Nedd4L suppression in lung fibroblasts facilitates pathogenesis of lung fibrosis

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Abstract

Ubiquitination-mediated protein degradation is associated with the development of pulmonary fibrosis. We and others have shown that Nedd4L plays anti-inflammatory and anti-fibrotic roles by targeting lysophosphatidic acid receptor 1 (LPAR1), p-Smad2/3, and β-catenin, and other molecules for their degradation in lung epithelial cells and fibroblasts. However, the molecular regulation of Nedd4L expression in lung fibroblasts has not been studied. In this study, we find that Nedd4L levels are significantly suppressed in lung myofibroblasts in IPF patients and in experimental pulmonary fibrosis, and in TGF-β1-treated lung fibroblasts. Nedd4L knockdown promotes TGF-β1-mediated phosphorylation of Smad2/3 and lung myofibroblast differentiation. Mechanistically, Nedd4L targets TGF-β receptor II (TβRII), the first key enzyme of TGF-β1-mediated signaling, for its ubiquitination and degradation. Further, we show that inhibition of transcriptional factor E2F rescues Nedd4L levels and mitigates experimental pulmonary fibrosis. Together, our data reveal insight into mechanisms by which E2F-mediated Nedd4L suppression contributes to the pathogenesis of lung fibrosis. This study provides evidence showing that upregulation of Nedd4L is a potential therapeutic strategy to treat fibrotic disorders including lung fibrosis.

Introduction

Idiopathic pulmonary fibrosis (IPF) is an aging-associated, chronic, irreversible, and ultimately fatal lung disease, which is characterized by the excessive accumulation of differentiated myofibroblasts and extracellular matrix (ECM) deposition, resulting in

scarring of the lungs.[1–3] Repetitive lung epithelial cell injury contributes to the initiation phase of fibrosis, while abnormal activation of fibroblasts/myofibroblasts leads to the development and progression of pulmonary fibrosis (PF).[4–6] TGF-β1 is a master regulator in the progression of PF. TGF-β1 binds to and activates the TGF-β receptor complex (TβRII activation of TβR1) to signal via canonical (Smad-mediated pathways) and non-canonical pathways to promote myofibroblast differentiation, activation, and survival.[3,7–9] Because of its central role in fibroblast biology and the pathogenesis of fibrosis, TGF-β1 signaling has been targeted as a therapeutic strategy in human diseases including cancer and fibrosis.

Ubiquitination-mediated protein degradation is dependent on ubiquitin E3 ligases.[10,11] Different linkages of polyubiquitin chains regulate distinct protein functions, such as degradation, cellular localization, protein-protein interactions, and enzyme activity. The ubiquitin E3 ligase Nedd4L has been shown to exhibit anti-fibrotic properties by promoting degradation of TβRI, p-Smad2/3, LPAR1, and ENaC.[12–15] Recent studies demonstrated that reduced Nedd4L levels in lung epithelial cells in IPF patients showed that the reduction of Nedd4L promoted chronic lung epithelial injury and subsequent PF[16] and identified β-catenin as a novel substrate of Nedd4L.[17] Taken together, these studies suggest that Nedd4L plays an anti-fibrotic role by targeting multiple substrates. However, the role of Nedd4L in TβRII stability and molecular regulation of Nedd4L suppression have not been revealed.

The E2F family regulates cellular functions by acting as an activator or repressor of transcription.[18–22] In this study, we revealed that E2F4 suppresses Nedd4L transcription. Additionally, the current study discovered new molecular mechanisms by which Nedd4L regulates TGF-β1-mediated signaling through ubiquitinating and degrading TβRII. This study provides molecular mechanistic evidence that restoring Nedd4L levels in the lungs may reduce the severity of PF.

Material and methods

Cells and reagents

Human lung fibroblast cell lines (Mrc5 and IMR90) were purchased from ATCC (Manassas, VA). Human primary lung fibroblasts (HLF) and lung myofibroblasts (IPF-LF) from adult normal human subjects and IPF patients were obtained from the Center for Organ Recovery and Education and Lung Transplantation at the University of Pittsburgh. The study was approved by the institutional Review Board at the University of Pittsburgh (STUDY18100070). Cells were cultured in Eagle's Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS) in 5% CO_2 cell culture incubator. V5 antibody, mammalian expressional plasmid pcDNA3.1/His-V5-topo, and *Escherichia coli* Top10 competent cells were purchased from Life technologies (Grand Island, NY). Nedd4L, TβRII, collagen I, ubiquitin, smurf1, Smad4, p-Smad2/3, and Smad2/3 antibodies were purchased from Cell Signaling (Danvers, MA). Fibronectin (FN), alpha-smooth muscle actin (α-SMA), E2F4 antibodies, immobilized protein A/G beads, and control IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HLM006474, bleomycin, and β-actin antibody were purchased from Sigma (St. Louis, MO). Human recombinant

TGF-β1 was purchased from R&D systems (Minneapolis, MN). All materials used in the experiments are the highest grade commercially available.

Plasmid and siRNA transfection

Human *Nedd4L* and human *TβRII* cDNA were inserted into pCDNA3.1/V5-His-Topo vector or pCDNA3.1/HA-Topo vector. Plasmids and siRNAs transfections in fibroblast cells were performed using Lipo-Jet or PepMute siRNA transfection reagent (SignaGen Laboratories, Frederick, MD). Overexpression or downregulation of genes was confirmed by immunoblotting.

Immunoblotting and co-immunoprecipitation (co-IP)

Lung tissue or lung fibroblast cells were lysed in 1x lysis buffer (Cell signaling, Danvers, MA). Equal amounts of total protein (20 μg) were subjected to SDS-PAGE, transferred to nitrocellulose, and then incubated with primary and secondary antibodies, sequentially. For co-IP, 1 mg of protein in 1 ml of cell lysis buffer was incubated with a primary antibody overnight at 48C, followed by incubation with protein A/G beads for an additional 2 hour in room temperature. The beads were rinsed with PBS and lysis buffer 3 times. Proteins on the beads were eluted by boiling in 2x SDS sample buffer. Eluted samples were subjected to immunoblotting. Imaging was conducted using Azura Biosystems Imaging Station C600.

In vivo ubiquitination assay

Fibroblasts were collected in PBS and cell pellets will be obtained by centrifuge. Cell pellets were then resuspended with ubiquitin aldehyde and N-ethylmaleinimid (NEM) in 50–80 μl of 2% SDS lysis buffer, followed by heating at 100°C for 10 minutes. This procedure disrupts any non-covalent protein-protein interaction. The denatured cell lysates were then sonicated on ice, followed by 10-fold dilution with TBS. Cell lysates were subjected to IP and immunoblotting.

Immunofluorescence staining

Fibroblasts were cultured in glass-bottom dishes. After indicated treatment, cells were fixed with 3.7% formaldehyde for 20 minutes. Cells were blocked by 1% BSA in TBST buffer. Cells were then exposed to primary antibodies, followed by incubation with fluorescence-labeled secondary antibodies and 4′,6-diamindino-2-phenylindole (DAPI). Immunofluorescent cell imaging was conducted using a Nikon A1R confocal microscope.

Animals and flow cytometry

C57/BL6 mice were housed in the specific pathogen-free animal care facility at the Ohio State University in accordance with institutional guidelines and guidelines of the US National Institutes of Health. All animal experiments were approved by the Ohio State University Animal Resources Centers. Lentiviruses were administered to the lungs intratracheally. C57BL/6 mice (8–10 weeks, male and female) were given intratracheal (IT) bleomycin (2 mg/kg body weight) with or without intraperitoneal injection of HLM006474 as detailed in the Results section. Lung tissues were fixed for hematoxylin and eosin (H&E) and trichrome staining. Protein expression in lung tissues was examined by immunoblotting.

Collagen levels were determined by measuring the hydroxyproline content. Lung tissues were minced and incubated in a dissociation medium containing collagenase and DNase I at 37°C for 20 minutes. The digested lung tissue mixture was filtered with 70 μm nylon mesh strainer to obtain a single-cell suspension. Nedd4L expression was analyzed with flow cytometry with antibodies specific to Nedd4L and cell-type markers.

Statistical analysis

Statistical analysis was carried out by 1 or 2-way analysis of variance or unpaired *t* test, with a *P* value of < 0.05 considered indicative of significance.

Results

Nedd4L levels are reduced in fibrotic lungs and isolated myofibroblast cells

We have shown that Nedd4L mediates the ubiquitin-proteasomal degradation of LPAR1[14], which has been implicated in lung injury and fibrosis. We hypothesized that Nedd4L activity may be associated with the pathogenesis of PF, and focused on investigation the role of Nedd4L in lung fibroblast activation because these cells are the ultimate effector cells of fibrosis. We reanalyzed microarray data from the Lung Genomics Research Consortium (http://www.lung-genomics.org/) including 160 patients with IPF and 108 donor control subjects. Consistent with recent studies[16,17], *Nedd4L* mRNA levels in lungs were significantly lower in IPF cohorts compared to control subjects and the reduction of *Nedd4L* levels directly correlates with DLCO% and FVC% predicted (Fig. 1A–C). To further evaluate Nedd4L expression in a murine model of PF, Nedd4L levels in lungs from bleomycin-challenged mice were examined. IT injection of bleomycin (2 U/kg, 14 and 21 days) decreased Nedd4L protein levels in lungs (Fig. 1D–F). The Nedd4L levels showed an inverse correlation with fibronectin (FN) levels (Fig. 1G). Consistent with the findings from IPF patients, *Nedd4L* mRNA levels were also decreased in experimental fibrotic lungs (Fig. 1H), supporting a link between Nedd4L and fibroblast phenotypes in lung fibrosis. *Nedd4L* levels were decreased in lung fibroblasts/myofibroblasts from IPF patients compared to control subjects. Collagen I and α-SMA levels were examined as biomarkers for myofibroblasts from IPF patients (Fig. 1I). Nedd4L reduction was also observed in isolated fibroblasts from murine fibrotic lungs (Fig. 1J, K). The data indicates that in addition to epithelial cells, Nedd4L is also decreased in lung fibroblasts/myofibroblasts in IPF patients and experimental fibrotic lungs. Thus, we believe that *Nedd4L* reduction may have clinically relevant implications.

Nedd4L ubiquitinates and targets TβRII for degradation, thereby attenuating lung fibroblast differentiation to myofibroblasts

It has been shown that Nedd4L regulates TGF-β1 signaling through targeting phosphorylated Smad2/3 and TβRI.[12,13] We confirmed that overexpression of Nedd4L attenuates TGF-β1-induced phosphorylation of Smad2/3 and induction of collagen I, FN, and α-SMA. In contrast, downregulation of *Nedd4L* by siRNA transfection enhanced Smad2/3 phosphorylation and collagen expression (Fig. 2A–D), indicating that Nedd4L diminishes pro-fibrotic response in normal lung fibroblasts. Furthermore, we found that Nedd4L exhibited same phenomenon in A549 cells (Supple Fig. S1).

We have shown that Nedd4L is localized on the inner plasma membrane;[14] thus, we hypothesized that Nedd4L may target TβRII for its ubiquitination and degradation. We found that upregulation of Nedd4L reduced both endogenous and overexpressed TβRII (Fig. 2E, F). Association between the substrate and E3 ligase were confirmed by co-immunoprecipitation and co-immunofluorescence staining (Fig. 2G, H). The data from ubiquitination assay indicated that Nedd4L increased polyubiquitination of TβRII (Fig. 2I). TβRII directly binds to TGF-β1. Our study suggests that Nedd4L regulates TGF-β1-mediated fibrotic effects through targeting TβRII for its degradation.

Overexpression of Nedd4L ameliorates lung fibrosis in a bleomycin-induced model of PF

To elucidate the anti-fibrotic effect of Nedd4L in lung fibrosis, we delivered the *Nedd4L-V5* gene to C57BL/6 mouse lungs using an intratracheal (IT) lentiviral vector delivery system. After 7 days, mice were challenged with IT single-dose BLM (2 U/kg). Mice were sacrificed after additional 3 weeks of IT BLM. After 7 days of lentiviral transduction, expression of Nedd4L-V5 in isolated lung fibroblast cells was confirmed by immunoblotting (Fig. 3A). Immunohistochemical staining with a V5 antibody showed that Nedd4L-V5 is detected in most cell types including lung epithelial cells (Fig. 3B). Lenti-Nedd4L-V5 attenuated IT BLM challenge-increased FN, collagen levels, and lung fibrosis with extension into the adjacent alveolar parenchyma in mouse lungs (Fig. 3C–F), indicating an anti-fibrotic effect of Nedd4L against PF.

Nedd4L suppression is induced by TGF-β1

TGF-β1 signaling is critical for the development of fibrosis.[3,6] TGF-β1 treatment of Mrc5 and human lung fibroblast cells (HLF) decreased Nedd4L protein in a time- and dose-dependent manner (Fig 4A–C). TGF-β1 had no effect on levels of Smurf1, another TGF signaling-related E3 ligase (Fig. 4A–C). Similar to the changes in protein levels, *Nedd4L* mRNA levels were reduced by TGF-β1 in a dose- and time-dependent manner (Fig. 4D–F). Changes of mRNA levels are usually caused by modulation of gene transcription or mRNA stability. To investigate if TGF-β1 affects *Nedd4L* mRNA stability, HLF cells were treated with actinomycin D (an mRNA synthesis inhibitor) with or without TGF-β1. As shown in Fig. 4F, TGF-β1 did not alter actinomycin D-induced *Nedd4L* mRNA degradation, suggesting that TGF-β1 does not influence *Nedd4L* mRNA stability. To examine *Nedd4L* transcription, a 4.5 kbp proximal *Nedd4L* promoter region (p4500) was inserted into pGL3 vector. Luciferase assay showed that TGF-β1 treatment reduced *Nedd4L* promoter activity (Fig. 4G). Taken together, the data indicates that TGF-β1 reduces *Nedd4L* expression by inhibiting its transcription.

Transcription factor E2F1/4 suppresses Nedd4L expression

The role of the canonical Smad pathway in TGF-β1-reduced Nedd4L was examined. Knockdown of *Smad2, 3, and 4* had no effect on TGF-β1-reduced Nedd4L levels (Supple. Fig. S2A,B), suggesting the canonical Smad pathway is not involved in *Nedd4L* suppression. To screen which pathway regulates Nedd4L expression, we tested 30 non-canonical TGFβ signaling and transcriptional inhibitors. Nedd4L suppression was not altered by p38MAPK inhibitor (SB203580), mTOR inhibitor (rapamycin), MAPKK inhibitor (PD98059), HDAC inhibitor (TSA), acetyltransferase inhibitor (C646), DNA

methylation inhibitor (AZA), or histone methyltransferase inhibitor (BIX-01294) (Fig. S1D–H). DNA damage-binding protein 2 (DDB2) has been shown to suppress Nedd4L expression in ovarian cancer; however, downregulation of DDB2 did not alter *Nedd4L* mRNA levels in TGF-β1-treated Mrc5 cells (Supple. Fig. S3). E2F inhibitor HLM006474 exhibited the maximal effect on attenuation of Nedd4L reduction (Fig. 5A). By analysis of Nedd4L promoter region, multiple E2F1 binding sites on the human *Nedd4L* promoter region were identified (data not shown). HLM006474 pretreatment attenuated TGF-β1-reduced Nedd4L levels in lung fibroblasts (Fig. 5B), suggesting that TGF-β1-reduced Nedd4L levels through activation of E2F. Among E2F isoforms, overexpression of E2F4 decreased Nedd4L abundance, while downregulation of E2F4 increased Nedd4L levels (Fig. 5C–E), suggesting that E2F4 suppresses *Nedd4L* transcription.

Inhibitor of E2F dampens severity of PF

Next, we determined whether inhibition of E2F by HLM006474 promotes the de-differentiation of myofibroblasts to fibroblasts. TGF-β1-differentiated myofibroblast cells were treated with HLM006474 for 24 hour. HLM006474 significantly reduced FN and α-SMA levels in the differentiated cells (Fig. 6A, B). In IPF-LF cells, inhibition of E2F4 reduced FN levels, as well as cell migration (Fig. 6C, D). To examine the effect of HLM006474 on bleomycin-induced ECM accumulation, C57BL/6 mice were challenged with bleomycin (IT 2 U/kg body weight). Starting on day 9, HLM006474 (1 mg/kg body weight) was intraperitoneal (IP) injected at 2-day intervals. Mouse lungs were collected at day 21 (Fig. 7A). HLM006474 post-treatment dramatically reduced FN and collagen levels in mouse lung tissues (Fig. 7B). Histological analysis showed that HLM006474 significantly decreased collagen accumulation and severity of PF (Fig. 7C, D).

Discussion

Nedd4L E3 ligase regulates various cellular functions through ubiquitinating multiple substrates. Here we demonstrate an anti-fibrotic effect of Nedd4L that is mediated by its ability to diminish TGF-β1 signaling. Importantly, Nedd4L expression in lung fibroblasts from IPF patients is reduced, and the extent of reduction correlates with markers of disease severity, suggesting that this reduction in Nedd4L contributes to the pathogenesis of pulmonary fibrosis. Supporting this role, we also show that Nedd4L is reduced in murine lung tissue following in vivo administration of bleomycin and murine fibroblasts isolated from fibrotic murine lungs. Consistently, Nedd4L is reduced by TGF-β1 via non-canonical E2F4-mediated gene suppression. Further, we show that TβRII is a novel target protein of Nedd4L ubiquitination. Collectively, this study suggests that prevention of Nedd4L suppression by inhibition of E2F represents a novel new therapeutic strategy for treatment of patients with IPF and other fibrotic lung diseases.

The ubiquitin-proteasome or lysosome systems regulate the majority of protein degradation. Abnormal expression of key enzymes in the protein degradation systems lead to the pathogenesis of human disorders. Nedd4L E3 ligase has been shown to be downregulated in lung tissues from IPF patients, consistent with our current finding. Kimura T. et al. reported that Nedd4L was reduced in lung epithelial cells in IPF patients. Repetitive lung epithelial

injury and repair triggers initiation of fibrosis; however, myofibroblast differentiation is key step for the development of fibrosis. This study shows that Nedd4L levels are reduced in lung myofibroblasts from IPF patients, in an experimental animal model of PF, and in TGF-β1-differentiated myofibroblasts, indicating that Nedd4L reduction occurs in both lung epithelial and myofibroblast cells. This study also suggests that Nedd4L reduction contributes to both initiation and progression of PF.

Molecular regulation of Nedd4L has not been well studied. We screened multiple pathways and transcriptional factors and found that E2F4 potently suppressed Nedd4L in lung fibroblasts. The E2F family of transcription factors regulate cellular functions by modulating gene transcription.[18–22] E2Fs, including E2F4, mediate TGF-β1-induced cell cycle arrest, apoptosis, and gene suppression in cancer cells.[23–25] E2F has been shown to be novel fibrogenic genes that regulate cholestatic liver, renal, and pulmonary fibrosis.[26–29] In our study, the E2F family inhibitor HLM006474 diminished lung fibrosis, supporting a key role for E2F family proteins in lung fibrosis. However, this broad-spectrum inhibitor may cause non-specific effects and cytotoxicity, which limits its therapeutic potential and highlights the need to develop specific inhibitors of E2F4 for interventional trials in fibrotic disorders.

Nedd4L targets multiple proteins in the TGF-β1 pathway, with prior studies demonstrating effects on TβRI and p-Smad2/3. This study reveals that TβRII is also a target protein for Nedd4L ubiquitination. Our previous study showed that TβRII stability is regulated by USP11-mediated deubiquitination. Here we show that Nedd4L ubiquitinates TβRII for its degradation. USP11 is increased in myofibroblasts[30], while Nedd4L is reduced, suggesting that TβRII levels are regulated by the balance of Nedd4L and USP11. Together, Nedd4L/USP11-mediated TβRII stability may determine the activity TGF-β1 signaling pathways, thus, the development of PF. The interplay between Nedd4L and USP11 has not been studied. Our earlier study revealed that USP11 regulates E2F1 stability.[30] Further, Qiao L, et al. reported that E2F1 increases USP11 transcription.[31] The effect of USP11 on E2F4 stability has not been revealed. It is possible that E2F4 plays a role in the regulation of USP11 and Nedd4L expression.

Taken together, this study reveals that Nedd4L suppression in lung myofibroblast contributes to the development of PF, providing evidence that the development of a new strategy to rescue of Nedd4L may halt the pro-fibrotic progression of PF. E2F4 plays a central role in Nedd4L suppression in lung myofibroblast cells. Targeting E2F4 to rescue Nedd4L may be developed a new therapeutic strategy to treat IPF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

Disclosures

Abbreviations:

IPF	idiopathic pulmonary fibrosis
TGF-β1	transforming growth factor-β1
TβRII	TGF-β receptor II
ECM	extracellular matrix
FN	fibronectin
α-SMA	alpha-smooth muscle actin
BLM	bleomycin

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At A Glance Commentary

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Background

The ubiquitin E3 ligase, Nedd4L, exhibits an anti-fibrotic property via targeting multiple substrates for their degradation; however, the regulation of Nedd4L expression in lung fibroblasts has not been studied. Mechanisms for regulation of TGF-β signaling pathway by Nedd4L have not been well revealed.

Translational Significance

Our study revealed that Nedd4L expression is suppressed in lung myofibroblasts, which are effector cells during lung fibrosis. E2F4 negatively regulates Nedd4L expression and contributes to pathogenesis of lung fibrosis. Further, we revealed that Nedd4L targets and ubiquitinates TGF-β receptor II, thus, attenuating TGF-β1 signaling in lung fibroblasts. This study provides evidence showing that upregulation of Nedd4L is a potential therapeutic strategy to treat fibrotic disorders including lung fibrosis.
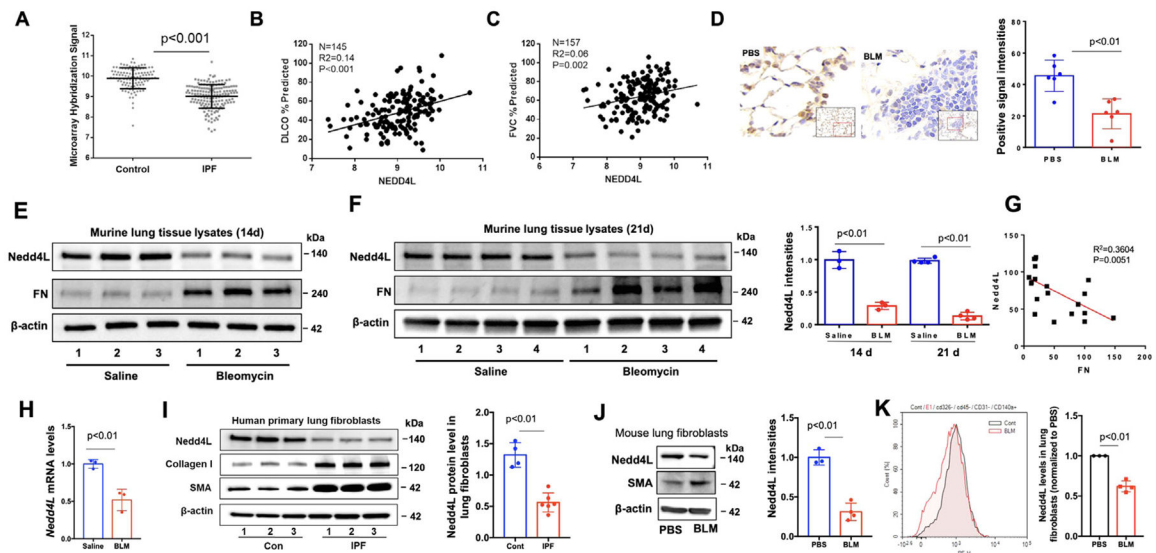
Fig 1. Nedd4L levels are reduced in fibrotic lungs and myofibroblast cells.
A. *Nedd4L* mRNA levels in controls (n=108) compared to IPF group (n=160). Mann Whitney test, *P*<0.001. **B**. Expression of Nedd4L in arbitrary units as a function of diffusing capacity of carbon monoxide (DLCO)% predicated (IPF patients, n=145). **C.** Expression of *Nedd4L* in arbitrary units as a function of FVC% predicated. (IPF patients, n=157). **D.** IHC staining of Nedd4L in lung tissues from IT PBS and IT bleomycin (BLM)-challenged mice. Nedd4L positive intensities were analyzed by Image J. **E.F.,** Immunoblotting analysis of lung tissues of BLM-challenged mice. Nedd4L intensities were analyzed by Image J (n=3−4). **G.** A negative correlation between expression of Nedd4L and FN in lung tissues from BLM-challenged mice. **H.** *Nedd4L* mRNA levels from lungs of IT PBS- or IT BLM (2 U/kg, 14 d)-challenged mice were examined by realtime PCR. **I.** Representative immunoblots of lung fibroblasts from normal control subjects and IPF patients. Nedd4L intensities were analyzed by Image J (n=4−6). **J.** Representative immunoblots in isolated lung fibroblast cells from IT PBS and IT BLM-challenged mice (n=3−4). **K.** Flow cytometry analysis of Nedd4L in isolated lung fibroblasts (cd326−/cd45−/cd31−/cd140a+) from IT PBS (cont)- or IT BLM-challenged mice. Gray: Cont; red: IT BLM-treated cells. Shown are representative flow cytometry histograms. n=3−4, *P*<0.01.
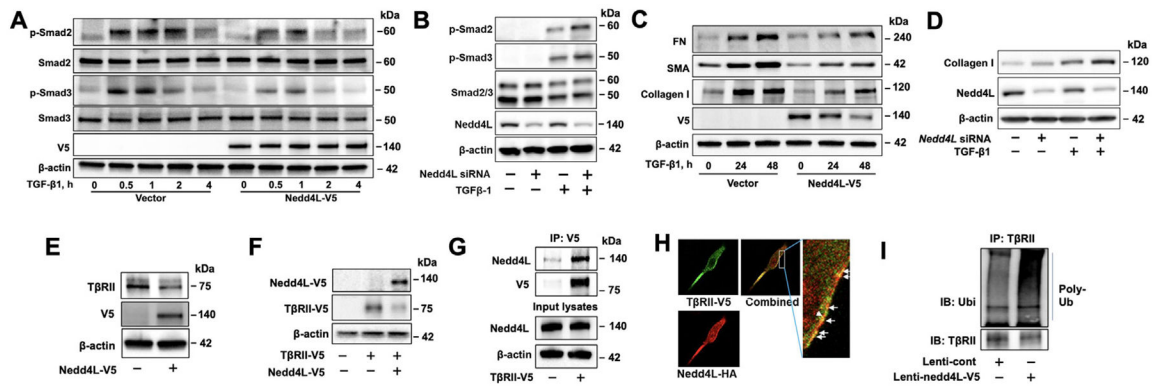
Fig 2. Nedd4L attenuates TGF-β1-induced pro-fibrotic responses through targeting TβRII in lung fibroblasts.

A. Immunoblotting analysis of TGF-β1 (10 ng/ml, 0–4 hour)-treated Nedd4L-V5-transfected HLF cells. **B.** Immunoblotting analysis of TGF-β1 (10 ng/ml, 15 minutes)-treated *Nedd4L* siRNA-transfected HLF cells. **C.** Immunoblotting analysis of TGF-β1 (10 ng/ml, 0, 24, and 48 hour)-treated Nedd4L-V5-transfected Mrc5 cells. **D.** Immunoblotting analysis of TGF-β1 (10 ng/ml, 24 hour)-treated *Nedd4L* siRNA-transfected HLF cells. **E.** Immunoblotting analysis of Nedd4L-V5-transfected Mrc5 cells. **F.** Immunoblotting analysis of Nedd4L-V5 and TβRII-V5-transfected Mrc5 cells. **G.** Immunoblotting analysis of Nedd4L and TβRII-V5 levels in the immunoprecipitated complex pulled down by a V5 antibody. **H.** Coimmunostaining of Nedd4L-HA and TβRII-V5 in double plasmid-transfected Mrc5 cells. Arrows indicate co-localization. **I.** Immunoblotting analysis of poly-ubiquitinated TβRII levels in the immunoprecipitated complex pulled down by a TβRII antibody in a denatured condition by an *in vivo* ubiquitination assay. Immunoblots are representative of three independent experiments.

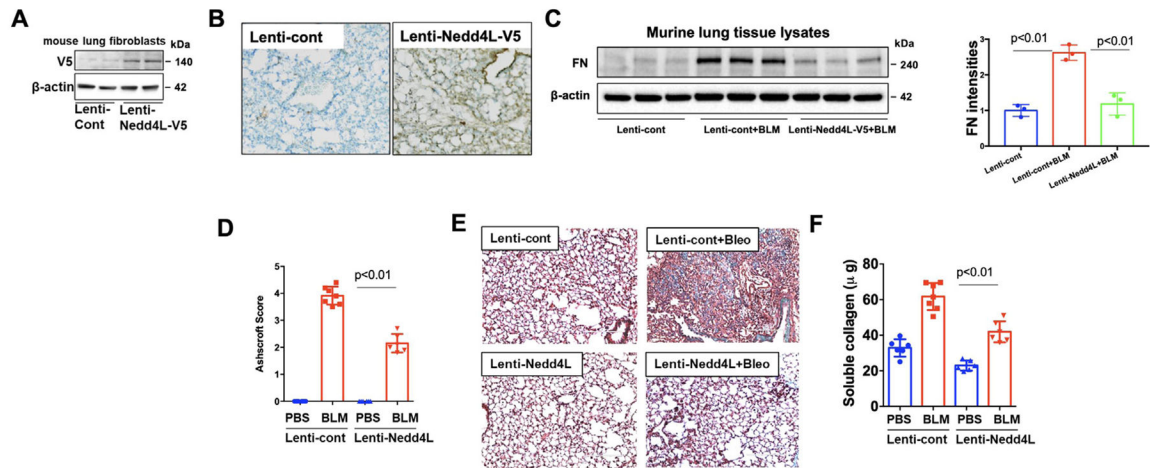**Fig 3. Nedd4L overexpression dampens severity of experimental PF.**
A. Immunoblotting analysis of isolated lung fibroblast cells from IT lenti-Nedd4L-V5-transducted mice. **B.** IHC staining of mouse lung tissues from lenti-control and lenti-Nedd4L-V5-transduced mice with a V5 antibody. n=3. **C.** Immunoblotting analysis of lung tissue lysates. FN intensities were analyzed by Image J. (n=3). **D.** Ashcraft score of lung histology. n=6–8. **E.** Masson trichrome staining of lung tissues. n=4–5. **F.** Collagen content in the right lung was analyzed by Sircol collagen assay. n=6–8.
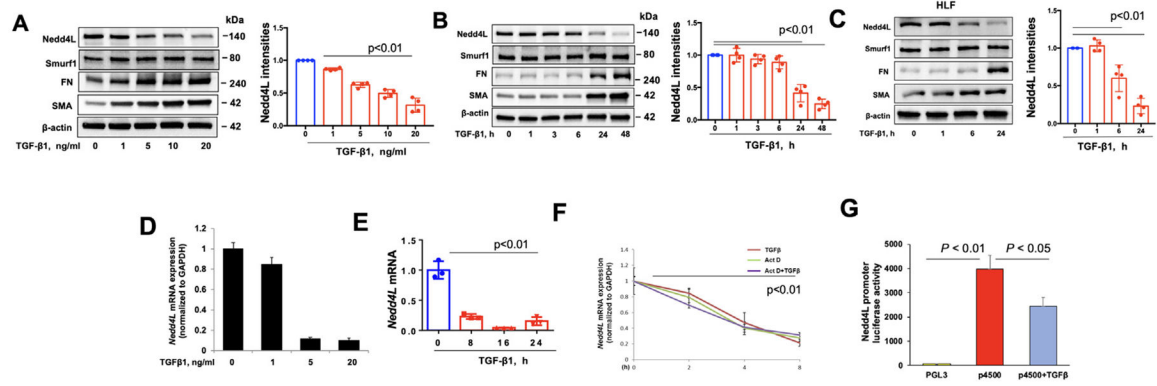
Fig 4. TGF-β1 suppresses Nedd4L transcription.
A-C. Immunoblotting analysis of TGF-β1 (10 ng/ml)-treated Mrc5 and HLF cells. Nedd4L intensities were analyzed by Image J (n=4). **D.E.** Realtime PCR analysis of TGF-β1 (10 ng/ml)-treated Mrc5 cells. n=3. **F.** *Nedd4L* mRNA levels were examined in HLF cells in response to actinomycin D (Act D), or TGF-β1+Act D, by real-time PCR (n=3). **G.** pGL3 empty vector or pGL3 vector containing *Nedd4L* promoter region (4.5 kbp length, p4500) were transfected into HLF cells for 48 h, and then cells were treated with TGF-β1 (10 ng/ml) for 6 h. *Nedd4L* promoter activity was assessed by luciferase assay (n=3).
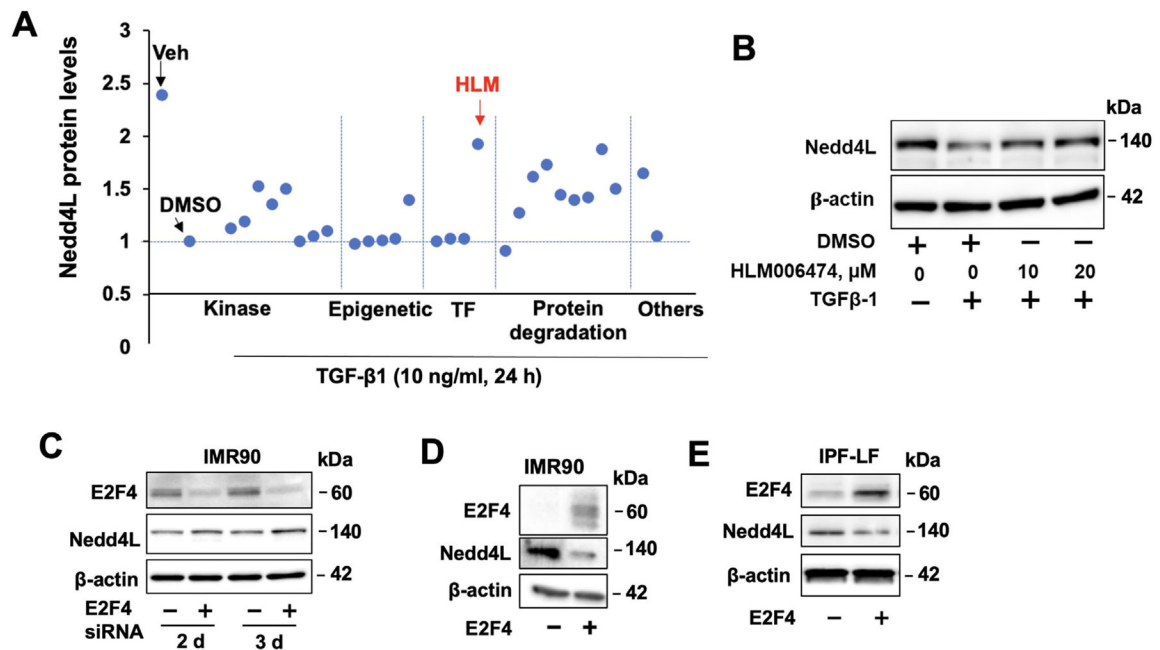
Fig 5. Inhibition of E2F4 rescues TGF-β1-rnduced Nedd4L expression.
A. HLF cells were pretreated with various inhibitors prior to TGF-β1 treatment. Nedd4L expression was determined by immunoblotting and normalized to TGF-β1+DMSO. **B.** Immunoblotting analysis of HLF cells treated with HLM006474 with or without TGF-β1. **C.** Immunoblotting analysis of *E2F4* siRNA-transfected HLF and IMR90 cells. **D.E.** Immunoblotting analysis of E2F4-transfected IMR90 and IPF-LF cells. Immunoblots are representative of three independent experiments.
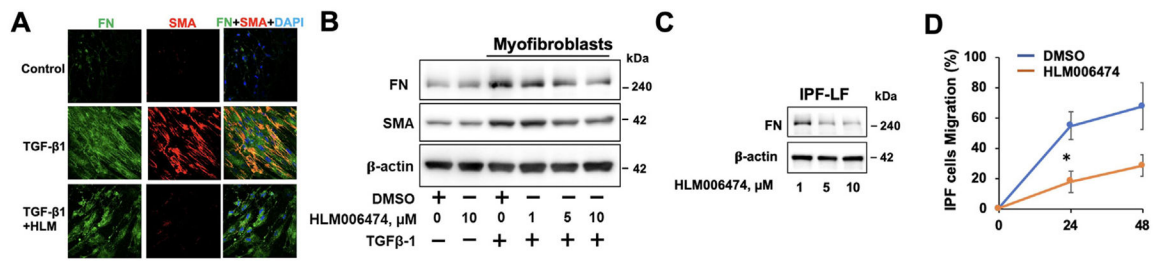
Fig6. HLM006474 attenuates TGF-β1-induced fibroblast differentiation.
A. HLF cells were treated with TGF-β1 for 48 hour, and then cells were post-treated with HLM006474 for an additional 24 hour. FN and a-SMA were examined by immunofluorescence saining. **B.** Immunoblotting analysis of HLM006474-treated TGF-β1-induced lung myofibroblast cells. **C.** Immunoblotting analysis of HLM006474-treated IPF-LF cells. Immunoblots are representative of three independent experiments. **D.** IPF-LF cell migration was measured by a cell scratch assay. n=6, * P<0.01, compared to DMSO-treated cells.
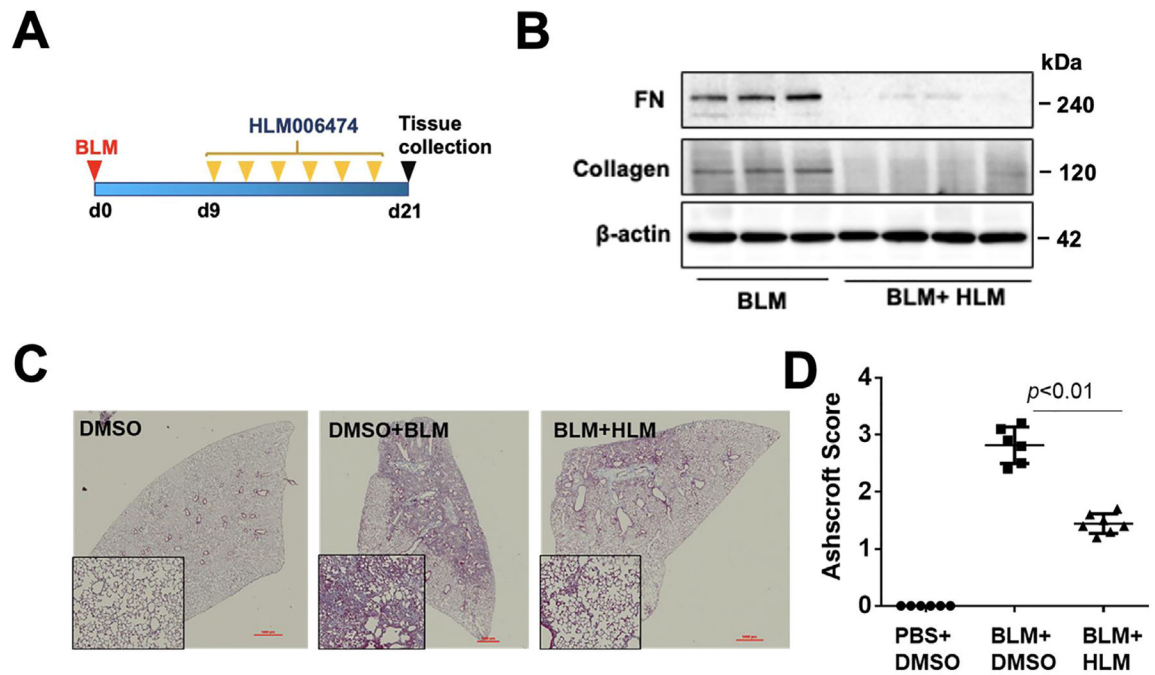
Fig 7. HLM006474 diminishes experimental PF.
A. Scheme of experimental design. **B.** Immunoblotting analysis of lung tissues from day 21. BLM: bleomycin; HLM: HLM006474. n=3–4. **C.** Masson trichrome staining of lung tissues from day 21. Images are representative from 5–6 mice/group. **D.** Summary of the histopathological score from H&E staining. n=5–6.