Discoidin Domain Receptor 1 Kinase Activity is required for regulating collagen IV synthesis

Corina M. Borza[1], Yan Su[1], Truc-Linh Tran[1], Ling Yu[1], Nick Steyns[2], Kayla J. Temple[2], Marcin J. Skwark[2], Jens Meiler[2] , Craig W. Lindsley[2], Brennan R. Hicks[1], Birgit Leitinger[3] ,Roy Zent[1,4,5], and Ambra Pozzi[1,5]

Departments of [1]Medicine (Division of Nephrology), [2]Chemistry, Pharmacology and [4]Cell and Developmental Biology; Vanderbilt University, Nashville, TN; [3]National Heart and Lung Institute, Imperial College of London, London UK [5]Veterans Affairs Hospitals, Nashville, Tennessee

Running Title: DDR1 controls collagen IV synthesis

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Highlights

- Loss of Discoidin Domain Receptor (DDR)1 improves renal function and reduces glomerulosclerosis in the partial renal ablation model of kidney injury.
- DDR1 positively regulates collagen IV production and this effect requires collagen binding and kinase activity of the receptor.
- Inhibition of DDR1 kinase activity with an ATP-competitive small molecule inhibitor reduces collagen production which suggests that blocking DDR1 activation may be beneficial in fibrotic diseases.

Correspondence should be addressed to
Ambra Pozzi, PhD or Corina M. Borza, PhD
Department of Medicine
Division of Nephrology and Hypertension
Medical Center North, B3115
Vanderbilt University
Nashville, TN, 37215
Phone: 615-322-4637
email: corina.borza@vanderbilt.edu
e-mail: ambra.pozzi@vanderbilt.edu

Abstract

Discoidin domain receptor 1 (DDR1) is a receptor tyrosine kinase that binds to and is activated by collagens. DDR1 expression increases following kidney injury and accumulating evidence suggests that it contributes to the progression of injury. To this end, deletion of DDR1 is beneficial in ameliorating kidney injury induced by angiotensin infusion, unilateral ureteral obstruction, or nephrotoxic nephritis. Most of the beneficial effects observed in the DDR1-null mice are attributed to reduced inflammatory cell infiltration to the site of injury, suggesting that DDR1 plays a pro-inflammatory effect. The goal of this study was to determine whether, in addition to its pro-inflammatory effect, DDR1 plays a deleterious effect in kidney injury by directly regulating extracellular matrix production. We show that DDR1-null mice have reduced deposition of glomerular collagens I and IV as well as decreased proteinuria following the partial renal ablation model of kidney injury. Using mesangial cells isolated from DDR1-null mice, we show that these cells produce significantly less collagen compared to DDR1-null cells reconstituted with wild type DDR1. Moreover, mutagenesis analysis revealed that mutations in the collagen binding site or in the kinase domain significantly reduce DDR1-mediated collagen production. Finally, we provide evidence that blocking DDR1 kinase activity with an ATP-competitive small molecule inhibitor reduces collagen production. In conclusion, our studies indicate that the kinase activity of DDR1 plays a key role in DDR1-induced collagen synthesis and suggest that blocking collagen-mediated DDR1 activation may be beneficial in fibrotic diseases.

Introduction

Discoidin Domain Receptor 1 (DDR1) is a receptor tyrosine kinase that binds collagens [1] and has been implicated in the regulation of multiple cellular functions including migration [2-5], cytokine secretion [6-8], and extracellular matrix homeostasis/remodeling [9-11]. DDR1 is required for normal tissue development, but the function of DDR1 in adult tissues particularly in diseased tissues is poorly understood [12, 13]. DDR1 contributes to cancer [14, 15] and promotes inflammation in models of atherosclerosis [10, 16] and lung fibrosis [17], but the mechanisms whereby DDR1 contributes to disease progression are not clear.

DDR1 upregulation in patients with kidney diseases such as lupus nephritis and Goodpasture's syndrome [8] as well as in animal models of kidney injury [7, 8, 18] suggests that DDR1 plays an important role in kidney disease. Studies in various mouse models of kidney injury indicate that DDR1 deletion results in improved renal function and reduced inflammation and fibrosis. In this context, angiotensin-induced abnormal extracellular matrix (ECM) deposition, perivascular and glomerular infiltrate and albuminuria were reduced in DDR1-null mice [19]. Similarly, collagen deposition, macrophage infiltration and pro-inflammatory cytokine levels were significantly reduced in DDR1 null mice following unilateral ureteral obstruction [7]. In genetic models of chronic kidney disease, such as the Alport mice, DDR1 deletion improves survival and reduces fibrosis and inflammation [9]. Moreover, inhibition of DDR1 expression by using antisense oligodeoxynucleotides in a model of crescentic glomerulonephritis attenuates glomerular and tubular injuries decreases proteinuria and reduces inflammatory cells infiltration and fibrosis [8]. Overall, loss of DDR1 protects mice from kidney injury and the beneficial effects seems to reside in reduced inflammatory cells infiltration due to impaired migration and/or reduced inflammatory cytokines production in the DDR1-null mice.

In addition to its pro-inflammatory action, DDR1 controls collagen synthesis. Mice lacking DDR1 show increased fibrillar collagen deposition in the mammary gland [20] and DDR1-null smooth muscle show increased mRNA levels of fibrillar collagens I and III [21], suggesting that DDR1 is a negative regulator of fibrillar collagen synthesis. In contrast to these findings, DDR1-null smooth muscle cells showed decreased mRNA levels of non-fibrillar collagen, such as the basement membrane collagen IV [21]. Thus, DDR1 can promote and/or inhibit collagen synthesis and this effect is dependent on the type of collagen.

DDR1 is normally expressed at low levels by cells comprising the glomerulus, the functional filtration unit of the kidney, but its expression increases following glomerular injury [8]. The glomerulus consists of three major cell types, namely endothelial cells, podocytes and mesangial cells that are kept together by an extracellular network composed primarily of collagen IV. Insult to these cells can lead to glomerulosclerosis, the process by which glomerular tissue is replaced by ECM (mainly collagens I and IV) and the final common pathway for loss of functioning glomeruli [22, 23]. Podocytes and endothelial cells are likely critical for initiation of sclerosis; however, mesangial cells are the major contributor to progression. Mesangial cells express DDR1 *in vitro* [24] and DDR1 expression increases in glomerular injury [19]. However, whether DDR1 contributes to glomerulosclerosis by directly regulating collagens I and/or IV production is unknown.

We provide evidence that following partial renal ablation, DDR1-null mice have reduced proteinuria and glomerulosclerosis with reduced collagen I and collagen IV deposition. We show that mesangial cells isolated from DDR1-null mice cells produce significantly less collagen compared to DDR1-null cells reconstituted with wild type DDR1. Moreover, mutagenesis analysis revealed that mutations in the collagen binding site or in the kinase domain significantly reduce DDR1-mediated collagen production. Finally, we provide evidence that blocking DDR1 kinase activity with an ATP-competitive small molecule inhibitor reduces collagen production. In conclusion, our studies indicate that the kinase activity of DDR1 plays a key role in DDR1-induced collagen synthesis and suggest that blocking collagen-mediated DDR1 activation may be beneficial in fibrotic diseases.

Results

Attenuated glomerulosclerosis and proteinuria in DDR1KO mice after renal injury. To determine the role of DDR1 in the response to glomerular injury we subjected littermates 129Sv/Ev wild type (WT) and DDR1-null (DDR1KO) mice to partial renal ablation (5/6 nephrectomy) which induces proteinuria and glomerulosclerosis beginning at 6-8 weeks after injury [25]. Histologic examination of kidney sections showed more severe glomerular injury in WT compared to DDR1KO mice 12 weeks after partial renal ablation (**Fig. 1A**). There was also a significant difference in the degree of glomerulosclerosis, analyzed by scoring the mesangial sclerosis index (MSI), between the two genotypes with a significant reduction in MSI in the injured DDR1KO mice (**Fig. 1B**). Consistent with reduced glomerular injury, DDR1KO mice also developed significantly less proteinuria than injured WT mice at 8 and 12 weeks post-injury (**Fig 1C**). These findings are consistent with previous studies showing that DDR1KO mice have less severe glomerular lesions and improved renal function following angiotensin-induced hypertensive nephropathy [19] or nephrotoxic serum induced nephritis [8].

Decreased collagen deposition in the DDR1KO mice after 5/6 nephrectomy. Since increased collagen deposition is a hallmark of glomerulosclerosis, we examined the levels of fibrillar (e.g. collagen I) and non-fibrillar (e.g. collagen IV) collagens in the glomeruli of 12 weeks injured WT and DDR1KO mice. Fibrillar collagen levels, assessed by Picro Sirius Red and Masson staining, increased in both WT and DDR1KO mice, although this effect was more evident in the kidneys of WT mice (**Figs. 2A, B**). We also examined the levels of glomerular collagen IV by staining kidney sections with anti-collagen IV antibody. There was a clear increase in the levels of collagen IV in both WT and DDR1KO mice (**Figs. 2A, C**). However, collagen IV levels were significantly lower in the DDR1KO mice compared to WT mice (**Figs. 2A, C**). These results suggest that DDR1 contributes to the increased collagen deposition in the course of glomerular injury.

DDR1 positively regulates collagen IV production in mesangial cells. To determine whether DDR1 directly regulates collagen IV synthesis, we firstly isolated DDR1KO mesangial cells and then we transfected them with either empty vector (DDR1KO) or with the human FLAG-tagged DDR1b cDNA (Rec-DDR1b). We focused on mesangial cells as 1) we detected increased collagen IV in levels in the mesangium following 5/6 nephrectomy (**Fig. 2A**); and 2) DDR1 expression increases in the mesangium following kidney injury [19]. To ensure that DDR1b is

properly expressed, we performed FACS analysis using anti-DDR1 antibody raised against the extracellular domain of the human DDR1 and found that the receptor is properly expressed on the cell surface (**Fig. 3A**). Moreover, to ensure that the receptor is functional, we analyzed collagen I-mediated DDR1b tyrosine auto-phosphorylation by performing an immunoprecipitation with cell lysates from collagen I-stimulated DDR1KO and Rec-DDR1b cells using anti-FLAG antibody. The immunoprecipitates were then immunoblotted with anti-DDR1 or anti-DDR1 phosphotyrosine 792 located in the activation loop of the receptor [26]. DDR1b auto-phosphorylation was evident in Rec-DDR1b cells 30 minutes after collagen I stimulation and persisted up to 8 hours (**Figs. 3B, C**). Thus, in mesangial cells DDR1 can be activated by collagen and follows the long and sustained activation reported for DDR1 in other cell types [1].

We next analyzed collagen IV levels in DDR1KO and Rec-DDR1b cells and found that, at baseline, mesangial cells expressing DDR1b produce higher levels of collagen IV than DDR1KO cells (**Figs. 4A, B**). Collagen IV levels were further significantly increased in Rec-DDR1b, but not DDR1KO mesangial cells, stimulated for 24 hours with collagen I (**Figs. 4A, B**), suggesting that DDR1 expression and activation directly upregulates collagen IV production in mesangial cells.

DDR1-mediated increase in collagen IV production requires collagen binding and receptor kinase activity. Although DDR1 auto-phosphorylation and activation require collagen I binding, some of DDR1-mediated effects are ligand independent or do not require kinase activity of the receptor. In this context, DDR1-mediated collective cells migration does not require DDR1-collagen binding activity [3] and DDR1-mediated linear invadosome formation does not require kinase activity [27]. To gain mechanistic insight on how DDR1 regulates collagen IV production in mesangial cells, we determined whether collagen binding and/or receptor kinase activity are required. To do so, we transfected DDR1KO mesangial cells with human DDR1b carrying the R105A mutation (Rec-DDR1b-R105A) which abolish the ability of the receptor to bind to collagen I [28] or carrying the K655A mutation (Rec-DDR1b-K655A) which abolish receptor kinase activity [29]. Cell populations expressing mutated forms of DDR1b at levels similar to wild type DDR1b (**Fig. 5A**) were then analyzed for collagen levels. We found that, unlike Rec-DDR1b cells, Rec-DDR1b-R105A and Rec-DDR1b-K655A expressed collagen levels similar to DDR1KO cells transfected with empty vector both at baseline or following collagen I stimulation (**Figs. 5B-D**). These results indicate that both collagen binding and receptor kinase activity are required for the pro-fibrotic effect of DDR1 in mesangial cells.

DDR1-mediated increase in collagen IV production is inhibited by DDR1 kinase ATP-competitor inhibitors. As DDR1 emerged as a potential therapeutic target in fibrotic diseases and cancer significant efforts have been made to develop inhibitors that block DDR1 activity. Recently, several ATP competitive small molecule inhibitors with good selectivity have been developed, including compound 1 [30]. This compound was characterized as a DDR2 inhibitor, although it also targets DDR1 with an even lower IC50 (IC50 18.6 nM for DDR2 vs 12.4 nM for DDR1). For this reason, we synthetized compound 1 (**Fig. 6A**) and confirmed that it inhibits DDR1 auto-phosphorylation using the LanthaScreen Eu kinase Binding assay (**Fig. 6B**). We determined an IC50 of 11.98 ± 0.89 nM which is consistent with the one reported for this receptor [30]. Next, we analyzed the ability of compound 1 to block collagen I-mediated DDR1 auto-phosphorylation

in mesangial cells. To do this, we treated mesangial cells with collagen I for 24 hours in the presence or absence of various concentrations of compound 1. Cell lysates were then immunoprecipitated using anti-FLAG antibody and the immunoprecipitates were immunoblotted with anti-DDR1 or anti-phosphotyrosine 792. DDR1b auto-phosphorylation was reduced in Rec-DDR1b treated with compound 1 (**Fig. 6C**). Finally, we analyzed collagen IV production in mesangial cells treated with compound 1 and found that DDR1-mediated increase in collagen IV production was significantly reduced upon compound 1 treatment (**Figs. 6D, E**). In contrast, collagen production was not reduced in DDR1KO cells treated with the compound 1 (**Figs. 6D, E**). These results suggest that targeting DDR1 kinase activity reduced the DDR1 pro-fibrotic effect in a DDR1-dependent manner.

Discussion

The finding that DDR1 is upregulated in both glomeruli and tubules of injured kidneys [7, 8, 18, 19] together with the finding that mice lacking DDR1 are protected from the progression to chronic kidney disease ([9] and this study), clearly implies that DDR1 is an important contributor to kidney disease. However, how DDR1 contributes to kidney diseases is poorly understood. The goal of this study was to determine whether the collagen binding receptor DDR1 contributes to kidney fibrosis by directly regulating collagen production.

DDR1 has been shown to have a pro-inflammatory role as it promotes inflammatory cell infiltration [7, 19] and production of pro-inflammatory cytokines like TGF-β, CTGF and IL-1 [8, 9]. Thus, it is conceivable that the protective effect observed in the DDR1-null mice is due to reduced inflammatory cell infiltration to the site of injury. Another explanation is that loss of DDR1 might lead to reduced production of TGF-β and CTGF, two well established pro-fibrotic cytokines, thus indirectly contributing to reduced fibrosis following injury.

DDR1 expression is upregulated in the glomeruli and tubules of injured kidneys [7, 8, 19]; however, what role this receptor plays in kidney resident cells is unclear. A plausible hypothesis is that DDR1 might contribute to kidney injury by directly exerting a pro-fibrotic action. In favor to this hypothesis, smooth muscle cells lacking DDR1 show decreased mRNA levels of non-fibrillar collagen, such as the basement membrane collagen IV [21], suggesting a pro-fibrotic effect of DDR1. However, mice lacking DDR1 also show increased fibrillar collagen deposition in the mammary gland [20] and DDR1-null smooth muscle show increased mRNA levels of fibrillar collagens I and III [21], suggesting that DDR1 is a negative regulator of fibrillar collagen synthesis. Thus, DDR1 can promote and/or inhibit collagen synthesis and this effect is dependent on the type of collagen. We provide evidence that in the glomerulus of the kidneys, DDR1 plays a deleterious effect by promoting the synthesis of both fibrillar and non-fibrillar collagens. We show that following 5/6 nephrectomy-induced injury DDR1KO mice have reduced glomerulosclerosis characterized by decreased collagen I and collagen IV deposition. Thus, our study suggests that, in contrast to breast cancer and/or smooth muscle cells, in the kidney DDR1 positively regulates extracellular matrix production thus playing a pro-fibrotic action.

We also provide evidence that in order to promote collagen production, DDR1 requires collagen binding as mutation of R105 in the collagen binding site significantly decreases the ability of DDR1 to stimulate collagen production at baseline and/or following collagen stimulation. Thus, it is conceivable that in the course of kidney injury increased expression of collagen within the glomeruli results in increased DDR1 activation and further production of collagen. In addition, increased TGF-β production by injured resident cells and/or inflammatory cells could lead to increased DDR1 expression in a Smad4-depenent manner, as recently proposed for hepatocellular carcinoma cells [31]. Finally, endothelial stress, hypoxia and shear stress could also contribute to upregulation of DDR1 expression [32], thus creating a vicious cycle leading to chronic kidney disease. This statement seems to agree with the idea that in the context of hypertensive nephropathy, DDR1 functions as an amplifier of the initial lesion, which occurs independently of the initiating cause, and leads to the development of and progression to chronic kidney disease [32].

Another key point of this study is that DDR1 requires its kinase activity in order to promote collagen production as mutation of K655A in the kinase domain results in impaired ability of DDR1 to stimulate collagen production. To further confirm that the kinase domain is important in DDR1-mediated collagen production, we show that inhibition of DDR1 kinase with the ATP-competitive inhibitor compound 1 decreases collagen IV production in a DDR1 dependent manner. To the best of our knowledge, this study is the first to show that blocking DDR1 kinase activity attenuates DDR1-mediated collagen deposition.

Because of DDR1 contribution to fibrotic diseases and cancer progression the interest in targeting DDR1 has significantly increased in the recent years. Approaches to block DDR1-mediated effects include: reduction of DDR1 expression by using antisense nucleotide [8, 33], inhibition of DDR1 binding to collagen with recombinant engineered bacterial collagen [34], inhibition of DDR1 oligomerization with selective monoclonal antibodies [35], and inhibition of DDR1 tyrosine kinase activity (see also [12]). As DDR1 ATP-competitive inhibitors with good selectivity have been developed [26, 36-39] targeting the kinase activity of DDR1 has become an appealing strategy. The majority of tyrosine kinase inhibitors synthetized are ATP-competitive inhibitors which target either the kinase domains in the active form (type I inhibitors) or in the inactive form (type II inhibitors). While type I inhibitors tend to be promiscuous, because they usually target well-conserved active kinase binding sites, type II inhibitors tend to be more selective because they can interact with not-well-conserved exposed hydrophobic sites within the inactive kinase domain [38].

For our inhibition studies we used compound 1 discovered first as an inhibitor of tyrosine kinases of the Ephrin family [40]. Compound 1 is a type II kinase inhibitor as it targets the inactive conformation of the kinase [30]. In a screen against a panel of over 350 kinases, compound 1 was found to target a limited number of kinases with DDR1 and DDR2 among the top hits [30]. Inhibition of DDR1/2 was also confirmed in enzymatic assays with compound 1 inhibiting DDR1/2 in the nanomolar range, while requiring micromolar concentrations for other tyrosine kinases [30]. Thus compound 1 is highly potent against DDR1/2 and has good selectivity. Here, we confirm that compound 1 has high affinity for DDR1, (IC50 11.98 nM) and we show that it blocks collagen-

induced DDR1 autophosphorylation and collagen IV production in mesangial cells expressing human DDR1. In contrast, compound 1 had no significant effect on collagen production in cells lacking DDR1 although these cells express DDR2 (data not shown), suggesting that DDR1, but not DDR2, mediates collagen IV production in mesangial cells.

In conclusion, our study shows that deletion of DDR1 is beneficial in the remnant model of kidney injury and that DDR1 expression in resident glomerular cells directly regulates collagen production, thus directly contributing to progression of disease. Moreover, our study shows that blocking DDR1 kinase activity with an ATP-competitive small molecule inhibitor reduces collagen production and suggests that blocking DDR1 kinase activity may be beneficial in fibrotic diseases.

Experimental procedures
Partial renal ablation. All the *in vivo* experiments were performed according to institutional animal care guidelines and conducted in Association for Assessment and Accreditation of Laboratory Animal Care accredited facilities**.** The DDR1KO mice were backcrossed onto the 129Sv/Ev background for nine generations. For the remnant kidney model (5/6 nephrectomy), we used 129Sv/Ev littermates WT and DDR1KO generated from DDR1het × DDR1het mating. Male mice (6–8 weeks old, 20–23g b.w.) underwent 5/6 nephrectomy or sham operation as previously described [41]. Mice were sacrificed 12 weeks after injury, 6-9 mice/genotype were used.
Clinical Parameters and Morphologic Analysis. For analysis of albuminuria, spot urine was collected at 0, 8 and 12 weeks after injury and the concentration of albumin and creatinine was measured using ELISA Albuwell M test and the Creatinine companion kit (Exocel Inc., Philadelphia, PA). The albumin creatinine ratio (ACR) was expressed as micrograms per milligram. Urine from 5-9 mice/treatment was analyzed.
For histologic analysis, 12 weeks after partial renal ablation, the remnant kidney was fixed in 4% formaldehyde and embedded in paraffin. Paraffin tissue sections were stained with hematoxylin/eosin (H&E), periodic acid-Schiff (PAS), Masson's Trichrome and PicroSirius Red for the evaluation of glomerular injury and fibrillar collagen deposition. The mesangial sclerosis index (MSI) was evaluated in a blinded fashion as described [42]. The percentage of mesangial matrix occupying each glomerulus was scored as 1 (0-24%), 2 (25-49%), 3 (50-74%), and 4 (>75%). Kidneys from 3-7 mice with a total of 60-140 glomeruli were analyzed and MSI was expressed as mean ± SEM.

Immunohistochemistry. Collagen IV deposition in uninjured and 5/6 nephrectomy-injured mice was analyzed on paraffin kidney sections using anti-collagen IV antibodies recognizes an epitope in the α1 chain of collagen IV (1:400; Rockland, Limerick, PA) followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA) and Sigma Fast DAB chromogenic tablets (Sigma, St. Louis, MO). Glomerular collagen IV levels were quantified using Image J, as described (https://imagej.nih.gov/ij/docs/examples/stained-sections/index.html). Collagen IV was expressed as % area occupied by collagen IV positive structures/glomerulus. Kidneys from 5 mice with a total of 30-50 glomeruli were analyzed and data were expressed as mean ± SEM.

Plasmids. To generate full length pIRES-DDR1b, human DDR1b cDNA was released from pRK5- DDR1b with *EcoRI* and *BamHI* and cloned between the same sites in pIRES-puro. In some experiments, we used pIRES-DDR1-FLAG which contains a FLAG tag at the C-terminus. pIRES-DDR1b-R105A and -DDR1b-K655A were generated with QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) following the manufacturer instructions. We have used the human DDR1 cDNA as human and mouse DDR1b are 94% identical, and key residues involved in collagen binding (R105) and kinase activity (K655) are conserved between the two species.

Cell Culture and Transfection. Immortalized DDR1KO mesangial cells were isolated from DDR1KO mice crossed with the immorto mouse, as previously described [41]. For propagation, immortalized mesangial cells were grown at 33°C in the presence of 100 IU/ml IFN-γ (Sigma) in DMEM supplemented with penicillin/streptomycin, insulin (10μg/ml) (Sigma), non-essential amino acids (Sigma) and 20% fetal bovine serum (FBS). For experiments, cells were cultured at 37°C without IFN-γ for at least 3 days as this time is optimal for conditionally immortalized mesangial cells to acquire a phenotype similar to the one of freshly isolated primary mesangial cells. To generate DDR1KO, Rec-DDR1b, Rec-DDR1b-R105A, Rec-DDR1b-K655A, and Rec-DDR1b-FLAG cells, immortalized mesangial cells isolated from DDR1KO mice were transfected with 1-2 μg of empty vector or the corresponding plasmid using Lipofectamine 2000 (Life Technologies) and stable clones were isolated under puromycin (Sigma) selection.   Successful generation of DDR1 expressing cells was evaluated by Western blot or FACS analysis.

Flow cytometry. To sort cells for comparable DDR1 surface expression, DDR1KO mesangial cells expressing wild type or mutated DDR1b were collected with trypsin and recovered in 10% FBS media. $2-3 \times 10^6$ cells were incubated with antibody to the extracellular domain of DDR1 (mAb 7A9) [35] at 4°C for 1 hour followed by incubation with PE-conjugated secondary antibody and sorted for comparable DDR1 levels using a FACS Ariall sorter (BD Biosciences) available through the Research Flow Cytometry Core Laboratory at the Nashville VA Medical Center.

Immunoprecipitation and Western blot analysis. Serum starved DDR1KO cells reconstituted with DDR1b-FLAG or empty vector were treated with vehicle (20 mM acetic acid) or with collagen I (Corning, 50 μg/ml in 20 mM acetic acid) for different times. In some experiments cells were incubated with collagen I in the presence or absence of the DDR1/2 inhibitor, compound 1, (1, 3, 10 μM) [30]. Cells were then lysed in Cell Signaling lysis buffer supplemented with protease inhibitors (Roche Applied Science). Equal amounts of lysates (200 μg) were clarified and pre-cleaned with Protein A beads and then incubated with anti-FLAG affinity gel (Sigma). After 12 hours, the immunoprecipitates were washed with wash buffer (50 mM Tris, pH 7.2, 150 mm NaCl, and 1% Triton X-100), eluted in sample buffer and then analyzed by Western blot for levels of phosphorylated (anti-pY792, Cell Signaling, 1:1000) and total DDR1 (anti-DDR1, Santa Cruz, 1:3000) followed by horseradish peroxidase-conjugated secondary antibodies and the immunoreactive bands were detected using an enhanced chemiluminescence substrate (PerkinElmer) following the manufacturer's instructions. To analyze the levels of collagen IV, 3×10^5 mesangial cells were plated in 6 well plates in medium containing 10% FCS. After 12 hours, the cells were incubated in serum free medium in the presence or absence of collagen I (10μg/ml

in 20 mM acetic acid) and in the presence or absence of the DDR1/2 inhibitor compound 1 [3 µM]. After 24 hours, cells were lysed in collagen lysis buffer (50 mM, Tris pH 7.5), 150 mM NaCl, 1% Triton X-100, and 1% sodium deoxycholate), as previously described (Borza CM, 2012). Equal amounts of total proteins (~20 µg/lane) were analyzed by Western blot for levels of collagen IV using anti-collagen IV antibodies that recognize an epitope in the NC1 domain in the α2 chain of collagen IV (gift of Dr. Dorin Borza, Meharry University, 1:2000), as well as well FAK (Santa Cruz, 1:1000) and AKT levels (Cell Signaling 1:1000) followed by horseradish peroxidase-conjugated secondary antibodies. Collagen IV and FAK or AKT were quantified by densitometry analysis using Image J and the collagen IV signal was expressed as collagen IV/FAK or collagen IV/AKT ratio. Values were expressed as mean ± SEM.

Recombinant His-tagged DDR1b cytoplasmic tail expression and purification. The DDR1b cytoplasmic domain was expressed using Bac-to-Bac baculovirus expression system following the manufacturer instructions (Life Technologies). Briefly, DDR1b cytoplasmic domain (a.a. 485-913) was amplified and cloned between the *EcoRI* and *KpnI* sites of the pFastBac HT A donor vector. The DDR1 construct in the donor vector was transformed in competent DH10Bac *Escherichia coli* (*E. coli*) bacterial cells to allow transposition and recombinant bacmid DNA was isolated using high molecular weight Mini-prep kits (Life Technologies and Qiagen) and transfected in Sf9 cells using CellFectin (Life Technologies) following manufacturer instructions. Recombinant virus was collected 5 days post-transfection, amplified and titrated using Bac PAK Baculovirus Rapid Titer kit (Clontech). For recombinant protein expression, Sf9 cells grown in suspension, were infected at multiplicity of infection of 1, collected 72 hours post-infection, resuspended in lysis buffer (Tris 20 mM pH8, NaCl 0.3M, Igepal 0.1%, glycerol 10%, DTT 1mM supplemented with protease inhibitors) and lysed using a tissue homogenizer. Lysates were clarified by centrifugation at 16,000g and HIS-tagged recombinant proteins were purified using a Ni-NTA agarose (Qiagen) affinity chromatography following manufacturer instructions. To remove imidazole, eluted proteins were applied on a Zeba Spin desalting columns (Thermo scientific) and buffer was exchanged to Tris 50 mM pH 7.2, glycerol was added to 50% and DTT (Sigma) to 2 mM and the proteins were stored in -80°C.

Time-Resolved fluorescence resonance energy transfer (TR-FRET) binding assay. The LanthaScreen Eu kinase binding assay format was used to determine the DDR1b IC50 for the DDR1/2 inhibitor compound 1, following manufacturer instructions (Life Technologies). This is a biochemical assay based on the displacement of an Alexa 647-conjugated ATP-competitive kinase tracer. In the absence of kinase inhibitors, concurrent binding of Alexa-conjugated tracer and Eu-conjugated anti-His antibody to the purified his-tagged kinase results in a high degree of TR-FRET. This is calculated as the ratio of the signal from the Alexa 647 acceptor fluorophore to the signal from the Eu donor. Kinase inhibitors compete with the tracer for binding to the ATP binding site of the kinase thus resulting in lower values of TR-FRET. In order to perform this assay, purified His-tagged DDR1b cytoplasmic domain (2nM), Eu-conjugated anti-HIS antibody (2nM, Invitrogen) and kinase tracer 178 (10 nM, Invitrogen) in kinase buffer (Invitrogen), were incubated in a final volume of 15 µl in 384-well plates at room temperature. After 1 hour, the plate was read on the multi-mode micro plate reader (Bio Tek NEO) using a 330/80 excitation filter, a 620/10 emission filter 1 (donor) and 665/8 emission filter 2 (acceptor). DDR1b cytoplasmic tail

was incubated with various concentrations of the DDR1/2 inhibitor Compound 1 prior to the addition of the tracer. For data analysis we calculated the emission ratio as the ratio of acceptor/tracer emission (665) to the antibody/donor emission (620) and reported as % binding relative to the emission ratio in the absence of the inhibitor.

Synthesis of the DDR1/2 inhibitor 5-((2-methyl-5-((3-(trifluoromethyl)phenyl)carbamoyl)phenyl)amino)nicotinamide (a.k.a. compound 1). Compound 1, shown to inhibit DDR1 (18.6 nM) and DDR2 kinase (12.4 nM) was synthesized based on the published method by Terai et al. [30]. To a stirred solution of 4-methyl-3-nitrobenzoic acid (10.87 g, 60 mmol) and 3-(trifluoromethyl)aniline (8.11 g, 50 mmol) in 100 mL of dichloromethane was added HATU (22.81 g, 60 mmol), DMAP (7.33 g, 60 mmol) and DIEA (19.40, 150 mmol). The reaction mixture was allowed to stand for 24 hours at room temperature, then eluted with ethyl acetate and washed with water. The organic phase was concentrated and purified with column chromatography (dichloromethane: methanol 15:1) to give a colorless oil. MS m/z 325 [M+1]. $SnCl_2$ (30.37 g, 160 mmol) was then added to a stirred solution of the obtained oil (13.01 g, 40 mmol) in ethyl acetate (50 mL) and ethanol (5 mL). The reaction mixture was allowed to stand for 5 hours at 80°C. The reaction mixture was then cooled to room temperature, and incubated with a saturated $NaHCO_3$ solution, stirred for 30 minutes and extracted with ethyl acetate. The organic phase was concentrated and dried. MS m/z 295 [M+1]. 5-Bromo-*N*-nicotinamide (0.2 mmol) and K_2CO_3 (0.6 mmol) were added to a solution of the organic phase product (0.2 mmol) in *t*-butanol (2 mL). The reaction mixture was degassed for 5 minutes with Argon and then $Pd_2(dba)_3$ (0.03 mmol) and XPhos (0.036 mmol) were added. The reaction flask was stirred for 5 hours at 75°C. After cooling to room temperature, the reaction mixture was filtered through a pad of celite and concentrated. Purification by HPLC gave 19 mg (TFA salt,18% yield) as a pale yellow solid. ^{1}H-NMR spectra were recorded on 400 MHz (Bruker), and chemical shifts are reported in part per milion (ppm) relative to internal CD3OD.^{1}H-NMR (400 MHz, CD_3OD, δ in ppm, rotamers denoted with *): 9.00* (s, 0.25H), 8.76* (s, 0.25H), 8.64* (m, 0.25H), 8.53* (s, 0.25H), 8.43 (s, 1H), 8.36* (s, 0.25H), 8.30 (s, 1H), 8.13 (s, 1H), 7.91 (d, $J = 8.1$ Hz, 1H) 7.83 (m, 1H), 7.74 (m, 1H), 7.68 (dd, $J = 7.9, 1.6$ Hz, 1H), 7.52 (m, 1H), 7.44 (d, $J = 8.0$ Hz, 1H), 7.41 (d, $J = 7.7$ Hz, 1H), 2.34* (s, 3H). MS m/z 415 [M+1]. The characterization matched that of the previously reported compound [30].

Statistical analysis. Data are shown as mean± SEM. Statistical analysis was performed using GraphPad Prism software (7.01 version). To evaluate the statistical significance of the differences between groups we used unpaired two tailed *t-test* for two groups. $p \leq 0.05$ was considered statistically significant**.**

Figure Legend

Figure 1. Loss of DDR1 reduces partial renal ablation-mediated glomerular injury. Kidney injury was induced by performing partial renal ablation (5/6 Nephr) in wild type (WT) and DDR1KO mice. Mice were sacrificed 12 weeks after injury and kidneys were collected for histological analysis. (**A**) Representative light micrographs of Hematoxylin and Eosin (H&E) and periodic-acid-Schiff (PAS) stained kidneys from uninjured (Cnt) or 12 weeks post 5/6 Nephr. Loss of DDR1 rescues the severe glomerular damage observed in injured WT mice. Original magnification, 40x. (**B**) Mesangial sclerosis index (MSI) was evaluated 12 weeks after 5/6 Nephr and scored as described in the Methods. Values represent the mean ± SEM of the number of mice indicated. (**C**) Urine albumin excretion, expressed as albumin-to-creatinine ratio (ACR), was examined on the number of mice indicated at 0, 8, and 12 weeks after 5/6 Nephr. Mice lacking DDR1 show significantly less proteinuria following injury.

Figure 2. Loss of DDR1 improves partial renal ablation-induced matrix deposition. (**A**) *Upper and middle panels*: PicroSirius Red and Masson's Trichrome staining of kidneys from uninjured (Cnt) or 12 weeks post partial renal ablation (5/6 Neph) WT and DDR1KO mice. Note the reduced presence of fibrillar collagen (red and blue) in glomeruli of injured DDR1KO mice. *Lower panel*: Collagen IV staining of kidney sections from the mice indicated above revealed reduced glomerular collagen deposition in injured DDR1KO mice. Original magnification, 40x. (**B, C**) The levels of glomerular collagen I (**B**) and IV (**C**) were quantified in the number of glomeruli indicated using Image J analysis as described in Experimental procedures. Values represent the mean ± SEM of the number of glomeruli indicated.

Figure 3. Generation of DDR1KO mesangial cells expressing human DDR1b. (**A**) DDR1KO mesangial cells were transfected with either empty vector (DDR1KO) or human DDR1b-FLAG cDNA (Rec-DDR1b) and cell populations expressing DDR1b were sorted by FACS. PE, phycoerythrin. (**B, C**) Serum starved DDR1KO and Rec-DDR1b cells (**B**) or Rec-DDR1b in duplicates (**C**) were treated with collagen I (50 µg/ml in 20 mM acetic acid) for the time indicated. Time 0 represents cells incubated with 20 mM acetic acid for 2 (**B**) or 8 (**C**) hours. Equal amount of cell lysates were then immunoprecipitated with anti-FLAG antibody and the immunoprecipitates were analyzed by Western blot for levels of total (IB: DDR1) or tyrosine phosphorylated (IB: pY792) DDR1. Note that DDR1 phosphorylation is evident 30 minutes after collagen I stimulation and persists up to 8 hours.

Figure 4. Loss of DDR1 reduces basal and collagen I-mediated collagen IV production. (**A**) Serum-starved DDR1KO and Rec-DDR1b mesangial cells were treated with vehicle (20mM acetic acid) or Collagen I (10 µg/ml in 20 mM acetic acid) for 24 hours and the levels of collagen IV or total FAK (to ensure equal loading) were analyzed by Western blot. (**B**) Collagen IV (CIV) and FAK bands were quantified by densitometry. Values represent CIV/FAK ratio and are expressed as fold changes relative to vehicle treated DDR1KO cells. Values are the mean ± SEM of 3-5 experiments performed at least in duplicate.

Figure 5. Ligand binding and DDR1b kinase activity are required for collagen IV production. (**A**) DDR1KO mesangial cells were transfected with either empty vector (DDR1KO) or human DDR1b (Rec-DDR1b), DDR1b-R105A (Rec-DDR1b-R105A), or DDR1b-K655A (Rec-DDR1b-K655A) cDNA and cell populations expressing comparable levels of DDR1b were sorted by FACS. (**B, C**) The serum-starved mesangial cells indicated were treated with vehicle (20mM acetic acid) or Collagen I (10 µg/ml in 20 mM acetic acid) for 24 hours and the levels of collagen IV or total AKT (to ensure equal loading) were analyzed by Western blot. (**D**) Collagen IV (CIV) and AKT bands were quantified by densitometry. Values represent CIV/AKT ratio and are expressed as fold changes relative to vehicle treated DDR1KO cells. Values are the mean ± SEM of 3-6 experiments performed at least in duplicate.

Figure 6. Inhibition of DDR1 kinase activity prevents collagen IV production. (**A**) Synthesis of compound 1, **r**eagents and conditions: a) HATU, DIEA, CH_2Cl_2 24 hours at room temperature ; b) $SnCl_2$, EtOAC, EtOH 5 hours at 80 °C; c) 5-bromonicotinamide, $Pd_2(dba)_3$, XPhos, K_2CO_3, t-BuOH, 5 hours at 75°C as indicated in the Experimental Procedures. (**B**) Inhibition of DDR1 by compound 1 measured with the LanthaScreen binding assay as described in the Experimental Procedures. Values represent the ratio of acceptor/tracer emission (665) to the antibody/donor emission (620), normalized to the value in the absence of the inhibitor. The IC_{50} value was 11.98 ± 0.89 nM. (**C**) Mesangial cells expressing FLAG-tagged DDR1b were treated with vehicle (20 mM acetic acid) or collagen I (50µg/ml in 20 mM acetic acid) in the presence or absence of compound 1 at the indicated concentrations for 24 hours. Equal amounts of cell lysates were then immunoprecipitated with anti-FLAG antibody and the immunoprecipitates were analyzed by Western blot for levels of total (IB: DDR1) or tyrosine phosphorylated (IB: pY792) DDR1. Note that compound 1 inhibits collagen I-mediated DDR1 phosphorylation at all doses analyzed. (**D**) Serum-starved DDR1KO or Rec-DDR1b mesangial cells were left untreated or treated with compound 1 (3 µM) for 24 hours then the levels of collagen IV or FAK (to ensure equal loading) were analyzed by Western blot. (**D**) Collagen IV (CIV) and FAK bands were quantified by densitometry. Values represent CIV/FAK ratio and are expressed as fold change relative to Rec-DDR1b in the absence of the inhibitor. Values are the mean ± SEM of 2-3 experiments performed at least in duplicate.

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Borza et al., Figure 1

A

B

C

A

B

C

D

Borza et al., Figure 6

A

compound 1

B

IC50: 11.98 ± 0.89 nM

% Binding vs compound 1 [nM]

C

| | 0 | 1 | 3 | 10 | compound 1 [μM] |

Collagen I: − + + + +

125 kDa − IB: pY792

125 kDa − IB: DDR1

IP: anti-FLAG

D

DDR1KO   Rec-DDR1b

compound 1 [3 μM]: − − − + + + − − − + + +

200 kDa − CIV

125 kDa − FAK

E

CIV/FAK

p<0.05   p<0.05

□ DDR1KO
■ Rec-DDR1b

compound 1 [3 μM]