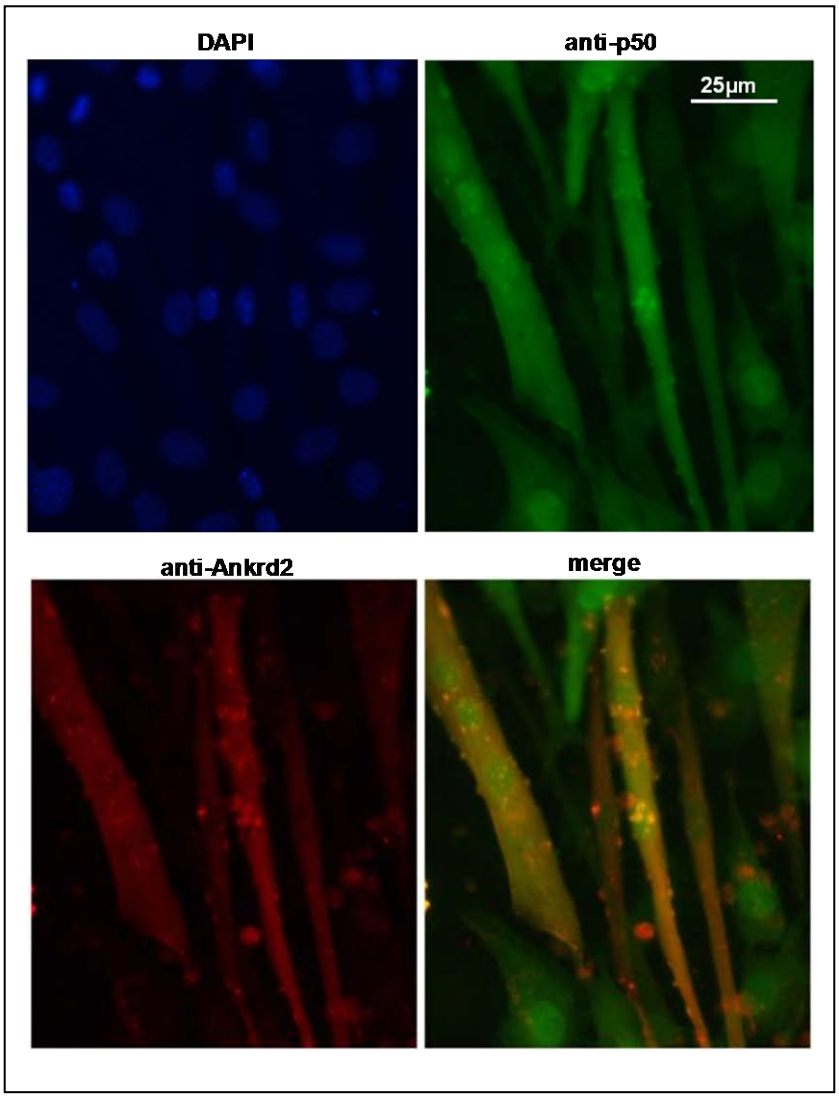

Figure 5.9 Ankrd2 colocalizes with p50 after overexpresssion in fully differentiated myotubes. Immunostainings of differentiating C2C12 cells for Ankrd2 and p50. Both proteins were visible in a speckled pattern in the nuclei of differentiated myoblasts (panels IV). MT: differentiating myotubes 6 days (d) after induction.

As shown in (Figure 5.9), exogenous Ankrd2 localized both to the nucleus and the cytoplasm of myotubes. Thus, the forced overexpression of Ankrd2 induces its translocation to the nucleus where it may interact with p50 and inhibit NF-kB activity, thereby regulating inflammation-related genes.

5.4. Ankrd2 regulates Gsk3b

5.4.1. Gsk3ß is a direct target of the Ankrd2:p50 repressor complex

To validate the Ankrd2:p50 complex as a novel repressor, we searched for putative targets among those genes whose expression levels were anti-correlated with that of Ankrd2. Interestingly, the only gene that was strongly downregulated upon overexpression of Ankrd2 at all stages was Gsk3ß, a powerful governor of inflammatory signaling (ranging from 2.3 to 6 fold in logarithmic scale) as shown in (Figure 5.10).

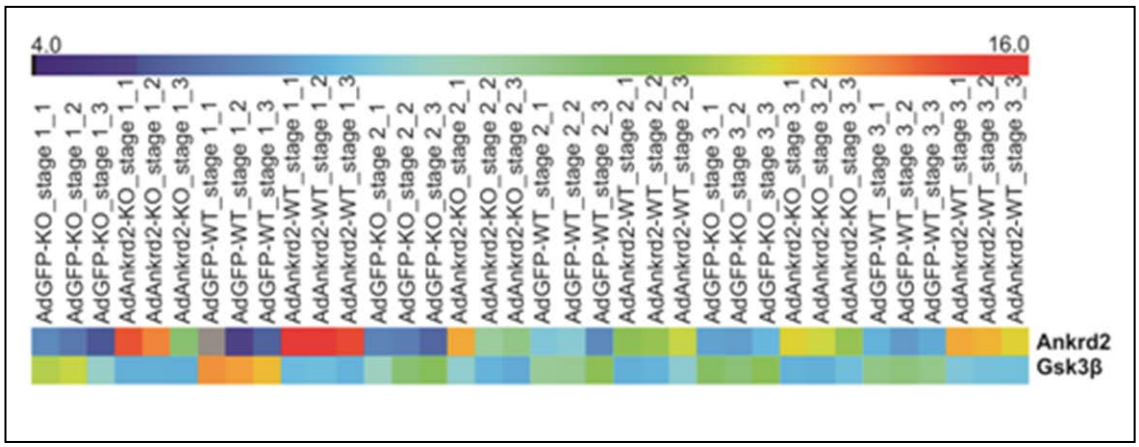

Figure 5.10 Ankrd2 decreases the Gsk3β expression. Heat map of gene expression values for Ankrd2 and Gsk3β in proliferating (stage 1), fusing (stage 2) and differentiated (stage 3) primary myoblasts derived from Ankrd2 KO and WT mice infected with AdAnkrd2 or control AdGFP (n = 3).

Further we quantified the Gsk3b expression level (both Phospho protein level and total protein level), we found down regulation of phospho-Gsk3b protein levels after overexpresion of Ankrd2 both in Ankrd2 KO and WT as well (Figure 5.11).

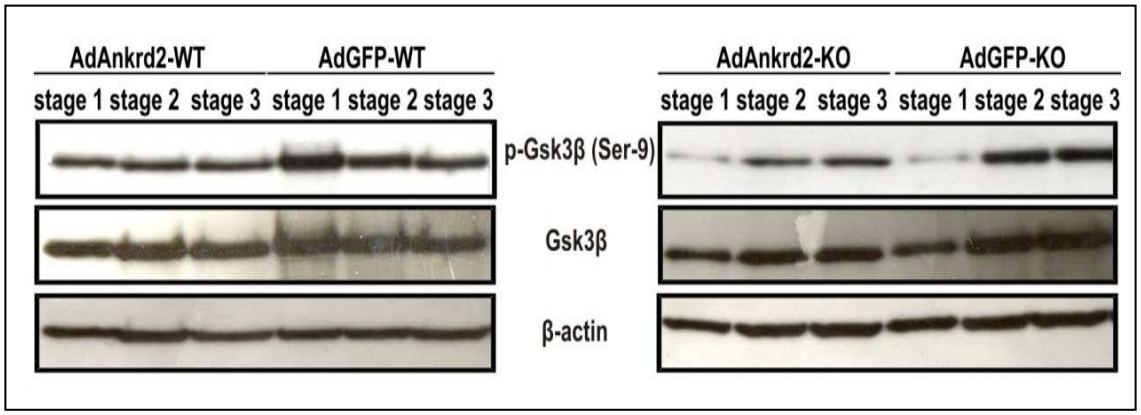

Figure 5.11 Representative western blots showing Gsk3β protein and phosphorylation levels in the same samples as in (Figure 6.10) proliferating (stage 1), fusing (stage 2) and differentiated (stage 3) primary myoblasts derived from Ankrd2 KO and WT mice infected with AdAnkrd2 or control AdGFP.

These results were confirmed in C2C12 cells where infection with AdAnkrd2 resulted in decreased phosphorylation levels of Gsk3ß at all stages (Figure 5.12a), while the protein level of Gsk3ß was significantly reduced only in fully differentiated cells. To test whether Akt2-mediated phosphorylation of Ankrd2 affects Gsk3ß activity, C2C12 myoblasts were transfected with WT or mutant Ser99Ala Ankrd2 (Figure 5.12b). Overexpression of WT Ankrd2 caused downregulation of Gsk3ß phosphorylated levels, while Ser99Ala Ankrd2 had no effect, demonstrating that the effect of Ankrd2 on Gsk3ß activity is dependent on its phosphorylation by Akt2. In conclusion, our results identify Gsk3ß as a downstream target of the anti-inflammatory signaling cascade activated by Akt2-phosphorylated Ankrd2.

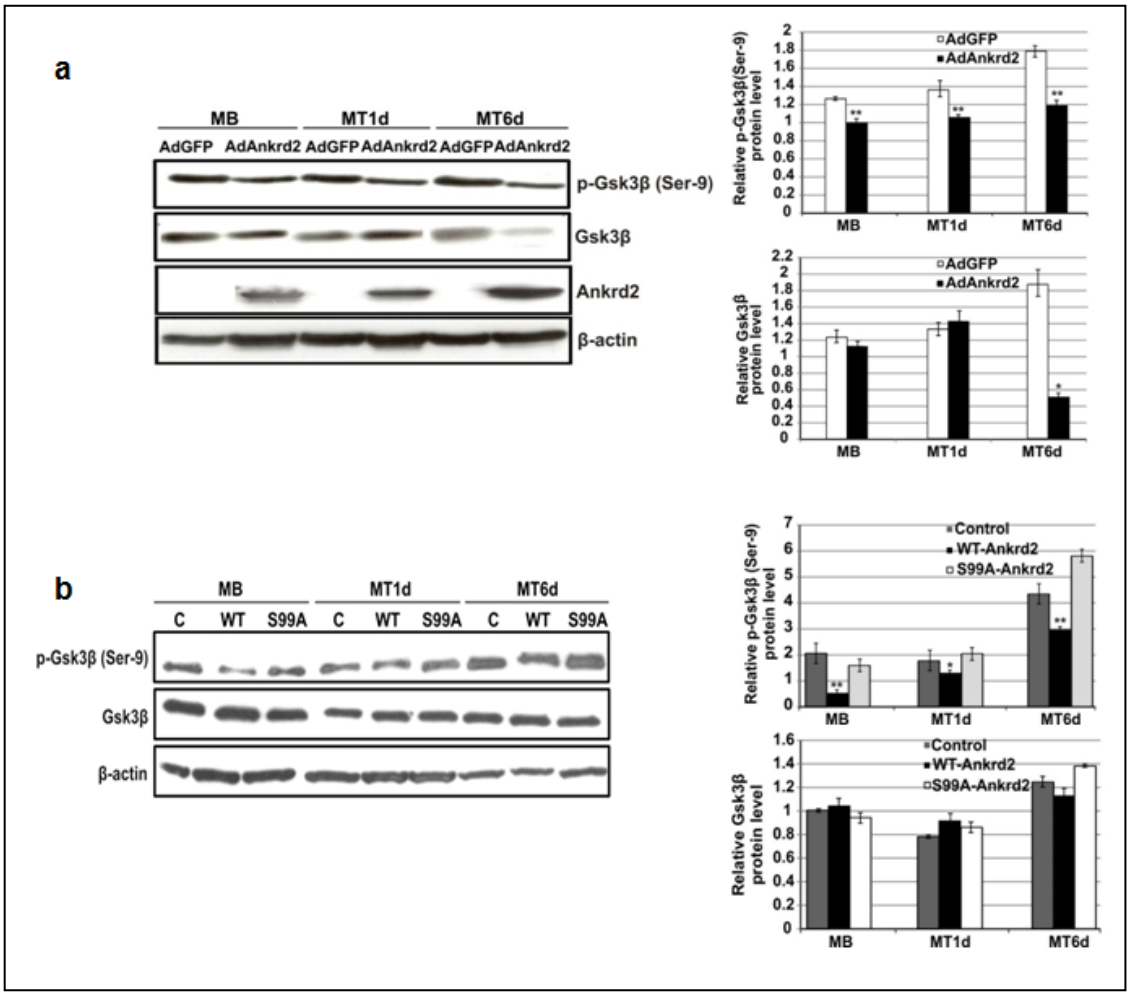

Figure 5.12 Ankrd2 decreases the Gsk3β protein expression level and inhibits its activity. **(a)** Western blot analysis for Ankrd2 and Gsk3β protein and phosphorylation levels on lysate from proliferating and differentiating C2C12 myoblasts infected with AdAnkrd2 or AdGFP. Densitometry analysis for total and phosphorylated Gsk3β normalized to β-actin protein levels was performed. **(b)** Western blot and relative densitometry analysis of active and total Gsk3β normalized to β-actin protein levels from proliferating and differentiating C2C12 cells transfected or not with WT or Ser99Ala mutant (S99A) Ankrd2. MB: proliferating myoblasts, MT: differentiating myotubes 1 and 6 days (d) after induction. Data are represented as mean ± SD (n=3). *P < 0.05; **P < 0.01.

5.4.2. GsK3ß is transcriptionally regulated by the Ankrd2:p50 repressor complex

Using the Genomatix and Patch tools, we identified several putative binding sites for NF-kB transcription factors in the Gsk3ß upstream promoter region (Figure 5.13).

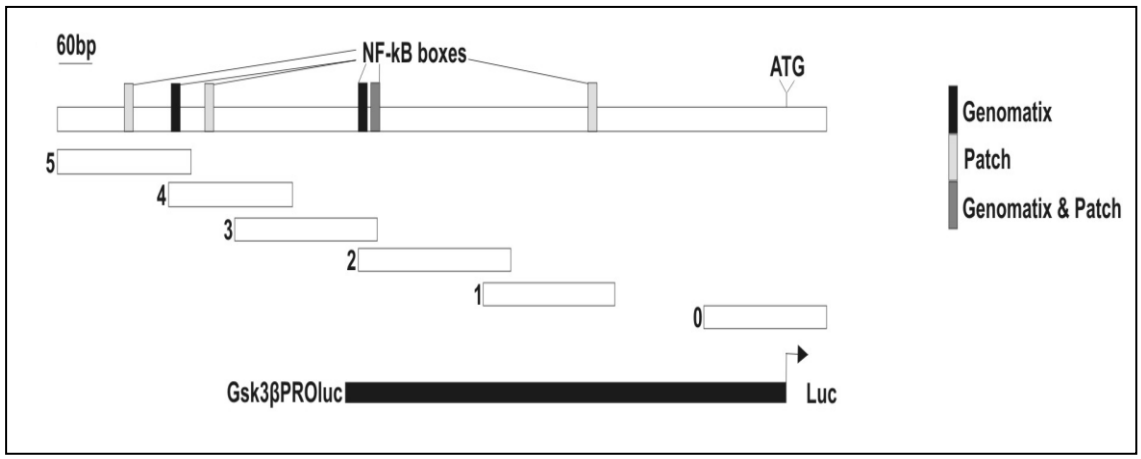

Figure 5.13 Schematic representation of putative NF-kB binding elements within the Gsk3β promoter identified using Genomatix and Patch software. The white bars depict the regions of the Gsk3β promoter amplified by primer sets covering the putative NF-kB response elements. The Gsk3β luciferase reporter construct is represented on the bottom.

To determine whether Ankrd2 and NF-kB are recruited to the Gsk3ß promoter, chromatin immunoprecipitation (ChIP) was performed on proliferating and differentiated C2C12 cells using antibodies against Ankrd2, p50, or p65, after which the captured genomic DNA was analyzed by qRT-PCR using primers spanning the Gsk3ß promoter. As shown in (Figure 5.14a and b), specific binding sites for Ankrd2 and p50 were found within the Gsk3ß promoter.

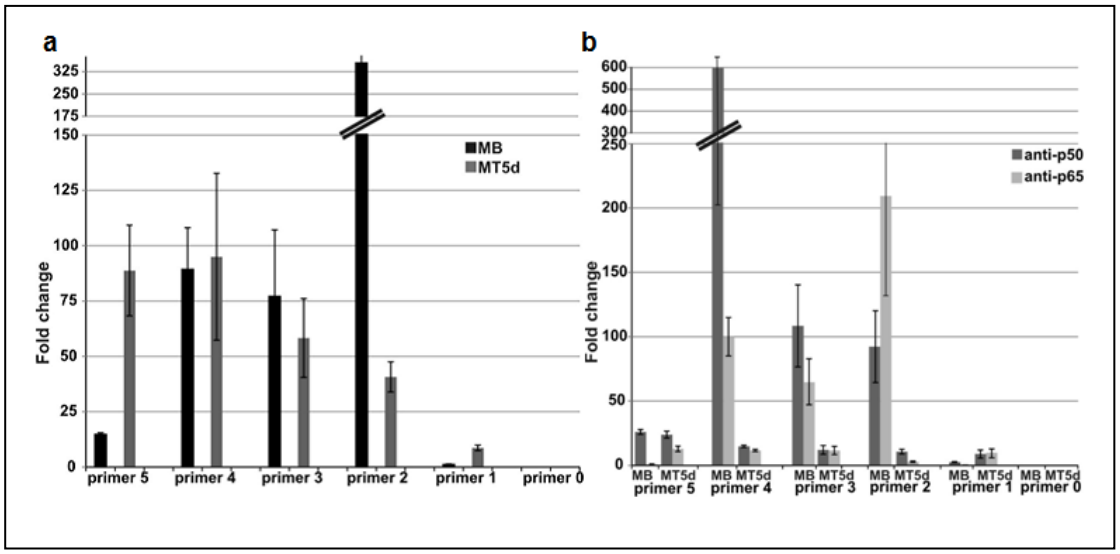

Figure 5.14 Ankrd2 regulates the transcription of Gsk3β. (a) Using anti-Ankrd2 antibody, ChIP was performed on nuclear extracts from proliferating and differentiating C2C12 myoblasts. qRT-PCR was performed on input and immunoprecipitated DNA with the Gsk3β promoter specific primers shown in panel (Figure 5.13). The bars represent the fold enrichment of Ankrd2 occupancy of the Gsk3β promoter relative to that of control IgG samples. **(b)** ChIP was performed using anti-p50 and anti-p65 antibodies followed by qRT-PCR analysis as described in (a) MB: proliferating myoblasts, MT: differentiating myotubes 1, 5 and 6 days (d) after induction.

To functionally validate these interactions, C2C12 cells were cotransfected with a luciferase reporter construct containing selected Ankrd2 and p50 binding sites within the Gsk3ß promoter and expression vectors encoding FLAG-tagged WT or Ser99Ala mutant human Ankrd2 or empty expression plasmid as a negative control. Cotransfection with WT Ankrd2 caused significant repression of Gsk3ß promoter activity, while mutant Ankrd2 resulted in a much lower repression (Figure 5.15d).

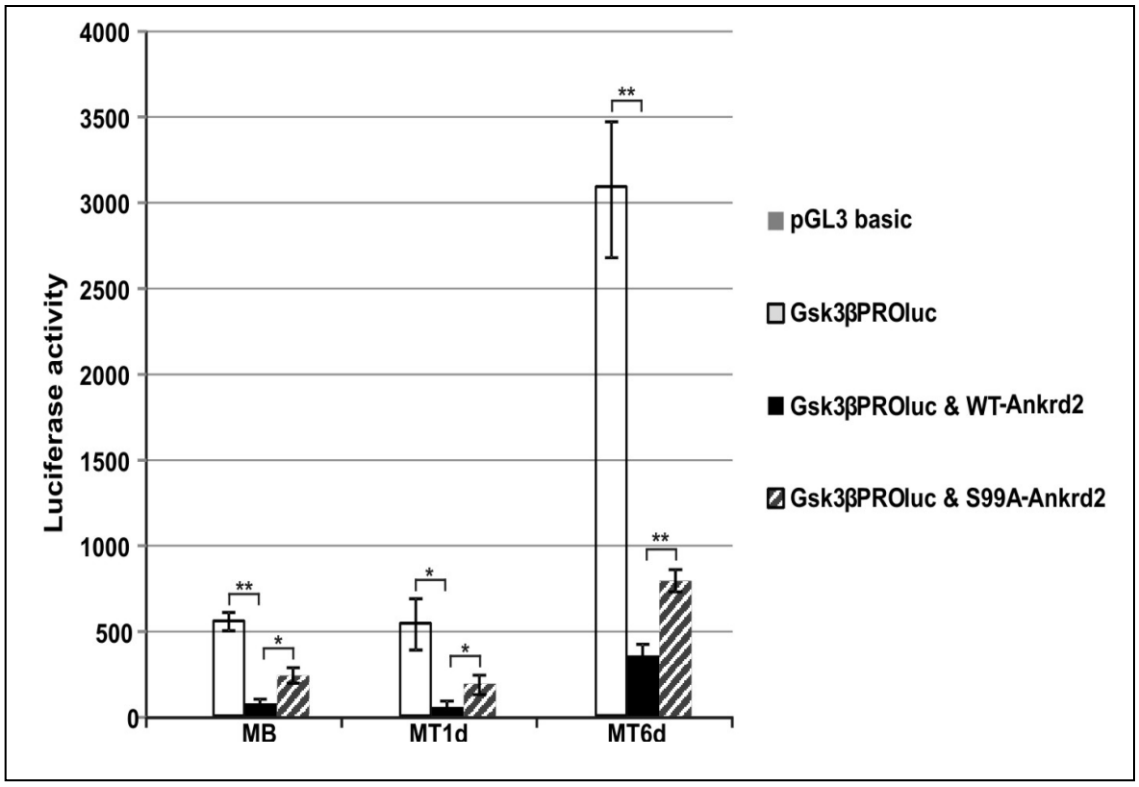

Figure 5.15 Luciferase activity assay following cotransfection of C2C12 cells with a luciferase reporter plasmid encoding the identified Ankrd2 and p50 binding region within the Gsk3β promoter (765 bp, panel A) and WT or S99A mutant Ankrd2. pGL4.74 was used for normalization of transfection efficiency. MB: proliferating myoblasts, MT: differentiating myotubes 1, 5 and 6 days (d) after induction. All data are represented as mean ± SD (n = 3). *P < 0.05; **P < 0.01.

Thus, the complex formed by p50 and phosphorylated Ankrd2 can repress Gsk3ß through direct binding to its promoter in an Akt2-phosphorylation dependent manner.

5.5. NF-kB regulates Ankrd2 transcriptional activity

5.5.1. Identification of Ankrd2 binding sites

The Ankrd2 promoter was recently shown to be activated by longitudinal stretch through the recruitment of the NF-kB heterodimer p50:p65 to the Ankrd2 promoter, which contains two putative NF-kB consensus binding sites (Mohamed et.al, 2010). Ankrd2 contains conserved bindings motifs especially for AP-1 and two putative NF-kB boxes, referred as proximal and the distal NF-kB box according to their relative position with respect to the transcription start site of the Ankrd2 gene (Figure 5.16).

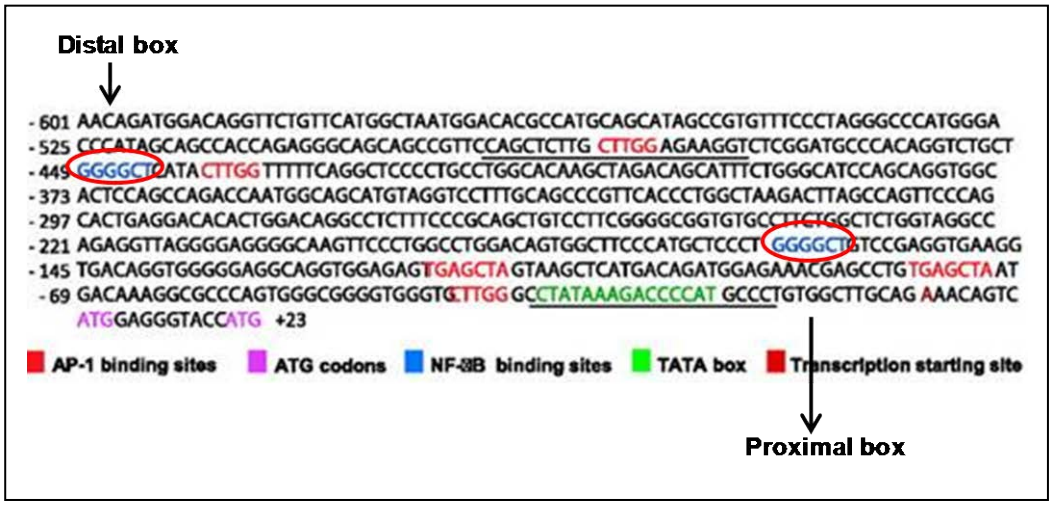

Figure 5.16 Ankrd2 gene promoter is activated via NFkB and AP-1 transcription factors under stretch. the position of the NFkB binding boxes both proximal and distal. (Figure adapted from Mohamed et.al, 2010).

5.5.2. p50 regulates the expression of Ankrd2 in a classic feedback-inhibition way

To investigate whether the NF-kB boxes are functional, we performed ChIP on proliferating and fully differentiated C2C12 myoblasts using antibodies against p50 and p65.

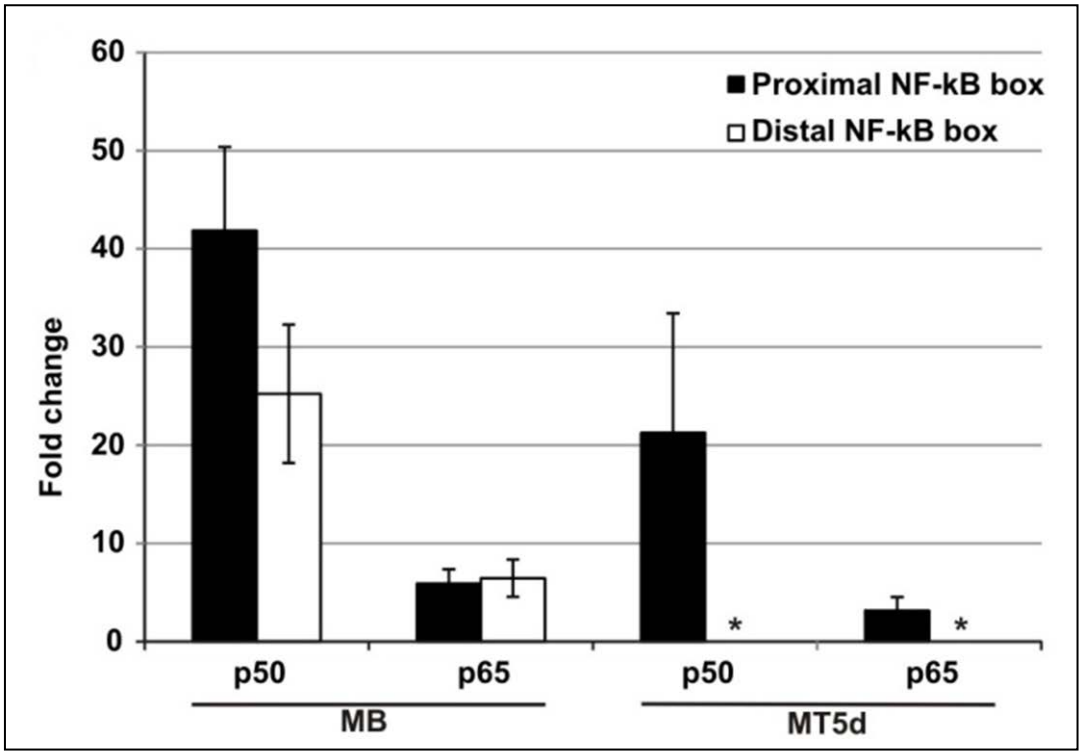

Figure 5.17 ChIP with antibodies against p50 and p65 was performed on nuclear extracts from proliferating and differentiating C2C12 cells followed by qRT-PCR using primers spanning the putative NF-kB binding elements. The bars represent the fold enrichment of p50 and p65 occupancy of the Ankrd2 promoter relative to that of control IgG samples. All data are represented as mean±SD (n = 3). MB: proliferating myoblasts, MT: differentiating myotubes 1 and 5 days (d) after induction. *P < 0.05; **P < 0.01.

The relative occupancy of the boxes (referred to as proximal and distal NF-kB box according to their position relative to the transcription start site) was determined by qRT-PCR using specific primers encompassing the NF-kB binding sites. Our results showed strong binding of p50 to both the proximal and distal NF-kB boxes in myoblasts, while in differentiated cells reduced binding to the proximal box and a

complete abrogation of binding to the distal box was found (Figure 5.17). Interestingly, only weak binding of p65 to the NF-kB boxes in the Ankrd2 promoter was detected both in myoblasts and differentiated cells, again with a reduction to baseline for the distal box in myotubes.

5.5.3. NF-kB p50 represses Ankrd2 gene transcription

To investigate the functionality of the two NF-kB boxes within the Ankrd2 promoter, we performed a series of luciferase assays with the constructs shown in (Figure 5.18a). The short-PROwt-luc vector contains 317 bp upstream of the Ankrd2 ATG start codon spanning two paired E-boxes and the proximal NF-kB box. The long-PROwt-luc construct (-663 bp upstream of ATG) includes also the distal NF-kB box. Mutant reporters with deletion of the proximal box (shortPRO-NF-kB-luc and longPRO-NF-kB-luc) were obtained by site-directed mutagenesis. The reporter gene activity for each construct was measured both in proliferating and differentiated myoblasts as summarized in (Figure 5.18b). Consistent with our previous data (Bean et.al, 2008) both WT constructs resulted in increased luciferase activity and thus increased Ankrd2 expression during differentiation. On the other hand, activation of luciferase activity by the short-PROwt-luc vector was about three times stronger than the long-PROwt-luc construct, suggesting that a transcription factor may bind to the distal region of the Ankrd2 promoter negatively regulating its expression.

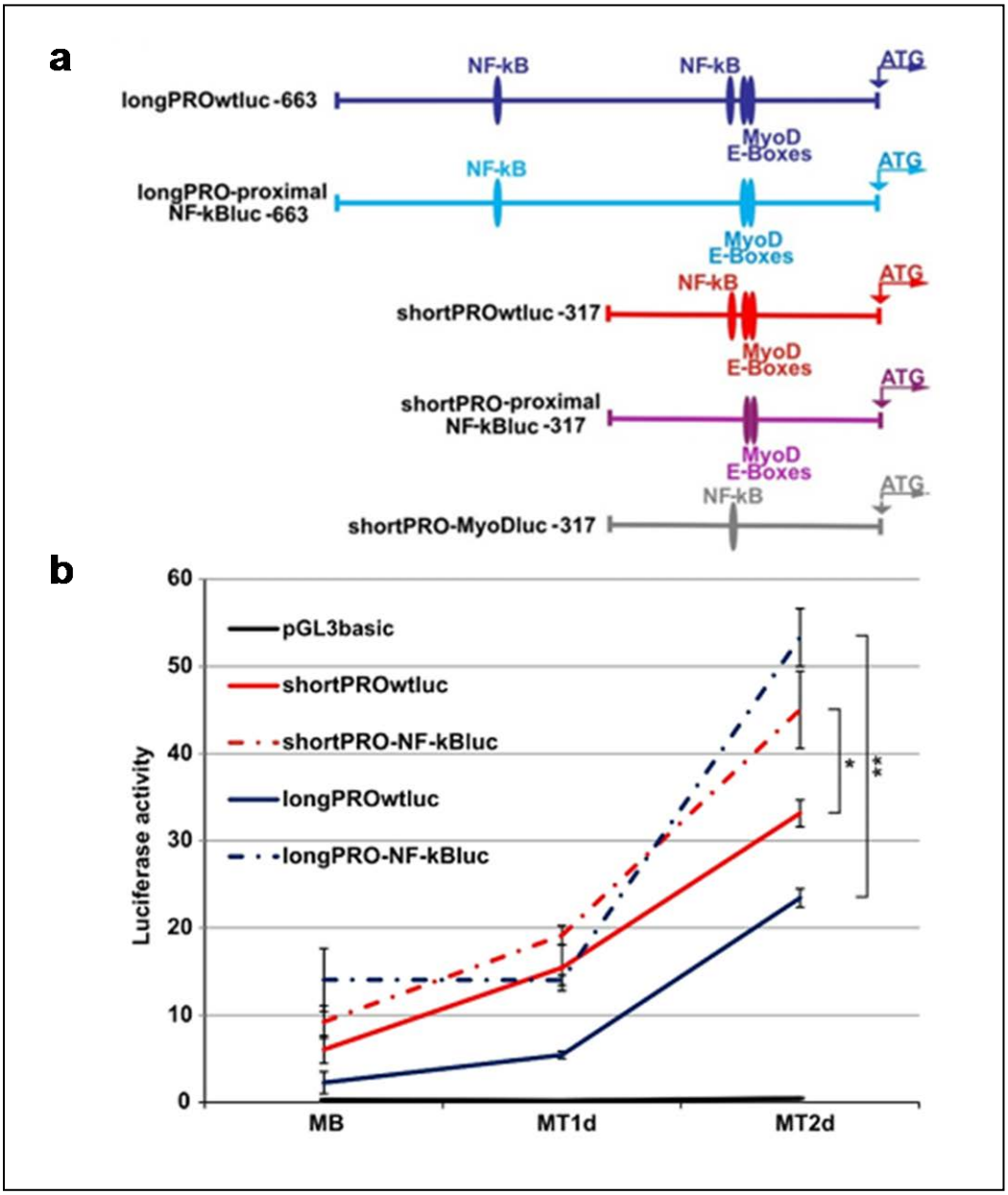

Figure 5.18 Ankrd2 expression is negatively regulated by p50. (a) Graphical representation of the 5'-mouse Ankrd2 promoter region and the conserved portions in the constructs used for transfection experiments. **(b)** Luciferase activity assay following cotransfection of C2C12 cells with the first four luciferase constructs in panel a and the pGL4.74 control vector.

Bioinformatic analysis suggested NF-kB as a repressor of the Ankrd2 promoter (Mohamed et.al, 2010), which is consistent with our finding that p50 can directly bind to the Ankrd2 promoter and the fact that p50 lacks a transactivation domain and thus is mainly associated with transcriptional repression (Bonizzi et.al, 2004).

These data, together with our finding that deletion of the proximal NF-kB regulatory element strongly increases Ankrd2-driven luciferase expression, suggest that the p50:p50 homodimer acts as a transcriptional repressor of Ankrd2 expression by binding its promoter.

It is well-known that treatment of cells with proinflammatory cytokines (TNFα and IL1ß), or bacterial lipopolysaccharides (LPS), leads to degradation of I-kB, allowing p50:p65 dimers to translocate to the nucleus and act as transcription factors (Karin et.al, 2000). Consistent with this, activation of NF-kB by LPS was confirmed by an about 2-fold increase in luciferase activity in LPS-treated proliferating and differentiating C2C12 cells transfected with the NF-kB reporter vector (Figure 5.19).

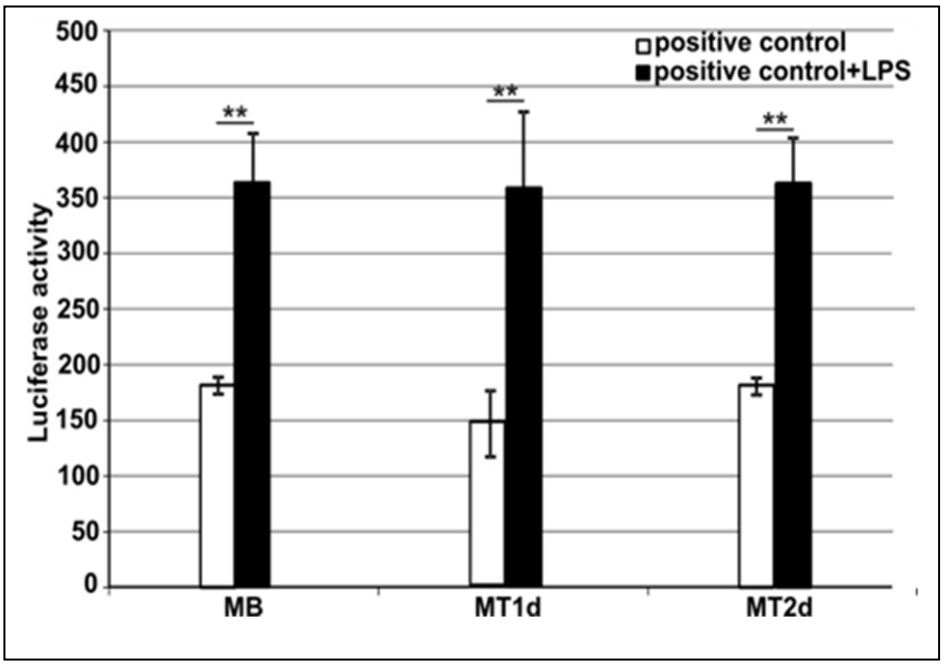

Figure 5.19 NF-kB activity is stimulated by bacterial lipopolysaccharides (LPS) treatment in C2C12 cells. The responsiveness to LPS treatment was determined in proliferating and differentiating C2C12 myoblasts after 1 and 2 days of induction by measurement of luciferase activity following transfection with a 3xNF-kB-luciferase vector. An about 2-fold induction in NF-kB (luciferase) activity was found in LPS-treated compared to untreated cells at all stages. All data are represented as mean±SD of three independent experiments. **P < 0.01.

Therefore, to study the effect of NF-kB activation on Ankrd2 expression, proliferating and differentiating C2C12 myoblasts transected with the different luciferase constructs were treated with LPS.

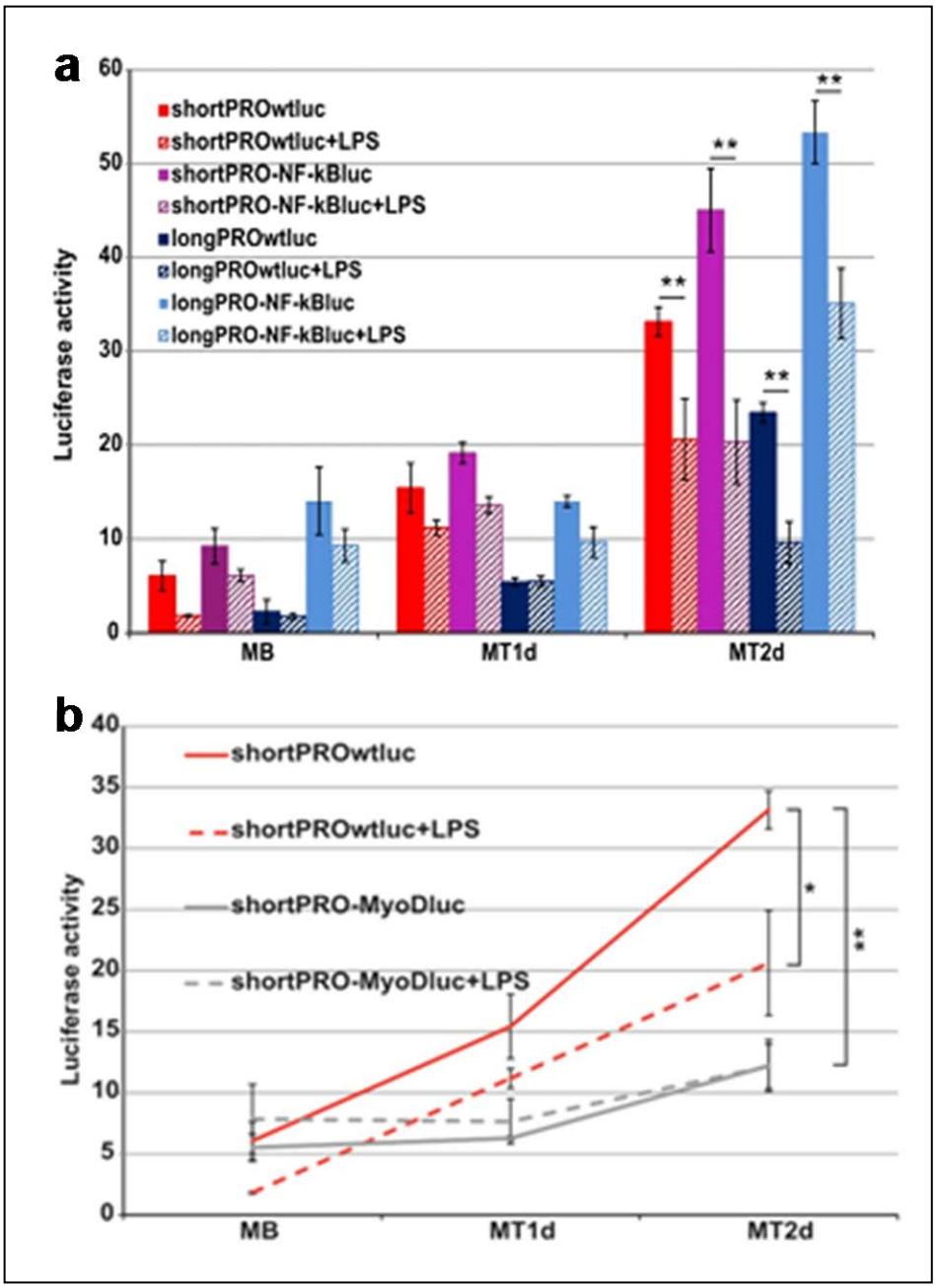

Figure 5.20 Luciferase activity assay in LPS-treated (2 µg/ml LPS for 24h) compared to untreated cells transfected with the reporter vectors in panel b.

As shown in (Figure 5.20), luciferase activity was repressed following LPS-treatment independent from the deletion of the proximal NF-kB box, suggesting that the p50:p65 dimer is not involved in the regulation of Ankrd2 expression.

We have previously shown that Ankrd2 expression is under the control of MyoD (Bean et.al, 2005), which is downregulated in response to TNFα-induced NF-kB activation (Guttridge et.al, 2000) consistently, a ChIP assay revealed direct binding of MyoD to the Ankrd2 promoter.

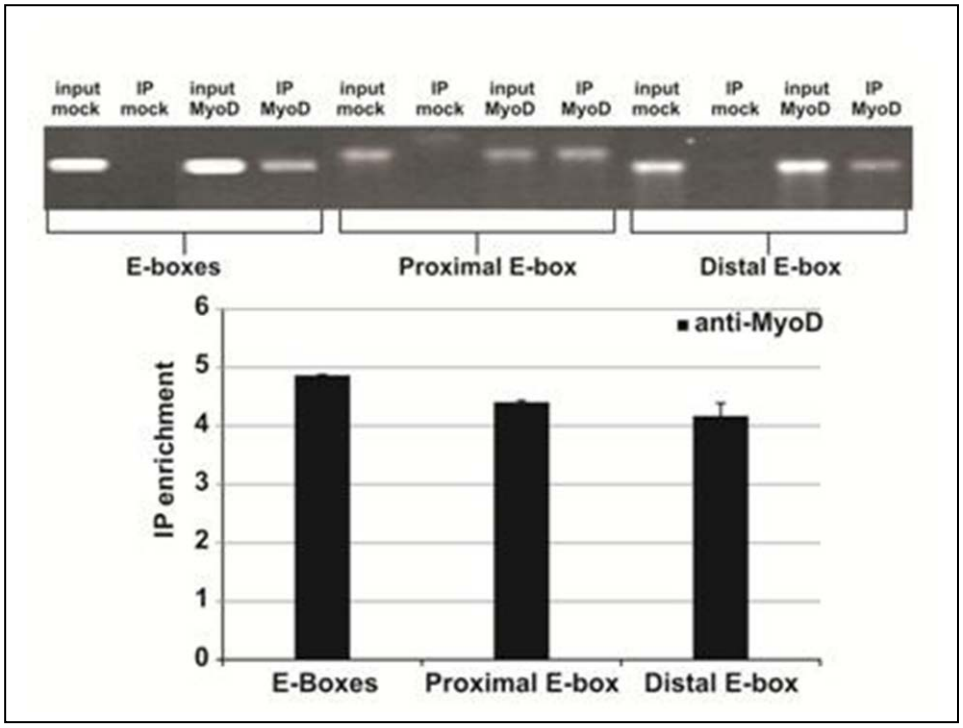

Figure 5.21 Ankrd2 expression is positively regulated by MyoD. Following cotransfection of HEK293A cells with FLAG-tagged MyoD and Ankrd2 promoter-driven luciferase vectors, ChIP was performed using anti-FLAG antibody. The immunoprecipitates were analyzed by standard PCR (top) and qRT-PCR (bottom) using specific primers for amplifying regions within the Ankrd2 promoter region containing the proximal or distal MyoD-binding site or both. IPed chromatin from HEK293A cells transfected with FLAG-tagged empty vector (IP mock) represents the negative control. The enrichment of each specific region of the Ankrd2 promoter compared to mock IP DNA was determined by qRT-PCR. All data are represented as mean±SD (n = 3). MB: proliferating myoblasts, MT: differentiating myotubes 1, 2, and 5 days (d) after induction. *P < 0.05; **P < 0.01.

Moreover, transfection with the shortPRO-MyoD-luc vector in which the MyoD binding site was deleted, resulted in a 2.7 fold reduction in luciferase activity compared to the WT construct (Figure 5.20), demonstrating that the Ankrd2 promoter is positively regulated by MyoD. Thus, the inhibition of the Ankrd2 promoter following LPS treatment during myoblast differentiation may be due to degradation of the Ankrd2 transcriptional activator MyoD both at the mRNA and protein level, induced by activation of the canonical NF-kB pathway (Guttridge et.al, 2000). This hypothesis is supported by the fact that luciferase activity of the shortPRO-MyoD-luc vector was not repressed following LPS-treatment (Figure 5.20).

5.6. Ankrd2 affects the level of proinflammatory cytokines *in vivo*

Our experiments on differentiating mouse primary and C2C12 myoblasts demonstrate a complex role of Ankrd2 in the pathways modulating inflammatory responses. To determine whether Ankrd2 may affect cytokine expression *in vivo*, mRNA and protein levels of NF-kB-regulated genes were determined by qRT-PCR and Western blot analyses on slow oxidative soleus and fast glycolytic extensor digitorum longus (EDL) muscles from WT and Ankrd2 KO mice. Consistent with the previously reported preferential expression of Ankrd2 in slow twitch fibers (Chemello et.al, 2011), Ankrd2 was expressed at higher amounts in soleus compared to EDL muscle (Figure 5.22a, Figure 5.23). Furthermore, knockout of Ankrd2 was not compensated for by upregulation of other MARP family members (Figure 5.22a).

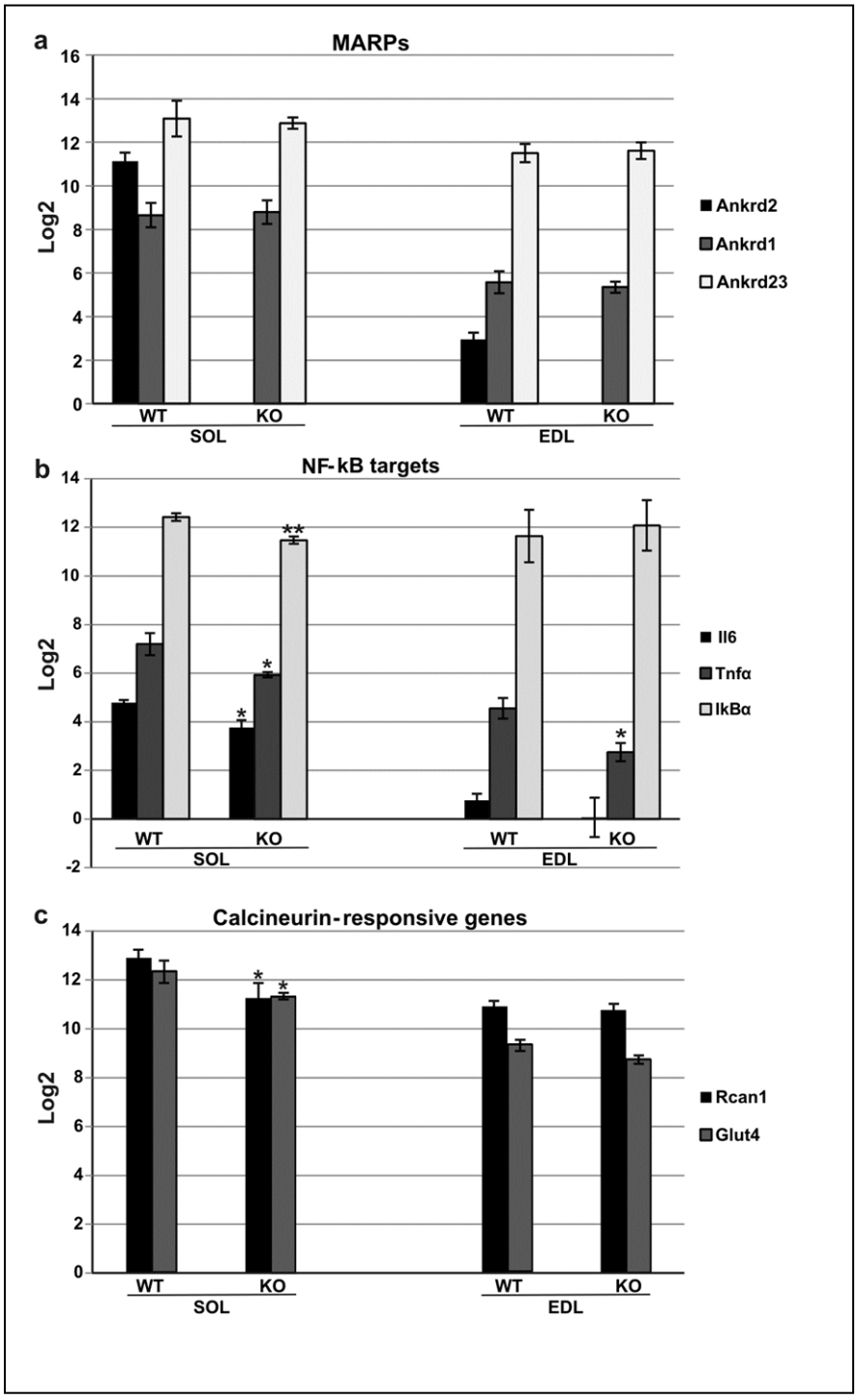

Figure 5.22 Ankrd2 affects the expression of inflammatory genes in a fiber type manner *in vivo*. qRT-PCR analysis of the MARP family members Ankrd2, Ankrd1 and Ankrd23, NF-kB dependent, and calcineurin-responsive genes in slow oxidative soleus and fast glycolytic EDL muscle from WT and Ankrd2 KO mice. *P < 0.05.

Unexpectedly decreased transcript levels of Il-6, Tnfα, and IkBα were found in soleus muscle in the absence of Ankrd2 (Figure 5.22b), although Akt, Gsk3β, and NF-kB activities were similar between WT and Ankrd2 KO mice (Figure 5.23).

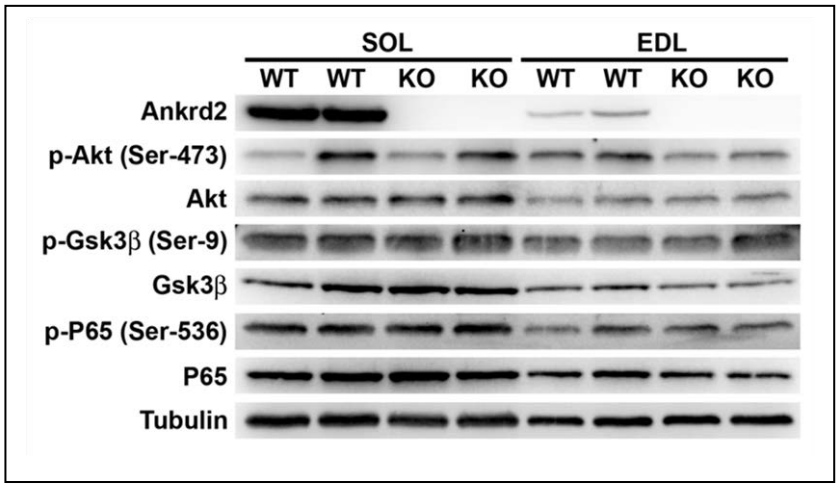

Figure 5.23 Ankrd2 knockout does not affect Akt, Gsk3ß, and NF-kB activity *in vivo*. Western blot analysis on soleus and EDL muscle from 3 month old WT and Ankrd2 KO mice.

Since NF-kB activity is physiologically higher in slow than in fast muscle (Shi et.al, 2009), (Figure 5.23), we investigated whether Ankrd2 deletion may make muscle prone to fiber type switching by changing the muscle transcriptional signature. As shown in (Figure 5.22c), although skeletal muscle from Ankrd2 KO mice appeared morphologically equal to WT muscle and no significant changes in fiber type distribution were found (Barash et.al 2007), the slow gene expression program was significantly modified. In particular, the calcineurin-dependent genes Il-6, Glut4, and Rcan1(Benzet et.al,2005, Smith et.al, 2007, Yang et.al, 2000) were significantly downregulated in soleus muscle from Ankrd2 KO mice. Thus, our *in vivo* results suggest a role of Ankrd2 in the regulation of basal inflammatory cytokine production in a fiber type dependent manner.

Ankrd2 is strongly and rapidly upregulated under different muscle stress conditions, but the reason for this response has remained unclear, in particular because we did not find any obvious skeletal muscle phenotype in Ankrd2 KO mice (Barash et.al, 2007). Based on our previous findings that Ankrd2 plays a role in myogenic differentiation (Bean et .al, 2008) and possibly provides a link between myofibrillar stress and muscle gene expression (Miller et.al, 2003), we performed genome-wide gene expression analyses on proliferating and differentiating primary myoblast cultures derived from WT and Ankrd2 KO mice. In addition, to explore the gene networks that are disturbed by the physiological strong upregulation of the Ankrd2 transcript in response to different types of mechanical stress, we studied the effect of acute overexpression of Ankrd2 induced by adenoviral expression. Since Ankrd2 is expressed in a fiber type specific manner (Chemello et.al, 2011), we chose to perform our study on primary cultures as gene expression analysis on whole muscle with non-homogeneous fibers composition is likely to produce averaged information with loss of the real contribution of Ankrd2 at the fiber level. Our analyses revealed that Ankrd2 negatively regulates the expression of a large number of genes known to be involved in inflammatory NF-kB mediated pathways. Ankrd2 was recently found to be upregulated in diaphragm muscle in response to longitudinal stretch through Akt-dependent activation of the NF-kB pathway (Mohamed et.al, 2010). Our work expands on these findings, by identifying the molecular mechanism through which Ankrd2 interacts with the NF-kB pathway and affects inflammatory responses. More specifically, we demonstrate a role of Ankrd2 as an inhibitor of NF-kB activity through interaction with the transcriptional repressive.

NF-kB subunit p50 and inhibition of the expression of crucial inflammation-related genes. This is consistent with its striking sequence and structural similarity to I-kB, the specific NF-kB inhibitor (Pallavicini et.al, 2001). Similarly, overexpression of the MARP protein family member Ankrd1 has been shown to result in decreased DNA-binding activity of the transcription factor NF-kB p65 through an unknown mechanism (Laure et.al, 2010). The NF-kB subunit p50 can operate either as pro-inflammatory or as a negative regulator of NF-kB-directed inflammation depending on the co-factors that interact with the complex on the DNA. Our results reveal a role of Ankrd2 as a selective regulator of p50:p50 NF-kB responsive genes in muscle cells. In particular, we found colocalization of Ankrd2 and p50 in a speckled intra-nuclear pattern, consistent with the presence of Ankrd2 in PML-NB multiprotein nuclear structures, where it interacts with PML (Kojic et.al, 2004), the major protein in PML-NBs, where transcriptional factors (i.e. NF-kB) and co-factors (i.e. Ankrd2) associate to regulate gene expression (Zhong et.al, 2000). This is interesting since PML has been shown to repress the transactivating function of NF-kB by recruiting p65 to PML-NBs and interfere with the binding of NF-kB to its cognate DNA recognition sequence (Wu et.al, 2003). In addition to upregulation of NF-kB target genes, PPARγ and other members of the PPAR pathway were found to be downregulated in Ankrd2 KO cells. Activated PPARs can exert anti-inflammatory activity in several cell types, including skeletal muscle cells. In particular, PPARγ was recently shown to suppress cytokine-induced NF-kB transcriptional activity and target gene expression independent from p65 nuclear translocation and DNA binding (Remels et.al, 2009). Thus, our findings suggest that PPAR-dependent inhibition of NF-kB activity may be mediated through the recruitment of Ankrd2 as a transcriptional cofactor of the NF-kB-DNA complex.

As a bona-fide target of the Ankrd2:p50 repressor complex, we identified Gsk3β, a crucial regulator of inflammatory processes (Jope et.al, 2007). Thus, Ankrd2 reinforces its anti-inflammatory protection by negatively regulating the expression of Gsk3β, which is required for the production of pro-inflammatory cytokines, such as IL-6, IL-1, and TNFα (Martin et.al, 2005). Consistently, Gsk3β, IL-6, IL-1, and TNFα were all downregulated in response to Ankrd2 overexpression. In accordance with the paradigm: "Genes whose expression is anti-correlated with those studied might include members of a pathway whose action is opposed" (Qian et.al, 2001), Gsk3β and Ankrd2 are both substrates of Akt2, but have opposite effects on myogenic differentiation, i.e. under stress conditions triggering the production of ROS, Akt2 phosphorylation of Ankrd2 at Ser-99 causes its translocation to the nucleus where it negatively regulates muscle differentiation pathways (Cenni et.al, 2011). On the other hand, Akt2 phosphorylation of Gsk3β causes its inactivation, thereby removing its inhibitory effect on differentiation and hypertrophy (Rochat et.al, 2004). We found that the inhibitory effect of the Ankrd2:p50 complex on Gsk3β is dependent on Akt2-mediated phosphorylation of Ankrd2 induced by hydrogen peroxide. Thus, based on our results we propose a novel model in which activation of Akt2 in response to oxidative stress counteracts the toxic effects of ROS through inhibition of NF-kB activity via the repressor complex Ankrd2:p50 (Figure 12). Thus, when there is risk for tissue damage and resolution of inflammation needs to be assured, Ankrd2 may represent an important anti-inflammatory agent.

This is particularly interesting since Ankrd2 expression has been shown to be positively correlated with physical exercise (Lehti et.al, 2009; Mckoy et.al, 2005; Barash et.al, 2004; Roberts et.al, 2012), which induces an anti-inflammatory environment, counteracting the development of various chronic diseases (Pedersen

et.al, 2011), including dementia, diabetes, cardiovascular disease, stroke, and cancer. Thus, our results suggest a role of Ankrd2 in the protective effect of exercise through its ability to repress inflammatory responses.

Constitutively expressed inflammatory cytokines participate in the regulation of many important aspects of skeletal muscle function, including regeneration, hypertrophy, and whole body metabolism (Pedersen & Febbraio; 2008; Serrano et.al; 2008). For example although a chronic increase in the IL-6 protein level is associated with several pathological conditions, such as atrophy (Haddad et.al; 2004), contracting muscle also produces and releases IL-6 to regulate glucose homeostasis (Febbraio & Pedersen; 2002), our *in vivo* data suggest a further role of Ankrd2 in modulating the basal expression of cytokines under physiological conditions. In soleus muscle from Ankrd2 KO, calcineurin-stimulated transactivation of cytokines was found to be affected in a fiber type specific manner as indicated by the downregulation of key calcineurin responsive genes, i.e. IL-6, Glut4, and Rcan1. This is particularly interesting since Ankrd2 mRNA is preferentially expressed in adult slow-twitch muscle fibers (Chemello et.al, 2011) and although we found no significant differences in fiber type distribution between Ankrd2 KO and WT mice (Barash et.al, 2007), our data reveal that slow muscle fibers of Ankrd2 KO mice are transcriptionally adapted to fiber type switching towards a faster phenotype. This may appear contradictory, but in fact an increasing number of studies have shown mismatches in the relative proportion of mRNA and protein expression levels of myosin isoforms detected in slow to fast or vice versa transitional fibers (Andersen & Schiaffino, 1997; Peuker & Pette, 1997; Stevens et.al, 1999).

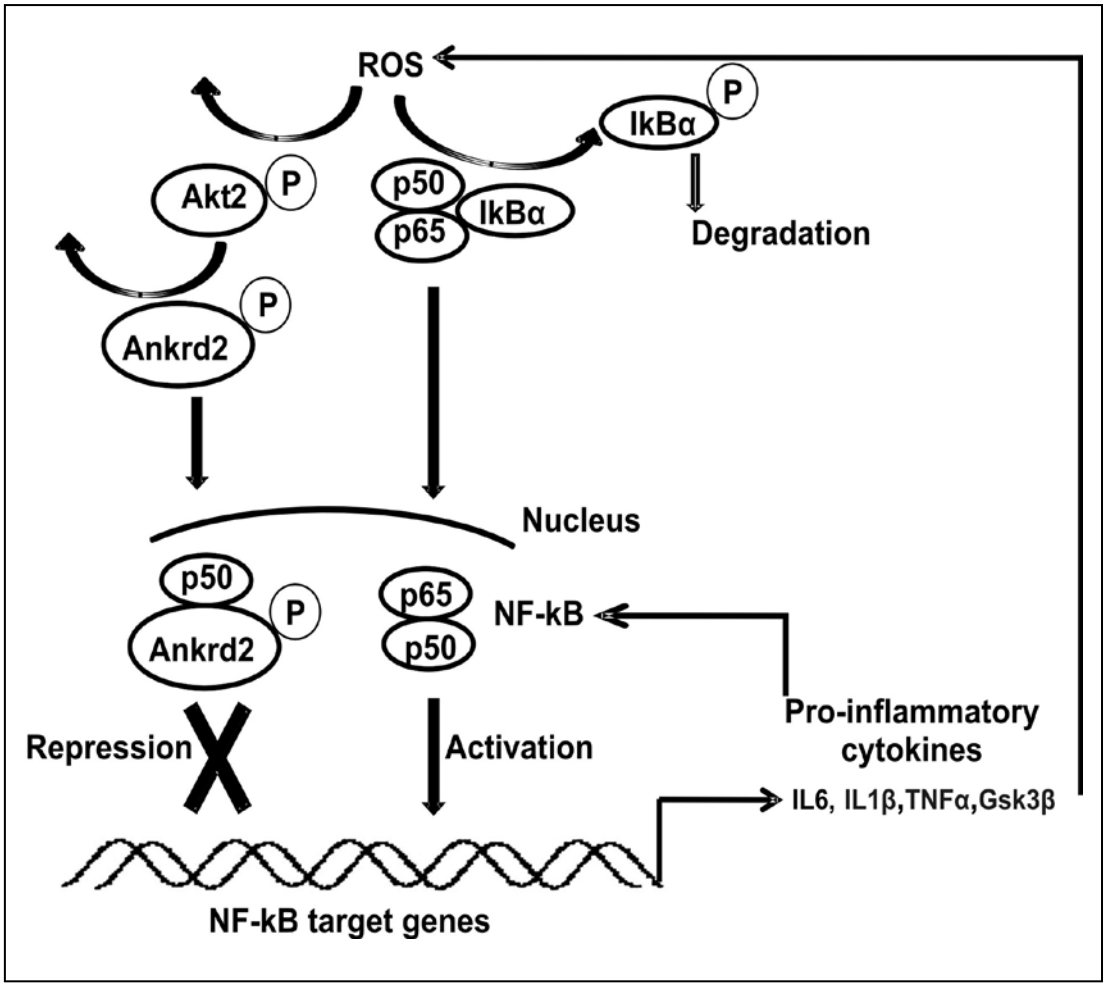

Figure 12. Proposed model for a novel antioxidant system. Ankrd2 counteracts the toxic effects of ROS through inhibition of NF-kB activity in resp onse to oxidative stress.

Although, Ankrd2 has been the focus of many studies, its role in signaling pathways has remained poorly understood. This report provides the first evidence and the underlying molecular mechanism for signal integration between Ankrd2 and NF-kB pathways, contributing to the understanding of the role of Ankrd2 in skeletal muscle. In particular, the novel role of Ankrd2 as an anti-inflammatory agent through suppression of NF-kB transcriptional activity suggest its potential use as a target for the development of specific therapeutic strategies for resolution of inflammation in muscle. Our findings indicate that Ankrd2 is a novel potent modulator of inflammation during the muscle differentiation. However, how these multiple signaling pathways converge

on NF-kB, and sorting out the underlying mechanisms by which the specificity of the response is maintained in the muscle is a daunting task. In particular, the novel role of Ankrd2 as an anti-inflammatory agent through suppression of NF-kB transcriptional activity suggest its potential use as a target for the development of specific therapeutic strategies for resolution of inflammation in muscle. Moreover, exact nature of these signaling complexes and the scenarios that drive their interaction remain a focus of the current research.

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