Prolyl Hydroxylase EGLN3 Regulates Skeletal Myoblast Differentiation through an NF-κB-dependent Pathway

Jian Fu and Mark B. Taubman
From the Aab Cardiovascular Research Institute and Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

The egg-laying abnormal-9 (EGLN) prolyl hydroxylases have been shown to regulate the stability and thereby the activity of the α subunits of hypoxia-inducible factor (HIF) through its ability to catalyze their hydroxylation. We have previously shown that EGLN3 promotes differentiation of C2C12 skeletal myoblasts. However, the mechanism underlying this effect remains to be fully elucidated. Here, we report that exposure of C2C12 cells to dimethyl oxalylglycine (DMOG), desferrioxamine, and hypoxia, all inhibitors of prolyl hydroxylase activity, led to repression of C2C12 myogenic differentiation. Inactivation of HIF by expression of a HIF dominant-negative mutant or deletion of HIF-1α by RNA interference did not affect the inhibitory effect of DMOG, suggesting that the effect of DMOG is HIF-independent. Pharmacologic inactivation of EGLN3 hydroxylase resulted in activation of the canonical NF-κB pathway. The inhibitory effect of DMOG on myogenic differentiation was markedly impaired in C2C12 cells expressing a dominant-negative mutant of IκBα. Exogenous expression of wild-type EGLN3, but not its catalytically inactive mutant, significantly inhibited NF-κB activation induced by overexpressed TRAF2 or IκB kinase 2. In contrast, deletion of EGLN3 by small interfering RNAs led to activation of NF-κB. These data suggest that EGLN3 is a negative regulator of NF-κB, and its prolyl hydroxylase activity is required for this effect. Furthermore, wild-type EGLN3, but not its catalytically inactive mutant, potentiated myogenic differentiation. This study demonstrates a novel role for EGLN3 in the regulation of NF-κB and suggests that it is involved in mediating myogenic differentiation, which is HIF-independent.

EGLN2 prolyl hydroxylases belong to the superfamily of oxygenases that require O2, 2-oxoglutarate, and Fe2+ for their enzymatic activity (1–5). There are three mammalian EGLN prolyl hydroxylases, termed EGLN1, -2, and -3 (6, 7). Although their biologic functions remain to be fully defined, the EGLN prolyl hydroxylases have been shown to regulate the transcription factor hypoxia-inducible factor (HIF)-α (2, 3). Inhibition of EGLN3 prolyl hydroxylase activity with dimethyl oxalylglycine (DMOG), an analog of 2-oxoglutarate, desferrioxamine (DFX), an iron chelator, or hypoxia stabilizes HIF proteins (2, 3, 8). It is thought that the consequences of inadequate tissue oxygenation, such as seen in anemia, stroke, and ischemic cardiovascular diseases, might be mitigated by increased levels of HIF (9), and therefore, pharmacologic inhibitors of EGLN prolyl hydroxylase activity might have therapeutic benefits. Preclinical studies have shown that prolyl hydroxylase inhibitors are useful for treatment of anemia (10) and may protect against ischemic renal disease (11), myocardial infarction (12), stroke (13), and whole body hypoxia (14).

We previously reported that EGLN3 is up-regulated during C2C12 myogenic differentiation and promotes skeletal muscle differentiation, in part by binding to and enhancing myogenin protein stability (15). Skeletal muscle differentiation is primarily orchestrated by the MyoD family of transcription factors, including MyoD, Myf5, myogenin, and MRF4. These factors heterodimerize with ubiquitously expressed basic helix-loop-helix proteins called E proteins, and the resultant heterodimers bind to the E box present in the promoters and enhancers of muscle-specific genes, such as myosin heavy chain (MHC), activating muscle gene transcription (16–19). These myogenic regulatory factors also induce myoblasts to exit the cell cycle and fuse into multinucleated myotubes (17).

The NF-κB family consists of five members, RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) (20). In unstimulated cells, these components exist as homo- or heterodimers bound to the IκB family of inhibitors. Binding to IκBs masks the NF-κB nuclear localization signal, thereby sequestering NF-κB in the cytoplasm and maintaining it in an inactive state (21). Degradation of IκB is a prerequisite for NF-κB activation, which is initiated upon phosphorylation by activated IκB kinase (IKK). The liberated NF-κB translocates to the nucleus, where it binds to specific sequences in the promoter or enhancer regions of target genes. In addition to its well characterized functions in inflammatory and immune responses, cell proliferation, and apoptosis (22), NF-κB has been demonstrated to act as an inhibitor of skeletal muscle differentiation. NF-κB DNA binding and transcriptional activity decreases with differentiation (23, 24). Inhibition of NF-κB via expression of an IκBα dominant-negative mutant accelerates differentiation of C2C12 skeletal myoblasts (24). Activators of the NF-κB pathway such as RIP2, tumor necrotic factor-α, and interleukin-1β are potent inhibitors of myogenesis.
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(23, 25, 26). In addition, recent studies have indicated that the lack of RelA or p50 enhances skeletal myoblast differentiation (27). The underlying mechanisms for NF-κB-mediated repression of myogenesis remain to be completely elucidated. Among the mechanisms proposed have been the induction of cyclin D1 (24), the suppression of MyoD synthesis (26, 28), and the up-regulation of the transcription factor YY1, resulting in transcriptional silencing of myofibrillar genes (29, 30).

In this study, we demonstrate that EGLN3 negatively regulates NF-κB activation and that pharmacologic inhibition of hydroxylase activity enhances NF-κB activity. Most importantly, the regulation of NF-κB by EGLN3 appears to be essential for its effect on skeletal muscle differentiation. These data provide novel insights into the regulation of the NF-κB pathway and shed light on the molecular mechanism by which EGLN3 regulates myogenic differentiation.

MATERIALS AND METHODS

Antibodies and Reagents—Anti-EGLN3 and anti-HIF-1α were from Novus Biologicals (Littleton, CO). Anti-myogenin, anti-IkBα, anti-RelA, anti-IKK2, anti-IKKγ, anti-IY1, and anti-cyclin D1 were from Sigma. Anti-RhoD was from Pharmingen and Santa Cruz Biotechnology. Anti-MF20 monoclonal antibody was from the Developmental Studies Hybridoma Bank (University of Iowa). Anti-lamin A/C, anti-phospho-IkBα (Ser-32/36), anti-phospho RelA (Ser-536), anti-IKK2, anti-phospho IKK1/2, and anti-p100/p52 were from Cell Signaling Technology (Beverly, MA). DMOG was from Cayman Chemical and Santa Cruz Biotechnology. Anti-MF20 monoclonal antibody was from the Developmental Studies Hybridoma Bank (University of Iowa). Anti-lamin A/C, anti-phospho-IkBα (Ser-32/36), anti-phospho RelA (Ser-536), anti-IKK2, anti-phospho IKK1/2, and anti-p100/p52 were from Cell Signaling Technology (Beverly, MA). DMOG was from Cayman Chemical and Frontier Scientific Inc. DFX was from Calbiochem. Protease inhibitors were from Sigma and Roche Applied Science. DMOG was from Cayman Chemical and Santa Cruz Biotechnology. Anti-MF20 monoclonal antibody was from the Developmental Studies Hybridoma Bank (University of Iowa). Anti-lamin A/C, anti-phospho-IkBα (Ser-32/36), anti-phospho RelA (Ser-536), anti-IKK2, anti-phospho IKK1/2, and anti-p100/p52 were from Cell Signaling Technology (Beverly, MA). DMOG was from Cayman Chemical and Frontier Scientific Inc. DFX was from Calbiochem. Protease inhibitors were from Sigma and Roche Applied Science. Tumor necrotic factor-α was from R & D Systems (Minneapolis, MN).

DNA Constructs—The EGLN3 expression plasmids were described previously (15). Expression plasmids for the EGLN3H196R mutant, TRAF2, RIP1, IKK2, IkBα mutant, HIF-1α, and dominant-negative HIF-β mutant were kindly provided by Dr. Y. Guoo (University of Rochester), Dr. W. Min (Yale University), Dr. M. Kelliher (University of Massachusetts), Dr. W. Greene (University of California San Francisco), Dr. S. Linardopoulos (Cancer Research UK), and Dr. Y. Jin (University of Rochester), respectively. The reporter plasmids muscle creatine kinase-Luc, NF-κB-Luc, and HRE-Luc were obtained from Dr. A. Lassar (Harvard Medical School), Dr. J. Li (University of Rochester) and Dr. Y. Jin (University of Rochester), respectively.

siRNAs—EGLN3 siRNAs were described previously (15). HIF-1α siRNAs were obtained from Dharmacon and Santa Cruz Biotechnology. Control nontargeting siRNAs were purchased from Dharmacon and Ambion. siRNAs were transfected into cells by using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s protocol.

Cell Culture and Transfection—Murine C2C12 skeletal muscle myoblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal bovine serum and antibiotics (growth medium). To induce myogenic differentiation, C2C12 myoblasts at ~70–90% confluence were shifted from growth medium into Dulbecco’s modified Eagle’s medium containing 2% horse serum and antibiotics (differentiation medium). Cells were transfected with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Hypoxia was achieved by exposing cells to 1% O2, 5% CO2, and 94% N2 in an O2-regulated incubator.

Cellular Fractionation—Cells were harvested, washed with PBS, and resuspended in Buffer A (10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, and protease inhibitors). After incubation on ice for 15 min, cells were homogenized using a Dounce homogenizer. The nuclei were separated by centrifugation at 500 × g for 5 min. The supernatant was collected as cytosolic fraction. The pellet, containing the nuclei, was washed with PBS, then resuspended in radioimmune precipitation assay buffer containing 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 50 mM NaF, 1 mM Na2VO3, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture, 1 μg/ml pepstatin for 10 min on ice, centrifuged, and collected the supernatant (nuclear fraction). The cytosolic and nuclear fractions were analyzed by using Western blot.

Western Blotting Analysis—Cells were harvested in Triton X-100-based lysis buffer or radioimmune precipitation assay buffer as described previously (15, 31). The whole cell lysates were clarified, and the soluble fractions were recovered and quantified using the DC protein assay kit (Bio-Rad). The proteins were fractionated by SDS-PAGE and subjected to Western blot analysis as described previously (31).

In Vitro Kinase Assay—Whole cell extracts (250 μg) were precleared by normal rabbit IgG plus protein A-agarose, followed by immunoprecipitation with IKKγ antibody and protein A-agarose. Precipitates were washed three times in cell lysis buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na2VO3, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) and once in kinase buffer containing 25 mM Tris/HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 10 mM MgCl2. IKK activity was determined in the kinase buffer supplemented with 1 μg of GST-IkBα and 0.2 mM ATP. After 30 min of incubation at 30 °C, the reaction was stopped and analyzed by Western blotting assay for the extent of phosphorylation of IkBα with an antibody specific for phospho-IκBα.

Immunofluorescence—Cells were fixed in 4% paraformaldehyde for 15 min, permeabilized in PBST (PBS containing 0.1% Triton X-100) for 10 min, and then blocked in PBS with 10% goat serum. Cells were incubated with the primary antibody at 4 °C overnight, followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse IgG (Santa Cruz Biotechnology) or by Alexa Fluor 488 goat anti-mouse or -rabbit (H+L) for green fluorescence (Molecular Probes, Inc.) in PBS for 45 min at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (0.1 μg/ml). About 800–1000 nuclei were counted from several random fields. The fusion index was calculated as follows: (the number of nuclei within MF20-positive myocytes containing two or more nuclei/total number of nuclei analyzed) × 100. Statistical significance (p < 0.05) was determined using a two-tailed Student’s t test.
Luciferase Reporter Assay—Cells were transfected as detailed in the figure legends. Luciferase expression was detected using the Dual-Luciferase reporter assay system (Promega, Madison, WI) following the manufacturer’s instructions. The expression of firefly luciferase driven by the NF-κB-responsive element or hypoxia-responsive element (HRE) was used as reporter. pRL-tk (Renilla luciferase) was co-transfected to normalize for the transfection efficiency. Luciferase activity was expressed as a ratio of firefly luciferase activity to Renilla luciferase activity. Normalized values are reported as the mean ± S.D. from triplicate transfection. Student’s t test for paired samples was used to determine statistical significance.

RESULTS

Inhibitors of Prolyl Hydroxylases Repress C2C12 Myogenic Differentiation—Our previous report indicated that EGLN3 prolyl hydroxylase facilitated differentiation of C2C12 skeletal myoblasts (15). However, it remained to be determined whether EGLN3 catalytic activity was required.

As shown in Fig. 1A, DFX inhibited the expression of myogenin, MyoD, and MHC in a concentration-dependent manner. DFX also inhibited the expression of MyoD, myogenin, and caveolin-3, a downstream target of myogenin (32), in a time-dependent manner (Fig. 1B and C). Consistent with these observations, DFX significantly blocked myotube formation (Fig. 1D). Similarly, DMOG inhibited the expression of MyoD and myogenin in a concentration- and time-dependent manner (Fig. 2, A–D), reduced MHC expression (Fig. 2D), and dramatically blocked myotube formation (Fig. 2E).

Like the inhibitors, hypoxia also substantially down-regulated the expression levels of myogenin and MHC (supplemental Fig. 1A) and significantly suppressed myotube formation (supplemental Fig. 1B). These data suggest that the prolyl hydroxylase activity is critical for C2C12 myogenic differentiation. Because DMOG is most widely utilized to inactivate EGLN3 prolyl hydroxylases (2, 33–35), we chose to focus on this inhibitor in the remainder of this study.

HIF-1 Is Not Involved in the DMOG-mediated Inhibition of C2C12 Differentiation—Because HIFs are the best identified substrate for EGLN, we determined whether HIFs mediated the effect of prolyl hydroxylase inhibition on C2C12 differentiation. Exposure of C2C12 cells to DMOG led to increased levels of HIF-1α (Fig. 3A) and enhanced HIF transcriptional activity (Fig. 3B). The dominant-negative mutant of HIF β subunit (HIF-β DN) inhibits the transcriptional activity of both HIF-1 and HIF-2 (36). Expression of HIF-β DN had minimal effect on DMOG-induced suppression of myogenin and MHC (Fig. 3C).

In addition, two different HIF-1α siRNAs efficiently decreased HIF-1α expression (Fig. 3, D and E) but failed to abrogate the inhibitory effect of DMOG on the expression of myogenin and MHC (Fig. 3F). These results suggest that HIFs are not involved. In support of our finding, a recent study demonstrated that HIF is not involved in C2C12 differentiation (37).

Pharmacologic Inactivation of EGLN Prolyl Hydroxylase Results in Activation of the NF-κB Pathway—Having demonstrated that HIFs were not involved, we sought to identify a signal pathway that was sensitive to prolyl hydroxylase inhibitors and regulated skeletal muscle differentiation.

Short-term treatment of C2C12 cells with DMOG activated the NF-κB signaling pathway, as judged by the increase in the following: 1) IκBα degradation (supplemental Fig. 2A); 2) phosphorylation of IKK and RelA (supplemental Fig. 2B); 3) nuclear retention of RelA (supplemental Fig. 2C); and 4) expression of NF-κB target genes YY1 and cyclin D1 (supplemental Fig. 2D).

We next addressed whether longer term treatment resulted in sustained activation of NF-κB. NF-κB activation requires activation of IKK2 or IKK1 (21). As shown in Fig. 4A, treatment with DMOG greatly enhanced phosphorylation of GST-IκBα protein, indicating that it potentiated IKK2 activity. Activation of IKK2 is dependent on its phosphorylation (38) and IKK2 also catalyzes phosphorylation of RelA (39–41). As shown in Fig. 4B, treatment with DMOG induced phosphorylation of IKK and RelA and increased the nuclear accumulation of RelA protein (Fig. 4C). As expected, DMOG enhanced NF-κB transcriptional activity (Fig. 4D), as determined by luciferase assay. In keeping with this, DMOG increased the expression of cyclin D1 and YY1 (Fig. 4E), both of which have been demonstrated to
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DMOG Inhibits Myogenic Differentiation through the NF-κB Pathway—There are two well characterized NF-κB pathways as follows: the canonical pathway and the noncanonical pathway (21). IKK2 is essential and sufficient for the activation of the canonical pathway (39, 40), whereas IKK1 is required for the noncanonical pathway (20). The activation of the noncanonical pathway is characterized by the processing of p100 into p52 (42). DMOG failed to induce the processing of p100 in C2C12, as demonstrated by Western blot assay using anti-p100/p52 antibody, which can recognize both full-length p100 and the cleaved product p52 (Fig. 5A), indicating that DMOG has no effect on the activation of the noncanonical NF-κB pathway in C2C12.

To determine whether DMOG inhibits myogenic differentiation via the canonical NF-κB pathway, we employed a dominant-negative mutant of IκBα (IκBα S32A/S36A, IκBαDN) that blocks this pathway (24). Consistent with our earlier observation, DMOG inhibited the expression of myogenin, an early differentiation marker (43), and MHC, a later differentiation marker (15), in C2C12 transfected with the control vector (Fig. 5B). However, this inhibitory effect was markedly impaired in C2C12 expressing IκBαDN (Fig. 5B), indicating that activation of the canonical NF-κB pathway was involved.

Although our study and others have not supported a role for HIF-1 in the modulation of C2C12 differentiation, several studies have suggested that NF-κB is subject to regulation by HIF-1 in neutrophils or keratinocytes (44, 45). As shown in Fig. 5C, DMOG treatment induced NF-κB luciferase activity. However, this effect was not significantly altered in cells transfected with HIF-1α siRNA, suggesting that DMOG did not regulate NF-κB activity through activation of HIF-1A.
EGLN3 Functions as a Negative Regulator of the NF-κB Pathway—The above studies demonstrated that inhibitors of prolyl hydroxylase activity regulated NF-κB activation. We next sought to demonstrate more specifically that this was due to EGLN3. As shown in Fig. 6 (A and B), depletion of EGLN3 with two different siRNAs resulted in an increase in NF-κB reporter activity. Knockdown of EGLN3 also potentiated NF-κB activation by tumor necrotic factor-α (Fig. 6B), a widely used activator of NF-κB. TRAF2, RIP1, and IKK2 are important regulators of NF-κB (46, 47). As reported previously (46, 47), ectopic expression of TRAF2, RIP1, or IKK2 robustly induced the activation of NF-κB (Fig. 6, C and D).

Their effects were markedly reduced by co-expressed EGLN3. In addition, forced expression of EGLN3 led to a decrease in expression of the NF-κB target genes cyclin D1 and YY1. As shown in Fig. 6 (A and B), depletion of EGLN3 with two different siRNAs also resulted in an increase in NF-κB reporter activity. Knockdown of EGLN3 also potentiated NF-κB activation by tumor necrotic factor-α (Fig. 6B), a widely used activator of NF-κB. TRAF2, RIP1, and IKK2 are important regulators of NF-κB (46, 47). As reported previously (46, 47), ectopic expression of TRAF2, RIP1, or IKK2 robustly induced the activation of NF-κB (Fig. 6, C and D).
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Activation of NF-κB was significantly reversed by DMOG (Fig. 7A), suggesting that EGLN3 activity is required for its functionality.

EGLN3 contains a conserved iron-binding motif, of which two histidine residues (His-135 and His-196) are critical for its enzymatic activity (1); mutation of His-196 to alanine leads to loss of EGLN3 enzymatic activity (33). We employed an EGLN3 mutant, H196R, in which His-196 was replaced by arginine. The prolyl hydroxylase activity of H196R was evaluated by an HRE luciferase activity assay. This assay is based on the fact that hydroxylated HIF-1α is degraded and loses its ability to activate the HRE luciferase (48). Ectopic expression of HIF-1α in C2C12 activated the HRE luciferase reporter, whereas H196R had minimal effect (Fig. 7B), indicating that the H196R mutant has minimal hydroxylase activity.

Ectopic expression of wild-type EGLN3 greatly blunted the activity of NF-κB luciferase induced by either TRAF2 or IKK2 (Fig. 7C, top panel), whereas H196R had minimal effect (Fig. 7C, bottom panel). Most significantly, ectopic expression of wild-type EGLN3, but not H196R, increased the expression of myogenic differentiation markers such as myogenin, troponin T, and MHC in C2C12 cells (Fig. 7D). Consistent with this, EGLN3 enhanced the transcriptional activity of muscle creatine kinase luciferase, whereas H196R failed to do so (Fig. 7E). These data strongly suggest that EGLN3 enzymatic activity plays an essential role in the regulation of NF-κB and in myogenic differentiation.

DISCUSSION

Intensive studies have led to a profound understanding of the regulatory mechanisms through which muscle precursor myoblasts differentiate into myotubes (16–19). Nonetheless, more remains to be defined. In our previous study, we showed that EGLN3 promotes skeletal muscle differentiation by stabilization of myogenin protein, a crucial determinant of skeletal muscle differentiation, in part by antagonizing its polyubiquitylation and subsequent proteolysis (15). However, we did not address whether the intrinsic activity of this hydroxylase was required for this process. In this study, we show that three well documented inhibitors of prolyl hydroxylase (DMOG, DFX, and hypoxia) inhibit myogenic differentiation, as evidenced by repression of muscle-specific gene expression and suppression of myotube formation. Although advances in EGLN prolyl hydroxylase-inhibiting drug development have been made, our study suggests that they may have adverse effects on skeletal muscle. In future clinical trials, this side effect should be taken into consideration.

Several lines of evidence have suggested that skeletal myogenic differentiation is substantially inhibited under hypoxia (37, 49, 50). However, the signaling pathway for hypoxia-induced inhibition of myogenesis remains to be defined. Our data also suggest that hypoxia regulates myogenic differentiation and raise the possibility that this is due to inhibition of EGLN3 prolyl hydroxylase activity. Like EGLN prolyl hydroxylases, factor inhibiting-HIF 1 (FIH-1) asparaginyl hydroxylase belongs to the 2-oxoglutarate-dependent dioxygenase superfamily (51, 52). Intriguingly, a recent study demonstrated that FIH-1 promoted C2C12 differentiation independently of its enzymatic activity (53). Therefore, it is unlikely that the effect of DMOG on C2C12 differentiation is due to inhibition of FIH-1. HIFs are the best characterized targets for EGLN3 prolyl hydroxylase. We found that inactivation or ablation of HIF had no significant impact on DMOG-mediated inhibition of C2C12 differentiation. These data would support the hypothesis that inhibition of prolyl hydroxylase activity may regulate skeletal muscle differ-
entiation through an EGLN3-dependent but HIF-independent mechanism. Interestingly, EGLN3 mediates apoptosis of neurons through a mechanism that is dependent upon its hydroxylase activity but independent of HIF-1 or -2 (33).

Many activators of the NF-κB pathway have been identified thus far, whereas relatively few negative regulators were reported. A major finding of this study is that EGLN3 is a novel negative regulator of NF-κB in C2C12. This negative regulation is dependent upon its prolyl hydroxylase activity, because the prolyl hydroxylase inhibitor DMOG activated the canonical NF-κB pathway and, unlike wild-type EGLN3, a catalytically inactive mutant H196R had no significant effect on NF-κB activity. The molecular mechanism by which EGLN3 prolyl hydroxylase regulates the canonical NF-κB pathway remains to be determined. Of note, EGLN2 was suggested to be an inhibitor of the NF-κB pathway in cancer cells by an undefined mechanism (54). A detailed understanding of how EGLN hydroxylase negatively modulates the NF-κB pathway may provide important insight into the complex regulation of this pathway. It may also shed light on how this regulation affects the function of NF-κB in a variety of diseases that are associated with constitutive NF-κB activity.

NF-κB has been shown to be a negative regulator of skeletal muscle differentiation (23–28). This study provides a direct link between EGLN3-mediated NF-κB repression and myogenic differentiation. We have previously reported that EGLN3 promotes skeletal muscle differentiation in part by binding to and stabilizing myogenin protein (15). We therefore propose that EGLN3 promotes myogenic differentiation through two different mechanisms, as summarized in Fig. 8. One is through stabilization of myogenin, a positive regulator of myogenesis, and the other is through suppression of NF-κB, a negative regulator of myogenic differentiation. Further studies will be

![FIGURE 7. Effect of the EGLN3 catalytic-inactive mutant on NF-κB activity and myogenesis. A, C2C12 cells were co-transfected with an NF-κB reporter, pRL-tk, and the indicated expression plasmids. After 24 h, cells were treated with 1 mM DMOG as indicated for 24 h. The luciferase activities were measured and normalized to that of Renilla luciferase (top panel). *, *p < 0.05; **, *p < 0.01. The expression of transfected cDNAs was measured by immunoblotting (IB) (bottom panel). B, C2C12 cells were co-transfected with an HRE-luc reporter, pRL-tk, and the indicated expression plasmids for 28 h. The luciferase activities were measured and normalized (top panel). The expression of the transfected genes was evaluated by immunoblotting (bottom panel). C, C2C12 cells were co-transfected with an NF-κB luciferase reporter, pRL-tk, and the indicated expression plasmids for 28 h. The luciferase activities were measured and normalized (left panel). The protein levels of transfected genes were determined by immunoblotting (right panel). D, C2C12 cells were transfected with FLAG-tagged wild-type EGLN3 or EGLN3 H196R mutant as indicated. One day later, cells were shifted into differentiation medium for additional 48 h. Western blot analysis of whole cell lysates was performed with anti-MF20 antibody (for MHC), anti-myogenin, anti-troponin T, or anti-FLAG antibody (for EGLN3 or H196R). Tubulin was used as the loading control. E, C2C12 cells were co-transfected with a muscle creatine kinase luciferase reporter, pRL-tk and the indicated expression plasmids, followed by incubation in differentiation medium for 40 h. The luciferase activities were measured and normalized. The protein levels of transfected genes were determined by immunoblotting.](https://example.com/fig7.png)
necessary to elucidate the relative contributions of these pathways and to determine under what circumstances these pathways are activated in skeletal muscle.

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