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Discoidin Domain Receptor 2 Signaling Regulates Fibroblast Apoptosis Through PDK1/Akt.

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Abstract:

Progressive fibrosis is a complication of many chronic diseases and collectively, organ fibrosis is the leading cause of death in the US. Fibrosis is characterized by accumulation of activated fibroblasts and excessive deposition of extracellular matrix proteins, especially type I collagen. Extensive research has supported a role for matrix signaling in propagating fibrosis but type I collagen itself is often considered an end product of fibrosis rather than an important regulator of continued collagen deposition. Type I collagen can activate several cell surface receptors including α2β1 integrin and discoidin domain receptor2 (DDR2). We have previously shown that mice deficient in type I collagen have reduced activation of DDR2 and reduced accumulation of activated myofibroblasts. In the present study, we find that DDR2-null mice are protected from fibrosis. Surprisingly, DDR2-null fibroblasts have a normal and possibly exaggerated activation response to TGFβ and do not have diminished proliferation compared to WT fibroblasts. DDR2-null fibroblasts are significantly more prone to apoptosis, in vitro and in vivo, compared to WT fibroblasts, supporting a paradigm in which fibroblast resistance to apoptosis is critical for progression of fibrosis. We identify a novel molecular mechanism in which DDR2 can promote the activation of a PDK1/Akt survival pathway and find that inhibition of PDK1 can augment fibroblast apoptosis. Furthermore, our studies demonstrate that DDR2 expression is heavily skewed to mesenchymal cells compared to epithelial cells and that IPF cells and tissue demonstrate increased activation of DDR2 and PDK1. Collectively these findings identify a promising target for fibrosis therapy.

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Introduction

Progressive fibrosis is a devastating consequence of a wide variety insults, affecting nearly every organ system and collectively accounts for over 45% of deaths in the developed world (1, 2). Fibrosis in the lung can result from an acute injury, chronic inflammation, or primary diseases such as Idiopathic Pulmonary Fibrosis. Regardless of the cause, fibrosis can lead to a devastating clinical course due to a lack of good therapeutic treatment (3-5).

Pulmonary fibrosis is characterized by accumulation of activated fibroblasts and deposition of fibrotic extracellular matrix (ECM) proteins (3, 4). Progressive fibrosis is thought to be the result of a dysregulated repair mechanism in response to injury, or some other inciting event, which leads to excessive and continued deposition of fibrotic matrix proteins (1). Activated fibroblasts are thought to accumulate in the fibrotic tissue through increased proliferation, differentiation into the activated phenotype and resistance to apoptosis. In addition to direct deposition of ECM proteins, especially type I collagen, activated fibroblasts secrete a number of profibrotic signaling ligands which lead to paracrine activation of neighboring cells (6).

While primarily regarded as the scaffolding proteins of fibrotic matrix, type I collagen can initiate cell signaling through activation of a number of cell surface receptors with significant influence on cell behavior (7-9). Type I collagen signaling has been shown to be upregulated in cancer progression and inhibition of collagen I synthesis can lead to decreased tumor invasion. In the lung, type I collagen has been shown to be upregulated quickly after acute injury both in human patients and in animal models, implicating a role for collagen I signaling as a regulator of progressive fibrosis rather than serving solely as the end product of fibrosis . Finally, we have previously shown that selective deletion of collagen I from alveolar epithelial cells surprisingly led to robust protection from fibrosis (10). We found that early production of collagen activates collagen receptors in vivo which promoted further fibroblast activation potentially establishing an

important positive feedback loop in progressive fibrosis (10, 11). Thus, type I collagen is both an important physical component of the fibrotic matrix as well as a potential pro-fibrotic signaling molecule.

Collagens can signal through several different families of cell surface receptors, including several integrins, especially α2β1 integrin, and the discoidin domain receptors (DDRs) (7, 8, 12). The DDRs are receptor tyrosine kinases that are activated upon binding to collagens. DDR1 primarily binds type IV collagen and has already been implicated in mediating the fibrotic response (13). Fibrosis is characterized by increased production of fibrillar collagens, especially type I collagen, but a role for type I collagen signaling during progressive fibrosis has not been well characterized. DDR2 is the primary receptor for fibrillar collagens including type I and type III collagen (7, 8, 12). There is increased recognition for an important role of DDR2 in a variety of malignancies leading to interest in generating DDR2-specific inhibitors (14). The role of DDR2 in fibrosis is mixed. Mice deficient in DDR2 have an increased propensity for liver fibrosis (15), but knockdown of DDR2 reduced angiotensin induced cardiac fibrosis (16). A recent report found attenuated lung fibrosis in mice deficient in DDR2, however, the molecular mechanisms involved in DDR2 regulation of fibrosis remain poorly explored (17). Most of the research has focused on DDR2-mediated fibroblast activation. Notably, nintedanib, one of the approved therapies for IPF, is a non-specific tyrosine kinase inhibitor with known activity against DDR2 (18). Several other tyrosine kinase inhibitors which are known to block DDR2 have also been shown to attenuate experimental fibrosis (19).

The present study explores the mechanisms by which collagen signaling through DDR2, regulates fibroblast behavior during lung fibrosis. We find that DDR2 is a major regulator of fibroblast apoptosis through activation of a PDK1/Akt pathways. Finally, we find that DDR2 expression is skewed to a mesenchymal population and that IPF fibroblasts demonstrate upregulation of this pathway offering a potentially attractive target for fibrosis therapy(20, 21).

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Methods and Materials

Reagents. Phospho-DDR2 (Y740) antibody and α2β1 integrin inhibitor, TC-I15, were purchased from R&D Systems. Phospho-Tyrosine9-PDK1, DDR2 and integrin- α2 antibodies were from Abcam. Col1a1 antibody was from ThermoFisher. Hydroxyproline reagent, cycloheximide, and SMA, SMA-FITC, β-actin, and FLAG M2 antibodies were from Sigma. AKT, phospho-AKT (Ser473), phospho-AKT (Thr308), ERK1/2, phospho-ERK1/2 (T202/Y204), Src, phospho-Src, XIAP, GAPDH, phospho-SMAD3, phospho-Tyr, and phospho-Tyr-HRP antibodies were from Cell Signaling. SMAD2/3 primary antibody and HRP-conjugated secondary antibodies were from Santa Cruz. Phospho-Tyrosine376-PDK1 antibody was from Signalway Antibody. Phospho-SMAD2 antibody, phosphatase inhibitor cocktail, and bleomycin were purchased from Millipore. [^{3}H]thymidine was purchased from GE Healthcare PerkinElmer. Collagen I and anti-mouse CD95 were from BD Biosciences (San Jose, CA). Protein-A and protein-G conjugated agarose beads and In Situ Cell Death Detection Kit (TMR Red) were from Roche. Caspase-Glo 3/7 reagent was from Promega. IncuCyte Caspase-3/7 Apoptosis Assay Reagent was from Essen BioScience. PDK1 inhibitors BX-795 and BX-912 were from Selleckchem. Phosphatase inhibitor cocktail and bleomycin were purchased from Millipore. FLAG-tagged PDK1 cloned into a retroviral expression vector was purchased from AddGene.

Mice. DDR2-null mice (slie/slie) are previously described (22) and were purchased from Jackson Laboratories. Floxed col1a1 mice are previously described (10). Six- to eight-week-old wild type and DDR2-null mice were injured with 40 uL of 2 unit/kg of bleomycin dissolved in saline versus saline alone by oropharyngeal aspiration as previously described (23). Mice were euthanized at the specified time points after treatment in each respective experiment and analyzed as described. All mice were maintained in a specific-pathogen-free environment until the time of sacrifice. All animal experiments were approved by the Animal Care and Use Committee at the University of Michigan.

Bronchoalveolar Lavage (BAL) and Lung myeloid cell isolation. For BAL, mice were sacrificed and lavaged with 1 mL of PBS. Total cells were quantified by counting with a hemocytometer. For cell differential calculation, cells were fixed, cytofuged onto glass slides, stained with hematoxylin and eosin and visualized by light microscopy. Lung myeloid cell were isolated as previosly described(24, 25). Briefly, lungs were removed from sacrificed mice, minced and digested with collagenase and dispase. Erythrocytes were lysed and the myeloid cells isolated by filtration and centrifugation in Percoll.

Hydroxyproline Assay. Whole lung hydroxyproline was measured as previously described (26, 27). Mice were euthanized at the specified time points in each experiment. Whole lung was harvested, homogenized in water and baked in 12N hydrochloric acid at 120°C overnight. Samples were then neutralized with citrate buffer and incubated in chloramine T solution at room temperature. Erlich's solution was added prior to incubation at 65°C. The absorbance at 540 nm was measured and the hydroxyproline concentration was quantified against a standard.

Histology. Lung section trichrome staining was previously described (10). Briefly, whole lungs harvested from euthanized mice were fixed by inflation with formaldehyde to a pressure of 25 cm H_2O. Lungs were then embedded in paraffin. Sections were stained with Masson's trichrome by the McClinchey Histology Laboratory (Stockbridge, MI).

Immunofluorescent Staining. Lungs were perfused with PBS, inflated and embedded in OCT, and frozen in a dry-ice alcohol bath. Ten-micron lung sections were then stained as previously described (10). Briefly, lung sections were fixed and permeabilzed with methanol and blocked with 5% goat serum and 1% BSA in PBS. The slides were stained with primary antibody overnight at 4°C. After washing slides were stained with appropriate secondary antibody and for TUNEL by using the In Situ Cell Death Detection Kit TMR Red (Roche). Slides were mounted in Prolong Gold containing DAPI (Molecular Probes). Lung sections were visualized on an

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Olympus BX-51 fluorescence microscope (Olympus, Tokyo, Japan), and images were captured with an Olympus DP-70 camera and analyzed with DP controller software version 3.1.1.267. Quantification of co-stained cells was completed in a blinded fashion.

Mouse and human lung fibroblast isolation and culture. Primary murine lung fibroblasts were isolated and cultured as previously described (10). Ten- to 24-week-old mice were sacrificed and whole lungs were removed after perfusion and lavage with PBS. Whole lung samples were finely minced and incubated in DMEM with 10% FBS, penicillin, and streptomycin until fibroblasts adhered to the plate. Human lung fibroblasts were isolated from IPF and normal patients as previously described (28) and cultured in DMEM with 10% FBS, penicillin, streptomycin, and amphotericin B. All cells were cultured in a 37°C incubator supplemented with 5% CO_2.

For cell expression experiments, equal numbers of cells were seeded on tissue culture plates in serum-containing media and allowed to adhere overnight. Cells were rinsed and serum-starved for 24 hours. Cells were then treated with TGFβ (4ng/mL) or vehicle control for the time points indicated. Some cells were treated with TC-I15 (2.5μM) versus vehicle control one hour followed by treatment with TGFβ.

Retroviral and Lentiviral Infection of Cells. Murine DDR2 was cloned into a retroviral expression vector (pWZL-blast) by PCR and standard cloning techniques. Retroviral supernatants were generated using Phoenix-E cells as previosly described (29). Briefly, one day after seeding, primary WT or DDR2-null murine lung fibroblasts were treated with retroviral supernatants to overexpress DDR2 or FLAG-PDK1 (versus vehicle control) as indicated. After two days, the media was replaced and cells treated and analyzed as indicated. For DDR2 shRNA, lentivirus was generated by the University of Michigan Vector Core using RNA interference vectors purchased from OpenBiosystems. One day after seeding, primary WT

murine fibroblasts treated with DDR2 shRNA lentivirus (5 PFU/cell) or scrambled control lentivirus. After two days, the media was replaced and cells were treated and analyzed as described (30).

Immunoblot and Immunoprecipitation. Immunoblot and immunoprecipitation of samples were performed as previously described (10). Immunoblots are representative of a minimum of three separate experiments.

Cell Proliferation Assays. Cellular proliferation was measured by the [^{3}H]thymidine incorporation method as previously described (31). Briefly, equal numbers of stimulated cells were incubated with the addition of 5 uL of 0.1 mCi/mL [^{3}H]thymidine per well in a 96-well plate overnight. Sixteen hours after addition of [^{3}H]thymidine cells were then harvested for incorporated [^{3}H]thymidine using scintillation fluid in a beta-scintillation counter.

Cell Death Assays. Equal numbers of WT and DDR2 null cells were seeded on 24 well plates in serum containing media. The next day the cells were washed and covered with serum-free media. After an additional 24 hours cells were treated with vehicle control or Fas activating antibody (0.5μg/mL) and cycloheximide (5μg/mL) to induce apoptosis. Some cells were pretreated for 1 hour with PDK1 inhibitors BX-795 (1nM) or BX-912 (2nM) prior to addition of Fas activating antibody or cyclohexamide. After the time points indicated cell death was assessed by caspase 3/7 activity using Caspase-Glo 3/7 Assay (Promega) per product protocol and as previously described (23). Briefly, treated cells were washed with PBS and lysed in RIPA. Active caspase 3/7 activity was captured using a Veritas Microplate Luminometer, normalized to protein concentration per sample, and expressed as fold differences in relative luminescence. Cell death by caspase 3/7 activity was also captured using the IncuCyte Live Cell Analysis System (Essen BioScience) by incubating treated cells with IncuCyte Caspase-3/7 Apoptosis Assay Reagent. At least four images were captured per well in a 96-well plate.

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Discrete fluorescent apoptotic cells were counted using the IncuCyte basic analyzer and expressed as fold differences in relative numbers over time.

Gene Expression Analysis. Quantitative RT-PCR was performed as previously described (10). Briefly, RNA was isolated from lung tissue and cells with TRIzol (Invitrogen) per manufacturer's protocol. Reverse transcription was performed with the SuperScript III First-strand synthesis kit (Invitrogen) and RT-PCR was performed using POWER SYBR Green PCR MasterMix Kit (Applied Biosystems) and the Applied Biosystems 7000 sequence detection system. Relative expression of genes were normalized to β-actin and GAPDH levels as previously described (30) using the following primers: α-actin FW: 5'-CCGTGAAAAGATGACCCAGATC-3', β-actin RV: 5'-CACAGCCTGGATGGCTACGT-3', GAPDH FW: 5'-AACTTTGGCATTGTGGAAGG-3', GAPDH RV: 5'-ACACATTGGGGGTAGGAACA-3', COL1A1 FW: 5'-GCCAAGAAGACAAACTTT-3', COL1A1 RV: 5'-GGCCTTGGAAACCTTGTGGAC-3',

COL1A2 FW: 5'-GGAGGGAACGGTCCACGAT-3', COL1A2 RV: 5'-GAGTCCGCGTATCCACAA-3', COL3A1 FW: 5'-CAAGGTCTTCCTGGTCAGCCT-3', COL3A1 RV: 5'-TGCCACGAGATCCATCTC-3', α-SMA FW: 5'-AGAGACTCTCTTCCAGCCATC-3', α-SMA RV: 5'-GACGTTGTTAGCATAGAGATC-3', TGFβ1 FW: 5'-ATCCTGTCCAAACTAAGGCTCG-3', TGFβ1 RV: 5'-ACCTCTTTAGCATAGTAGTCCGC-3', IL1β FW: 5'-TGTTCTTTGAAGTTGACGGAC-3', IL1β RV: 5'-GATACTGCCTGCCTGAAGCTC-3', CCL2 FW; 5-TTCTGGGCCTGCTGTTCACAG-3', CCL2 RV: 5'-CTACTCATTGGGATCATCTTGC-3', CCL7 FW: 5'- TGCTTTCAGCATCCAAGTGTG-3', CCL7 RV: 5'-GGACACCGACTACTGGTGATC-3', CCL12 FW: 5'-TAGCTACCACCATCAGTCCTC-3', CCL12 RV; 5'-GGGACACTGGCTGCTTGTGAT3', TNFα FW: 5'-CCAAAGGGATGAG AAGTTCCC-3', TNFα RV: 5'-GCTCCTCCACTTGGTGGTTTG-3'.

Statistical Analysis. Data are expressed as means and error bars indicate the SEM. For evaluation of group differences, the 2-tailed Student's t-test was used. A P value of less than 0.05 was accepted as significant.

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Results.

Mice deficient in DDR2, are protected from fibrosis. We have previously shown that mice deficient in type I collagen expression have decreased fibrosis and decreased numbers of active fibroblasts suggesting an important role for collagen signaling in propagating progressive fibrosis (10). We first examined the relative expression of two of the most well described collagen I receptors, α2β1 and DDR2 in alveolar epithelial cells and fibroblasts (Figure 1A). DDR2 was expressed almost exclusively on fibroblasts compared to AECs consistent with prior reports (7, 8, 18). Integrin α2β1 was expressed in both cell types, but in higher amounts in fibroblasts. We have previously shown increased activation and total levels of DDR2 in mouse lungs after bleomycin injury(10). We therefore focused on DDR2 as a potential mediator of collagen induced fibroblast activation. To determine if DDR2 regulated the fibrotic response, DDR2-null and WT mice were injured with bleomycin. After 21 days, DDR2 mice had no increased in fibrosis determined by trichrome stained lung section or by hydroxyproline assay indicating that collagen signaling through the DDR2 receptor regulates subsequent collagen deposition and fibrosis (Figure 1 and Supplemental Figure 1). Notably, DDR2 is not highly expressed on myeloid cells and the absence of DDR2 did not affect myeloid cell recruitment or expression of several inflammatory and profibrotic cytokines and chemokines (Supplemental Figure 2).

DDR2 does not regulates fibroblast activation or proliferation. We have previously shown that fibroblasts with deletion of col1a1 have reduced expression of fibroblast activation markers including col1a2, col3a1 and α-smooth muscle actin (α-SMA)(10). DDR2 is highly expressed on fibroblasts which accumulate during fibrosis and are thought to be the primary fibrotic effector cells. To determine if DDR2 regulates fibroblast activation, WT and DDR2-null primary lung fibroblasts were isolated and treated with TGFβ. After 24 hours, cells were analyzed by qPCR and immunoblot for several well described markers of fibroblast activation including type I

collagen and α-SMA. Contrary to our expectations, DDR2-null fibroblasts did not have decreased levels of fibroblast activation markers and indeed showed a trend toward increased levels of α-SMA and collagen I (Figure 2A&B). We also found no differences in TGFβ-induced activation of Smad2 and Smad3 (Figure 2C).

To determine if collagen I/DDR2 signaling regulates expression of the other major fibroblast fibrillar collagen receptor, fibroblasts from floxed type I collagen mice treated with AdCre or control adenovirus were analyzed for expression of α2 integrin. Indeed, fibroblasts deficient in col1a1 had marked increased expression of α2 integrin (Figure 3A). Similarly, DDR2-null fibroblasts had increased expression of α2 integrin suggesting collagen I/DDR2 signaling negatively regulates expression of α2 integrin and that higher expression of α2 integrin might regulate the increased expression of profibrotic markers (Figure 3B). WT fibroblasts were treated with TGFβ1 with or without pretreatment with an α2 integrin specific inhibitor, TC-I15 (2.5μM). Indeed, fibroblasts treated with TC-I15 had reduced expression of type I collagen and α-SMA (Figure 3C). These results demonstrate one aspect by which type I collagen signaling regulates accumulation of activated fibroblasts but does not clarify the mechanism by which DDR2-null mice are protected from fibrosis.

Fibroblast proliferation has been implicated in the progression of fibrosis. We cultured WT and DDR2-null fibroblasts in serum-free or 10% serum containing conditions and measured proliferation with the incorporation of ^{3}H-thymadine. Again, contrary to our expectations, the presence of DDR2 did not influence fibroblast proliferation (Figure 4A).

DDR2 regulates fibroblast apoptosis. Fibroblast resistance to apoptosis has emerged as a critical regulator of fibrosis. WT and DDR2-null fibroblasts were treated with cycloheximide and a Fas activating antibody (or vehicle control) to induce robust apoptosis. To track the induction of apoptosis over time, WT and DDR2-null fibroblasts were cultured with a reagent that

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fluoresces when cleaved by caspase3/7. Cells were imaged over time using the IncuCyte system and numbers of fluorescent cells quantified over time. Greater induction of apoptosis in the DDR2-null fibroblasts was apparent within 20 hours after addition of Fas/CHX (Figure 4B). To confirm increased activation of caspase3/7 in DDR2-null fibroblasts, WT and DDR2-null cells were treated with Fas/CHX as before then lysed after 24 hours. Caspase3/7 activity was measured in cell lysates using a luciferase based CaspaseGlo system. As expected DDR2-null fibroblasts demonstrated a more robust activation of caspase3/7 compared to WT fibroblasts.

To determine if DDR2 contributes to fibroblast survival in vivo, WT and DDR2-null mice were treated with bleomycin. After 14 days, lung sections were stained for TUNEL and counterstained with α-SMA to mark the active myofibroblasts. Consistent with the in vitro data, the numbers of TUNEL-positive myofibroblasts in bleomycin treated DDR2-null lungs was significantly greater than bleomycin injured WT lungs (Figure 5).

DDR2 regulates fibroblast apoptosis through a PDK1/Akt pathway. We next interrogated several well described pathways involved in fibroblast survival that might predispose DDR2-null cells for greater apoptosis. Some of these pathways, most notably Src signaling, has been linked to DDR2 activation as well as fibroblast survival (19). However, we found no differences between WT and DDR2-null fibroblasts in levels of total or phospho-Src. We recently reported that XIAP is a major regulator of fibroblast survival in the context of lung fibrosis (32, 33) but again found no differences between WT and DDR2-null fibroblasts (Figure 6A). Erk and Akt signaling are among the most well studied survival pathways. WT and DDR2 fibroblasts had no differences in Erk phosphorylation. Akt phosphorylation at Serine473 (Ser473) and Threonine308 (Thr308) lead to Akt activation and increased survival in several cell types. We found no difference in pSer473-Akt, however, DDR2-null fibroblasts had marked reduction in pThr308-Akt suggesting that decreased Akt activation may be linked to the observed increased apoptosis (Figure 6B).

PDK1 is the most well described kinase to phosphorylate Akt at Thr308 and PDK1 activity is known to be regulated by Tyrosine phosphorylation (34). DDR2 has not previously been described to regulate PDK activity. To determine if the presence of DDR2 regulates tyrosine phosphorylation of PDK1 we utilized a FLAG-tagged PDK1 vector. WT and DDR2-null fibroblasts were treated with retrovirus encoding control vector or FLAG-PDK1. FLAG-PDK1 was immunoprecipitated and the tyrosine phosphorylation levels were determined by immunoblot for phospho-Tyrosine. DDR2-null fibroblasts exhibited markedly decreased levels of phospho-tyrosine PDK1 (Figure 7A). We next obtained antibodies specific for PDK1 phosphorylation at its two major regulatory tyrosine residues, Tyrosine9 (Tyr9) and Tyrosine376 (Tyr376). Consistent with the FLAG-PDK1 overexpression experiment, immunoblot of WT and DDR2-null fibroblasts demonstrated reduced levels of pTyr9-PDK1 in DDR2 fibroblasts compared to WT fibroblasts (Figure 7B).

To our knowledge, PDK1 has not been previously been reported to be involve in fibroblast survival. To confirm that PDK1 mediated Akt phosphorylation at Thr308 regulates fibroblast apoptosis, WT lung fibroblasts were treated with two different PDK1 inhibitors. PDK1 inhibition demonstrated rapid and sustained reduction in pThr308-Akt compared to fibroblasts treated with vehicle alone control (Figure 7C). Consistent with DDR2 regulation of Akt phosphorylation, WT lung fibroblasts in which expression of DDR2 was knocked-down by shRNA had reduced levels of pThr308-Akt (Figure 7D) and DDR2-null lung fibroblasts with retroviral-mediated overexpression of DDR2 had increased levels of pThr308-Akt (Figure 7E) compared to DDR2-null fibroblasts treated with control retrovirus. Next, WT lung fibroblasts were treated with PDK1 inhibitors, or vehicle control and stimulated with Fas/CHX to induce apoptosis. As before, Fas/CHX induced robust activation of caspase 3/7, however the level of caspase activation was much greater in the cells treated with the PDK1 inhibitors (Figure 7F).

Human fibroblasts have increased levels of activation of DDR2 and PDK.

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IPF lungs are characterized, in part, by the accumulation of fibroblasts and type I collagen. Since DDR2 is known to be highly expressed by fibroblasts and phosphorylated in response to type I collagen, we confirmed that IPF lungs demonstrate increased levels of DDR2 and pDDR2 (Figure 8A). IPF fibroblasts also have greater collagen production and are resistant to apoptosis compared to normal human lung fibroblasts. To assess whether IPF fibroblasts exhibit greater activation DDR2 and PDK1 we used primary human lung fibroblasts isolated from IPF lungs or normal human lungs. Consistent with the murine data, IPF fibroblasts exhibited increased levels of p-DDR2 and pTyr9-PDK1 (Figure 8B). Finally, frozen OCT-embedded lung tissue from IPF and normal human lungs were stained for pTyr9-PDK1 and α-SMA. As expected, IPF lungs demonstrated increased staining for pTyr9-PDK1, primarily within α-SMA-positive myofibroblasts (Figure 8). Collectively, these results suggest that collagen I signaling mediated through pDDR2/pTyr9-PDK1/pThr308-Akt contributes to increased fibroblast survival in progressive lung fibrosis.

Discussion

Our results support a model in which fibroblast resistance to apoptosis is a major driver of sustained and progressive fibrosis (32, 33). The current paradigm suggests in normal wound healing after injury there is measured activation of fibroblasts with appropriate scar formation and disappearance of activated myofibroblasts through apoptosis or de-differentiation. In contrast, in fibrosis there is exuberant activation of fibroblasts and resistance to apoptosis leading to exaggerated and progressive fibrosis. The fibrosis itself can propagate this sustained fibrotic response (35, 36). Prior research has focused primarily on the mechanical properties of the fibrotic matrix leading to activation of intracellular pathways such as FAK and Rho leading to greater fibroblast activation (37-39). We have recently shown that resistance to fibroblast apoptosis may actually be the more significant component of this progressive fibrotic response (32, 33). In support of this model, we find that DDR2-null fibroblasts actually have a tendency toward greater activation even though DDR2-mice have a significantly attenuated fibrosis.

The role of signaling through the extracellular matrix has been well studied as a regulator of fibrosis (4, 39). Prior studies have focused primarily on provisional matrix proteins such as fibronectin (40, 41). The role of collagen signaling during progressive fibrosis is not well studied. There is clearly upregulation of fibrillar collagens including types I type III and others. The role of type IV collagen is less clear, although one of its main receptors, DDR1 has been shown to regulate lung fibrosis (13). Type I collagen is the most abundant protein in the fibrotic matrix and we and others have found that collagen I expression is induced early after injury suggesting that collagen signaling might have a role in regulating continued collagen expression and the development of fibrosis (10). Like many matrix proteins, collagen can activate a number of integrins, especially α2β1(42, 43). Collagen is fairly unique among matrix proteins in its ability to activate a receptor tyrosine kinase (8, 44). Our results suggest that these receptors have differing roles in regulating the cell response to type I collagen with DDR2 regulating cell

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survival and α2β1 regulating myofibroblast activation. It is noteworthy that deletion of DDR2 or type I collagen led to increased levels of α2 integrin which may complicate attempts to target this pathway for therapy. Consistent with collagen I mediated inhibition of α2 integrin expression, a recent report found reduced levels of α2 integrin among IPF fibroblasts(45). Notably, there is growing evidence for matrix/integrin signaling having a key role in translating matrix mechanotransduction signaling to cell behavior including fibroblast activation and survival through well-known integrin intracellular signaling pathways involving FAK, Rho and MRTF(46). DDR2 signaling may involve an independent survival mechanism in response to early collagen production that might precede formation of a mature dense collagen matrix. Although our interest in DDR2 signaling arose from our prior studies of type I collagen deletion (10), DDR2 can be activated by a number of collagens including type III collagen which is also upregulated during fibrosis (47). The extent to which type I and other collagens promote fibroblast DDR2 activation during fibrosis is not well characterized.

A unique attractive feature of DDR2 is its extremely skewed high expression on mesenchymal cells and limited expression on other cell types including epithelial cells and myeloid cells. Attempts to target survival and activation pathways have been challenging due to commonality of these pathways among multiple cells types. For example, imatinib was shown to be a potent inhibitor of fibroblast activation and survival, through inhibition of PDGFR and Abl, but was later shown to influence epithelial cell survival as well (48-50). Similarly, we recently reported that inhibition of FAK signaling, which has been proposed as another potential fibrosis therapeutic target, can also influence epithelial cell survival, potentially leading to sustained injury and fibrosis (51, 52). DDR2 expression is not limited to fibroblasts and mesenchymal cells. DDR2 expression on myeloid cells has been reported (53, 54) although we found very low expression on myeloid cells isolated from WT murine lungs (Supplemental Figure 2). Recently, mutations in DDR2 have been linked to a subset of squamous cell lung cancers and DDR2 signaling has

been linked to epithelial-mesenchymal transition in other malignancies (55, 56). It is possible that DDR2 expression can be induced in alveolar epithelial cells, however, this population is likely to be profibrotic as well.

Based on the lung cancer findings, there has been a concerted effort to develop DDR2-specific inhibitors (21). Indeed, many tyrosine kinase inhibitors are fairly nonspecific. As noted above, imatinib has activity against Abl, PDGFR and many other tyrosine kinases. It is worth noting that dasatinib which has been shown to block fibrosis in vivo has significant activity against DDR2 (57, 58). Furthermore, nintedanib, which is approved therapy for IPF, is a fairly non-specific tyrosine kinase inhibitor with activity against DDR2 (18, 59).

Although DDR1 and DDR2 have previously been studied in different fibrosis models, the results have been mixed and the direct downstream targets not well described (13, 15, 17, 60). These mixed results may be due to the complexity of the signaling networks that intersect with the DDRs. As noted above, deletion of DDR2 or collagen led to increased levels of α2 integrin which may lead to greater myofibroblast activation. Indeed, other studies have found a complex regulation of collagen receptor signaling influencing the expression and function of other collagen receptors and other intracellular signaling pathways (7, 8, 61, 62). Our findings also link collagen/DDR2 signaling to a signaling network that intersects with other pathways implicated in fibrosis. We find that the presence of DDR2 signaling promotes PDK phosphorylation at tyrosine9. Since DDR2 is a well described tyrosine kinase it is possible that PDK is a direct downstream target of DDR2. Alternatively, DDR2 is well known to interact with a number of other kinases and PDK phosphorylation may be indirectly regulated by DDR2(44, 63). More detailed in vitro studies are under way to help distinguish between these possibilities. The role of different tyrosine sites in regulating PDK activity at remains controversial with some reports suggesting a more prominent role for tyrosine376 and others suggesting an important role for tyrosine9 (64-66). It may be that regulation of PDK activity is dependent on different cell

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types or other cell culture conditions. Inhibition of Akt has also been shown to promote myofibroblast apoptosis and block fibrosis (36, 67, 68). Akt can be activated at two main sites, Ser473, which is primarily regulated by mTOR, and Thr308, which is regulated primarily by PDK1(34). Notably, mTOR inhibition is actively being pursued as a potential therapy for fibrosis (69) and it is possible that dual inhibition of Akt with PDK1 inhibition will augment the effect.

In summary, we find that collagen/DDR2 signaling can activate the Akt pro-survival pathway through activation of PDK1, leading to fibroblast survival and progressive fibrosis. DDR2 expression is skewed to the mesenchymal cell population making it an attractive target for therapy.

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Figure Legends

Figure 1. DDR2 Regulates Lung Fibrosis. A. Levels of collagen I receptors, DDR2 and α2 integrin, in primary murine alveolar epithelial cells (AEC) and primary murine lung fibroblasts were determined by immunoblot **B.** Hydroxyproline assay of total lungs 21 days after intratracheal saline or bleomycin in DDR2-null or littermate control mice. DDR2-null mice have less fibrosis after bleomycin compared to control mice treated with bleomycin (n=6-10 per group), *$p < 0.05$. **C&D.** Trichrome stain of WT **(C)** and DDR2-null **(D)** lung sections (20x) 21 days after injury with intratracheal bleomycin. DDR2-null mice are protected from bleomycin induced fibrosis compared to control.

Figure 2. DDR2 does not regulate lung fibroblast activation. A. Real-time qPCR of primary lung fibroblasts from control (WT) and DDR2-null (null) mice with and or without treatment with TGFβ. Loss of DDR2 does not significantly alter expression of col1a1, col1a2, col3a1, and smooth muscle actin (sma), n=4. **B.** Immunoblot for collagen I and α-smooth muscle actin (α-SMA) of primary lung fibroblasts from WT and null mice. Presence of DDR2 did not influence fibroblast production of collage I and α-SMA. **C.** Immunoblot of primary lung fibroblasts from WT and null mice, after treatments with TGFβ at 0, 1, 6, and 24 hours. Phospho-Smad2 (pSmad2), Smad2, phospho-Smad3 (pSmad3) and Smad3 are shown. The presence of DDR2 does not influence Smad phosphorylation.

Figure 3. Collagen I/DDR2 signaling regulates expression of α2 integrin. A. Lung fibroblasts from floxed col1a1 were treated with an adenovirus expressing Cre (AdCre) or a control adenovirus expressing GFP (AdGFP). After one week, cells were analyzed from expression of collagen I and α2 integrin by immunoblot. **B.** Primary lung fibroblasts from WT and DDR2-null mice were analyzed from expression of α2 integrin by immunoblot. **C.** WT primary

lung fibroblasts were treated with or without TGFβ and an α2 integrin inhibitor, TC-I15, and analyzed for expression of collagen I and α-smooth muscle actin (α-SMA) by immunoblot.

Figure 4. DDR2 regulates fibroblast apoptosis but not proliferation. A. Cellular proliferation assessed by [3H]thymidine incorporation for 16 hours in WT and DDR2-null primary lung fibroblasts under serum-free or 10% serum containing media. DDR2 does not regulate lung fibroblast proliferation; n=4. **B.** Activation of caspase3/7 using a fluorescent reagent and the IncuCyte System was trended over time in WT or DDR2-null primary lung fibroblasts cultured with and without a Fas activating antibody and cyclohexamide (Fas/CHX). Values are expressed as numbers of fluorescent cells per image field; n = 4. **C.** Activation of caspase 3/7 in WT or DDR2-null primary lung fibroblasts upon induction of apoptosis with Fas/CHX was measured by the CaspaseGlo luciferase based assay 24 hours after treatment with Fas/CHX. Values are expressed as fold differences in relative luminescent units; n = 5, *$p<0.01$ compared with WT fibroblasts.

Figure 5. DDR2 regulates fibroblast apoptosis in vivo. Fourteen days after bleomycin injury lung sections from WT **(A-C)** and DDR2-null **(D-G)** mice were stained for α-SMA (green, **A&D**) TUNEL staining (red, **B&E**) and images merged **(C&G).** Compared to WT mice, the DDR2-null lung sections had more TUNEL-positive myofibroblasts **(H)**; n=4, *$p<0.05$ compared to WT.

Figure 6. DDR2 regulates fibroblast Akt pathway. A. Immunoblot for phospho-ERK (p-ERK), ERK, phospho-Src (p-Src), Src and XIAP in primary lung fibroblasts isolated from DDR2-null (null) and littermate control (WT) mice. **B.** Immunoblot for phospho-Serine473-Akt (pSer473-Akt), phospho-Threonine308-Akt (pThr308-Akt), and total Akt in primary lung fibroblasts isolated from null and WT mice.

Figure 7. DDR2 regulates fibroblast survival through PDK1/Akt. A. Lysate from WT and DDR2-null (null) lung fibroblasts overexpressing FLAG-PDK1 were immunopreciptated using a

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FLAG antibody and immunoblotted for phospho-tyrosine. Null cells have less tyrosine phosphorylation of FLAG-PDK1. **B.** Null cells have less phospho-Tyrosine9-PDK1 (pTyr9-PDK1) and equal levels of phospho-Tyrosine376-PDK1 (pTyr376-PDK1) and total PDK1 compared to WT primary lung fibroblasts assessed by immunoblot. **C.** Immunoblot for pThr308-Akt and total Akt in wildtype primary lung fibroblasts treated with two small molecule inhibitors of PDK1 (BX795 and BX912) for 0, 1, 3, and 6 hours. **D.** Immunoblot of WT lung fibroblasts treated with lentiviral mediated shRNA to DDR2 (shDDR2) or scrambled control (shScr) for DDR2 and pThr308-Akt. **E.** DDR2-null lung fibroblasts treated with retroviral mediated overexpression of DDR2 (DDR2) or empty control retrovirus (ctrl) and immunoblotted for DDR2 and pThr308-Akt. **F.** Activation of caspase 3/7 in wildtype primary lung fibroblasts upon induction of apoptosis by treatment with a Fas activating antibody and cyclohexamide (Fas/CHX) with or without BX795 and BX912. Caspase 3/7 activity was significantly higher in lung fibroblasts with additional inhibition of PDK1 than with Fas/CHX induction of apoptosis alone. Values are expressed as fold differences in relative luminescent units; n=6, *$p<0.01$ compared with treatment with Fas/CHX and vehicle control.

Figure 8. IPF fibroblasts have greater levels of pDDR2 and pPDK1. A. IPF and normal human lung tissue was analyzed by immunoblot for pDDR2 and DDR2. **B.** IPF and normal human primary lung fibroblasts were analyzed by immunoblot for pDDR2, DDR2, phospho-Tyrosine9-PDK1 (pTyr9-PKD1) and PDK1. **C-H.** Normal human **(C-E)** and IPF **(F-H)** lungs were stained for pTyr9-PDK1 (red, **C&F**), α-smooth muscle actin (α-SMA, green, **D&G**) and images merged (**E&H**).

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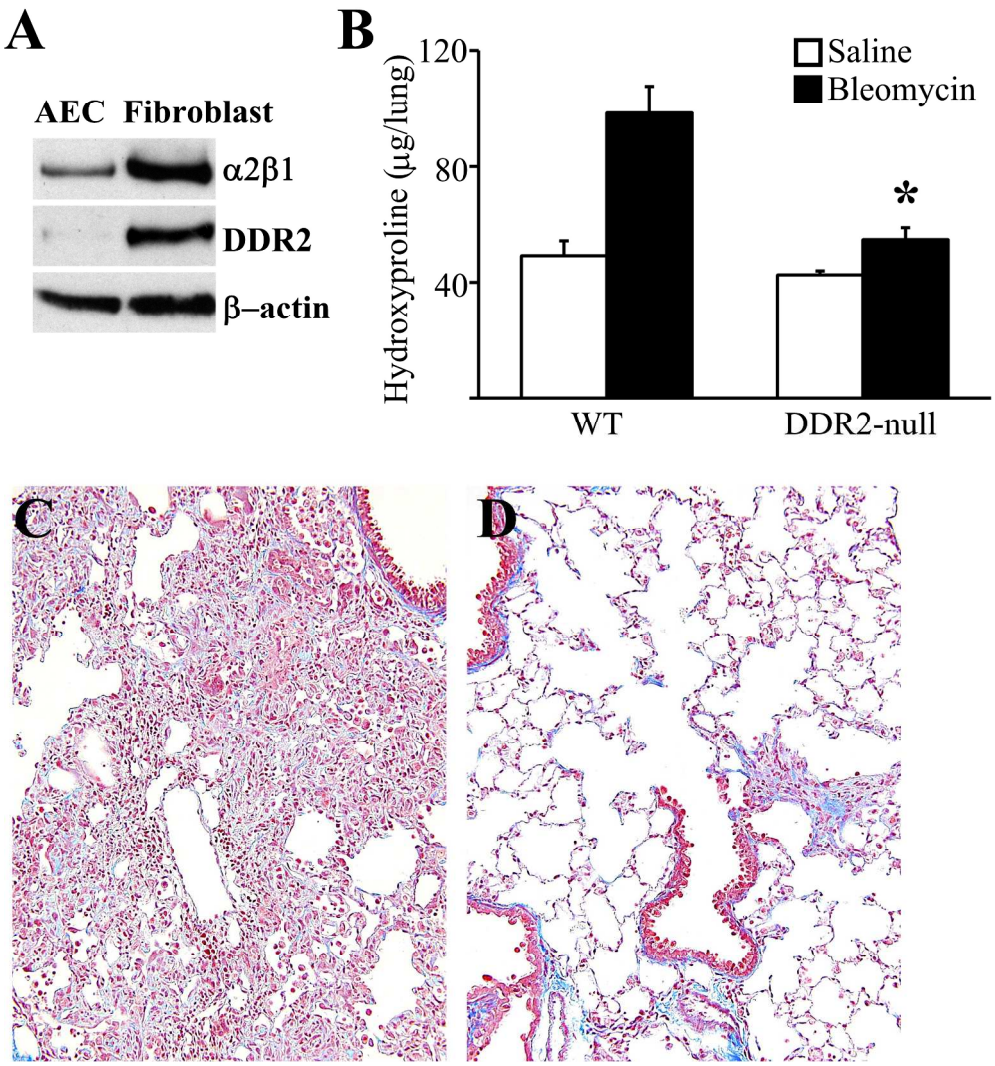

Figure 1

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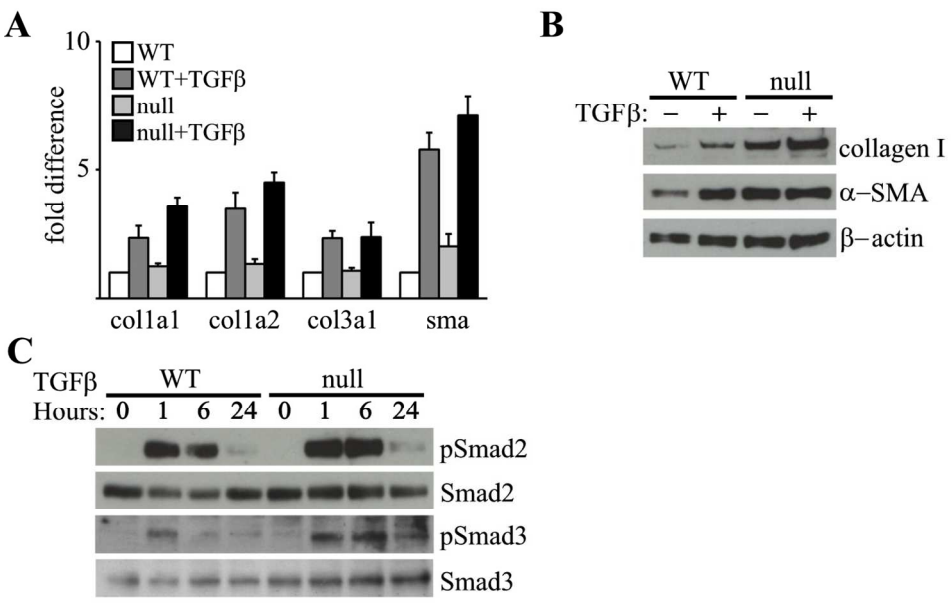

Figure 2

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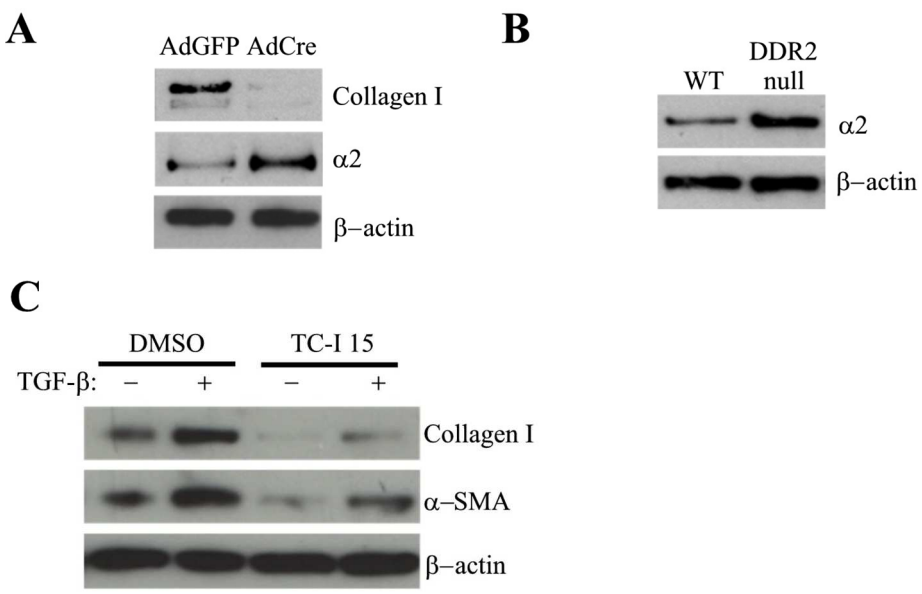

Figure 3

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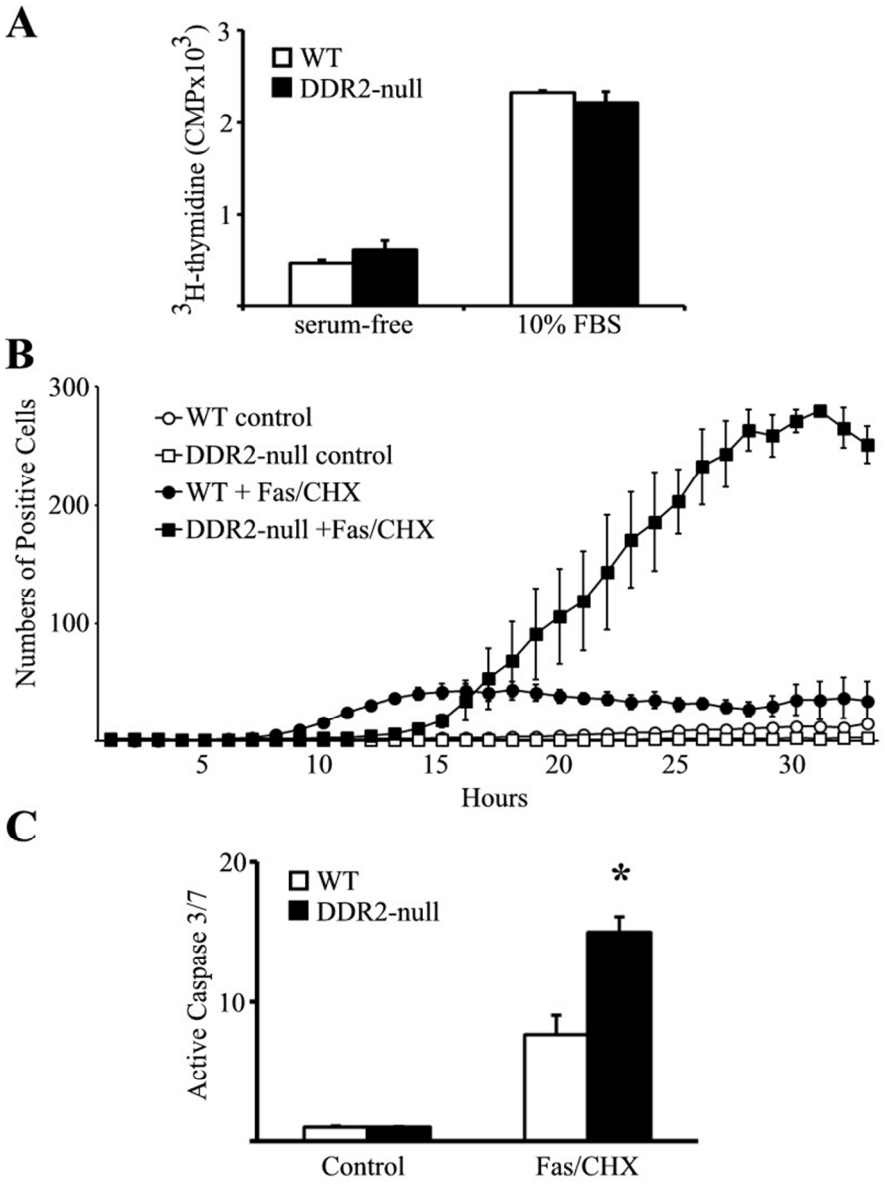

Figure 4

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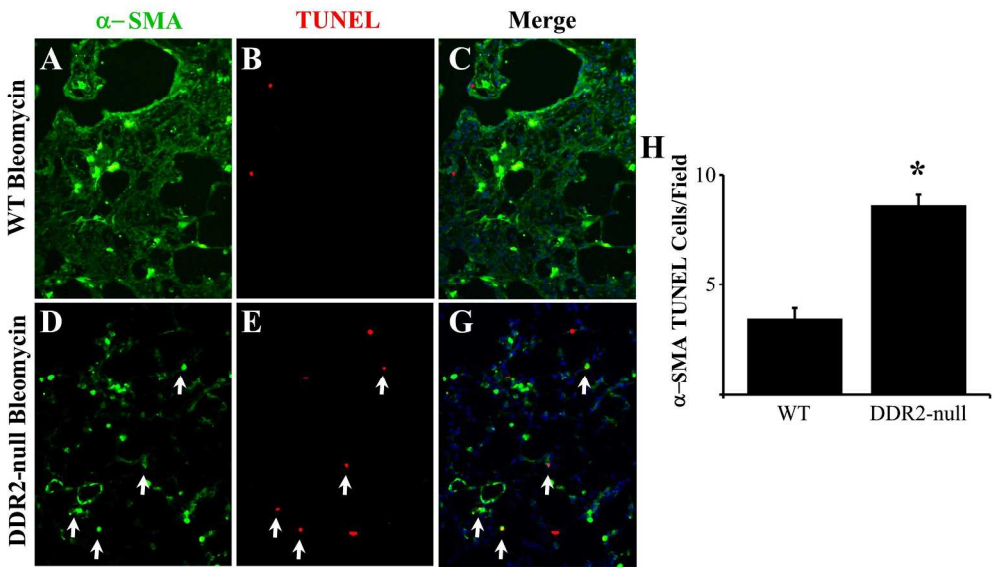

Figure 5

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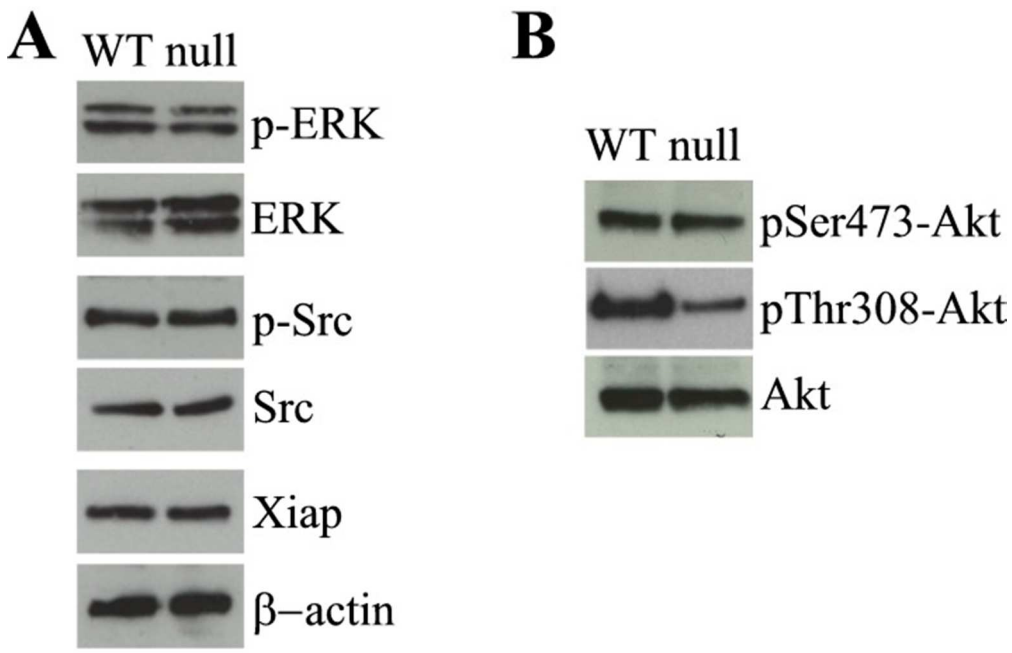

Figure 6

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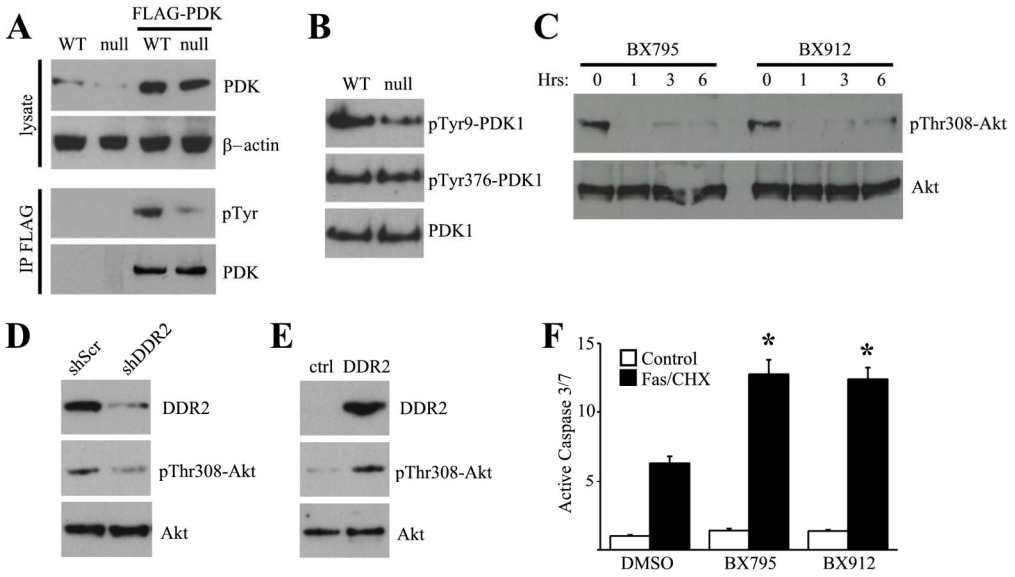

Figure 7

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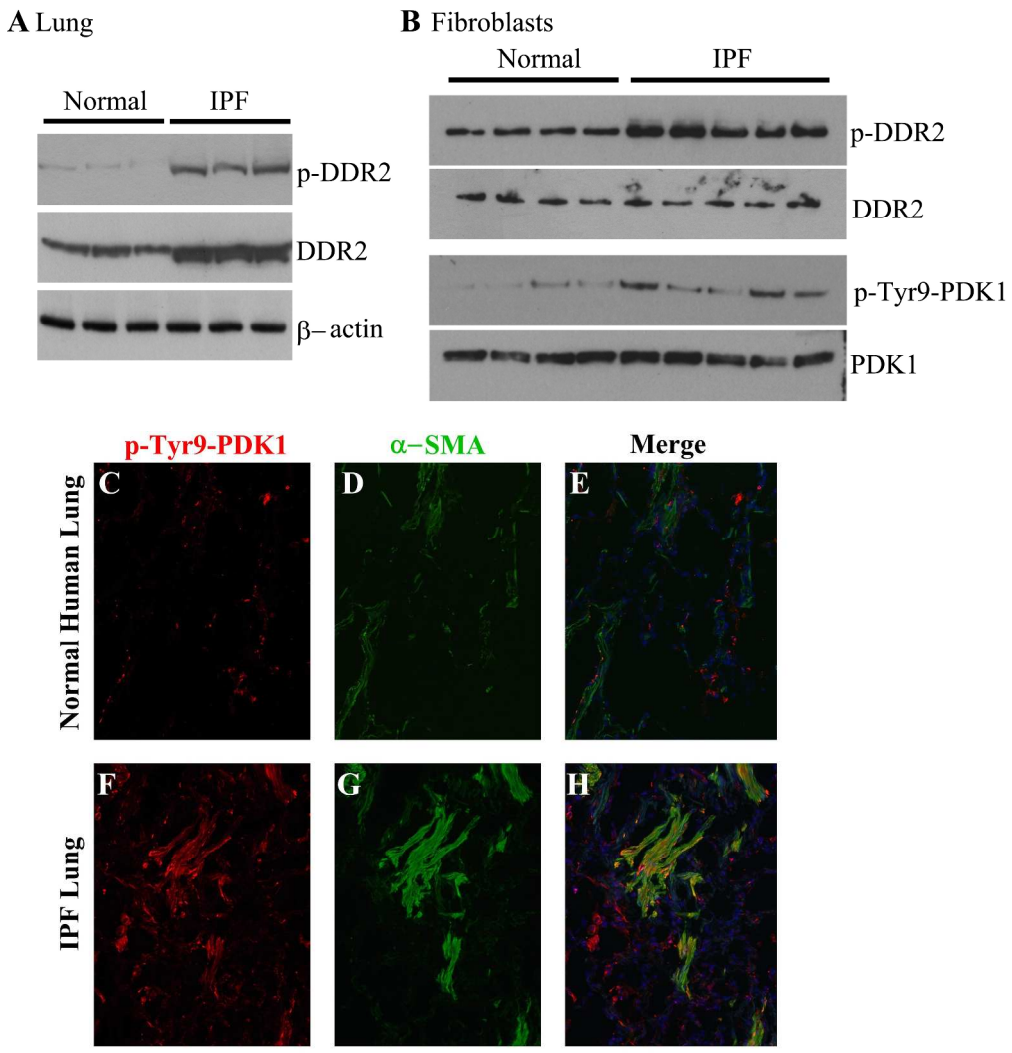

Figure 8

629x671mm (150 x 150 DPI)

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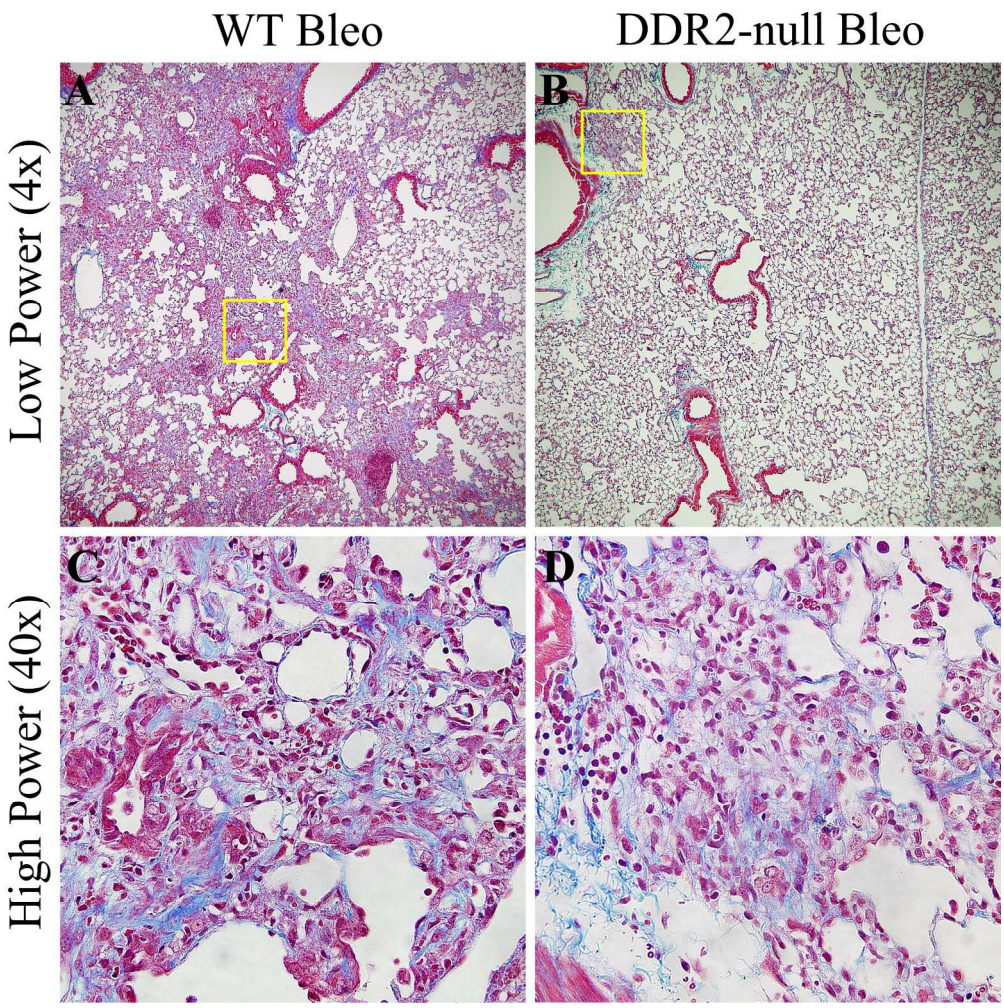
Supplemental Figure Legends

Supplemental Figure 1. DDR2 Regulates Lung Fibrosis. A.&B Low power (4x) trichrome stained lung sections of WT **(A)** and DDR2-null **(B)** lung sections 21 days after injury with intratracheal bleomycin and high power magnification (40x) from the indicated area (yellow box) from WT **(C)** and DDR2-null **(D)** mice demonstrate fewer and less dense areas of fibrosis in DDR2-null mice.

Supplemental Figure 2. DDR2 Regulation of Myeloid Cells. A. Expression of DDR2 in myeloid cells derived from WT and DDR2-null mice one week after bleomycin injury. **B.** Bronchoalveolar lavage cells from WT and DDR2-null mice one week after saline or bleomycin injury. **C.** Quantitative RT-PCR of mRNA isolated from lungs of WT and DDR2 mice one week after saline or bleomycin injury.



Supplemental Figure 1

273x272mm (216 x 216 DPI)

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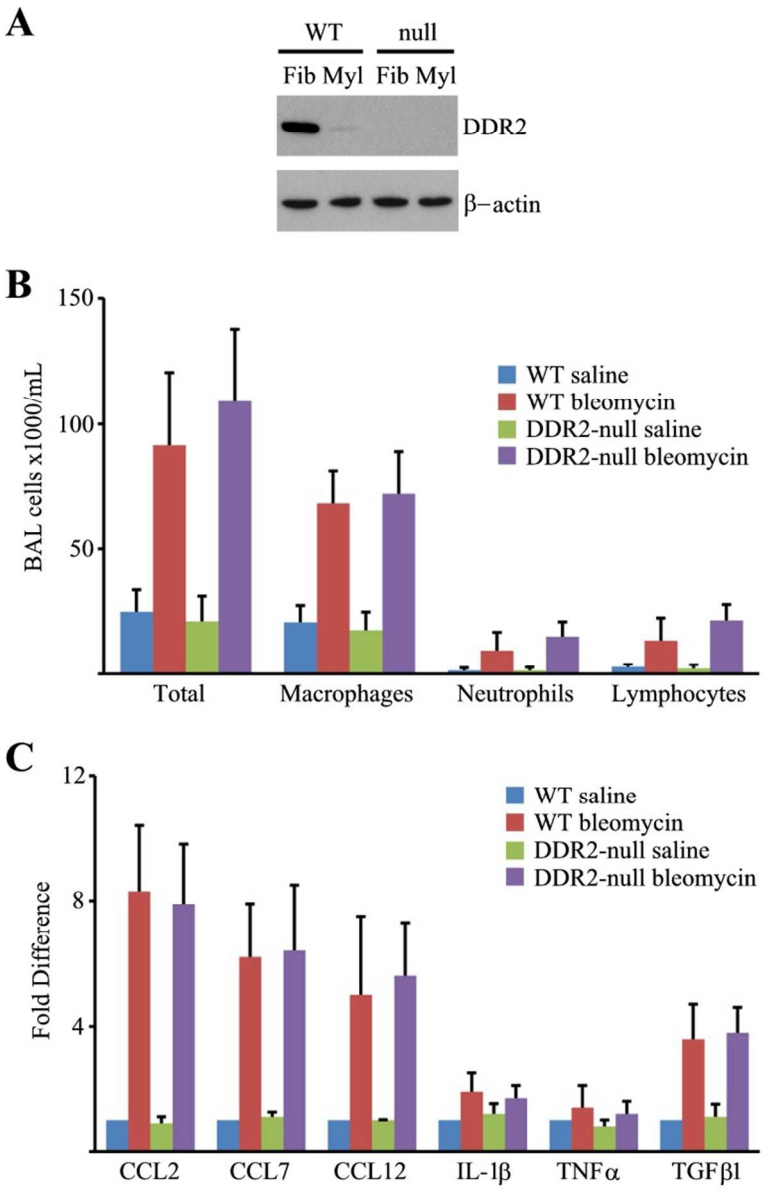

Supplemental Figure 2

132x204mm (150 x 150 DPI)