Fibromodulin Suppresses Nuclear Factor-κB Activity by Inducing the Delayed Degradation of IκBα via a JNK-dependent Pathway Coupled to Fibroblast Apoptosis

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Fibulin-5 (FBLN5) belongs to the Fibulin family of secreted extracellular matrix proteins, and our laboratory first established FBLN5 as a novel target for TGF-β in fibroblasts and endothelial cells. To better understand the pathophysiology of FBLN5, we carried out microarray analysis to identify fibroblast genes whose expressions were regulated by FBLN5 and TGF-β. In doing so, we identified fibromodulin (Fmod) as a novel target gene of FBLN5, and we validated the differential expression of Fmod and 12 other FBLN5-regulated genes by semi-quantitative real time PCR. Fmod belongs to the small leucine-rich repeat family of extracellular matrix (ECM) proteoglycans and glycoproteins, whose members also include decorin, biglycan, and sclera (1). Fmod belongs to the small leucine-rich repeat family of proteoglycans, which are important constituents of mammalian extracellular matrices. Interestingly, parental 3T3-L1 fibroblasts displayed high levels of nuclear factor-κB (NF-κB) activity, although those engineered to express Fmod constitutively exhibited significantly reduced NF-κB activity, suggesting that Fmod functions to inhibit NF-κB signaling. By monitoring alterations in the activation of NF-κB and the degradation of its inhibitor, IκBα, we demonstrate for the first time that Fmod contributes to the constitutive degradation of IκBα protein in 3T3-L1 fibroblasts. Mechanistically, we observed Fmod to delay the degradation of IκBα by promoting the following: (i) activation of c-Jun N-terminal kinase; (ii) inhibition of calpain and casein kinase 2 activity; and (iii) induction of fibroblast apoptosis. Taken together, our study identified a novel function for Fmod in directing extracellular signaling, particularly the regulation of NF-κB activity and cell survival.

Fibromodulin (Fmod) is a keratan sulfate proteoglycan that is highly concentrated in cartilage, tendon, skin, cornea and sclera (1). Fmod belongs to the small leucine-rich repeat family of extracellular matrix (ECM) proteoglycans and glycoproteins, whose members also include decorin, biglycan, and lumican (2, 3). Fmod plays a central role in organizing the structure of type I and II collagen fibrils, and its expression is ubiquitous in a variety of connective tissues, such as cartilage, tendons, ligaments, and dermal tissues (4, 5). Along these lines, transforming growth factor-β (TGF-β) isoforms interact with Fmod physically to regulate the fibrotic responses of several tissues, including the kidney, lung, and skin (6–8). Moreover, Fmod also affects the differentiation of tendon stem/progenitor cells by modulating the signals transduced by bone morphogenetic proteins, ultimately enhancing tendon repair and formation in vivo (9). Thus, Fmod expression figures prominently in regulating tissue homeostasis in part by impacting the activities of TGF-β superfamily members.

TGF-β itself is a multifunctional cytokine that regulates tissue morphogenesis and differentiation by effecting cell proliferation and survival and by altering the production of ECM proteins within cell and tissue microenvironments (10, 11). In addition, our laboratory first identified the ECM protein Fibulin-5 (FBLN5) as a novel target gene for TGF-β in fibroblasts (12) and endothelial cells (12, 13). Moreover, we established FBLN5 as a multifunctional signaling molecule that does the following: (i) regulates the proliferation, motility, and invasion of normal and malignant cells both in vitro and in vivo (12–14); (ii) antagonizes endothelial cell activities coupled to angiogenesis both in vitro and in vivo (13, 14); (iii) inhibits the growth of fibrosarcomas in mice (14); and (iv) induces epithelial-mesenchymal transition (EMT) in normal and malignant mammary epithelial cells via a matrix metalloproteinase-dependent mechanism (15). In an attempt to further our understanding of the pathophysiological functions of FBLN5, we characterized changes in the fibroblast transcriptome induced by constitutive FBLN5 expression. In doing so, we identified Fmod as a novel target gene of FBLN5 in fibroblast cells. Whereas previous studies of Fmod action have been performed primarily in connective tissues (16) and endothelial cells (17), we show here that Fmod expression in fibroblasts potently suppressed their activation of nuclear factor-κB (NF-κB) by preventing the degradation of IκBα via a c-Jun N-terminal kinase (JNK)-, casein kinase-2 (CK2)-, and calpain-dependent pathway that serves in diminishing fibroblast survival.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Reagents**—Normal murine 3T3-L1 fibroblasts were obtained from ATCC (Manassas, VA) and cultured as described previously (12). 3T3-L1 fibroblasts were engineered to stably express either GFP or Fmod using a bi-
cistronic retroviral vector (pMSCV-IRES-GFP) as described previously (12). The NF-κB promoter-driven luciferase reporter was provided by Dr. John M. Routes (Medical College of Wisconsin, Milwaukee). The generation of 3T3-L1 fibroblasts that lacked Fmod expression was accomplished by their transduction with lentiviral particles that encoded for either a scrambled (i.e. nonsilencing) or Fmod-directed shRNA in pLKO.1 (Thermo Scientific, Huntsville, AL). The generation of pLKO.1 lentiviral particles and their transduction into 3T3-L1 fibroblasts were accomplished as described previously (18, 19), although the extent of Fmod depletion was monitored by immunoblotting whole-cell extracts prepared from parental (i.e. nonsilencing shRNA) and Fmod-deficient 3T3-L1 fibroblasts with anti-Fmod antibodies.

Microarray Analysis and Semi-quantitative Real Time PCR Assays—Total RNA from parental (i.e. GFP) and FBLN5-expressing 3T3-L1 fibroblast cells was purified using the RNeasy plus mini kit (Qiagen, Valencia, CA) according to the manufacturer’s recommendations. The creation of polyclonal populations of 3T3-L1 cells that constitutively expressed FBLN5 and its role in promoting fibroblast proliferation and MAPK activation were described previously (12). In some experiments, GFP- or FBLN5-expressing 3T3-L1 fibroblasts were stimulated with TGF-β1 (5 ng/ml). RNA samples were reverse-transcribed and labeled for microarray analysis using standard techniques (20, 21). For real time PCR, cDNAs were synthesized by iScript reverse transcription (Bio-Rad), which then were diluted 10-fold in H2O and employed in semi-quantitative real time PCRs (25 μl) using the SYBR Green system (Bio-Rad) supplemented with 5 μl of diluted cDNA and 0.1 μM of oligonucleotide pairs provided in supplemental Table S1. PCRs were performed and analyzed on a Bio-Rad Mini-Opticon detection system, and differences in RNA concentrations were monitored by normalizing individual gene signals to their corresponding GAPDH signals.

Immunoblot Assays—Parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts were lysed and solubilized in Buffer H/Triton X-100 (22) for 30 min on ice. Clarified whole-cell extracts were resolved on 10% SDS-polyacrylamide gels, transferred electrophoretically onto nitrocellulose membranes, and blocked in 5% milk before incubation with the following primary antibodies (dilutions): (a) anti-Fmod (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); (b) anti-IκBα (1:3000; Santa Cruz Biotechnology); (c) anti-phospho-IκBα (1:1000; Cell Signaling, Danvers, MA); (d) anti-phospho-ERK (1:500; Cell Signaling); (e) anti-phospho-p38 (1:500; Cell Signaling); (f) anti-phospho-JNK (1:500; Cell Signaling); (g) anti-p65 (1:1000; Santa Cruz Biotechnology); and (h) anti-histone H1 (1:200; Santa Cruz Biotechnology). The resulting immunocomplexes were visualized by enhanced chemiluminescence. Differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin antibodies (1:1000; Rockland Immunochemicals, Gilbertsville, PA). Where specified, parental (i.e. GFP), Fmod-expressing, or Fmod-deficient (Fmod shRNA) 3T3-L1 fibroblasts were incubated for various times in the absence or presence of the following: (i) protein synthesis inhibitor, cycloheximide (CHX); (ii) proteosome inhibitor, MG-132; (iii) JNK inhibitor, SP600125; (iv) calpain inhibitor, EST (Calbiochem); and (v) CK2 inhibitor, TBBz (Calbiochem) at the provided concentrations.

Luciferase Reporter Gene Assays—Analysis of luciferase activity driven by a synthetic NF-κB promoter was accomplished as described previously (23). Briefly, parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts (25–30,000 per well) were cultured overnight onto 24-well plates and subsequently were transiently transfected the following morning by overnight exposure to LT1 liposomes (Mirus, Madison, WI) containing pNF-κB-luciferase cDNA (300 ng/well) and CMV-β-gal cDNA (50 ng/well) which was used to control for differences in transfection efficiency. Afterward, the resulting luciferase and β-gal activities contained in detergent-solubilized cell extracts were determined. As above and where specified, parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of the inhibitors to JNK (SP600125), calpain (EST), and CK2 (TBBz). Data are the mean (±S.E.) luciferase activities of at least three independent experiments normalized to untreated cells.

NF-κB-biotinylated Oligonucleotide Capture Assay—DNA binding activity of NF-κB was monitored in parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts as described previously (23). Briefly, the cells were incubated for 24 h in the absence or presence of JNK inhibitor (SP600125) and subsequently were collected and fractionated into cytoplasmic and nuclear extracts using a nuclear extraction kit according to the manufacturer’s instructions (Chemicon). NF-κB binding activity was determined by incubating 60 μg of nuclear extract with 1 μg of biotinylated double-stranded DNA oligonucleotides that contained an NF-κB consensus sequence site under continuous rotation at 4°C (forward probe, 5’-GATT-AAGGGACTTTCCGCTGGGGACTTTCCAGTCGA; reverse probe, 5’-TCGACCTGAAAGTCCGCCAGGAAGATCGTCCCTAGATC). The resulting NF-κB-oligonucleotide complexes were captured by addition of streptavidin-agarose beads (Pierce) and collected by microcentrifugation. Washed complexes were fractionated through 10% SDS-PAGE before their immobilization to nitrocellulose membranes, which were subsequently probed with anti-p65 antibodies (1:500). Differences in extract loading were monitored by immunoblotting 25 μg of resolved nuclear extract aliquots with antibodies against histone H1 (1:200).

Cell Biological Assays—The effect of Fmod on calpain activity in 3T3-L1 fibroblasts was determined using the Calpain-Glo™ protease assay kit (Promega, Madison, WI) according to the manufacturer’s recommendations. Briefly, 3T3-L1 fibroblasts (30,000 cells/well in 96-well plates) were lysed and mixed with proluminescent calpain substrate, and the resulting luminescence was measured as described previously (12). Along these lines, the effect of Fmod on 3T3-L1 fibroblast apoptosis was determined using two complementary methods. First, 3T3-L1 fibroblasts (10,000 cells/well) were incubated for 24 h in the absence or presence of the JNK inhibitor SP600125 prior to addition of the proluminescent caspase-3/7 DEVD-aminoluciferin substrate as instructed by the Caspase-Glo® assay kit (Promega) (24). Second, parental (i.e. GFP),
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TABLE 1
Validation of select gene targets regulated by FBLN5 expression in 3T3-L1 fibroblasts
Among the genes whose expression was controlled by FBLN5 as detected in microarray analyses, the expression of 13 genes was confirmed by semi-quantitative real-time PCR.

| Gene name                  | NCBI no.   | Microarray expression | Real time PCR expression |
|----------------------------|------------|-----------------------|-------------------------|
| Unknown                    | BS503935   | 9.2-Fold decrease     | 22.2-Fold decrease      |
| Unknown                    | BS533736   | 5.4-Fold increase     | 3.2-Fold increase       |
| Unknown                    | BR831146   | 9.2-Fold increase     | 2.1-Fold increase       |
| Pleckstrin homology domain-containing A member | BC010215 | 7.5-Fold decrease     | 5.8-Fold decrease       |
| Transglutaminase 2         | BC016492   | 5.8-Fold decrease     | 33.6-Fold decrease      |
| Homeobox D9                 | BC019150   | 9.4-Fold decrease     | 2.1-Fold increase       |
| Rho GTPase-activating protein 24 | BC025502 | 5.5-Fold decrease     | 3.3-Fold decrease       |
| Thrombospondin 1           | M87276     | 10.7-Fold increase    | 10.5-Fold increase      |
| Procollagen, type XI, a 1 | NM_007729  | 9.9-Fold increase     | 3.3-Fold increase       |
| Angiopoietin 1             | NM_009640  | 8.3-Fold increase     | 10.5-Fold increase      |
| Cysteine-rich protein 61   | NM_010516  | 10.4-Fold increase    | 3.3-Fold increase       |
| Dickkopf homolog 3         | NM_015814  | 6.9-Fold increase     | 1.8-Fold decrease       |
| Fibromodulin               | NM_021355  | 9.6-Fold increase     | 10.6-Fold increase      |

Fmod-expressing, or Fmod-deficient (i.e. Fmod shRNA) 3T3-L1 cells were again subjected to TNK inhibitor prior to their processing for TUNEL assays according to the manufacturer’s recommendations (Invitrogen).

RESULTS

Fmod Is a Novel Target Gene of FBLN5 in 3T3-L1 Fibroblasts—Our laboratory first established FBLN5 as a novel gene target for TGF-β in fibroblasts and endothelial cells (12). Moreover, we demonstrated that constitutive FBLN5 expression was sufficient to enhance 3T3-L1 cell proliferation and activation of MAPKs (12). In an effort to enhance our understanding of the pathophysiological actions of FBLN5, we characterized changes in the fibroblast transcriptome induced by constitutive FBLN5 expression or by constitutive FBLN5 expression plus added TGF-β1. Afterward, total RNA was collected and prepared for hybridization to Affymetrix microarrays, which enabled us to identify ~1200 genes whose expression was controlled by constitutive FBLN5 expression. The altered expression of 13 genes was confirmed by semi-quantitative real time PCR (Table 1), including that of Fmod (NCBI accession number NM_021355). Here, we limited our analyses to Fmod because this ECM protein and FBLN5 both play prominent roles in regulating cell-cell and cell-matrix signaling in ECM. Interestingly, FBLN5 only coupled to Fmod expression in 3T3-L1 fibroblasts but not in normal (NMuMG) or malignant (MDA-MB-231) mammary epithelial cells or in endothelial (MB114) cells (see supplemental Fig. S1). Collectively, these findings identify Fmod as a novel and fibroblast-specific gene target of FBLN5.

Fmod Suppresses NF-κB Activity by Stabilizing IκBα—To study the function of Fmod, we first established Fmod-expressing fibroblasts using a retroviral expression system according to our published procedures (12). As expected, fibroblasts transduced with Fmod-encoding viral particles constitutively expressed Fmod proteins at significantly higher levels as compared with their parental (i.e. GFP) counterparts (Fig. 1A). We then measured the production of luciferase whose expression was driven by Smad2/3 or AP1 transcription factors in these cells before and after their stimulation of with TGF-β1 because this cytokine is known to readily interact with Fmod (25). In doing so, we observed Fmod-expressing fibroblasts to exhibit significantly elevated luciferase expression driven by the Smad2/3 and AP1 transcription factors both basally and in response to TGF-β (data not shown). We also examined the effects of Fmod in altering the expression of luciferase driven by NF-κB. In comparison with parental (i.e. GFP) fibroblasts, those engineered to express Fmod exhibited a 75% reduction of luciferase activity directed by NF-κB (Fig. 1B). Along these lines, the ability of NF-κB to mediate and induce the expression of MMP-2 and −9 in fibroblasts has previously been established (26), and as such, Fmod-mediated inhibition of NF-κB activity would be anticipated to reduce fibroblast expression of MMP-2 and −9. Accordingly, Fmod-expressing fibroblasts did indeed express significantly reduced levels of MMP-2 and −9 as compared with their parental (i.e. GFP) counterparts (see supplemental Fig. S2).

NF-κB activity is regulated primarily by its interaction with inhibitory κB (IκB) proteins (27). Extracellular signals that stimulate NF-κB must first induce the release of IκB proteins from NF-κB, thereby allowing for its nuclear translocation and regulation of gene expression. This initial dissociation event requires the activation of IκB kinases, which phosphorylate IκB proteins and lead to their subsequent release from NF-κB, ubiquitination, and proteosomal degradation (27). In light of the dramatic reduction in NF-κB measured in Fmod-expressing fibroblasts, we immunoblotted whole-cell extracts prepared from parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts with anti-IκBα antibodies. Fig. 1C shows that Fmod-expressing fibroblasts contain significantly higher quantities of IκBα as compared with their parental counterparts, a finding consistent with the diminished NF-κB activity measured in Fmod-expressing fibroblasts. Interestingly, although IκBα levels were considerably higher in Fmod-expressing versus parental fibroblasts, we observed the phosphorylation status of IκBα to be equivalent between these cell lines (Fig. 1C). This finding suggests that the ability of Fmod to inhibit NF-κB activity may reflect dysregulated IκBα dynamics. As such, we monitored the stability of IκBα proteins in parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts before and after their treatment with CHX, which inhibits protein synthesis and enables assessment of the rate of IκBα turnover. As shown in Fig. 2A, IκBα was rapidly degraded in parental (i.e. GFP) 3T3-L1 fibroblasts treated with CHX; how-
however, this same experimental treatment elicited little to no change in the levels of IκBα detected in Fmod-expressing fibroblasts. Likewise, administration of MG-132, which inhibits proteosomally mediated protein degradation, also failed to alter the levels IκBα in Fmod-expressing fibroblasts (Fig. 2B). Collectively, these findings suggest that Fmod inhibits NF-κB activity by preventing the degradation of IκBα. To more thoroughly investigate the merits of this supposition, we transduced Fmod-expressing 3T3-L1 fibroblasts with scrambled or Fmod-directed shRNAs to neutralize the activities of constitutive Fmod expression. The extent of Fmod deficiency mediated by individual Fmod-directed shRNAs was monitored by immunoblotting with antibodies against Fmod (see supplemental Fig. S3 and Table S2). Based on these analyses, polyclonal populations of 3T3-L1 cells that stably expressed Fmod shRNA2 were selected and used for all subsequent Fmod deficiency studies. As shown in Fig. 2C, elevating Fmod expression (i.e. Fmod) prevented IκBα degradation in CHX-treated fibroblasts, a reaction that was readily reversed in fibroblasts depleted of Fmod expression (i.e. shFmod). Moreover, the enhanced stability of IκBα was specific to Fmod expression because the introduction of nonsilencing shRNA (i.e. scram) failed to restore IκBα degradation (Fig. 2C). Taken together, these findings indicate that Fmod repressed the activation of NF-κB by increasing the stability of IκBα.

### Fmod Stabilizes IκBα by Activating JNK—Mammalian MAPKs play important roles in many different signaling pathways, including the control of gene expression, and of cell proliferation, differentiation, and apoptosis (28, 29). NF-κB and JNK are two key regulators of the pathophysiology of cells, and cross-talk between these two signaling molecules has been reported in the scientific literature (30–32). These connections prompted us to test whether the ability of Fmod to inhibit NF-κB transpired through MAPKs, particularly JNK. In doing so, we monitored the activation status of individual MAPKs by immunoblotting extracts prepared from parental (i.e. GFP) or Fmod-expressing fibroblasts with phospho-specific anti-MAPK antibodies. Fig. 3A shows that although Fmod expression failed to activate ERK1/2 and elic-
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FIGURE 3. Fmod stabilizes IκBα by activating JNK. A, whole-cell extracts prepared from parental (i.e. GFP) and Fmod-expressing cells were fractionated through 10% SDS-PAGE and immobilized onto nitrocellulose. Immunoblot analysis with antibodies against IκBα, phospho-ERK (p-ERK), phospho-JNK (p-JNK), and phospho-p38 MAPK (p-p38) showed that Fmod specifically couples to JNK activation. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Data are from a representative experiment that was performed three times with identical results. B, parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated in the absence or presence of the JNK inhibitor SP600125 (10 μM) for 24 h. Afterward, the expression levels of IκBα were detected by immunoblot analysis using anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Data are from a representative experiment that was performed three times with similar results. C, parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts were transiently transfected with pNF-κB-luciferase and pCMV-β-gal, and subsequently were incubated for 24 h with the JNK inhibitor SP600125 as indicated. Data are the mean (± S.E.) ratios of luciferase-β-gal activity observed in three independent experiments completed in triplicate (*, p < 0.05). D, parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated for 24 h with the JNK inhibitor (SP600125) as indicated, and nuclear extracts were prepared and incubated with biotinylated IκBα oligonucleotides probes. Afterward, p65/RelA-oligonucleotide complexes were captured from nuclear fractions of Fmod-expressing 3T3-L1 fibroblasts incubated with streptavidin-agarose beads and visualized by immunoblotting with antibodies against total p65/RelA. Images are from a single experiment that was performed four times with identical results. F, control (i.e. pcDNA) and constitutively active MEKK1 (CAMEKK1) were co-transfected with pNF-κB-luciferase and pCMV-β-gal and subsequently were incubated for 24 h with the JNK inhibitor SP600125 (25 μM) as indicated. Data are the mean (± S.E.) ratios of luciferase-β-gal activity observed in three independent experiments completed in triplicate (*, p < 0.05). G, Fmod-expressing (Fmod, scramp) and Fmod-depleted (shFmod and GFP) 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of JNK inhibitor SP600125 (25 μM). The expression levels of IκBα were analyzed by immunoblotting with anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin antibodies. Data are from a representative experiment that was performed three times with similar results. Con, control.

ited only modest activation of p38 MAPK, this ECM protein did significantly stimulate the phosphorylation of JNK in a manner that correlated with elevated IκBα expression. Thus, JNK activation may underlie diminished NF-κB activity in Fmod-expressing fibroblasts. To address this hypothesis, we treated parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts with the JNK inhibitor, SP600125, prior to monitoring changes in IκBα expression. Interestingly, inhibiting JNK activity elicited only modest alterations in IκBα levels in parental (i.e. GFP) fibroblasts; however, this same experimental treatment induced a dramatic reduction of IκBα in Fmod-expressing cells (Fig. 3B), suggesting that Fmod-mediated activation of JNK prevents IκBα degradation. Moreover, this finding contrasts sharply with the failure of inhibitors against p38 MAPK (SB203580) or MEK1/2 (U0126) to alter IκBα expression in Fmod-expressing 3T3-L1 fibroblasts (data not shown). Along these lines, we observed SP600125 to dose-depen-
pared with mock (i.e. empty pcDNA)-transfected cells (Fig. 3E). Moreover, expression of CAMEKK1 in 3T3-L1 fibroblasts reduced their NF-κB activity by ~75%, a response that was partially neutralized by administration of SP600125 (Fig. 3F). Thus, enforced activation of JNK by expression of CAMEKK1 in parental (i.e. GFP) fibroblasts recapitulated the actions of Fmod on NF-κB signaling in fibroblasts. Finally, to determine the role of Fmod in promoting JNK-mediated IκBα stabilization, we once again depleted constitutive Fmod expression in 3T3-L1 fibroblasts using shRNAs directed against this ECM protein. As shown in Fig. 3G, administering SP600125 to Fmod-expressing cells (i.e. Fmod and Fmod/scram) readily promoted IκBα degradation; however, this same treatment protocol failed to effect IκBα levels in cells depleted of Fmod. Taken together, these findings strongly suggest that Fmod expression stimulates JNK and its ability to prolong the half-life of IκBα, leading to reduced NF-κB activity in fibroblasts.

Constitutive Degradation of IκBα in Fibroblasts Is Mediated by Calpain—Several reports established calpain as a mediator of constitutive IκBα degradation (34–36). In light of our findings that demonstrated the ability of Fmod to suppress IκBα degradation, we next determined whether calpain activity was differentially regulated between parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts using the Calpain-Glo™ protease assay. As shown in Fig. 4A, Fmod-expressing 3T3-L1 fibroblasts exhibited 40% less calpain activity as compared with their parental (i.e. GFP) counterparts, a finding consistent with the ability of Fmod to enhance IκBα stability (Figs. 1–3). As such, we determined whether administering the calpain inhibitor, EST, could alter NF-κB activity in a JNK-dependent manner. Fig. 4, B and C, shows that inhibiting calpain alone or in combination with JNK had no effect on NF-κB activity in parental (i.e. GFP) fibroblasts. In stark contrast, NF-κB activity was readily induced in Fmod-expressing cells treated with the JNK inhibitor SP600125. More importantly, the effects of JNK inactivation on NF-κB activity were readily reversed by administration of the calpain inhibitor, EST (Fig. 4, B and C). Identical findings were obtained by treating Fmod-expressing fibroblasts with a second independent calpain inhibitor, calpain inhibitor III (see supplemental Fig. S5). Taken together, these findings suggest that the elevated expression of Fmod inhibited calpain protease activity and its degradation of IκBα, which manifests significantly reduced NF-κB activity in 3T3-L1 fibroblasts.

Along these lines, the ability of calpain to degrade IκBα has been associated with the activation of CK2 (36, 37). Our findings thus far suggest that parental 3T3-L1 fibroblasts likely contain high levels of active CK2, which couples to calpain activation and the rapid degradation of IκBα, resulting in constitutive NF-κB activity. Thus, constitutively active NF-κB activity in parental (i.e. GFP) fibroblasts is predicted to be inhibited by administration of CK2 antagonists, although their Fmod-expressing counterparts are predicted to be refractory to CK2 inhibition. To determine the validity of these predictions, we measured NF-κB luciferase activity in parental (i.e. GFP) and Fmod-expressing 3T3-L1 fibroblasts before and after their treatment with the CK2 inhibitor, TBBz. Fig. 4D

![Figure 4](https://example.com/figure4.png)
shows that NF-κB activity in Fmod-expressing fibroblasts was insensitive to the administration of TBBz; however, this same experimental regimen significantly inhibited the transcriptional activity of NF-κB in parental (i.e., GFP) fibroblasts. Moreover, identical findings were obtained by treating parental (i.e., GFP) and Fmod-expressing fibroblasts with a second independent CK2 inhibitor, TBCA (see supplemental Fig. S6). Collectively, these findings suggest that the constitutive turnover of IκBα is mediated by a CK2-calpain signaling axis that is inactivated in Fmod-expressing fibroblasts in a JNK-dependent manner.

**Fmod Induces Fibroblast Apoptosis**—Recent findings have linked CK2 to the regulation of cell survival in part via its ability to promote IκBα degradation and NF-κB activity (38). Because constitutive Fmod expression promotes JNK activation coupled to reduced calpain activity and enhanced IκBα stability, we investigated the effect of Fmod on fibroblast survival using the Caspase-Glo™ 3/7 assay kit, which showed that Fmod-expressing fibroblasts possessed significantly higher caspase-3/7 activities as compared with parental (i.e., GFP) fibroblasts (Fig. 5A). In contrast to parental (i.e., GFP) fibroblasts, SP600125 administration significantly reduced caspase-3/7 activity in Fmod-expressing fibroblasts, suggesting that Fmod-mediated activation of JNK couples to caspase-3/7 activation. We also determined the relative reliance of caspase-3/7 activation on Fmod by once again depleting its expression in Fmod-expressing cells via introduction of Fmod-directed shRNA. Fig. 5B clearly shows that Fmod expression was essential for JNK-dependent caspase-3/7 activation in 3T3-L1 fibroblasts. Along these lines, we observed that Fmod-mediated activation of JNK couples to caspase-3/7 activity in Fmod-expressing fibroblasts, SP600125 administration significantly reduced caspase-3/7 activity as compared with parental and Fmod-depleted counterparts (Fig. 5C). As above, inhibiting JNK activity significantly reduced TUNEL staining only in Fmod-expressing 3T3-L1 cells (Fig. 5C), suggesting that Fmod-mediated activation of JNK promotes apoptosis in fibroblasts. Collectively, these findings demonstrate that Fmod expression is necessary for the activation of JNK (Fig. 3) and, consequently, for activation of caspase-3/7 to initiate fibroblast apoptosis (Fig. 5).

**DISCUSSION**

NF-κB is a ubiquitously expressed transcription factor that governs the transcription of genes involved in immune responses, angiogenesis, cell transformation, invasion, apoptosis, and cell cycle (39). As such, the inappropriate activation of NF-κB has been linked to a variety of inflammatory and autoimmune diseases and of human malignancies (40). The signaling of NF-κB is normally suppressed by a family of inhibitory molecules termed IκB proteins, such as IκBα that binds NF-κB and prevents its activation in the cytoplasm. In response to a variety of stimuli, including proinflammatory cytokines (e.g. TNF-α or IL-1), T and B cell mitogens, lipo-polysaccharide, viral infections, and cellular stresses (e.g. ionizing radiation or chemotherapies) (41, 42), IκBα molecules are rapidly degraded to facilitate the activation and nuclear translocation of NF-κB (39). The extent to which ECM molecules regulate NF-κB activity and the molecular mechanisms whereby these events transpire remain incompletely understood. To this end, we show here for the first time that Fmod is a novel gene target of FBLN5 (Table 1), which itself func-
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Our findings, together with those mentioned above, suggest that these players may represent a general mechanism to fine-tune NF-κB signaling in resting cells and tissues, particularly their ability to adapt to altered survival signals. Up-regulated expression of Fmod and other proteoglycans has been detected during the repair and remodeling of wounded collagen matrices, a response that transpires in the absence of obvious inflammatory reactions (54). Moreover, we previously associated FBLN5 to enhanced wound healing in vivo (55), and as such, future studies need to investigate the relative contribution of Fmod to these events and to the initiation and resolution of tissue inflammation and fibrotic responses.

As mentioned previously, Fmod belongs to the LLR family of ECM proteoglycans and glycoproteins typically found in connective tissues, including cartilage, tendon, skin, cornea, and sclera (1). Fmod plays a significant role in collagen assembly and maintenance, and Fmod-deficient mice display abnormally thin type I collagen fibrils in their tendons, which increases their occurrence of arthritis (56). Besides its structural functions, Fmod also transduces signals by interacting with a variety of ECM and secreted molecules. Indeed, recent work demonstrated the ability of Fmod to stimulate the complement cascade through its physical interaction with C1q (57). Interestingly, although other ECM molecules such as laminin and decorin also bind C1q, only Fmod is capable of inducing complement activation and sustained inflammatory reactions. Thus, studies aimed at determining the interplay between Fmod and NF-κB in directing inflammation also appear warranted. Likewise, the regulatory roles of NF-κB in cancer biology, including its coupling to tissue invasion, migration, and metastasis, have been thoroughly established (58, 59). Interestingly, the expression of Fmod transcripts are significantly reduced in metastatic tumors as compared with their non-metastatic counterparts, particularly tumors originating in the breast (60, 61) and prostate (62–64). In addition, Oncomine analyses of Fmod expression showed the expression of this proteoglycan to be reduced at metastatic sites relative to non-metastatic lesions in gastric cancers, head and neck cancers, and sarcomas (data not shown). These analyses also found aberrantly low Fmod expression to associate with reduced overall survival rates in patients with cancers of the brain, breast, lung, and blood (data not shown). Thus, future studies need to investigate the specific contributions of diminished Fmod expression that elicit the acquisition of metastatic phenotypes in human tumors.

Finally, Fmod clearly functions in regulating the dynamics between developing carcinoma and their accompanying stroma (65). In particular, desmoplastic reactions in carcinomas elicit elevated interstitial fluid pressure that diminishes efficient nutrient and gaseous transfer, as well as reduces the delivery of cancer chemotherapeutics (47). The inflammatory environment of developing carcinomas is thought to induce stromal production of Fmod, leading to the acquisition of dense and rigid tissue architectures (65). Future studies clearly need to address the chemotherapeutic potential of augmenting or attenuating Fmod action in developing carcinomas as a novel means to improve the efficacy of cancer chemotherapeutics.
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