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Interactions between NF-κB and SP3 Connect Inflammatory Signaling with Reduced FGF-10 Expression*

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Background: Inflammation and NF-κB activation inhibit FGF-10 expression and lung development by an unknown mechanism.
Results: In the presence of NF-κB activation, SP3 functions as a transcriptional repressor, inhibiting SP1-mediated FGF-10 transcription.
Conclusion: NF-κB can regulate developmental gene expression by recruiting inhibitory transcription factors to gene promoters.
Significance: Interaction between NF-κB and Sp proteins identifies a mechanism by which inflammation inhibits normal developmental programs.

Inflammation inhibits normal lung morphogenesis in preterm infants. Soluble inflammatory mediators present in the lungs of patients developing bronchopulmonary dysplasia disrupt expression of multiple genes critical for development. However, the mechanisms linking innate immune signaling and developmental programs are not clear. NF-κB activation inhibits its expression of the critical morphogen FGF-10. Here, we show that interactions between the RELA subunit of NF-κB and SP3 suppress SP1-mediated FGF-10 expression. SP3 co-expression reduced SP1-mediated *Fgf-10* promoter activity, suggesting antagonistic interactions between SP1 and SP3. Chromatin immunoprecipitation of LPS-treated primary mouse fetal lung mesenchymal cells detected increased interactions between SP3, RELA, and the *Fgf-10* promoter. Expression of a constitutively active IκB kinase β mutant not only decreased *Fgf-10* promoter activity but also increased RELA-SP3 nuclear interactions. Expression of a dominant-negative IκB, which blocks NF-κB nuclear translocation, prevented inhibition of FGF-10 by SP3. The inhibitory functions of SP3 required sequences located in the N-terminal region of the protein. These data suggested that inhibition of FGF-10 by inflammatory signaling involves the NF-κB-dependent interactions between RELA, SP3, and the *Fgf-10* promoter. NF-κB activation may therefore lead to reduced gene expression by recruiting inhibitory factors to specific gene promoters following exposure to inflammatory stimuli.

During fetal lung development, spatially restricted expression of mesenchymal growth factors stimulates cell proliferation, elongation of epithelial tubes, and expansion of newly formed airways (1). FGF-10 is expressed in the lung mesenchyme from the earliest stages of development and is critical for lung formation. By activating its receptor FGFR2b on adjacent epithelial cells, FGF-10 stimulates both proliferation and airway branching (2, 3). Mice lacking either FGF-10 or FGFR2b develop only rudimentary lung structures containing tracheas but lacking bronchial airways (2, 4). During later stages of lung development, transgenic expression of an FGF-10 antagonist or addition of inhibitory antibodies disrupts normal airway elongation and branching (5–7). Although many genes play important roles in lung development, data clearly implicate FGF-10 as a major regulator of lung morphogenesis.

Defects in *Fgf-10* expression contribute to lung disease in both children and adults. Preterm infants with severe bronchopulmonary dysplasia have reduced saccular airway and alveolar duct formation and have lower FGF-10 expression in their lungs (8, 9). In adult patients, *Fgf-10* haploinsufficiency can lead to abnormal pulmonary function and chronic obstructive pulmonary disease (10). In both chronic obstructive pulmonary disease and bronchopulmonary dysplasia, lung inflammation plays a key role in disease pathogenesis and also inhibits FGF-10 expression (11–13). Inflammatory signaling may therefore interfere with the mechanisms regulating FGF-10 expression and formation and maintenance of normal lung architecture.

Microbial products and inflammatory mediators stimulate lung inflammation by binding pattern recognition receptors on the surface of cells (14, 15). Although the expression of multiple receptors provides a diverse detection repertoire at the cell surface, many of these receptors signal through overlapping intracellular pathways that activate the transcription factor NF-κB (10, 16). In quiescent cells, NF-κB resides in the cytoplasm bound to IκB. When cell surface receptors detect inflammatory

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stimuli, IκB kinase β (IKKβ)[2] phosphorylates IκB, displacing it from NF-κB and leading to IκB degradation (17, 18). NF-κB is then free to traffic into the nucleus, where it regulates gene transcription. In addition to driving the acute innate immune response, the NF-κB signaling pathway also influences wound repair, tumor formation, and tissue morphogenesis (19–23).

Because inflammation plays a major role in the pathogenesis of bronchopulmonary dysplasia, we previously investigated how innate immunity and NF-κB activation in the fetal lung affects expression of genes important for normal development. NF-κB activation by microbial products or inflammatory mediators inhibits FGF-10 expression in the fetal mouse lung, leading to alterations in normal airway formation (13).

NF-κB most commonly acts as a transcriptional activator; the mechanisms by which NF-κB can reduce gene transcription are less well characterized. Inhibition of FGF-10 does not involve direct interaction between NF-κB and canonical DNA binding elements in the *Fgf-10* promoter (24). We hypothesized that NF-κB might therefore inhibit FGF-10 expression by regulating the activity of other transcription factors.

The *Fgf-10* promoter lacks a TATA box sequence but contains multiple conserved GC-rich regions predicted to bind Sp proteins. SP1 is a potent activator of TATA-less gene transcription. The related Sp family member SP3 can act as both a transcriptional activator and repressor, depending on cellular context (25). We previously showed that SP1 drives FGF-10 transcription and that this effect can be inhibited by NF-κB activation (8). However, it was not clear how NF-κB might inhibit SP1-mediated transcription in the absence of conserved predicted NF-κB binding sites. Here, we show that NF-κB activation recruits Sp3 to the *Fgf-10* promoter, where it functions as a transcription inhibitor. This novel mechanism may provide new insight into how inflammation can alter expression of developmentally important genes.

EXPERIMENTAL PROCEDURES

Reagents—Gel-purified *Escherichia coli* LPS (O55:B5) was purchased from Sigma-Aldrich. Recombinant IL-1β was purchased from R&D Systems. The IKKβ inhibitor BMS-345541 was purchased from EMD Biosciences (San Diego, CA). Antibodies against RELA, SP1, and SP3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-FLAG was purchased from Sigma. Rabbit anti-GFP was purchased from Millipore (Bedford, MA). Rat anti-E-cadherin and Alexa Fluor-conjugated secondary antibodies were procured from Invitrogen CHO-K1 cells were obtained from ATCC and cultured as directed in Ham's F-12 medium supplements with 10% fetal bovine serum from Atlanta Biologicals (Atlanta, GA).

Plasmids—The FGF-10 luciferase reporter in the pXPI vector was generously supplied by Benoit Bruneau (Gladstone Institute of Cardiovascular Disease, San Francisco, CA). This construct contains ~6-kb BamHI fragment from the murine *Fgf-10* gene placed immediately upstream of the luciferase coding sequence. Serial truncations were made using PCR mutagenesis. Deletions were confirmed by restriction digest and sequencing. The *Sp1* and *Sp3* expression plasmids were generated by inserting the murine cDNA for these genes into the pcDNA3.1/CT-GFP vector (Invitrogen). Mutations made within the *Sp3* coding sequence were produced using the QuikChange II XL mutagenesis system (Agilent, Palo Alto, CA) and were confirmed by sequencing. *Fgf-10* luciferase reporter constructs were co-transfected into CHO cells using SuperFect (Qiagen, Velencia, CA). pSV-β-Gal was used to control for transfection efficiency. Cells were lysed after 48 h, and luciferase activity measured by Steady-Glo system (Promega, Madison, WI) on a microtiter plate with a luminometer. Arbitrary light units were normalized to β-galactosidase activity.

Immunostaining, Imaging, and Analysis—To quantify mean fluorescent intensity in explanted fetal mouse lung, optical sections of explants immunostained for SP1 and SP3 were collected on an Olympus FV1000 laser scanning confocal microscope (Olympus, Melville, NY). The images were then imported into SlideBook (Intelligent Imaging Innovations, Inc., Ringsby, CT). Individual nuclei were selected, and their mean fluorescent intensity for both channels was quantified.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was performed using primary fetal mouse lung mesenchymal cells (26). Control and LPS-treated cells were fixed with 1% formaldehyde and lysed. DNA was sheared by sonication, phenol:chloroform extracted, and precipitated with ethanol. Samples were then incubated with anti-SP1, anti-SP3, and anti-RELA antibodies for immunoprecipitation. Following stringent washing, DNA-protein linkages were disrupted, and released DNA fragments were phenol:chloroform extracted and precipitated with ethanol. The 320-bp region of the *Fgf-10* promoter (upstream of and including the transcriptional start site) was detected by PCR. Products were analyzed by agarose gel electrophoresis.

DNA-based ELISA—Quantitative protein assay for SP1 and SP3 was performed using TransAm transcription factor ELISA (Active Motif, Carlsbad, CA). Control and LPS-treated 3T3 cells were grown in culture to 90% confluency, harvested, and lysed by hypotonic buffer and 0.5% Nonidet P-40. Nuclei were pelleted, and equal amounts of nuclear proteins were applied to wells coated with oligonucleotide containing consensus binding sequences for SP1 or SP3. After incubation with primary antibodies that recognize SP1 and SP3 only in their DNA-bound states, an HRP-conjugated secondary antibody provided quantitative detection of SP1 and SP3 by spectrophotometry. 5 μg of nuclear extract from MCF-7 cells, supplied by the manufacturer, was used as a positive control. Wells without nuclear extract were used as negative controls.

In Situ Proximity Ligation Assay—CHO cells were transfected with plasmids expressing FLAG-tagged RELA and constitutively active IKKβ mutant (cIKKβ), dnIκB, or empty vector. Cells were fixed 24 h after transfected in 4% paraformaldehyde before being permeabilized with 0.1% Triton X-100 (Pierce) and blocked with normal donkey serum (Sigma). Interactions between FLAG-RELA and SP3 were measured using the Red Duolink II *in situ* proximity ligation assay (PLA) kit (OLink Bioscience, Uppsala, Sweden). Cells were incubated with anti-FLAG and anti-SP3 primary antibodies overnight. After wash-

[2] The abbreviations used are: IKKβ, IκB kinase; cIKKβ, constitutively active IKKβ mutant β; dnIκB, dominant-negative isoform of IκB; PLA, proximity ligation assay; CHO, Chinese hamster ovary cells.

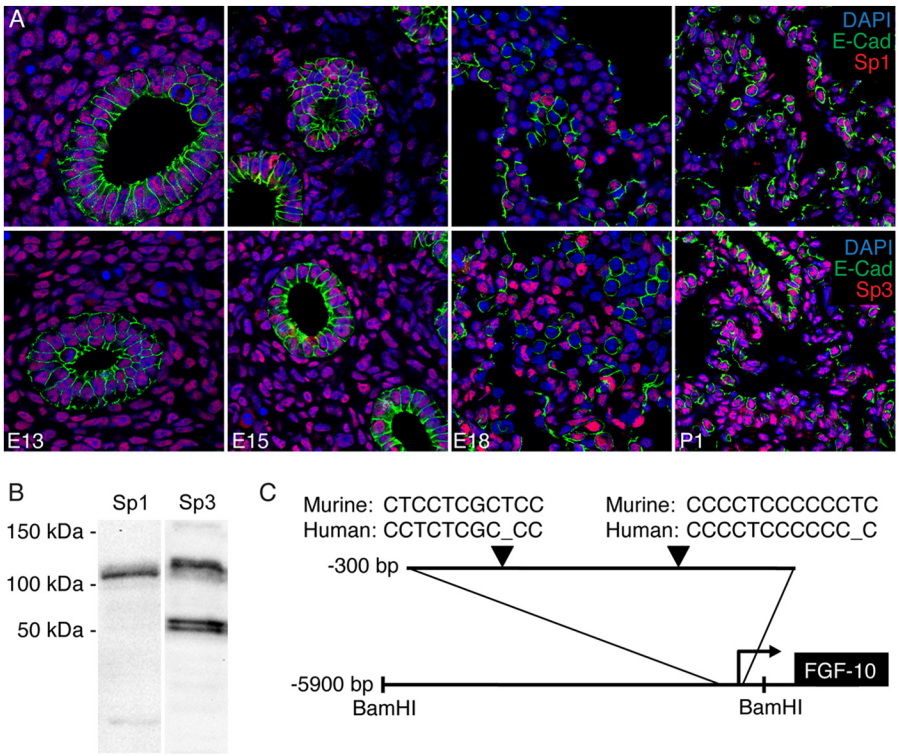

FIGURE 1. **SP1 and SP3 expression in the fetal mouse lung.** *A*, immunostaining of lung sections from embryonal day 13 (*E13*) through postnatal day 1 (*P1*) mice using antibodies against SP1 (*top panels*) or SP3 (*bottom panels*). Airway epithelial cells were immunostained using an antibody against E-cadherin (*E-Cad*), and nuclei were labeled with DAPI. *B*, immunoblot of total fetal mouse lung homogenate using antibodies against SP1 (*left*) and SP3 (*right*). *C*, sequence comparison of murine and human *Fgf-10* promoter regions, showing conserved GC-rich regions upstream of the transcriptional start site.

ing with PBS, the cells were incubated with PLA anti-mouse MINUS and PLA anti-rabbit PLUS for 1 h at 37 °C in a humidified chamber. After washing, the ligation, polymerization, and hybridization steps of the Duolink II protocol were carried out as instructed by the manufacturer. Slides were mounted in medium containing DAPI. Control cells were not incubated with the anti-FLAG antibody. Mounted cells were imaged using an inverted Olympus BX-81 fluorescence microscope and analyzed using SlideBook software.

RESULTS

SP3 Is Expressed throughout Development in the Fetal Lung—We hypothesized that NF-κB inhibits FGF-10 transcription by interfering with other transcriptional activators. Because SP1 interacts with GC-rich sequences, such as those found in the *Fgf-10* promoter (Fig. 1*C*) and stimulates *Fgf-10* promoter activity, we first tested whether SP1 and SP3 might both regulate FGF-10. SP3 binds similar GC-rich regions as SP1 but can function as a transcriptional inhibitor. Similar to SP1, SP3 was expressed throughout fetal mouse lung development in both epithelial and mesenchymal cell populations (Fig. 1*A*). Expression appeared heterogenous, with higher levels of nuclear staining in some individual cells compared with others. Immunoblotting confirmed expression in the fetal mouse lung with the four previously identified SP3 isoforms present in fetal lung homogenate and in primary fetal lung mesenchymal cell lysates (Fig. 1*B*).

SP3 Decreases SP1-mediated Fgf-10 Promoter Activity—To test whether SP3 could inhibit *Fgf-10* transcription, we employed an FGF-10 luciferase reporter plasmid containing a 6-kb region of the mouse *Fgf10* gene that included the transcriptional start site and 5′-UTR. In transfected CHO cells, expression of increasing amounts of mouse Sp3 cDNA did not change basal FGF-10 luciferase activity (Fig. 2*A*). As we demonstrated previously, Sp1 increased FGF-10 luciferase expression, but co-expression with increasing *Sp3* inhibited this activation in a concentration-dependent manner, returning reporter expression to basal levels (Fig. 2*A*). These data suggested that SP1 and SP3 have opposing functions on *Fgf-10* transcription.

NF-κB Activation Recruits SP3 to the Fgf-10 Promoter—Because LPS and inflammatory mediators that signal through NF-κB reduce FGF-10 expression, we tested whether NF-κB activation might increase the amount of SP3 present at the *Fgf-10* promoter. We treated primary fetal lung mesenchymal cells with LPS and performed ChIP analysis using antibodies against SP1, SP3, and the NF-κB subunit RELA (Fig. 2*B*). In control cells, we detected SP1-FGF-10 interactions; faint bands were also detected in samples precipitated with antibodies against SP3 and RELA. However, in LPS-treated cells, interactions between all three proteins and the *Fgf-10* promoter region were strongly detected. We obtained similar results using a molecular approach to increase NF-κB activation. Expressing a cIKKβ in which serines 177/181 are replaced with negatively charged glutamate residues) (27) increased SP3-FGF-10 interactions in CHO cells (Fig. 2*C*), further supporting the possible connection between SP3 and NF-κB in regulating the *Fgf-10*

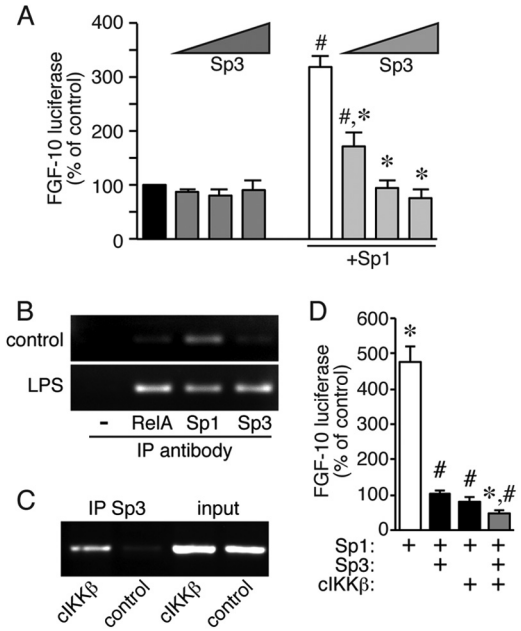

FIGURE 2. **SP1 and SP3 antagonistically regulate *Fgf-10* promoter activity.** *A*, CHO cells were transfected with FGF-10 luciferase and increasing concentrations of *Sp3* cDNA in the absence or presence of *Sp1* cDNA. SP1 expression increased FGF-10 luciferase activity, and this effect was inhibited by SP3. #, $p < 0.01$ compared with control. *, $p < 0.01$ compared with SP1 only ($n = 6$). *B*, ChIP analysis of the *Fgf-10* promoter in primary fetal mouse lung mesenchymal cells. Cells were cultured in the absence or presence of LPS (250 ng/ml) for 4 h. DNA·protein complexes were immunoprecipitated with antibodies against RELA, SP1, SP3, or rabbit IgG. The immunoprecipitated DNA was amplified by PCR using primers flanking 350 bp of the *Fgf-10* promoter immediately upstream of the transcriptional start site. *C*, chromatin immunoprecipitation from CHO cells expressing either cIKKβ or GFP control plasmid. Anti-SP3 antibodies used as in *B*. Input DNA are included on the *right*. *D*, expression of either SP3 or calKKβ (*black bars*) inhibited SP1-activated FGF-10 luciferase activity. Co-expression of both SP3 and calKKβ (*gray bar*) had an additive inhibitory effect. #, $p < 0.01$ compared with SP1 only. *, $p < 0.01$ compared with control ($n = 4$).

promoter. We next tested the functional effects of both SP3 and increased IKKβ activity. SP3 and cIKKβ separately inhibited SP1-mediated *Fgf-10* promoter activity and co-expressing SP3 and cIKKβ appeared to have an additive inhibitory effect (Fig. 2*C*). Collectively, the ChIP and co-expression data suggested that SP3 might play a role in NF-κB-dependent *Fgf-10* inhibition.

SP1 and SP3 Levels Are Not Altered by NF-κB Activation—LPS and NF-κB activation could reduce *Fgf-10* transcription by changing the relative levels of SP1 and SP3. As seen in Fig. 3, activation of NF-κB by treating fetal lung mesenchyme with LPS (Fig. 3*A*) or CHO cells with recombinant IL-1β (Fig. 3*B*) did not change the amount of SP3 in nuclear extracts based on immunoblotting. Importantly, we also did not observe any differences in the relative abundance of each of the four naturally occurring SP3 isoforms. Treating CHO cells with recombinant IL-1β also did not cause changes in SP1 or SP3 protein concentration in nuclear extracts as measured by DNA-based ELISA (Fig. 3*C*). We next tested this hypothesis by immunostaining control and LPS-treated lung explants from embryonic day 15 fetal mice. In Fig. 3*D*, SP1 and SP3 expression appear similar in control and LPS-treated explants. Quantification of nuclear fluorescence intensity in cells located in the mesenchyme showed that LPS caused small changes in both SP1 and SP3

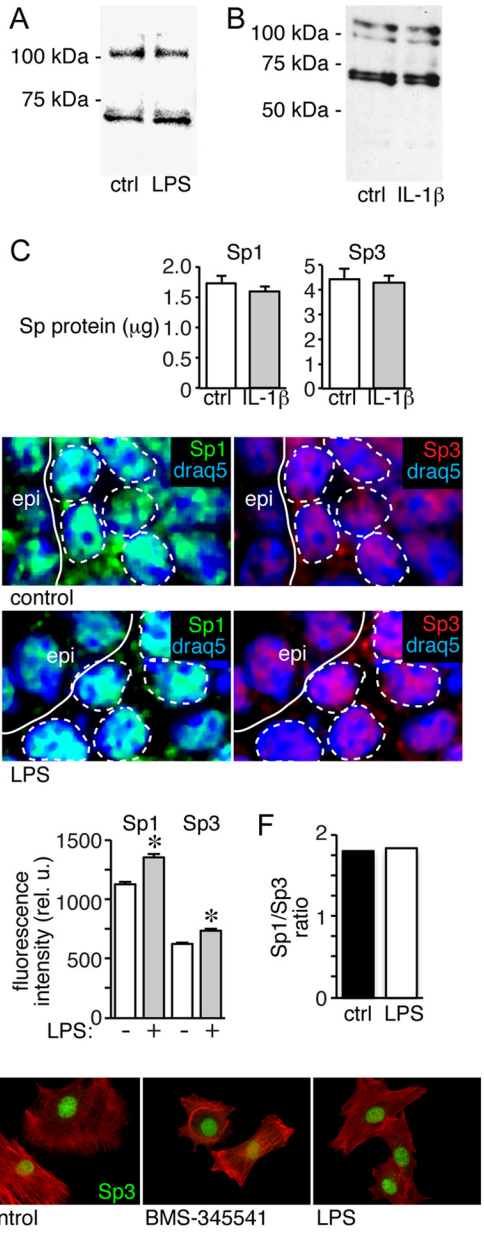

FIGURE 3. **NF-κB activation does not alter relative SP1 and SP3 expression levels.** *A* and *B*, representative SP3 immunoblot from control (*ctrl*) and LPS-treated mouse fetal lung mesenchymal cells (*A*) and control and IL-1β-treated CHO cells (*B*), showing no differences in SP3 expression level or relative isoform abundance. *C*, the protein levels of SP1 (*left*) and SP3 (*right*) in control and IL-1β-treated CHO cell nuclei were measured using DNA-binding ELISA. *D*--*F*, control and LPS-treated embryonic day 15 fetal mouse lung explants were immunolabeled with antibodies against SP1 and SP3. Cell nuclei within the mesenchyme were identified by confocal microscopy. Sum fluorescence intensity in ~700 cell nuclei was quantified in control and LPS-treated samples. LPS increased both SP1 and SP3 expression similarly (*E*; *, $p < 0.01$) and had no net effect on the SP1/SP3 ratio. epi, epithelial cells. *G*, NF-κB activation did not alter SP3 subcellular localization. Primary fetal mouse lung mesenchymal cells were immunolabeled with antibodies against endogenous SP3 following treatment with the IKKβ inhibitor BMS-345541 or LPS. Cells were counterstained with phalloidin (*red*) and DAPI (*blue*). *rel. u.*, relative units.

(Fig. 3, *E* and *F*) but did not appear to change the relative SP1:SP3 ratio. In addition, LPS and the IKKβ inhibitor BMS345541 also did not change the relative expression or subcellular localization of SP3 in primary fetal mouse lung mesenchymal cells (Fig. 3*G*). These data suggest the effects of NF-κB on SP-medi-

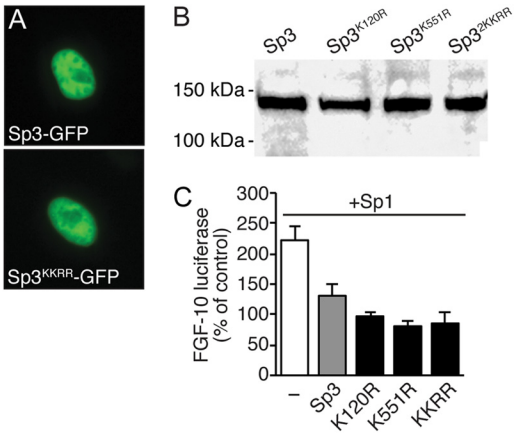

FIGURE 4. **Mutation of SP3 SUMOylation sites does not alter ability of SP3 to inhibit *Fgf-10* promoter activity.** CHO cells were transfected with SP3 mutants containing single amino acid mutations (K120R and K551R) or mutation of both SUMOylation sites (KKRR). *A*, SP3-GFP and $SP3^{KKRR}$-GFP localized to the cell nucleus when expressed in CHO cells. *B*, immunoblot of transfected CHO cells showed that each SP3 SUMOylation mutant was expressed in CHO cells at the predicted molecular weight. *C*, mutation of SUMOylated lysine residues in SP3 did not affect the ability of SP3 to inhibit SP1-mediated *Fgf-10* promoter activity ($n = 8$).

ated gene expression in the fetal lung mesenchyme did not involve dramatic changes in protein levels or subcellular localization.

Modification by SUMO Is Not Required for SP3 to Regulate FGF-10—The inhibitory effects of SP3 on the *Fgf-10* promoter activity could involve post-translational modifications. SUMOylation has been reported to switch SP3 from a transcriptional activator to a repressor (28). We therefore mutated the lysine residues in SP3 shown to be SUMO-modified and tested SP3 repressor function (Fig. 4, *A* and *B*). Mutant SP3 lacking either one or both SUMO sites was still able to inhibit *Fgf-10* promoter activity, suggesting that SUMOylation does not play a role in the ability of SP3 to repress FGF-10 expression (Fig. 4*C*).

SP3 Interacts with the RELA Subunit of NF-κB—We next tested whether activation and nuclear import of NF-κB subunits could recruit SP3 to the *Fgf-10* promoter and inhibit transcription. Using an *in situ* proximity ligation assay that detects colocalization of two proteins within 40 nm, we tested whether RELA and SP3 colocalize within cell nuclei (Fig. 5). RELA-SP3 colocalization was detected in control cells by quantifying fluorescent intensity in individual cell nuclei (Fig. 5*A*). Transfection with cIKKβ increased sum fluorescence ~3-fold (Fig. 5*B*). We next tested the effect of expressing a dominant-negative IκB, which cannot be phosphorylated by IKK and therefore prevents NF-κB nuclear import. In cells transfected with dnIκB, we did not detect RELA-SP3 colocalization above background levels (Fig. 5, *C–E*). Spatial interaction between RELA and SP3 therefore correlates with NF-κB activation.

To determine whether SP3 requires NF-κB to down-regulate FGF-10, we co-transfected Sp3 and dnIκB into CHO cells with an FGF-10 luciferase reporter. When NF-κB activation was inhibited with dnIκB expression, SP3 did not decrease *Fgf-10* promoter activity but instead activated FGF-10 expression (Fig. 5*F*). These data suggest that nuclear import of activated of RELA recruits SP3 to the FGF-10 promoter where it then inhibits FGF-10.

The N-terminal Region of SP3 Is Required for FGF-10 Inhibition—We next determined whether each of the four translation-dependent isoforms of SP3 were equally able to inhibit FGF-10 expression. The ATG start sites in *Sp3* were mutated so that each plasmid expressed only a single *Sp3* isoform. Each isoform had similar nuclear localization and was expressed at the appropriate molecular weight (Fig. 6, *A* and *B*). Although expression of the longest isoform reduced *Fgf-10* promoter activity, the shortest SP3 isoform, which lacks the N-terminal region, had no effect even at the highest cDNA concentration (Fig. 6*C*). We next tested two additional N-terminal SP3 mutants. SP3 lacking the N-terminal 149 amino acids ($SP3^{2.5}$) and SP3 lacking the conserved Sp region located between amino acids 44–90 ($SP3^{\Delta SP}$) localized to the cell nucleus. However, both $SP3^{2.5}$ and $SP3^{\Delta SPBox}$ had reduced ability to inhibit SP1-mediated *Fgf-10* promoter activity (Fig. 6, *D* and *E*). These data suggest that the inhibitory function of SP3 requires structural elements located at the N terminus of the protein.

DISCUSSION

Our data show that NF-κB activation decreases FGF-10 expression by altering Sp protein-mediated transcription. We determined that NF-κB activation increases interactions between the transcription factor SP3 and the *Fgf-10* promoter, decreasing FGF-10 expression. SP3 is incapable of down-regulating *Fgf-10* without NF-κB nuclear translocation, and the N terminus of the SP3 protein is required for this effect. These findings identify an important molecular mechanism regulating FGF-10 expression.

In extremely preterm infants that develop bronchopulmonary dysplasia, inflammation disrupts the normal developmental programs that control lung morphogenesis. Although the connections between inflammatory signals and altered development have long been suggested, the mechanisms linking immunity and development have been less clear. Within the developing lung mesenchyme, microbial products and cytokines activate NF-κB and inhibit FGF-10 expression and prevent normal epithelial-mesenchymal interactions during airway formation (8, 13). Our results here reveal a novel mechanism linking inflammation-mediated NF-κB activation and abnormal transcriptional regulation of *Fgf-10*.

The NF-κB subunit RELA interacts with SP3 to inhibit FGF-10 transcription. This inhibitory function could occur in one of several ways. RELA could act as a molecular chaperone within the nuclear microenvironment, recruiting SP3 to the *Fgf-10* locus. In cells expressing FGF-10 in the absence of inflammatory stimuli, RELA is mostly cytoplasmic. Interaction of SP3 with the *Fgf-10* promoter is minimal and FGF-10 expression is high due to the stimulatory function of SP1. However, when cells are exposed to inflammatory signals, the nuclear transport of RELA increases SP3 levels at the *Fgf-10* promoter, inhibiting Sp1-mediated transcription. As part of this model, RELA-SP3 complexes could specifically interact with the *Fgf-10* promoter (as well as other Sp-regulated genes also inhibited by NF-κB).

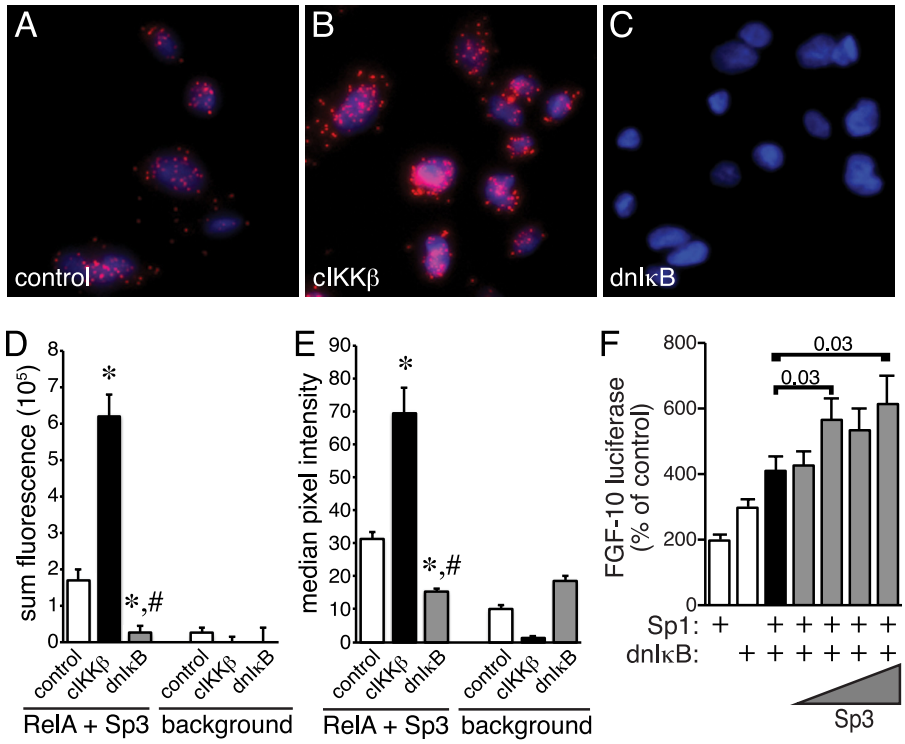

FIGURE 5. **Interactions between SP3 and nuclear RELA regulate *Fgf-10* promoter activity.** *A–C*, evidence for SP3-RELA interactions using an *in situ* PLA. CHO cells were transfected with FLAG-tagged RELA and either control empty vector (*A*), a cIKK*β* (*B*), or a dominant-negative IκB mutant that prevents nuclear RELA translocation (*C*). Following fixation and processing, red fluorescence was visualized at sites where SP3 and RELA-FLAG localized to within 40 nm of each other. Nuclei were labeled with DAPI. Fluorescence microscopy images were acquired from 60–70 cells within each group. Expression of cIKK*β* increased both the sum fluorescence intensity (*D*) and median pixel intensity (*E*), consistent with increased RELA-SP3 colocalization (*, $p < 0.01$). Expression of dnIκB reduced *in situ* PLA signal to background levels. *F*, increasing levels of *Sp3* (0.1, 0.2, 0.4, or 0.8 *μ*g) did not inhibit SP1-mediated *Fgf-10* promoter activity when CHO cells were co-transfected with dnIκB ($n = 6$).

Alternatively, SP3-RELA interactions could cause functional switching. SP3 appeared to activate *Fgf-10* transcription when NF-κB was inhibited. Therefore, interacting with RELA may turn SP3 into a transcriptional repressor. Additionally, NF-κB activation and binding of RELA to the *Fgf-10* gene could change the structure or modification of the *Fgf-10* promoter-enhancer region, leading to increased SP3 binding and perhaps preventing SP1 from promoting transcription. These types of interactions may help explain how transcription factors such as NF-κB can function as both activators and repressors given different genetic contexts.

Examples of transcription factors functioning both as activators and repressors are found throughout molecular biology (29). The retinoic acid receptor family of retinoid x receptor/retinoic acid receptor complexes normally functions as co-repressors, inhibiting transcription. In the presence of ligand, additional co-activators are recruited and promote gene expression. In this example, the retinoid x receptor/retinoic acid receptor acts somewhat as a molecular scaffold, allowing ligand-dependent recruitment of additional factors (30, 31). For genes regulated by Wnt and *β*-catenin signaling, activated *β*-catenin can switch T cell factor family transcription factors from repressors to activators (32, 33). *β*-Catenin also promotes exchange of repressor T cell factor proteins for activating family members (34). This exchange of activating and inhibiting transcription factors has similarities to the one we propose here. Activated RELA increases the localization of the repressor SP3 at the *Fgf-10* promoter that is occupied primarily by SP1 in the absence of inflammatory stimuli. As many genes contain GC boxes in their promoter regions, transcriptional repression by RELA-SP3 complexes could target more genes than just FGF-10.

The inhibitory function of SP3 requires the N-terminal peptide region of the protein. Expression of SP3 isoforms lacking the amino-terminal 302 amino acids did not inhibit *Fgf-10* promoter activity, and a smaller truncation of the 149 N-terminal region resulted in an intermediate inhibitory ability of SP3. Within the SP3 N-terminal domain, deletion of the conserved Sp box reduced but did not completely abolish inhibitory function. The differential function of the various SP3 isoforms is particularly interesting. Expressed from a single, full-length mRNA, internal ATG start sites produce peptides of 60, 62, 100, and 102 kDa (35). As each isoform appears to be expressed in all cell types examined to date, the differential roles of short and long SP3 peptides are unknown (36, 37). As Sp box domains are described in all Sp family members, the potentially unique properties of the SP3 Sp box are not clear (38). Future experiments will better define this region and how it might regulate interactions between SP3 and other transcription factors, including RELA and SP1.

NF-κB can activate and inhibit gene expression by distinct mechanisms. For activation of innate immunity genes, NF-κB directly binds to the gene promoter via a consensus NF-κB binding sequence (39). In addition, members of the NF-κB family possess a transactivating domain that can recruit additional transcription factors to discrete regulatory sites (40). NF-κB

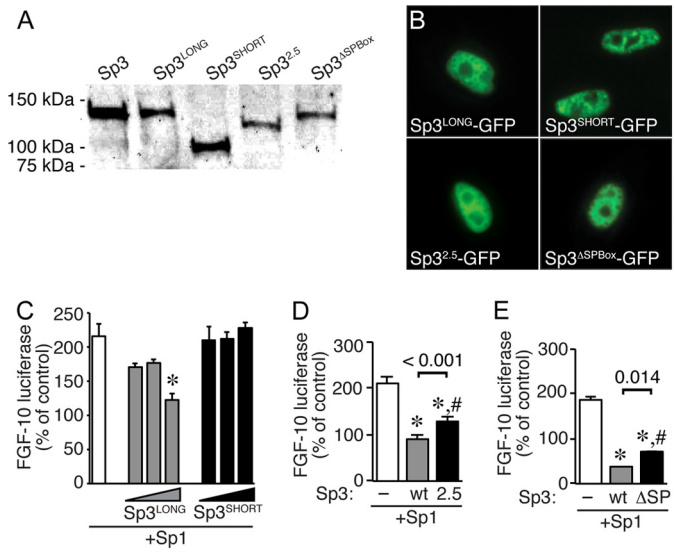

FIGURE 6. **The N terminus of SP3 is required for inhibition of SP1-mediated *Fgf-10* expression.** *A*, SP3-GFP fusion proteins containing N-terminal mutations were expressed in CHO cells. $SP3^{LONG}$ and $SP3^{SHORT}$ correspond to the longest and shortest SP3 isoforms that are expressed from the full-length *Sp3* mRNA. $SP3^{2.5}$ lacks the 149 N-terminal amino acids. $SP3^{\Delta SPBox}$ lacks the Sp box domain located between amino acids 44–90. Immunoblotting for GFP confirms that each mutant *Sp3* construct was expressed at the expected molecular weight. *B*, SP3-GFP mutant isoforms localized to the cell nucleus when expressed in CHO cells. *C*, increasing amounts (0.2, 0.4, 0.8 μg) of full-length SP3 ($SP3^{LONG}$, *gray bars*) inhibited SP1-mediated *Fgf-10* promoter activity (*, $p < 0.05$ compared with SP1 only; $n = 3$). The shortest SP3 isoform ($SP3^{SHORT}$, *black bars*) failed to inhibit SP1-mediated *Fgf-10* promoter activity. *D* and *E*, SP3 mutants lacking either the 149 N-terminal amino acids (*D*) or the Sp box (*E*) were less effective at inhibiting SP1-mediated *Fgf-10* promoter activity than wild type SP3 (*, *p* compared with SP1 alone; #, *p* compared with wild type SP3; $n = 6$).

also represses the expression of a smaller subset of genes, many of which are involved in development and repair (41–43). The mechanisms responsible for suppression by NF-κB are less well understood. NF-κB can stimulate the expression of transcriptional regulators that then inhibit downstream target genes. For instance, NF-κB interacts with histone deacetylases to guide these enzymes to targets of NF-κB suppression (44). This particular mechanism is thought to transcriptionally silence specific genes, such as the proapoptotic gene BNIP3 in cardiomyocytes (45), as well as tempering activation of NF-κB targets, including TNFα and IL-8 (44). NF-κB directly inhibits expression of intestinal trefoil factor (TFF3) (46), E-cadherin (42), and collagen 1A1 (41). These inhibitory functions of NF-κB suggest a regulatory role in development.

Many components of the NF-κB signaling pathway are required for normal fetal development and tissue morphogenesis. Mice deficient for IKKα die shortly after birth with profound developmental defects in skin, skeleton, and hematopoietic system (47–49). In addition to its role in lymphocyte survival, NF-κB prevents apoptosis during development. Mice null for RELA die *in utero* with massive liver apoptosis (50). An emerging body of literature also implicates NF-κB in regulating neural repair and neural synapse integrity. NF-κB influences neuronal excitability in the amygdala, protecting neurons in this critical region of the brain from excitoxicity while simultaneously promoting long term potentiation (51, 52). NF-κB has also been implicated in promoting neural regeneration and neural synapse pruning in the hippocampus (52–55). NF-κB signaling therefore regulates developmental processes in addition to the innate immune response. By interacting with other developmental transcription factors, NF-κB provides an additional level of expression control. These molecular mechanisms may provide unique insight into the connections between inflammation, immunity, and the pathogenesis of developmental diseases.

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