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Cross Talk between Id1 and Its Interactive Protein Dril1 Mediate Fibroblast Responses to Transforming Growth Factor- β in Pulmonary Fibrosis

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The presence of activated fibroblasts or myofibroblasts represents a hallmark of progressive lung fibrosis. Because the transcriptional response of fibroblasts to transforming growth factor- β_1 (TGF- β_1) is a determinant of disease progression, we investigated the role of the transcriptional regulator inhibitor of differentiation-1 (Id1) in the setting of lung fibrosis. Mice lacking the gene for Id1 had increased susceptibility to bleomycin-induced lung fibrosis, and fibroblasts lacking Id1 exhibited enhanced responses to TGF- β_1 . Because the effect of Id1 on fibrosis could not be explained by known mechanisms, we performed protein interaction screening and identified a novel binding partner for Id1, known as dead ringer-like-1 (Dril1). Dril1 shares structural similarities with Id1 and was recently implicated in TGF- β_1 signaling during embryogenesis. To date, little is known about the function of Dril1 in humans. Although it has not been previously implicated in fibrotic disease, we found that Dril1 was highly expressed in lungs from patients with idiopathic pulmonary fibrosis and was regulated by TGF- β_1 in human fibroblasts. Dril1 enhanced activation of TGF- β_1 target genes, whereas Id1 decreased expression of these same molecules. Id1 inhibited DNA binding by Dril1, and the two proteins co-localized *in vitro* and *in vivo*, providing a potential mechanism for suppression of fibrosis by Id1 through inhibition of the profibrotic function of Dril1. (Am J Pathol 2008, 173:337–346; DOI: 10.2353/ajpath.2008.070915)

Fibrosis is a feature of many chronic diseases, and the unfortunate consequence of losing normal organ tissue to

scar formation is functional impairment or outright organ failure. One determinant of the progression of lung fibrosis is the presence and extent of fibroblastic foci.¹ Most cells in the fibroblast foci are myofibroblasts, defined by the presence of α -smooth muscle actin (α -SMA) and increased contractile properties.^{1,2} Myofibroblasts exhibit elevated matrix protein synthesis and increased production of growth factors and cytokines such as transforming growth factor- β_1 (TGF- β_1).²

Inhibitor of differentiation-1 (Id1) is the first discovered member of an evolutionarily conserved family of four Id proteins (designated Id1 to Id4) that act as dominant-negative inhibitors of basic helix-loop-helix (bHLH) transcription factors.³ Id1 has been identified as a TGF- β -responsive gene, but its response appears to be cell-type-specific: Id1 was reported to be robustly, although transiently, induced in fibroblasts by TGF- β_1 ⁴ but suppressed in human epithelial cells.⁵ The suppression of Id1 in epithelial cells is thought to play an important role in the TGF- β_1 cytostatic program,⁵ just as the increase of Id1 in fibroblasts is thought to play a role in cell-cycle entry.³

Id proteins have been implicated in a variety of biological processes such as cellular growth, senescence, differentiation, apoptosis, angiogenesis, neoplastic transformation, and T-cell receptor signaling,^{4,6–10} although the role that each Id family member plays in the regulation of these functions and the mechanisms by which they act are still areas of active investigation. Biological responses that have so far been described for Id1 invite questions about its role in fibrosis, but to date very little is known. Global expression profiling of fibroblast responses to TGF- β_1 has shown that Id1 is highly induced by TGF- β_1 , raising the possibility that Id1 plays a part in determining fibroblast behavior in the setting of fibrosis.

We hypothesized that Id1 induced by TGF- β_1 would act in a negative regulatory manner to inhibit fibrosis *in*

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vivo and delay or halt myofibroblast differentiation *in vitro*. To test this hypothesis, we examined the effect of Id1 in a murine bleomycin model of lung fibrosis and in fibroblast responses to TGF- β_1 stimulation. We present evidence here that Id1 does in fact inhibit the progression of fibrosis in these models. Surprisingly, the inhibition of α -SMA promoter activation by Id1 did not rely on the presence of E-boxes, the known recognition sequence for bHLH family proteins. This finding suggested that Id1 was not acting via inhibition of traditional bHLH partners. As a result of yeast two-hybrid protein interaction screening, we identified a novel binding partner for Id1, known as dead ringer-like-1 (Dril1). Dril1 shares structural similarities with Id1 and other bHLH proteins, and Dril1 was also recently implicated in TGF- β_1 signaling during embryogenesis.¹¹ To date, little is known about the function of Dril1 in humans. Although it has not been implicated in fibrotic disease, our studies show that Dril1 is highly expressed in lungs of patients with idiopathic pulmonary fibrosis (IPF) and is regulated by TGF- β_1 in human fibroblasts. Our studies define the function of Id1 and Dril1 in fibroblast responses to TGF- β_1 and explore the relationship between these two molecules, providing evidence for a regulatory role of Id1 and Dril1 in the progression of fibrosis.

Materials and Methods

Animals and Treatments

Id1^{-/-} mice were provided by Dr. Robert Benezra (Sloan Kettering Institute, New York, NY) and were generated as previously described.¹² Mice were backcrossed with C57BL/6 for seven additional generations at the University of Pittsburgh; wild-type littermates were used as controls. All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols and were approved by the Animal Care and Use Committees of the Pittsburgh University School of Medicine. Gender- and age-matched mice were assigned to different treatment groups. Bleomycin was administered as previously described.¹³

Cell Culture Experiments

Human fetal lung fibroblasts (MRC-5; American Tissue Cell Culture, Rockville, MD), primary adult human lung fibroblasts, and mouse embryonic fibroblasts from Id1^{+/+} and Id1^{-/-} mice (kind gift of Dr. Robert Benezra) were maintained and grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.1% gentamicin. For siRNA inhibition, MRC-5 cells were transfected with control siRNA or target siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) at a final concentration of 20 nmol/L using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. For adenovirus infection, cells were seeded and grown to 50% confluence, then incubated with adenovirus in serum-free medium. Four hours later, adenovirus was re-

placed by complete medium and cells were harvested for Western blot analysis after 48 hours of incubation.

Immunofluorescence Staining and Microscopic Analysis

Lung tissues were processed as previously described.¹³ After blocking, incubation with primary antibody and three washes with bovine serum albumin, the sections were incubated with cy3-conjugated AffinPure (Jackson ImmunoResearch, West Grove, PA) goat anti-rabbit or anti-mouse IgG (H+L) (1:3000) for 1 hour, stained with Hoechst dye for 30 seconds, and mounted in Gelvatol (Sigma-Aldrich, Milwaukee, WI) after washing. Images were collected with an Olympus Provis microscope (Olympus, Tokyo, Japan) as well as an Olympus Floview scanning confocal microscope.

Yeast Two-Hybrid Assay

The Hybrid Hunter yeast two-hybrid system (Invitrogen) was used to identify Id1-interacting proteins. The full length Id1 was cloned into the yeast expression plasmid LexZeo and sequenced to confirm that the gene was cloned in frame with LexA. A human adult lung cDNA expression library (cloned into pYESTrp2) was purchased from Invitrogen. The bait plasmid was transformed into L40 yeast and expression was confirmed by immunoblot analysis. Bait plasmid was also tested for nonspecific activation. To identify Id1-interacting proteins, L40 was transformed with bait strain and library and Leu⁺ transformants were tested for β -galactosidase activity. A total of 88 colonies were screened by polymerase chain reaction (PCR).

Electrophoretic Mobility Shift Assay

Oligonucleotides were labeled with biotin for chemiluminescence using a biotin 3' end DNA labeling kit (Pierce, Rockford, IL). After labeling, equal amounts of complementary strands were mixed together and allowed to anneal for 1 hour at 37°C to form double-stranded probe. Gel mobility shift assays were performed using the Light-shift chemiluminescent electrophoretic mobility shift assay kit (Pierce) according to the manufacturer's instructions. Briefly, nuclear protein was extracted using a nuclear extract kit (Active Motif, Carlsbad, CA). Four to eight μ g of nuclear proteins were incubated in binding buffer with 50 fmol biotin end-labeled target DNA for 20 minutes at room temperature. Protein-DNA complexes were then separated from the free oligonucleotide by electrophoresis through 4% native polyacrylamide gels containing 0.5 $\times$ Tris-borate-ethylenediaminetetraacetic acid and transferred to a nylon membrane at 380 mA for 60 minutes. After cross-linking the transferred DNA to the membrane at 120 mJ/cm² using a UV-light cross-linker instrument equipped with 254-nm bulbs, we incubated the membrane in blocking buffer for 15 minutes and then conjugated/blocking buffer for 15 minutes. After four

washes, the membrane was incubated in substrate equilibration buffer for 5 minutes, then substrate working solution for 5 minutes, and then exposed to Biomax MR film (Kodak, Rochester, NY).

Biotinylated DNA Precipitation Assay

Streptavidin beads were incubated with biotin-labeled double-stranded target DNA oligonucleotide and negative control DNA oligonucleotide at 4°C for 3 hours, respectively. Cells were lysed by ice-cold phosphate-buffered saline (PBS) containing 0.5% Triton X-100, 5 mmol/L ethylenediaminetetraacetic acid, 125 μ mol/L phenylmethyl sulfonyl fluoride, protease inhibitor cocktail, and phosphatase inhibitor cocktail (1:100 dilution; Sigma, St. Louis, MO). Cell lysates were then centrifuged at 10,000 $\times$ g for 30 minutes. The supernatant was divided equally and added to the mixture of DNA oligonucleotide and streptavidin beads and further incubated overnight at 4°C. Beads were then washed with ice-cold PBS containing 0.5% Triton X-100 and 5 mmol/L ethylenediaminetetraacetic acid for four times. Proteins were eluted by adding 2 $\times$ sodium dodecyl sulfate loading buffer and boiling for 5 minutes, and then were analyzed by Western blotting using rabbit anti-Dril1 antibody.

α -SMA Promoter Constructs

The α -SMA constructs were kindly provided by Dr. Gary K. Owens (Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA). The E-box mutant constructs (E1 and E2) contained the following mutated sequences in bold: E1, CAAGCT; E2, GTACTGA. Details of the generation of the mutants has previously been published.¹⁴ Cells were seeded at 3 $\times$ 10⁴ cells/cm² into 6-well or 12-well plates the day before transfection and were transiently transfected (in triplicate) using Lipofectamine 2000 according to the manufacturer's instructions. Cell extracts were prepared 48 hours after transfection using a passive lysis buffer (Promega, Madison, WI), and β -galactosidase activity was measured using the Galacto-Star system (Applied Biosystems, Foster City, CA). β -Galactosidase activity was normalized to activity of Renilla luciferase measured using the dual luciferase assay system from Promega. Each construct was tested in triplicate per experiment, and the data shown represent three independent experiments.

Tissue Collagen Assay

Lung tissues were homogenized in 0.5 mol/L acetic acid and incubated with shaking for 24 hours at 4°C. Homogenate was spun at 15,000 $\times$ g, and supernatant was analyzed for soluble collagen by the Sircol collagen assay kit following the instructions of the manufacturer (Biorad Ltd., Newtownabbey, UK).

Northern Blotting and Quantitative Reverse Transcriptase (RT)-PCR

Lung tissue or cells was homogenized and RNA was extracted using the Trizol method (Invitrogen). Standard Northern blotting and quantitative RT-PCR were performed as described elsewhere.¹⁵ Probes and primers for the Id1, TIMP1, MMP9, procollagen, and fibronectin genes were obtained from Applied Biosystems. Real-time quantitative PCR was conducted by the TaqMan core facility of the Genomics and Proteomics Core Laboratories of the University of Pittsburgh. Gene expression was analyzed by the $\delta\delta$ Ct method, with 18s rRNA as the endogenous control, and one of the control samples was chosen as a calibrator sample.

Co-Immunoprecipitation

Cell extracts were prepared from 100-mm dishes in 800 μ l of RIPA (radio-immunoprecipitation assay) lysis buffer (Sigma) containing protease inhibitors. The protein extract was incubated with either Id1- or Dril1-specific rabbit polyclonal antibody at 4°C overnight with rotation. After the addition of protein A beads (Santa Cruz Biotechnology Inc.) for another 4 hours. The complexes were then washed with RIPA buffer, and the proteins were extracted in reducing sampling buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blot analysis.

Results

Id1 Is Increased in the Setting of Bleomycin-Induced Fibrosis and in Fibroblasts after TGF- β_1 Stimulation

To characterize Id1 expression in fibrosis, we began by examining lungs of mice after bleomycin treatment for Id1 mRNA and protein expression. Fourteen days after bleomycin treatment, whole lung mRNA levels for Id1 were increased; this corresponded with a time-dependent increase in Id1 protein levels as assessed by Western blotting of whole lung homogenate (Figure 1, A and B). Similarly, Id1 expression increased at early time points after TGF- β_1 treatment of both embryonic human lung fibroblasts (MRC-5) and primary adult human lung fibroblasts (Figure 1C and data not shown). The increase in protein levels correlated with an early increased expression of Id1 mRNA after TGF- β_1 treatment (Figure 1D). The rise in Id1 expression occurred predominantly in the nucleus, although a modest increase in cytoplasmic expression was also seen by immunostaining (Figure 1F). The image shown in Figure 1 was generated using mouse wild-type fibroblasts; Id1^{-/-} controls were used to confirm antibody specificity (not shown). Identical results were obtained using human fibroblasts (not shown). In both Western and Northern blots for Id1, the expression level appeared to fall below the baseline level after an initial early rise. We confirmed this observation using

Figure 1. Id1 is increased in mouse lung after bleomycin treatment and in fibroblasts after TGF-β₁ stimulation. **A** and **B**: Lungs of C57BL/6 mice treated with bleomycin exhibit a significant increase in Id1 mRNA expression and protein expression by 14 days. **C** and **D**: Stimulation of human lung fibroblasts with TGF-β₁ leads to an early increase in Id1 protein expression that correlates with a rise in Id1 mRNA as assessed by Northern blot. **E**: Quantitative RT-PCR demonstrates a biphasic pattern after TGF-β₁ stimulation with an early rise in Id1 mRNA followed by late suppression. **F**: Immunostaining of mouse fibroblasts shows an increase in predominantly nuclear Id1 expression 2 hours after TGF-β₁ treatment.

quantitative RT-PCR. After an initial sharp rise in Id1 mRNA expression, there is late suppression of Id1 mRNA levels (Figure 1E), indicating a biphasic response of Id1 to TGF-β₁ stimulation.

Mice Lacking the Gene for Id1 Demonstrate Increased Susceptibility to Bleomycin Treatment

To determine whether Id1 plays a functional role in experimental fibrosis, mice lacking the gene for Id1 were treated with intratracheal bleomycin. At a dose of 0.1 U per age- and weight-matched mouse, the Id1^{-/-} mice exhibited increased mortality when compared with WT mice (Figure 2A). The Id1^{-/-} mice displayed evidence of increased respiratory distress 10 days after bleomycin treatment and succumbed to death from the 12th day onwards. A lower dose of bleomycin (0.06 U per mouse) allowed for improved survival of the Id1^{-/-} mice, but lung

collagen content was increased in the Id1^{-/-} mice relative to wild-type 14 days after bleomycin treatment (Figure 2B). The expression of fibronectin and procollagen mRNA (assessed by real-time PCR) was also increased in the lungs of Id1^{-/-} mice treated with bleomycin relative to control mice (Figure 2, C and D). Lungs were also harvested for α-SMA staining 1 week after bleomycin treatment. Lungs of mice lacking the gene for Id1 did not have noticeably increased α-SMA expression at baseline, and histologically were indistinguishable from those of wild-type mice. After bleomycin treatment, Id1^{-/-} mice exhibited appreciably more parenchymal α-SMA staining than wild-type mice; representative images are shown in Figure 2E. Quantitation of α-SMA immunofluorescence for each group is shown in Figure 2F. In wild-type mice, there was a threefold induction over baseline, whereas in Id1^{-/-} mice the α-SMA staining increased fivefold over baseline.

Figure 2. Mice lacking the gene for Id1 have increased susceptibility to bleomycin-induced lung fibrosis. **A**: Id1^{-/-} mice had increased mortality in response to intratracheal bleomycin when compared with control mice. **B**: At a lower dose of bleomycin, Id1^{-/-} mouse survival was improved, but lung collagen was increased. **C** and **D**: Lung fibronectin and procollagen mRNA were similarly increased. **E**: α-SMA expression was increased in Id1^{-/-} mice relative to control mice. The left two images represent bleomycin-treated Id1^{-/-} and WT mice; the right pair of images shows the same. Red stain represents α-SMA expression. **F**: Quantitation of α-SMA lung immunostaining. For bleomycin-treated animals, n = 5 to 6; for untreated controls, n = 1. Error bars represent SD. Quantitation excluded staining in airways and blood vessels. Original magnifications: ×10 (**E**, left); ×40 (**E**, right).

Inhibition of Id1 Leads to an Exaggerated Fibroblast Response to TGF- β_1 Stimulation

Treatment of fibroblasts with TGF- β_1 results in a characteristic increase in the expression of proteins involved in fibrosis, including matrix proteins, α -SMA, and plasminogen activator inhibitor-1 (PAI-1). To determine whether the presence or absence of Id1 would affect this characteristic response *in vitro*, fibroblasts from Id1^{-/-} and control mice were compared. As expected, TGF- β_1 stimulation led to an increase in expression of fibronectin, α -SMA, and PAI-1 within 48 hours. Fibroblasts lacking the gene for Id1 had an even greater response to TGF- β_1 than did the wild-type fibroblasts (Figure 3A). This finding was confirmed in human lung fibroblasts using siRNA to inhibit the expression of Id1 (Figure 3, B–D). It should be mentioned that the level of α -SMA expression in Figure 3D is identical to the level of expression in the unstimulated lanes in Figure 3C; the same cellular protein was used to generate both figures. The apparently higher expression of α -SMA in Figure 3D is attributable to a longer film exposure time. This indicates that even in the absence of exogenous TGF- β_1 stimulation, Id1 may be acting to maintain a low baseline level of α -SMA expression. Taken together, these *in vitro* observations are consistent with our *in vivo* finding that Id1 acts to suppress fibrosis.

Figure 3. Absence of Id1 leads to an exaggerated fibroblast response to TGF- β_1 . **A:** Fibroblasts from Id1^{-/-} mice stimulated with TGF- β_1 (5 ng/ml) express higher levels of fibronectin, α -SMA, and PAI-1 than control-stimulated cells after 48 hours. **B:** Id1 expression is suppressed in human fibroblasts (MRC-5) treated with Id1 siRNA. **C:** Cells treated with Id1-specific siRNA followed by TGF- β_1 stimulation demonstrate an exaggerated rise in α -SMA, fibronectin, and PAI-1 expression. **D:** Id1 siRNA treatment alone leads to increased α -SMA expression relative to control siRNA-treated cells. (Note that film exposure time was longer for **D** than for **C** to resolve the bands corresponding to baseline α -SMA expression.) **E** and **F:** Densitometry ($n = 3$) for blots shown in **C** and **D**, respectively. * $P < 0.001$ compared with control(s). # $P < 0.001$ compared with TGF- β alone.

Modulation of α -SMA Expression by Id1 Does Not Require the E-Box Promoter Elements

Id1 is best known for its ability to bind bHLH proteins and prevent their association with DNA. The DNA binding recognition sequence of bHLH proteins is the E-box (CANNTG); this element is present in the promoter regions of a number of TGF- β_1 -regulated genes, including PAI-1, α -SMA, Smad7, and collagen-1.^{14,16–18} We postulated that Id1 might modulate the expression of these genes by a mechanism involving E-box binding. To test this hypothesis, we transfected Id1^{-/-} and wild-type fibroblasts with control and E-box mutant α -SMA promoter constructs fused with a β -galactosidase reporter (kindly provided by Dr. Gary K. Owens). The α -SMA promoter contains two E-boxes at –214 bp (E1) and –252 bp (E2); the constructs tested were mutated at one or both of these sites. After transfection, fibroblasts were stimulated with TGF- β_1 , and β -galactosidase activity was assessed. As shown in Figure 4A, mutation of either or both E-box elements did not alter the enhanced promoter responsiveness in the Id1^{-/-} fibroblasts, indicating that Id1 is likely acting through an E-box-independent mechanism.

Dril1 Is a Novel Binding Partner of Id1

Because the effect of Id1 on α -SMA promoter activation did not require the presence of intact E-boxes and therefore might not require traditional bHLH binding partners,

Figure 4. Dril1 is a novel binding partner for Id1 and a TGF- β_1 -responsive molecule. **A:** Responsiveness of α -SMA promoter constructs (control and E-box mutants) was assessed in WT and Id1^{-/-} fibroblasts. E1 refers to mutation of the E-box at –214 bp and E2 to the E-box at –252 bp. In the E1 + E2 construct, both sites are mutated. Galactosidase activity is expressed as mean $\pm$ SD of triplicate samples. Data are representative of three separate experiments. * $P < 0.05$ compared with WT baseline; # $P < 0.05$ compared with Id1^{-/-} baseline, and TGF- β_1 -treated WT. **B** and **C:** Human lung fibroblasts exhibit a time- and dose-dependent increased in Dril1 expression by Western blot after TGF- β_1 stimulation. **D:** Top: Immunoprecipitation with anti-Dril1 and immunoblotting with anti-Id1 in human lung fibroblasts. Lane 1, control IgG. The protein in lanes designated NS was precipitated with a control nonspecific monoclonal antibody. Bottom: Immunoprecipitation with anti-Id1 and immunoblotting with anti-Dril1. **E:** Densitometric analysis of three representative Western blots corresponding with the experiment shown in **B**.

we sought novel Id1-interacting proteins by yeast two-hybrid screening. Of the 12 individual interacting proteins identified (from a total of 73 independent clones sequenced), 3 were known Id1-interacting proteins (HEB, E2-2, and E2A), 3 were unknown proteins, and the remainder were novel proteins not previously known to interact with Id1. Of these, the most promising candidate for a fibrosis-related molecule was Dril1. Interaction between Dril1 and Id1 was confirmed by co-immunoprecipitation (Figure 4D). Dril1 is a DNA-binding protein containing an A/T rich interaction domain (ARID). ARID domain proteins are members of a highly conserved family involved in chromatin remodeling and cell-fate determination. Very little is known about the role of Dril1 in humans, but it shares some similarities with Id1, including a helix-loop-helix structure.¹⁹

Dril1 Is a TGF- β_1 Responsive Molecule

Little is known about control of Dril1 expression, and it is not known whether TGF- β_1 has any role in regulating its expression. To determine whether Dril1 is responsive to TGF- β_1 , human fibroblasts were treated with TGF- β_1 and analyzed by Western blotting for expression of Dril1. As shown in Figure 4, C and D, a time- and dose-dependent increase in Dril1 expression occurs after TGF- β_1 treatment, with maximal expression at the 6-hour time point. The protein for dose response was collected at the 6-hour time point. Densitometry of Western blots showing the time course of Dril1 response to TGF- β_1 is shown in Figure 4E.

Expression of Dril1 Is Increased in IPF

Whole tissue homogenate of lungs explanted from patients with IPF and control lungs were analyzed by Western blotting for Dril1 expression. Expression of Dril1 was uniformly increased in IPF lungs as compared with controls (Figure 5A), suggesting that Dril1 may play a role in human disease. This finding was confirmed by Dril1 immunostaining of control and IPF lungs (Figure 5B). Co-

staining of human IPF lung with antibodies against Dril1 and the fibroblast marker vimentin indicates that Dril1 is likely expressed in fibroblasts in IPF. Some of the co-stained cells have the typical morphology of a fibroblast (Figure 5C, left), whereas others have a more cuboidal morphology (Figure 5C, right).

Id1 and Dril1 Co-Localize in Vitro and in Vivo

Given that we had identified Dril1 as an Id1-binding protein, we tested whether Id1 and Dril1 co-localize in fibroblasts *in vitro* in response to TGF- β_1 stimulation. Human fibroblasts were stained for Id1 and Dril1 before and 2 hours after TGF- β_1 treatment. This time point corresponds with declining levels of Id1 expression and rising levels of Dril1 expression, providing the highest levels of both proteins according to our Western blot kinetics. Expression of both proteins increased as expected after TGF- β_1 treatment. Dril1 and Id1 both stained primarily in the nucleus, although cytoplasmic Id1 also increased after TGF- β_1 stimulation (Figure 5D). Co-localization of the two proteins occurred mainly in the nucleus. Co-staining was also performed in the IPF lung to demonstrate co-localization *in vivo*. Figure 5E shows co-expression of Id1 and Dril1 in end-stage IPF/UIP lung. The areas of greatest co-localization (yellow) again occur in the nucleus.

Id1 Expression Influences Dril1 Binding to DNA

Because Id1 and Dril1 interact physically and co-localize *in vitro* and *in vivo*, we hypothesized that Id1 might inhibit Dril1 DNA binding in the same way that Id1 is known to inhibit DNA binding of classical bHLH family members. Dril1 is known to bind AT-rich regions of DNA, and using a published consensus sequence for Dril1²⁰ (5'-AATAAATTAAGTTTAAATATTTT-3'), we tested the effect of increasing intracellular Id1 levels on binding of Dril1 to DNA probe by electrophoretic mobility shift assay. Intracellular Id1 expression levels were manipulated using Id1 adenovirus and Id1 siRNA to increase or decrease ex-

Figure 5. Dril1 expression is increased in IPF. **A:** Dril1 protein expression is increased in human whole lung homogenate of IPF patients (lanes 5 to 9) compared with normal donor lungs (lanes 1 to 4). **B:** Dril1 immunostaining in human end-stage IPF/UIP lung is also increased relative to normal lung. **C:** Co-immunostaining of IPF/UIP lung with Dril1 (red) and vimentin (green) demonstrates co-localization. The left image shows a cell with typical fibroblast morphology; other co-staining cells had a less typical appearance as shown at the right. **D and E:** Co-immunostaining for Id1 (red) and Dril1 (green) demonstrates co-localization in TGF- β_1 -stimulated fibroblasts and IPF/UIP lung, respectively. Arrows demonstrate areas of co-localization in IPF lung.

Figure 6. Changes in level of Id1 expression affect Dril1 DNA binding. **A:** Adenoviral transfer of Id1 successfully increases intracellular expression in a dose-dependent manner. Con, control untreated cells; LacZ, control adenovirus; Ad.Id1, adenovirus expressing Id1. **B:** Overexpression of Id1 using 10^7 PFU Id1 adenovirus results in diminished binding of Dril1 to its AT-rich consensus sequence. NS, nonspecific competitor; S, specific competitor. **C:** Conversely, inhibition of Id1 with Id1-specific siRNA results in increased Dril1 binding to DNA. NS, nonspecific competitor; S, specific competitor. 1 h and 2 h refer to the time after TGF- β_1 stimulation. **D:** After DNA precipitation and immunoblotting with anti-Dril1 antibody, a band of ~80 kDa can be appreciated (lane P). Lane represents control DNA precipitation using nonspecific oligonucleotide. Lanes lysate and R represent Dril1 immunoblots of TGF- β_1 -treated human fibroblast lysate and recombinant Dril1, respectively.

pression, respectively. As shown in Figure 6A, adenoviral infection of human fibroblasts increased Id1 expression in a dose-dependent manner. Adenovirus containing a LacZ expression plasmid was used as a control. Overexpression of Id1 *in vitro* led to decreased Dril1-DNA binding as assessed by electrophoretic mobility shift assay. A representative blot is shown in Figure 6B. When fibroblasts were transfected with siRNA specific for Id1, the opposite effect was seen: decreased Id1 expression led to increased of Dril1-DNA binding when compared with control siRNA-transfected cells (Figure 6C). To confirm that the observed band did represent Dril1 bound to DNA, DNA immunoprecipitation was performed using a

Figure 7. Inhibition of Dril1 results in a dampened fibroblast response to TGF- β_1 . **A:** Dril1-specific siRNA inhibits expression of Dril1 in human fibroblasts. **B:** Inhibition of Dril1 with siRNA results in a diminished response of α -SMA to TGF- β_1 stimulation. **C** and **D:** TGF- β_1 -stimulated fibronectin, PAI-1, and collagen-1 expression are also inhibited by Dril1 siRNA. **D:** Densitometry ($n = 3$) for the experiment shown in **C**. * $P < 0.001$ compared with control(s). # $P < 0.01$ compared with control siRNA and TGF- β -treated cells.

Dril1-specific sequence. This is shown in Figure 6D. The lane labeled P is the DNA precipitate blotted with anti-Dril1 antibody. The lanes labeled lysate and R represent a Dril1 immunoblot of TGF- β_1 -treated fibroblast lysate and recombinant Dril1, respectively.

Inhibition of Dril1 Results in Decreased Fibroblast Responsiveness to TGF- β_1

Because the function of Dril1 in fibrogenesis is unknown, we tested whether inhibition of Dril1 using siRNA would lead to changes in fibroblast responses to TGF- β_1 . Successful suppression of Dril1 expression *in vitro* using siRNA is shown in Figure 7A. Fibroblasts treated with Dril1 siRNA did not have the expected time-dependent increase in α -SMA expression in response to TGF- β_1 that was seen in control transfected cells (Figure 7B). The expression of collagen-1, fibronectin, and PAI-1 was also suppressed 2 days after TGF- β_1 treatment by inhibition of Dril1 (Figure 7, C and D).

Discussion

IPF is a disease that follows a relentless course of respiratory failure leading to death. There are currently no

effective therapies for patients afflicted with this disease. Although much recent progress has been made toward understanding the pathogenesis of IPF, our understanding of the mechanism by which IPF develops and progresses remains incomplete.

Dr. R.C. Chambers and colleagues⁴ have shown that Id1 expression is increased in a bleomycin model of fibrosis. These authors speculated that transient induction of Id1 might initially delay the myofibroblast differentiation program initiated in response to TGF- β_1 .⁴ To date, the function of Id proteins in a model of fibrosis has not been tested. We have shown evidence here that the progression of experimental lung fibrosis is altered by the presence of Id1. Our data indicate that mice lacking the gene for Id1 have increased susceptibility to bleomycin-induced lung injury and fibrosis, and fibroblasts lacking Id1 exhibit enhanced responses to TGF- β_1 stimulation. Although inhibition of fibroblast activation by Id1 may account for its ability to modulate fibrosis in the intact lung, there may also be contributory effects from other cell types. For instance, the loss of Id1 may result in diminished epithelial cell proliferation,⁵ limiting appropriate repair mechanisms. Loss of Id1 might also enable epithelial-mesenchymal transition, in analogy with the reported suppression of epithelial-mesenchymal transition by ectopic expression of Id2 and Id3.²¹ Of interest, one recently published study demonstrated worse lung injury and fibrosis in Id1^{-/-} animals compared with wild-type controls, and in this case the authors ascribed the difference to increased vascular permeability in the setting of lung injury.²² Findings included increased vascular leak in Id1^{-/-} mice after bleomycin-induced injury and increased susceptibility of cultured Id1^{-/-} lung microvascular endothelial cells to apoptosis. In addition, these authors demonstrated increased lung collagen in bleomycin-exposed Id1^{-/-} mice relative to control mice using hydroxyproline measurement as their metric.²² These data introduce an alternative and complimentary hypothesis for why Id1^{-/-} mice may have increased susceptibility to bleomycin-induced lung injury and fibrosis.

Id1 expression is regulated both at the level of transcription and protein stability. At the promoter level, TGF- β_1 -responsive transcription factors such as Smad proteins, Egr1, and ATF3 have been shown to affect Id1 expression.⁵ Id proteins behave as immediate early or primary response genes after growth factor stimulation²³; although the pattern of expression varies with stimulus and cell type, biphasic responses such as the one we have shown here for Id1 expression in fibroblasts are not uncommon.^{5,23} Proteasomal degradation accounts for the short intracellular half-lives of Id proteins, which may be as short as 20 minutes.^{24–26} We have found that prevention of Id1 degradation by proteasome inhibition abrogates TGF- β_1 -induced fibroblast α -SMA expression (unpublished data). Interestingly, even in the absence of TGF- β_1 stimulation, proteasomal inhibition led to increased intracellular levels of Id1. This suggests that there is active turnover of Id1 in the resting fibroblast, with production and proteasomal degradation occurring simultaneously. Our data, indicating that inhibition of Id1 by siRNA causes increased α -SMA expression in the ab-

sence of exogenous active TGF- β_1 , suggest that the low baseline level of Id1 in the fibroblast exerts an effect. It is possible that tonic low-level expression of Id1 serves an inhibitory function in the fibroblast, and that the late suppression of Id1 mRNA expression by TGF- β_1 disrupts this balance, releasing the fibroblast to differentiate toward a myofibroblast phenotype.

Id1 is best known as an inhibitor of bHLH transcription factors. By binding bHLH proteins, Id1 inhibits their ability to bind DNA as homodimeric or heterodimeric complexes. Id proteins have been shown to bind factors other than E-proteins, such as ternary complex factors²⁷ and retinoblastoma tumor suppressor protein.²⁸ We have now shown that Id1 is also capable of binding to Dril1, a protein that is evolutionarily conserved and ubiquitously expressed. Although little is known about its role in humans, Dril1 has been implicated in TGF- β signaling during embryonic development,¹¹ and we have shown here that Dril1 is a TGF- β -responsive molecule that is up-regulated in human fibrotic disease. Dril1 belongs to the Arid (AT-rich interaction domain) family of DNA-binding proteins; it shares structural similarities with Id1 including a modified helix-turn-helix motif domain. The known cellular functions of Arid proteins include regulation of cell growth, differentiation, and development,²⁹ similar to Id proteins. The mouse protein Bright was the first identified member of this family in 1995; it was subsequently renamed Arid3A and is known as the mouse counterpart of the more recently discovered human Dril1 (also named Arid3A).^{30,31} In recent years, however, it has become apparent that the tissue distribution of these two proteins is discordant. Bright is exclusively expressed in mature B cells,³² whereas Dril1 is ubiquitously expressed.³¹ The highly similar (and ubiquitously expressed) protein ARID3B (Bdp) may be the true murine counterpart of Dril1. In contrast to Bright, deletion of Bdp in the mouse is lethal,³³ as predicted for Dril1. The data presented here indicate that Dril1 can alter fibroblast responses to TGF- β_1 and is highly expressed in adult fibrotic lung tissue, suggesting that this molecule has a functional role in the adult lung.

Although we have identified Dril1 by screening for Id1 binding partners, it is probable that Id1 does not represent the only protein capable of binding to Dril1 and altering its function. Mice lacking the individual gene for Id1, Id2, or Id3 do not exhibit developmental abnormalities, but ablation of two of these Id genes in any combination leads to embryonic lethality.³⁴ This suggests that the Id proteins have some overlapping functions and that the absence of one Id gene may be compensated for by other family members. It is possible that Dril1 interacts with Id proteins other than Id1, and that other Dril1 binding partners such as E2F1³⁵ may alter its function in the setting of fibrosis.

The increase in lung collagen in Id1^{-/-} mice relative to control mice measured with the Sircol assay is quantitatively small. There are several potential explanations for this finding. The first relates to the problem of trying to show increased susceptibility (as opposed to protection) in the bleomycin model. Lung injury and repair in response to inhaled bleomycin is patchy, with some areas

of parenchyma showing dramatic architectural distortion and other areas appearing to be completely spared. Despite relatively low doses of bleomycin, severe injury and densely fibrotic regions of lung can still be appreciated in the lungs of wild-type animals, making it more difficult to appreciate increases in this degree of fibrosis in the Id1^{-/-} animals. Interestingly, a study by a separate group of investigators did demonstrate a greater difference in lung collagen content between Id1^{-/-} and wild-type animals after bleomycin treatment²² than we observed in our study. The reason for this discrepancy is unclear, although it may relate to the use of hydroxyproline measurement as opposed to the Sircol assay and to differences in animal treatment techniques. A second reason why Id1^{-/-} mice have a small increase in lung collagen relative to controls may relate to biological compensation by other Id proteins. As noted above, the Id1 gene belongs to a family of four homologous proteins, three of which are expressed in most tissues and organs and have overlapping distribution patterns.⁸ The deletion of any of these genes in mice does not affect survival, whereas deletion of a combination of two Id genes leads to embryonic lethality, suggesting that these proteins have compensatory function.³⁴ It is likely that the effect of the loss of Id1 on lung fibrosis is at least partially compensated by the other Id proteins, which are all intact in this mouse. Of note, we have examined the level of mRNA expression of other Id proteins in Id1^{-/-} fibroblasts in response to TGF- β_1 , and we have observed similar patterns of response in wild-type and Id1^{-/-} fibroblasts (unpublished data).

In summary, these data indicate that Id1 exerts an anti-fibrotic effect in the lung and negatively regulates fibroblast to myofibroblast transformation. We have identified a novel binding partner for Id1, known as Dril1. The expression of these two structurally similar molecules is regulated by TGF- β_1 , and they are co-expressed in human fibrotic lung, where it appears that Id1 may bind and therefore inhibit the profibrotic function of Dril1. Future studies of the regulation of Id1 and Dril1 expression in fibrosis and the interactions between these molecules and the well established Smad signaling pathway are warranted. These studies would be facilitated by identification of the true murine homolog of Dril1.

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