

NF- κ B Signaling: A Tale of Two Pathways in Skeletal Myogenesis

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Bakkar N, Guttridge DC. NF- κ B Signaling: A Tale of Two Pathways in Skeletal Myogenesis. *Physiol Rev* 90: 495–511, 2010; doi:10.1152/physrev.00040.2009.—NF- κ B is a ubiquitously expressed transcription factor that plays vital roles in innate immunity and other processes involving cellular survival, proliferation, and differentiation. Activation of NF- κ B is controlled by an I κ B kinase (IKK) complex that can direct either canonical (classical) NF- κ B signaling by degrading the I κ B inhibitor and releasing p65/p50 dimers to the nucleus, or causes p100 processing and nuclear translocation of RelB/p52 via a noncanonical (alternative) pathway. Under physiological conditions, NF- κ B activity is transiently regulated, whereas constitutive activation of this transcription factor typically in the classical pathway is associated with a multitude of disease conditions, including those related to skeletal muscle. How NF- κ B functions in muscle diseases is currently under intense investigation. Insight into this role of NF- κ B may be gained by understanding at a more basic level how this transcription factor contributes to skeletal muscle cell differentiation. Recent data from knockout mice support that the classical NF- κ B pathway functions as an inhibitor of skeletal myogenesis and muscle regeneration acting through multiple mechanisms. In contrast, alternative NF- κ B signaling does not appear to be required for myofiber conversion, but instead functions in myotube homeostasis by regulating mitochondrial biogenesis. Additional knowledge of these signaling pathways in skeletal myogenesis should aid in the development of specific inhibitors that may be useful in treatments of muscle disorders.

I. INTRODUCTION

First identified as a transcription factor important for the activation of kappa light chain genes in B cells, NF- κ B is now recognized as a ubiquitously expressed factor involved in regulating a wide array of cellular processes including immune response, cellular sur-

vival, proliferation, and differentiation (46, 134). This family of proteins includes several members that can be activated by an extensive number of extracellular signals, adding to the complexity of their regulation and function.

During the past decade there has been an increasing interest in studying the role of NF- κ B signaling in skeletal

muscle cell differentiation. This has led to the discovery that this signaling pathway is associated with multiple skeletal muscle abnormalities that comprise, but are not limited to, cachexia, muscular dystrophy, disuse atrophy, inflammatory myopathy, and more recently, rhabdomyosarcoma. Several current reviews have nicely summarized in detail the evidence linking NF- κ B in muscle diseases, so similar points will not be discussed here (35, 88, 104, 119). Instead, we will focus on the literature that more specifically addresses how NF- κ B participates in regulating the differentiation of skeletal muscle with respect to both classical and alternative signaling pathways and when appropriate provide our own views to help clarify some of the discrepancies surrounding NF- κ B control of myogenesis under physiological conditions.

II. AN OVERVIEW OF NF- κ B SIGNALING

A. NF- κ B and I κ B Family Members

NF- κ B is a family of evolutionary conserved dimeric proteins encoded by five gene members: RelA/p65, RelB, c-Rel, NF- κ B1/p50, and NF- κ B2/p52 (the last 2 of which derive from precursor subunits, p105 and p100, respectively). These proteins share a 300-amino acid region called the Rel homology domain (RHD) that is responsible for DNA binding, dimerization to other NF- κ B subunits, nuclear translocation, and physical interaction with its own I κ B family of inhibitors (Fig. 1). NF- κ B proteins exist as homo- or heterodimers that function as positive regulators of transcription, although p50/p50 and p52/p52 complexes are instead considered repressors of gene activation due to the absence of transactivation domains in each of their respective monomeric subunits (148). NF- κ B dimers bind with different affinities to DNA bearing the consensus sequence GGGRNYYCC, where R is a purine, Y is a pyrimidine and N is any base (58). The degenerate nature of this binding sequence and the diverse binding preferences of NF- κ B dimers lead to the recruitment of various coactivators and corepressors that result in expression of a wide variety of target genes (112). Transcription of these targets is further regulated through posttranslational modifications of NF- κ B that affect its interaction with transcriptional modulators. RelA/p65 (hereafter referred to as p65) can be phosphorylated on numerous amino acids including Ser-276, Ser-529, and Ser-536. These modifications are directed by various kinases such as protein kinase A (PKA), mitogen- and stress-activated protein kinase 1 (MSK1), Tank binding kinase 1 (TBK1), and casein kinase 2 (CK2) (19, 95, 111). Ser-311 is also phosphorylated *in vitro* directly by the ζ -isoform of protein kinase C and *in vivo* through tumor necrosis factor (TNF)- α stimulation (43). Such phosphorylation events function mainly to increase p65 transcriptional

activity either by enhancing binding to coactivators and basal transcription factors or by increasing p65 nuclear localization and stability (16). In more rare instances, phosphorylation such as on Ser-468 can be both stimulatory and inhibitory to NF- κ B, depending on the cellular signal and kinase involved (16, 96, 162). Conversely, dephosphorylation of Ser-536 by the phosphatase WIP1 was recently shown to inhibit NF- κ B transactivation function (29). The p65 subunit undergoes further posttranslational modifications that include acetylation at residues Lys-122, Lys-218, and Lys-310, mediated by p300/CBP and other proteins with histone acetyltransferase (HAT) activity to enhance p65 transcriptional activity (95). Furthermore, p65 activity can be negatively regulated through SOCS-1-dependent ubiquitination of residues 220 and 335, leading to p65 proteasomal degradation (58, 127).

The I κ B family that regulates NF- κ B includes members I κ B α , I κ B β , I κ B γ , I κ B ϵ , I κ B ζ , Bcl-3, and the NF- κ B precursor proteins p100 and p105 (Fig. 1) (58). I κ B α , - β , - ϵ , p100, and p105 contain a core of six or more ankyrin repeating subunits that bind the RHD of NF- κ B (47). This binding masks the nuclear localization signal (NLS) of NF- κ B dimers, retaining the complex in the cytoplasm of unstimulated cells. However, even in an inactive state, the p65/p50/I κ B α complex is able to shuttle between the cytoplasm and nucleus due to an exposed NLS within p50 that is not completely covered by I κ B interaction (46). The complex then returns to the cytoplasm due to nuclear export sequences contained in both I κ B α and p65 (56, 63). The resulting consequence of this shuttling has not been fully elucidated, but presumably this could represent a mechanism for cells to maintain basal expression of NF- κ B-dependent genes in the absence of extracellular activating signals. Nevertheless, it is the degradation of I κ B proteins that alters this dynamic balance of nuclear to cytoplasmic localization, favoring nuclear entry of NF- κ B dimers.

The I κ B kinase (IKK) complex controls the degradation of I κ B proteins by regulating the phosphorylation of Ser-32 and Ser-36 on I κ B α and Ser-19 and Ser-23 on I κ B β . Phosphorylation results in K48-linked polyubiquitination by the SCF^{TRCP} E3 ubiquitin ligase complex on Lys-21 and Lys-22 of I κ B α , an ATP-dependent event that rapidly targets these proteins for degradation via the 26S proteasome (116). Bcl-3 and I κ B ζ are inducibly expressed atypical I κ B proteins that regulate NF- κ B function by a distinct mechanism. I κ B ζ (also known as interleukin-1-inducible nuclear ankyrin repeat protein, inhibitor of nuclear factor κ B ζ , or MAIL) localizes to the nucleus, indicating that it regulates nuclear NF- κ B activity rather than its translocation from the cytoplasm (107). I κ B ζ expression is barely detectable in resting cells, but becomes strongly induced in response to toll like/IL-1 receptor activation, and associates primarily with p50 homodimers to positively or negatively regulate NF- κ B tar-

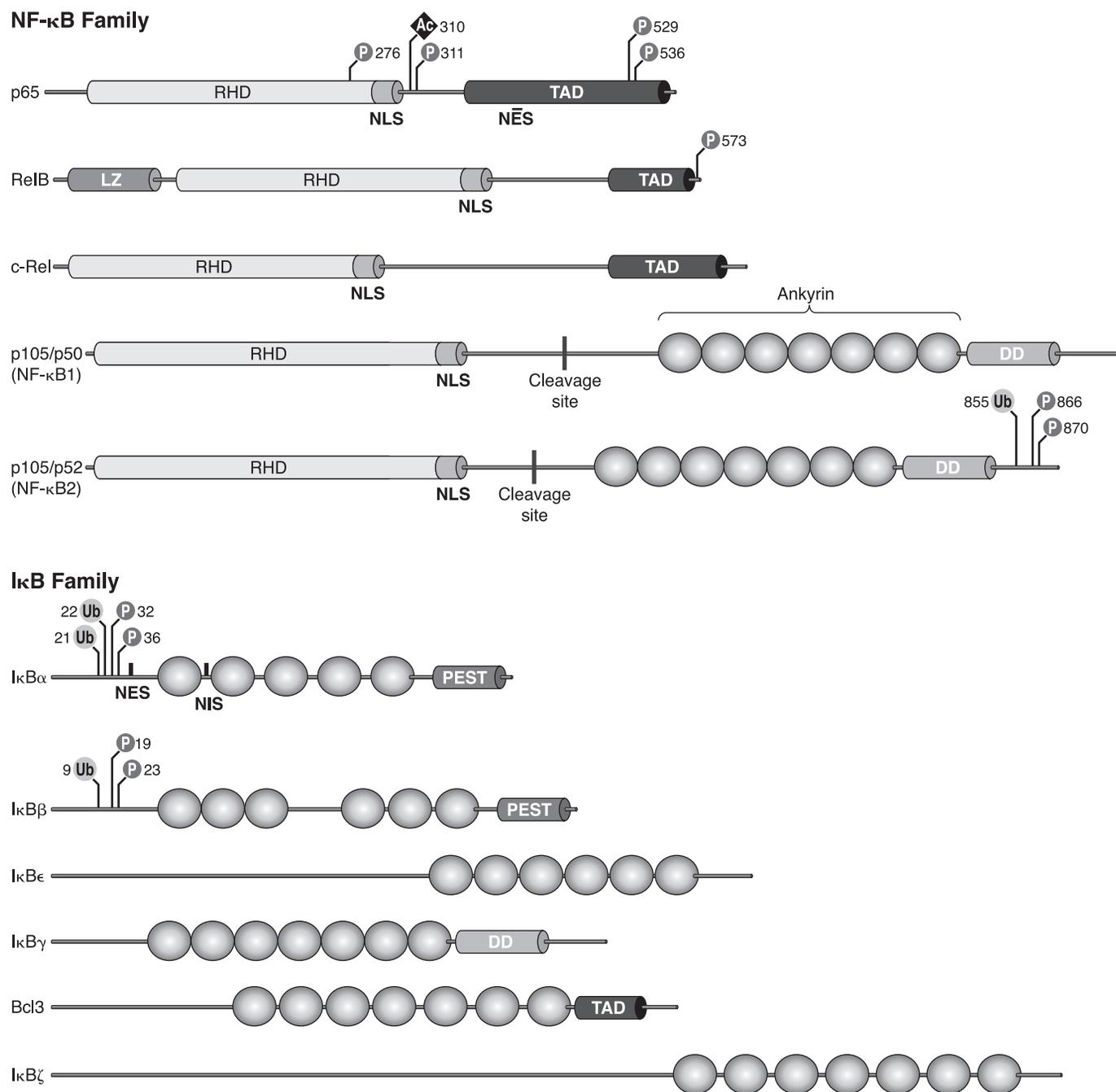


FIG. 1. NF- κ B and I κ B family members. Human NF- κ B and I κ B proteins are represented with their specific posttranslational modifications. P, phosphorylation; Ac, acetylation; Ub, ubiquitination; TAD, transactivation domain; RHD, Rel homology domain; NES, nuclear export signal; NLS, nuclear localization signal; NIS, nuclear import signal; LZ, leucine zipper; DD, death domain; Ankyrin, ankyrin repeats; PEST, polypeptide sequences enriched in proline (P), glutamate (E), serine (S), and threonine (T).

get genes (166, 168). Bcl-3, on the other hand, is a unique I κ B family member that contains a transactivation domain and functions to promote NF- κ B-dependent gene expression through its association with p50 homodimers that possess strong DNA binding activity (22). In addition, Bcl-3 has been shown to bind p52 homodimers to transactivate the expression of growth regulatory genes, cyclin D1 and p53 (69, 157).

B. The IKK Complex

The IKK complex, at times referred to as the IKK signalosome, contains two kinases, IKK α /IKK1/CHUK and IKK β /IKK2, as well as several copies of a regulatory protein, NEMO (NF- κ B essential modulator, also known as IKK γ , IKKAP-1, or FIP-3). IKK α and IKK β share a strong sequence homology particularly in their catalytic region

and contain helix-loop-helix domains (HLH) for protein interaction. In contrast, IKK γ is distinctly smaller and characterized by coiled-coil, leucine zipper, and Zn finger-like domains (169) (Fig. 2). IKK α and IKK β dimerize through their leucine zipper domain, and although these kinases can homodimerize, heterodimers are favored and are more catalytically active (100). Both IKK α and IKK β bind IKK γ through their COOH-terminal NEMO-binding domain (NBD), albeit with different affinities (98, 126). Activation of the IKK complex is mediated by IKK γ oligomerization and interaction with upstream signaling adapters leading to the subsequent phosphorylation of T-loop serines of at least one of the IKK catalytic subunits by an upstream kinase or by transautophosphorylation (94). Activation of IKK is a transient event that is terminated by deubiquitination of IKK γ through the action of the NF- κ B target gene A20, and the cylindromatosis (CYLD) deubiquitinases (55). This signaling shutdown is also mediated through dephosphorylation of IKK T-loop serines by the protein phosphatase 2A (PP2A) (114, 161). Other posttranslational modifications targeting IKK γ include K63-linked ubiquitination as well as SUMOylation and phosphorylation (132).

I κ B α and I κ B β are the prototypical IKK substrates, and findings from knockout models support that IKK β is more efficient than IKK α in phosphorylating these I κ B family members (55). Although IKK β was first described to phosphorylate p65 on Ser-536 *in vitro* (128), both IKK α and IKK β kinases appear capable *in vivo* to phosphorylate p65 to enhance its transactivation potential (17), and IKK β has been shown to further activate p65 via phosphorylation of Ser-468 (130). Other substrate preferences exist between these kinases. In addition to I κ B proteins, IKK β can preferentially phosphorylate the tuberous sclerosis complex (TSC1) to relieve repression on the mammalian target of rapamycin (mTOR) pathway, which in turn contributes to an inflammatory-induced transformation condition in breast cancer cells (81). Furthermore,

IKK β phosphorylation of FOXO3a causes nuclear export and subsequent proteolysis of this forkhead transcription factor to block apoptosis and facilitate breast tumorigenesis (62). Conversely, IKK α selectively phosphorylates p100 to stimulate its interaction with the NF- κ B inducing kinase (NIK), resulting in p100 ubiquitination and partial proteolysis to form p52 (164). IKK α has also been reported to function in an epigenetic fashion by acting in the nucleus to phosphorylate and thus regulate the activities of the transcriptional cofactors CBP and SMRT (60, 64), as well as the H3 chromatin histone protein (4), and the mitotic regulator Aurora A (122).

C. Upstream Activators of NF- κ B

NF- κ B is activated by a variety of upstream signals including bacterial products, inflammatory cytokines, oxidative stress, and mitogens. Such signals are then channeled through intracellular adapter proteins that allow for specific receptor-induced signaling events, starting with IKK activation and culminating with NF- κ B nuclear translocation in what is referred to as either the classical or the alternative signaling pathway. TNF receptor-associated factors (TRAF) are a family of such adapter proteins critical for NF- κ B signaling pathways. These adapters contain a conserved TRAF domain that regulates homo- and heterodimerization, as well as interaction with surface receptors, TNF, Toll like receptor (TLR), and interleukin (IL)-1 (83). The TRAF family consists of six members that function as E3 ubiquitin ligases. Upon ligand-induced receptor oligomerization, TRAFs are recruited to the cytoplasmic portion of cell surface receptors by interactions with “classical” adapter proteins such as TRADD, MyD88, and the IRAK kinase, resulting in the assembly of multi-protein signaling complexes (Fig. 3).

The receptor interacting proteins (RIPs) represent another family of “classical” adapter proteins that recruit

IKK Complex

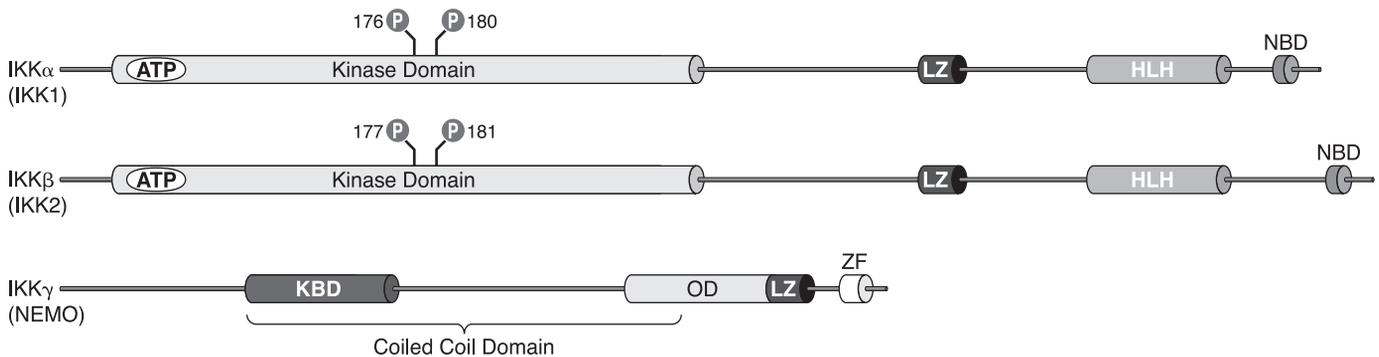


FIG. 2. IKK family members. Human IKK proteins are schematically represented including their critical posttranslational modifications as in Figure 1. HLH, helix loop helix; OD, oligomerization domain; NBD, Nemo binding domain; ZF, zinc finger; KBD, kinase binding domain; LZ, leucine zipper; ATP, ATP binding domain.

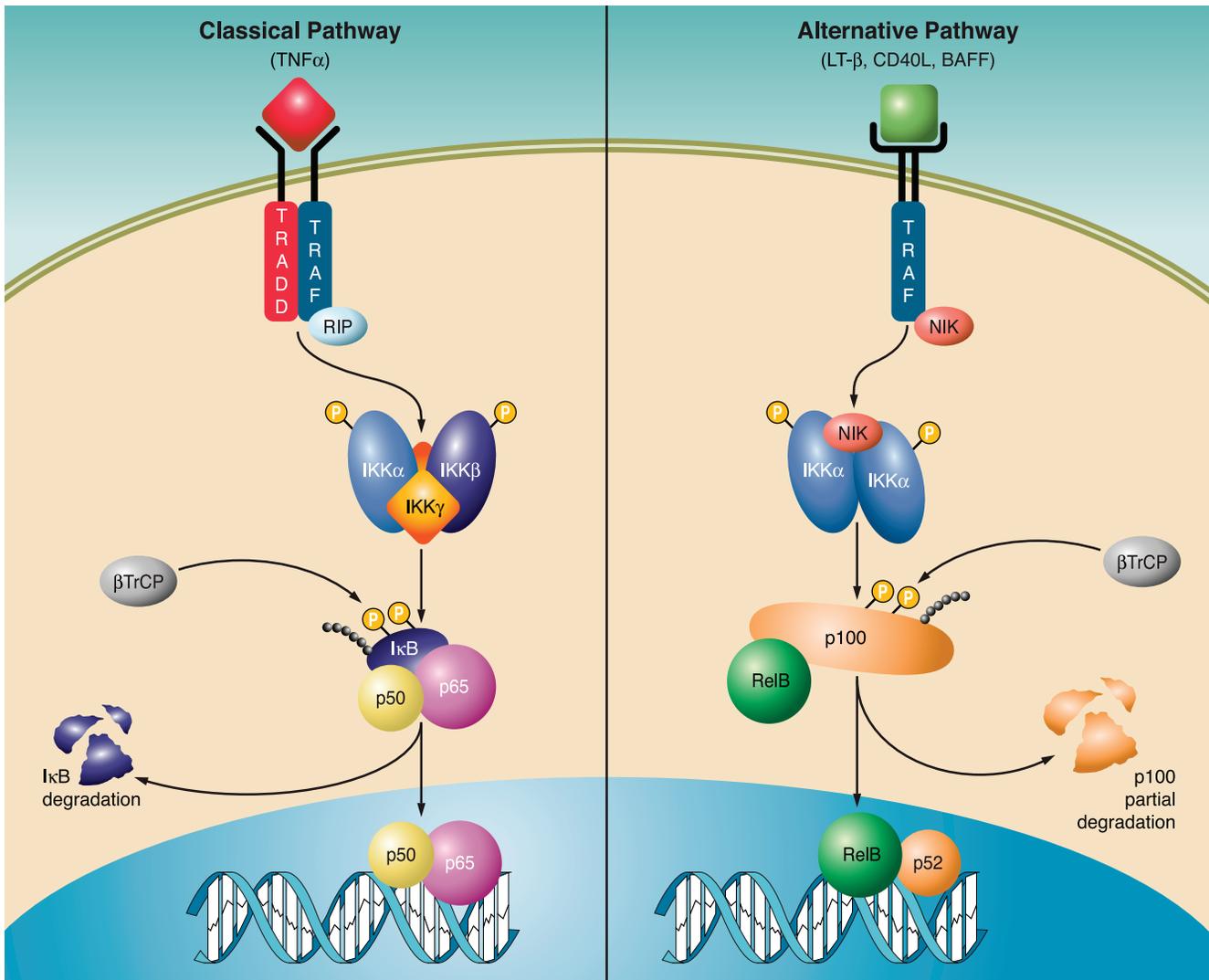


FIG. 3. Classical and alternative NF- κ B signaling pathways. The classical or canonical signaling pathway is represented using TNF- α as an activator. Signaling is initiated with the binding of TNF- α to its receptor and the subsequent sequential recruitment of the adaptors TRADD, RIP, and TRAF to the membrane. IKK complex assembly and recruitment to the cell membrane occurs between IKK α , IKK β , and IKK γ , resulting in IKK β phosphorylation and activation. IKK β then phosphorylates I κ B α to promote its polyubiquitination and subsequent immediate proteasomal degradation through β -TrCP. In turn, the classical p50/p50 heterodimer is freed to translocate to the nucleus and mediate transcription of NF- κ B target genes, including one of its own inhibitor I κ B α . Alternative NF- κ B signaling is instead activated by ligands, CD40L, and lymphotoxin β that triggers recruitment of TRAFs to the membrane-bound receptor and subsequently activates NIK. NIK then phosphorylates IKK α homodimer. Once active, IKK α phosphorylates p100, resulting in its partial proteolysis by β -TrCP to generate the p52 subunit. The resulting RelB/p52 complex then translocates to the nucleus to transcribe NF- κ B target genes that may be distinct from the classical pathway.

IKK through binding to IKK γ (102). RIP proteins function as serine/threonine kinases that, in the case of RIP1, associate with TRADD via its death domain (DD), and TRAFs1, -2 and -3 via their intermediate domains (61). RIP kinase activity is dispensable in some signaling pathways and needed for others, and the various RIP members exert nonredundant functions. Nevertheless, these RIP proteins act as scaffolds for IKK activation, while RIP1 signals through TNF- α , TLR3, and TLR4 and is essential for NF- κ B activation (102).

NF- κ B activators also include the IKK-related proteins TBK1 and IKK ϵ . TBK1 was originally identified as an

IKK complex-specific kinase that phosphorylates serines in the activation loop of IKK β to stimulate its I κ B kinase activity (147). TBK1 also interacts with TRAF2 and is involved in interferon gene induction in response to viral and bacterial infections (26). Similarly, IKK ϵ is essential for triggering host antiviral response by phosphorylating IRF3 and IRF7 and can phosphorylate I κ B α on Ser-36, but not Ser-32, in response to phorbol 12-myristate 13-acetate (PMA) and T-cell receptor activation (118, 137). IKK ϵ was also found to phosphorylate p65 on Ser-536, which is thought to contribute to the constitutive activation of NF- κ B in various cancer cells (2).

D. Classical and Alternative NF- κ B Signaling

Targeted disruption of the different IKKs has shown that IKK β and IKK γ are essential for p65/p50 activation via I κ B α phosphorylation (45), while IKK α is largely dispensable. This signaling pathway, referred to as the classical or canonical pathway, is mediated by the activation of IKK β and IKK γ , resulting in I κ B degradation and NF- κ B nuclear translocation (Fig. 3). Classical NF- κ B activation occurs in response to a myriad of extracellular and intracellular factors, including proinflammatory cytokines, bacterial products, growth factors, reactive oxygen species, genotoxic stress, and viruses. Conversely, distinct factors specifically signaling through IKK α are part of the alternative or noncanonical pathway involving p100 phosphorylation and its subsequent partial proteolysis to form the mature p52 subunit. Whereas the processing of p105 to p50 is constitutive, processing of p100 into p52 is tightly regulated by signals involved in B-cell maturation and lymphoid organogenesis, such as B-cell activating factor (BAFF), CD40, CD27, and lymphotoxin- β (120, 135) (Fig. 3). Alternative NF- κ B signaling does not require IKK β , IKK γ , or RIP, but rather proceeds through activation of NIK and under the negative regulation of TRAF3 (40, 57). NIK activation leads to IKK α phosphorylation in its activation loop, that in turn causes IKK α to bind and phosphorylate p100 on Ser-866 and Ser-870 (164). This phosphorylation stimulates recruitment of the SCF ^{β TrCP} E3 ligase complex that polyubiquitinates p100 at Lys-855 and subsequently causes its partial degradation by eliminating the COOH-terminal ankyrin repeat domains to form p52 (89). Such processing can occur constitutively (as in the case of some lymphomas) or at a cotranslational level (40).

In the absence of activating signals, p100 exists in the cytoplasm bound to the RelB subunit of NF- κ B. Once processed, RelB/p52 dimers are activated and translocate to the nucleus where they are thought to regulate a group of genes distinct from that of classically regulated promoters (11, 41, 87). It was also discovered that p100 is capable of binding to p65/p50 classical dimers, and that in response to induced lymphotoxin- β signaling in mouse embryonic fibroblasts, p65 undergoes nuclear translocation via the conversion of p100 to p52 (10). This regulation was further found to be dependent on NIK and IKK α , but independent of IKK γ , together suggesting for the first time that p100 represents a nodal point between classical and alternative signaling pathways. Whether p100 can act in a similar fashion in other cell types, or in response to lymphotoxin- β signaling, *in vivo* remains to be determined.

III. SKELETAL MUSCLE DIFFERENTIATION

Aside from its more commonly accepted role as a regulator of innate and adaptive immunity, and as a central mediator of cell survival, NF- κ B is also a prominent

factor in regulating cellular differentiation. Whereas most studies involving NF- κ B and differentiation have focused on bone, blood, and skin (50, 90, 129, 133, 150, 151, 165, 167), only more recently has attention been given to the differentiation of skeletal muscle cells, a process referred to as myogenesis. We thus start this section by giving a brief overview on the regulation of skeletal myogenesis before discussing NF- κ B regulation of this process.

A. Regulation of the Myogenic Program

Skeletal muscles derive from a subdivision of the paraxial mesoderm called the somites. Muscle progenitors within the maturing somite then become confined to the dorsolateral region called the dermomyotome, migrate to the limb buds, and differentiate to form muscle fibers (15). Migrating somites are characterized by the expression of Pax3 and Pax7, two members of the paired homeodomain transcription factors that need to be downregulated for the myogenic program to proceed (14, 159). A small fraction of these cells generates the satellite cells that then reside between the basal lamina and the sarcolemma of myofibers (80). Pax3 is critical for the delamination and migration of somatic muscle progenitors, as Pax3 null mice lack limb muscles (143). Pax7 knockout muscles, on the other hand, lack myogenic cells and exhibit smaller myofibers, although fetal and embryonic myogenesis are intact, indicating a specific requirement for Pax7 in the satellite cell lineage (or postnatal development) (131). Recent findings further reveal that Pax3 expression in somitic cells is sufficient to sustain embryonic myogenesis and that a developmentally distinct cell expressing both Pax3 and Pax7 contributes to fetal and subsequently postnatal myogenesis (66). Whether these same Pax3, Pax7 double-positive progenitor cells are required for adult myogenesis, at least beyond the juvenile stage of development, was put into question by recent provocative findings showing that inactivation of Pax3 and Pax7 expression in satellite cells in adult, but not early postnatal muscles, maintained their ability to contribute to muscle regeneration (86). Such studies underscore the distinct functions of Pax proteins during defined stages of skeletal muscle development.

Members of the basic HLH transcription factors, including MyoD, myogenin, Mrf4, and Myf5, are expressed downstream of Pax3 and control skeletal muscle differentiation. Such factors are necessary and sufficient for the determination of the myogenic lineage, with Myf5 and MyoD expression preceding that of myogenin (121). MyoD and myogenin are expressed during skeletal muscle differentiation, while Mrf4 is present in terminally differentiated cells (144). MyoD forms heterodimers with E protein subfamily E12 or E47 and binds to a consensus sequence termed the E-box present in the regulatory re-

gions of many skeletal muscle genes including its own and that of the Mef2 transcriptional regulator (76, 154). MyoD also initiates chromatin remodeling through recruitment of HATs and the SWI/SNF complex (51, 123, 138). Although MyoD and Myf5 are expressed in undifferentiated myoblasts, they are kept transcriptionally inactive due to their interaction with the Id1 suppressor protein (99). MyoD activity can also be silenced while bound to enhancer elements of muscle genes, sequestered in a complex with the Sir2 histone deacetylase and PCAF acetyltransferase (44, 113). When active, MyoD, bound to SRF and PCAF, can replace the Polycomb negative regulatory complex containing Ezh2 and YY1 in association with HDAC1 to stimulate contractile gene expression (21).

B. Signaling Pathways Positively Regulating Myogenesis

Myogenesis is positively or negatively regulated by a variety of factors and signaling pathways. The MAPK pathway involving p38 is one such regulator that activates differentiation by recruiting the SWI/SNF complex to muscle promoters, phosphorylating the E47 E-box protein to promote its association with MyoD and/or phosphorylating MEF2A, -C, and -D factors to enhance their transcriptional activity (70, 92, 138, 163). The p38 MAPK also phosphorylates MRF4 to inhibit its activity and thus repress the expression of selective myogenic genes late in the differentiation program, and to antagonize the JNK proliferation-promoting pathway, so as to again facilitate myogenesis (115, 141).

The PI(3)K/Akt pathway is activated in response to insulin/insulin-like growth factor (IGF) I and signals through its effectors mTOR and p70S6K to stimulate protein synthesis and promote myotube hypertrophy (125). Evidence suggests that Akt1 and Akt2 promote myogenesis by phosphorylating p300, which stimulates its association with MyoD and PCAF acetyltransferases (136).

Myogenesis is further stimulated by the Wnt class of secreted signaling proteins. Wnt signaling is necessary for muscle formation during embryogenesis (34), as well as myogenic differentiation (3), and functions by activating Myf5 (in the case of Wnt1) or MyoD (for Wnt7a) (142). Wnt7a was recently described to stimulate muscle regeneration via its receptor Fzd7 located on satellite cells (78). Furthermore, Wnt signaling promotes myogenic progenitor progression towards differentiation during muscle regeneration in a regulatory program accompanied by the functional decline in the inhibitory Notch signaling (13). Such a temporal switch converges on the GSK3 β kinase and its downstream target β -catenin that is necessary for muscle regeneration.

C. Negative Signaling Pathways of Myogenesis

Negative regulators of myogenesis include fibroblast growth factors (FGFs), mainly FGF6 (6, 110), transforming growth factor (TGF)- β (149), and the protooncogenes Ha-Ras, E1a, and *c-fos* (77, 145, 158). In addition, myostatin acts by suppressing the activity of MyoD, and its absence is associated with the double muscled phenotype in cattle, mice, and remarkably, even in humans (52, 72, 84). Notch signaling was similarly shown to inhibit myogenesis through the downstream RBP-J transcription factor, thus regulating the transcription of Hes1, which in turn represses MyoD (152), as well as through direct binding to MEF2c, blocking its DNA-binding site (160).

IV. NF- κ B IN MYOGENESIS AND MUSCLE REGENERATION

Early studies using the murine C2C12 myoblast line revealed that the p65 and p50 subunits of NF- κ B contain relatively abundant DNA binding activities (53). Similarly, RelB, p100/p52, and Bcl-3 subunits have been described in adult muscles, indicating that NF- κ B members are likely to play a role in the formation and/or function of skeletal muscles (65). However, this involvement may be subunit specific, since c-Rel in comparison is expressed at low levels in skeletal muscles, and mice lacking c-Rel or p50 contain normal fiber morphology (67). Nevertheless, given the role of NF- κ B in various cellular differentiation models as well as disease (88, 104, 119), numerous laboratories have focused on the potential relevance of this signaling pathway in myogenesis and muscle regeneration. Early results were however perplexing, as both promyogenic and antimyogenic roles of NF- κ B were described.

A. Evidence for NF- κ B as a Positive Regulator of Myogenesis

A number of reports have shown that activation of NF- κ B is associated with the promotion of myogenesis. These findings were described in part due to the response of the promyogenic factor p38 MAPK, which in muscle as well as nonmuscle cells stimulates the transactivation function of NF- κ B through phosphorylation of Ser-536 on the p65 subunit (7, 38, 93, 108). Using electrophoretic mobility gel shift assays (EMSA), Baeza-Raja and Munoz-Canoves (7) demonstrated a p38-dependent elevation of NF- κ B DNA binding activity during myogenic differentiation. The authors went on to reveal that both p38 and NF- κ B activation are required for IL-6 production, and that IL-6 itself is promyogenic (7). Although the authors did not clearly implicate classical NF- κ B signaling, they showed that NF- κ B DNA binding activity was associated

with p65/p50 classical subunits. Likewise, De Alvaro et al. (38) reported that NF- κ B activities were enhanced in response to overexpression of mutant Ras, a process that was also preceded by p38 MAPK signaling in muscle cells (38). Moreover, insulin or IGF-II signaling, which activates Akt, was shown in several studies to promote NF- κ B DNA binding during C2C12 differentiation (18, 20, 32, 33, 68). This latter activation of NF- κ B was found to restore differentiation of Ras-transformed cells (32). IGF-II-mediated induction of NF- κ B was accompanied by NIK and IKK α activation, as well as by I κ B α phosphorylation on Ser-32 and Ser-36 and its subsequent degradation (20). Since these findings were described prior to the discovery of the alternative pathway, it is not clear whether IGF-II-dependent activation of NF- κ B occurs through alternative signaling or if a more unique regulatory pathway is utilized that branches NIK and IKK α into the IKK signalsome to degrade I κ B α through the classical pathway. Further insight into this regulation is needed to ascertain the mechanism by which IGF-II-induced activation of NF- κ B promotes myofiber conversion.

Pharmacological agents have also been shown to block myogenesis in association with a negative regulation on NF- κ B. Specifically, pyrrolidine dithiocarbamate and the proteasomal inhibitor lactacystin abrogate NF- κ B in L6 rat myoblasts while hindering cellular fusion and expression of muscle-specific proteins (71, 82). Additionally, three-dimensional (3D) clinorotation, used as a model of microgravity, was shown to inhibit differentiation of L6 cells, which correlated with the prevention of I κ B ubiquitination and subsequent NF- κ B nuclear translocation (59).

B. NF- κ B Function as a Negative Regulator of Myogenesis

Contrary to these above findings, separate reports have associated NF- κ B with a negative regulatory role on skeletal muscle differentiation. It is interesting that in most cited cases, similar biochemical assays were used to reach vastly different conclusions. In several laboratories including our own, NF- κ B DNA binding activity was found to decline over the course of myogenic differentiation (8, 23, 39, 53, 85). This regulation was accompanied by a reduction of NF- κ B transcriptional activity as recorded from reporter assays and from expression of a bona fide NF- κ B target gene, I κ B α (53, 85). Additionally, inhibition of NF- κ B signaling by stable expression of the I κ B α -super repressor (SR) inhibitor mutant was found to accelerate myogenesis, increasing myogenin expression as well as myotube formation (53). Activators of NF- κ B such as TNF- α , the TNF family member TWEAK, IL-1 β , or the RIP homolog RIP2 strongly inhibit myogenesis (42, 48, 54, 75, 106). Furthermore, glutathione depletion (5) and cyclic

mechanical strain (73) impair myogenic differentiation through sustained activation of NF- κ B. Inhibition of NF- κ B further restored differentiation despite the depletion of glutathione, suggesting that such negative regulation was mediated by NF- κ B.

In all, these reports highlighted clear discrepancies as to whether NF- κ B is activated or repressed during myogenesis, and whether such regulation acts positively or negatively to regulate the myogenic program. Since many of these interpretations relied on EMSA analysis, it is possible that lab-to-lab variability in culturing conditions or during the shift assay itself may have contributed to alterations in NF- κ B DNA binding. In our experience, we have observed that NF- κ B activation in differentiating myoblasts is sensitive to a wide variety of conditions, such as extended cell passaging and culture density, which can both impact the timing and the degree to which cells permanently exit cell cycle. As a more extreme example, we found that simply switching culture media on murine C2C12 myoblasts from high (GM, growth medium) to low serum (DM, differentiation medium) induces a transient NF- κ B activity that peaks within the first 24 h (8). Since a similar regulation was not seen when myotubes were replenished with fresh DM, we attribute this transient activity to a stress response resulting from the rapid removal of mitogens contained in GM conditions. Considerations should also be given to how nuclear extracts are prepared for EMSA analysis. For example, the proper final concentration of NaCl present in the nuclear extract buffer is a critical factor that when altered can result in assay variability. This value should be maintained at 420 mM throughout the isolation of nuclear proteins, a simple step that can easily be miscalculated when cell volumes substantially expand due to the multinucleation of myotubes. Similar considerations exist for interpretations of transient reporter assays, where plasmids may be diluted out in myoblasts differentiated over an extended number of days, or reporter activity altered by the increase in cell size. Stable reporter cell lines should therefore be considered as an alternative approach to assay NF- κ B transcriptional activity.

To circumvent these potential technical limitations, an analysis of NF- κ B function in myogenesis was reexplored using genetic approaches. These findings revealed that differentiating *p65*^{-/-} cells exhibited a high degree of myogenic activity compared with the other NF- κ B subunits (9). In addition, hindlimb muscles from *p65*^{-/-} mice contained almost a twofold increase in fiber number compared with wild-type or *p50*^{-/-} mice. Furthermore, similar comparisons performed with the IKK complex demonstrated that cells lacking IKK β or IKK γ , but not IKK α , behaved similarly to p65 null cells in their propensity to increase their myogenic potential (9). These data supported the notion that NF- κ B functions as an inhibitor of skeletal muscle differentiation and that this property may

be mediated through components of the classical signaling pathway.

C. NF- κ B Inhibits Myogenesis Through Multiple Mechanisms

Mechanistically, NF- κ B can act at multiple levels to block muscle differentiation, and each of these activities seems to be mediated through the classical pathway. Cyclin D1, itself a reported repressor of myogenesis (140), is also a transcriptional target of NF- κ B (53). The cyclin D1 protein was also recently reported to interact and be stabilized by p65 (37). Furthermore, classical NF- κ B subunits can suppress the synthesis of MyoD by acting through a destabilization element in the MyoD transcript in response to TNF- α and TWEAK signaling (42, 54, 139). More recently, NF- κ B was shown to inhibit myogenesis in proliferating myoblasts through activation of the transcription factor Yin-Yang1 (YY1). In muscle cells, YY1 functions as a transcriptional repressor by associating with Ezh2 and the Polycomb group to silence myofibrillar genes that include, but may not be necessarily limited to, troponin I2, troponin C, myosin heavy chain IIb, and α -actin (156). Classical NF- κ B inhibition in myoblasts using the I κ B α -SR dominant negative was associated with a pronounced induction of the aforementioned myofibrillar genes even under proliferating conditions. During differentiation, YY1 expression decreases concomitant with a reduction of NF- κ B activity, thereby causing the derepression of myofibrillar genes (156). Aside from these terminally differentiated genes, a bioinformatic screen to identify putative microRNAs (miRNAs) capable of epigenetic regulation by YY1 uncovered miR-29, a miRNA family consisting of four members (29a, b1, b2, and c) with high sequence similarity (155). In myoblasts, miR-29 expression is repressed by p65 through the direct binding of YY1 to the miR-29 promoter. Upon differentiation, p65 activity decreases resulting in the subsequent increase in miR-29 levels that functionally was found to facilitate myogenesis. Part of the promyogenic activity of miR-29 is to target its own inhibitor, YY1, that in turn causes derepression of the myofibrillar genes (155). Interestingly, similar to other tumor types, expression of miR-29 is strongly reduced in the skeletal muscle-related cancer rhabdomyosarcoma (155). Such reduction is associated with an increase in p65 and YY1 levels. Inhibition of NF- κ B in rhabdomyosarcoma cells by stable expression of I κ B α -SR caused miR-29 induction and promoted the expression of myofibrillar differentiation genes, providing supporting evidence that classical NF- κ B signaling participates in rhabdomyosarcoma by favoring an undifferentiated phenotype. What role, if any, IKK α and the RelB/p52 alternative complex play in rhabdomyosarcomagenesis remains to be determined.

D. NF- κ B Function in Muscle Regeneration

Adult skeletal muscles are stable tissues that undergo little turnover. However, in response to acute toxin-induced muscle damage or degenerative muscle diseases, muscle fibers undergo a regeneration program due mainly to their resident muscle precursor satellite cells (25, 41). This process starts with a phase of degeneration characterized by muscle fiber necrosis and inflammatory cell infiltration and is followed by the activation of muscle repair. Satellite cells are mitotically and metabolically quiescent in the adult, but can be activated at the site of muscle injury by microenvironment-secreted growth factors such as FGF, TGF- β , and LIF (25, 153). These cells then begin proliferating and undergo rounds of cellular division and differentiation by initiating Myf5 and MyoD expression. At this stage they are called adult myoblasts and further differentiate to repair muscle by forming new myofibers or by fusing to preexisting fibers. Some of these satellite cells do not undergo differentiation and rather maintain a regenerative pool that can be used for successive repair processes (49, 79).

As a regulator of myogenesis, classical NF- κ B has also been found to modulate muscle regeneration both in response to damage and in degenerative muscle diseases. In a cardiotoxin injury model, lack of p65 from 4-wk-old mice was accompanied by increased numbers of centrally located nuclei, a hallmark of muscle regeneration (156). Similarly, mice lacking the classical kinase IKK β specifically in skeletal muscles showed enhanced regeneration as revealed by increased sizes of repaired fibers (103). Mechanistically, Mourkioti et al. (103) observed increased numbers of centrally located myonuclei per regenerated fiber in IKK β -deleted muscles. Furthermore, these muscles accumulated less fibrotic tissue and exhibited an earlier clearance of inflammatory infiltrates, correlating with enhanced muscle regeneration. Likewise, the increase in central nucleation and embryonic myosin heavy chain positive fibers in the Duchenne muscular dystrophy *mdx* mouse model confirmed that regeneration is increased in muscles lacking IKK β (1). Our laboratory linked the repair process to increased numbers of muscle progenitors, namely, a CD34⁺/Sca-1⁻ population coinciding with Pax7-positive satellite cells. We also reported that muscle-specific inhibition of IKK β led to decreased levels of TNF- α , thus implying that mature muscles are capable of producing this cytokine. Given that TNF- α has been found to be a potent inhibitor of skeletal myogenesis when administered at nonphysiological levels (54, 74), one can postulate that NF- κ B/IKK β represses regeneration in dystrophic muscles by promoting the secretion of TNF- α from myofibers that then signals to satellite cells or myoblasts to inhibit their differentiation. Taken together, these studies support that disruption of classical NF- κ B signaling in mature muscles enhances regenerative myo-

genesis and conversely that this pathway negatively regulates adult muscle differentiation. However, much still needs to be explored to rule out the contributions of various other cell types in regeneration. For example, studies where TNF- α signaling was genetically deleted using total TNF- α or TNF receptor I, II double knockouts models reported that muscle regeneration became compromised, suggesting that TNF- α might actually be required in some cell types for proper regeneration (27, 31). Although no links to NF- κ B signaling were drawn in these studies, such findings highlight the need for in-depth genetic investigation of the various factors involved in the regulation of regeneration within skeletal muscle. Future use of IKK signaling inactivation or overexpression models in the other cell types populating skeletal muscle may reveal specific roles of NF- κ B and its signaling pathways during physiological and pathological states of regeneration.

In addition to the genetic models where p65 or IKK β deletion modulated new muscle growth, similar findings were obtained using known general inhibitors of NF- κ B, including curcumin and pyrrolidine dithiocarbamate. Administration of either compound to dystrophic mice or freeze-injured muscles increased expression of biochemical markers associated with muscle regeneration (101, 146). Similar results were obtained with a cell-permeable NBD peptide (1) that functions by competitively inhibiting the interaction of IKK α or IKK β subunits with IKK γ (NEMO) in response to classical NF- κ B activating signals (97). These agents provide a new avenue of potential therapeutic intervention for muscular dystrophy and other muscle diseases associated with dysregulated classical NF- κ B signaling.

E. Modeling NF- κ B Function in Myogenic Differentiation

It has become clear that NF- κ B regulation of myogenesis is an intricate process, rendered even more complex by the various roles that the different NF- κ B subunits may play. Nevertheless, deletion studies in MyoD-converted fibroblasts, primary myoblasts, as well as in intact postnatal muscles indicate that components of the classical NF- κ B pathway act in muscle cells to prevent their premature differentiation (9). As discussed above, the ability of the classical pathway to inhibit differentiation occurs through multiple mechanisms. The reason for these functional redundancies is most likely to ensure that myoblasts are maintained in an undifferentiated state prior to receiving their extracellular cues to engage in their conversion to contractile myotubes. The timing during skeletal muscle development at which this regulation of NF- κ B manifests itself is not known, but recent findings from our laboratory indicate that this may be restricted to

postnatal muscle development, as the p65 subunit appears dispensable in primary myofiber formation during embryogenesis (Bakkar and Guttridge, unpublished observations) but required for maturation of neonatal muscles (36). Findings that adult muscles lacking IKK β undergo enhanced regeneration in response to acute (103) or chronic injury (1) seem to support this claim.

If classical NF- κ B signaling does function as a negative regulator of muscle differentiation, how then can we rectify those findings supporting NF- κ B as a promyogenic factor? In the context of an earlier report that overexpression of alternative complex members NIK and IKK α promote myofiber formation (20), and recent findings that IKK activity and p100 to p52 conversion are induced relatively late in a C2C12 myogenic program (9), we considered the possibility that such a myogenic promoting activity could be derived from the alternative NF- κ B signaling pathway. However, in our own attempts to either induce or knockdown alternative signaling in differentiating C2C12 myoblasts, we have been unable to see differences in the regulation of myogenic genes or in the formation of myofibers (9). More recently, our laboratory utilized IKK α ^{-/-} primary myoblasts in myogenic assays, and again were unable to detect differences in myofiber conversion (Bakkar and Guttridge, unpublished observations). This is in sharp contrast to the deletion of the classical signaling kinase component IKK β , which leads to enhanced myogenic activity in vitro and increased myofiber formation in vivo (9). Based on these genetic findings, we are left to conclude that if NF- κ B has an associated promyogenic activity, it does not derive from alternative signaling. Instead, the data argue that activation of this pathway, occurring late in the differentiation program of cultured skeletal muscle cells, plays a role distinct from myogenic differentiation, as defined by formation of a multinucleated myotube. This role may be in maintaining the homeostasis of myofibers as alternative NF- κ B signaling was found to promote ATP production in myotubes by inducing mitochondrial biogenesis (9). Regulation of mitochondrial biogenesis is not considered a part of myogenesis per se, but is nevertheless an integral component of a functional myotube. Myogenic differentiation is associated with a switch in metabolic pathways from glycolytic to oxidative phosphorylation where ATP production in the latter system occurs from resulting mitochondrial biogenesis (105). These findings thus indicate that the alternative pathway may be more of a regulator of myotube function rather than a regulator of myotube formation. Consistent with this notion, recent data performed in IKK α ^{-/-} muscles revealed defects in the expression of mitochondrial markers, but no effects on the expression of myogenic genes (Bakkar and Guttridge, unpublished observations).

Collectively, we view the role of NF- κ B in skeletal myogenesis as playing two distinct functions, specified by

the classical and alternative signaling pathways. In this model, undifferentiated myoblasts contain a basal level of activated classical signaling to promote myoblast proliferation and suppress premature differentiation. During this phase, IKK β phosphorylates I κ B α leading to the activation and nuclear translocation of p65 to bind DNA and regulate gene products with antimyogenic activity. As myoblasts activate their differentiation program and start fusing, the classical pathway is turned down, as evidenced by reduced phosphorylation of I κ B α and p65, nuclear export of p65, and loss of p65 binding to its target genes such as I κ B α and YY1 resulting in reduced mRNA expression of these NF- κ B target genes (Fig. 4) (9, 53, 156). The extracellular signals regulating the basal activity of NF- κ B

in myoblasts and causing its decrease in differentiating cells remain to be determined.

A further prediction of the model is that a decrease in classical signaling coincides with the induction of the alternative pathway, as distinguished by the conversion of p100 to p52. As with classical signaling, the signal that triggers IKK α activation and p100 processing remains unknown, nor to this point has RelB/p52 target genes been identified in muscle cells (Fig. 4). Although this regulatory switch between the classical and alternative was first described in a myogenic culture system, new evidence indicates that this same switch functions *in vivo* during early postnatal skeletal muscle development (36). However, unlike the classical pathway that functions to

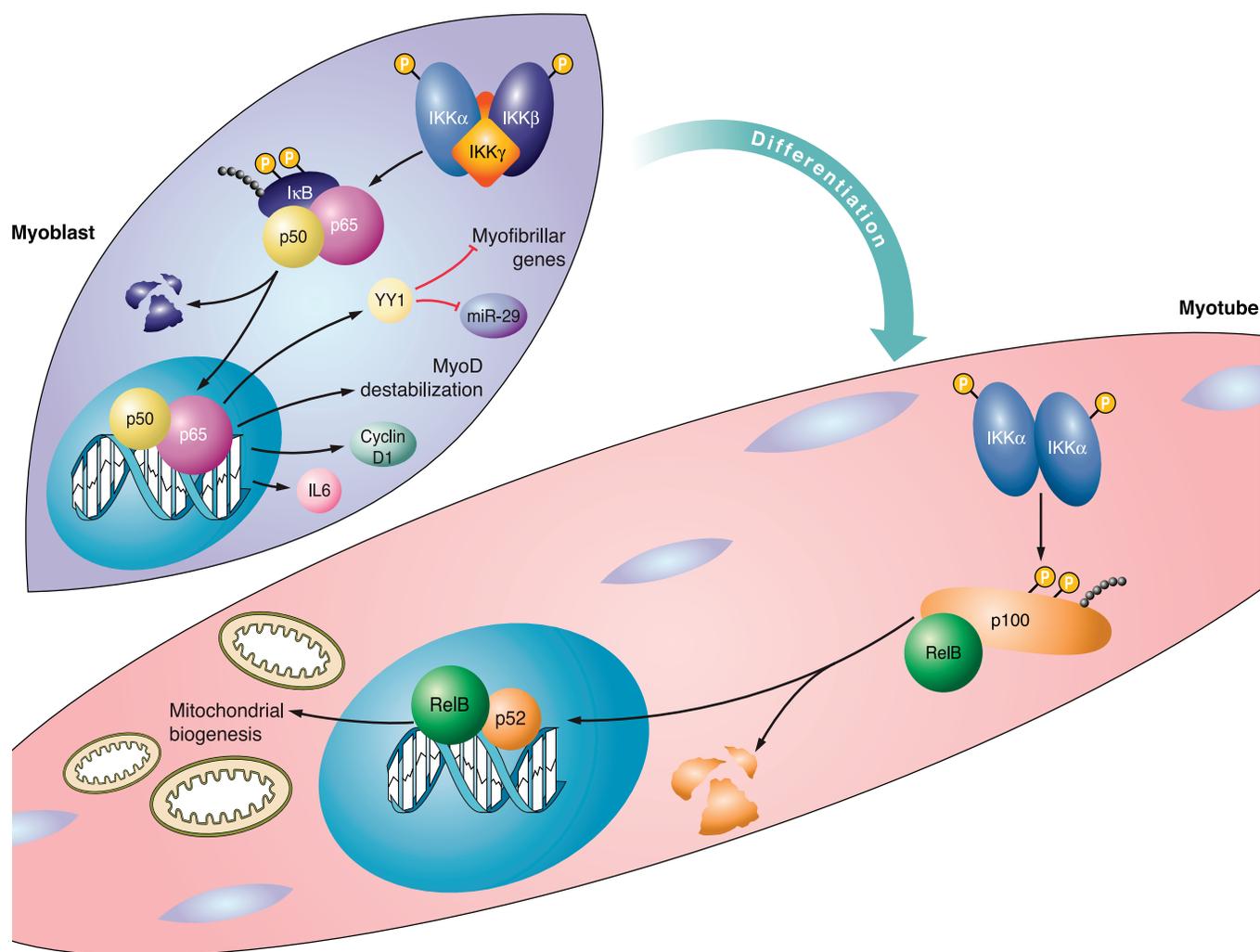


FIG. 4. A model for classical and alternative NF- κ B signaling pathways during myogenesis. In undifferentiated myoblasts, classical NF- κ B signaling, mediated by the IKK β subunit, phosphorylates the I κ B α inhibitor, targeting it for proteasomal degradation. The classical p65/p50 heterodimer can thus translocate to the nucleus to prevent premature myoblast differentiation by a number of mechanisms. Transcription of IL-6 also occurs, which serves as an opposing promyogenic signal. Once myogenic differentiation is initiated, the classical NF- κ B signaling is shut down and the alternative pathway is activated. Activated IKK α phosphorylates p100 to produce the RelB/p52 complex that functions to activate mitochondrial biogenesis and regulate the energy capacity of myotubes.

regulate the timing of myotube formation, induction of the alternative pathway appears to be dispensable for this process, and instead functions to promote mitochondrial biogenesis, presumably to ensure that sufficient ATP quantities are available to sustain myofiber contractile activity, as well as provide more efficient energy use in conditions of starvation stress. Current efforts in our laboratory are underway to elucidate the mechanism of this regulation, and further experimentation utilizing *in vivo* models of muscle regeneration will be needed to further investigate the contribution of alternative NF- κ B signaling during muscle repair.

It is noteworthy that despite the disparities among different laboratories to determine the regulation and function of NF- κ B during myogenesis, a consistent feature of all these studies is the residual amount of classical NF- κ B DNA binding and transcriptional activities that remain present in differentiated myotubes. This suggests, at least at the level of these biochemical assays, that components of this pathway might be involved in additional functions aside from negatively regulating the conversion of myoblasts to myotubes. The work from Baeza-Raja and Munoz-Canoves (7) has shown that classical NF- κ B drives the synthesis of IL-6 during myogenesis, which is necessary for proper myofiber formation (7). Along this same line, recent studies with TNF- α demonstrate that unlike high levels of this inflammatory cytokine that strongly block skeletal myogenesis and are associated with numerous muscle disorders (30, 54, 91, 109, 124), at physiological levels this cytokine actually promotes myogenesis in culture by acting through the pro-myogenic activity of the p38 MAPK (28). Since the TNF- α gene is a known transcriptional target of classical NF- κ B, it is interesting to contemplate whether the basal activity of NF- κ B found in early differentiating myoblasts may promote TNF- α production that in turn acts in an autocrine fashion through p38 to regulate the progression of the myogenic program. Such questions to test the requirement of classical NF- κ B in the regulation of these cytokines during myogenesis also await a formal in-depth study.

V. CONCLUDING REMARKS

It is now becoming clear that skeletal myogenesis is a unique differentiation model that requires a delicate balance and timing of two distinct NF- κ B signaling cascades, defined as the classical and alternative pathways. On the one hand, classical signaling maintains myoblasts in a proliferative stage and prevents their premature differentiation. Currently, it has not been formalized that this pathway is relevant during embryogenesis, although studies are underway to address this question. It will also be important to explore other components of this pathway

that function upstream of IKK, such as TBK1 and RIP. Similar to bona fide classical pathway members IKK β , IKK γ and p65, TBK1 mutant mice are embryonically lethal (12) but can be rescued with simultaneous deletion of the TNF receptor (117). It is expected that under these conditions increases in myogenesis and muscle regeneration would occur in TBK1-deficient muscle cells as was observed with p65 and IKK β (1, 9, 103, 156). Since TBK1 activates IKK in response to receptor-mediated signaling, the possibility remains that activation of classical NF- κ B signaling in myogenesis does not occur through TBK1, but rather through direct activation of the IKK complex by a yet-to-be-identified factor.

Evidence also indicates that downregulation of classical signaling coincides with the activation of the alternative pathway, which promotes mitochondrial biogenesis presumably to provide myotubes with a proper energy balance required for synthesis of myofibrillar proteins and maintenance of contractile function. The mechanism through which the alternative pathway regulates this process remains to be defined. In addition, it is unclear how the switch between classical and alternative NF- κ B signaling occurs, and whether such a switch is present during muscle regeneration, or is otherwise compromised during chronic muscle illness. Moreover, one will need to deduce whether these two signaling pathways are mutually exclusive in a differentiating muscle cell. Specifically, would inactivation of classical signaling need to occur to activate the alternative pathway, or could both pathways coexist?

One point made clear from various genetic analyses is the sharp contrast between the antidifferentiation property of classical NF- κ B in skeletal muscle and the pro-differentiation property of this transcription factor described in other differentiation systems such as hematopoiesis or keratinocyte differentiation (50, 90, 129, 133). One then wonders whether such a regulation is specific to myogenesis or if such a regulation could be extended to the differentiation of other tissues. Recent *in vitro* and *in vivo* evidence suggest that similarly to muscle formation, classical NF- κ B can also function as a repressor of differentiation in mesenchymal precursor osteoblasts (24). This suggests that the ability of the classical pathway to negatively regulate differentiation is not limited to muscle cells. As studies proceed, it will be interesting to see if parallels can be drawn between muscle differentiation and the differentiation of cell types of mesenchymal origin. Given the tight relationship between NF- κ B and skeletal muscle disorders, the hope is that information gained from understanding the role of NF- κ B in myogenesis can be translated to development of therapeutic compounds designed with the added specificity of distinguishing between classical and alternative signaling pathways.

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