

Targeting Phospholipase D4 Attenuates Kidney Fibrosis

Priyanka Trivedi,* Ramya K. Kumar,* Ashwin Iyer,* Sarah Boswell,[†] Casimiro Gerarduzzi,* Vivekkumar P. Dadhania,*[†] Zach Herbert,[‡] Nikita Joshi,[§] James P. Luyendyk,[§] Benjamin D. Humphreys,^{||} and Vishal S. Vaidya*^{†||}

*Renal Division, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts; [†]Harvard Program in Therapeutic Sciences, Harvard Medical School, Boston, Massachusetts; [‡]Molecular Biology Core Facilities, Dana-Farber Cancer Institute, Boston, Massachusetts; [§]Department of Pathobiology and Diagnostic Investigation, Michigan State University, East Lansing, Michigan; ^{||}Division of Nephrology, Washington University School of Medicine, St. Louis, Missouri; and ^{†||}Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, Massachusetts

ABSTRACT

Phospholipase D4 (PLD4), a single-pass transmembrane glycoprotein, is among the most highly upregulated genes in murine kidneys subjected to chronic progressive fibrosis, but the function of PLD4 in this process is unknown. Here, we found PLD4 to be overexpressed in the proximal and distal tubular epithelial cells of murine and human kidneys after fibrosis. Genetic silencing of PLD4, either globally or conditionally in proximal tubular epithelial cells, protected mice from the development of fibrosis. Mechanistically, global knockout of PLD4 modulated innate and adaptive immune responses and attenuated the upregulation of the TGF- β signaling pathway and α 1-antitrypsin protein (a serine protease inhibitor) expression and downregulation of neutrophil elastase (NE) expression induced by obstructive injury. *In vitro*, treatment with NE attenuated TGF- β -induced accumulation of fibrotic markers. Furthermore, therapeutic targeting of PLD4 using specific siRNA protected mice from folic acid-induced kidney fibrosis and inhibited the increase in TGF- β signaling, decrease in NE expression, and upregulation of mitogen-activated protein kinase signaling. Immunoprecipitation/mass spectrometry and coimmunoprecipitation experiments confirmed that PLD4 binds three proteins that interact with neurotrophic receptor tyrosine kinase 1, a receptor also known as TrkA that upregulates mitogen-activated protein kinase. PLD4 inhibition also prevented the folic acid-induced upregulation of this receptor in mouse kidneys. These results suggest inhibition of PLD4 as a novel therapeutic strategy to activate protease-mediated degradation of extracellular matrix and reverse fibrosis.

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Fibrosis is the underlying pathology of CKD, a complex debilitating condition affecting >70 million people worldwide.¹ Of several known mechanisms, it is often a balance between the immune components as well as the synthesis and degradation of extracellular matrix (ECM) components that determines whether the kidney injury resolves or progresses to fibrosis.^{2,3} Understanding the mechanistic basis of such multivariate interactions within the scar microenvironment, including interplay between the immune system and the proteases, may enable identification of novel target-based precision therapeutics for kidney fibrosis.

We previously reported phospholipase D4 (PLD4) to be among the most upregulated genes in a mouse model of folic acid (FA)-induced kidney fibrosis.⁴ PLD4 belongs to phospholipase D family,

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Correspondence: Dr. Vishal S. Vaidya, Harvard Institutes of Medicine, Rm 562, 77 Avenue Louis Pasteur, Boston, MA 02115. Email: vvaidya@bwh.harvard.edu

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which comprises six members, *i.e.*, PLD1–PLD6.⁵ Of these, PLD1 and PLD2 are well characterized and are known as classical PLDs. Unlike PLD1 and PLD2 proteins, which contain Phox homology (PX) and pleckstrin homology (PH) domains in the N-terminal region and two conserved His-x-Lys-x-x-x-Asp sequences (where x is any amino acid, HKD) motifs in the C-terminal region, PLD4, a nonclassical PLD, has a putative transmembrane domain instead of PX and PH domains.^{5–7} PLD1 and PLD2 are well known to play a vital role in the pathogenesis of various diseases such as cancer (breast, ovarian, lung, colon, renal, pancreatic, prostate, and brain cancer), infectious disorders (influenza viruses), and neurodegenerative disorders (encephalomyelitis, stroke, and Alzheimer disease)⁸ by modulating the mammalian target of rapamycin (mTOR) pathway,⁹ hypoxia inducible factor 1 α (HIF1 α) signaling pathway,¹⁰ angiogenesis,¹¹ and phagocyte cell migration.¹² Although much is known about the classic PLDs (PLD1 and PLD2), the functional role of PLD4 has not been investigated beyond its implication in systemic sclerosis,¹³ rheumatoid arthritis,¹⁴ and the phagocytosis of microglia.¹⁵ Our study was aimed at investigating the mechanistic role of PLD4 in kidney fibrosis and examining if modulating PLD4 by genetic and pharmacologic approaches alters the course of fibrosis progression.

RESULTS

PLD4 Expression Is Upregulated in Mice and Human Kidneys after Fibrosis

We found that among the PLD family members, PLD2 and PLD4 expression significantly ($P < 0.05$) increased at mRNA level when treated with FA (Figure 1, A and B) or subjected to unilateral ureteral obstruction (UUO) (Figure 1, D and E) two mechanistically distinct mouse models of kidney fibrosis.^{4,16–18} There was also a significant increase in the protein expression of PLD4 in both FA (Figure 1C) and UUO (Figure 1F) models. In order to examine the pan-fibrotic expression of PLD4, its levels were measured in a carbon tetrachloride (CCl₄)–induced liver fibrosis model. PLD4 expression significantly increased in mice with liver fibrosis (Supplemental Figure 1), extending the relevance of PLD4 in fibrosis beyond kidneys. Translatability of PLD4 expression in human disease was confirmed by observing a significant increase in PLD4 expression in patients with biopsy-proven tubulointerstitial fibrosis (Figure 1G).

PLD4 Global Knockout Mice Are Protected against Kidney Fibrosis

To decipher the functional role of PLD4 in kidney fibrosis, we conducted loss-of-function experiments using PLD4 global knockout (PLD4^{-/-}) mice. Knockdown of PLD4 was confirmed by the absence of PLD4 at baseline (Supplemental Figure 2, A and B) as well as lack of increase in PLD4 expression in the PLD4^{-/-} mice after fibrosis (Supplemental Figure 2, C and D). The PLD4^{-/-} mice are viable and did not show any histologic abnormality (Supplemental Figure 2E). There was a

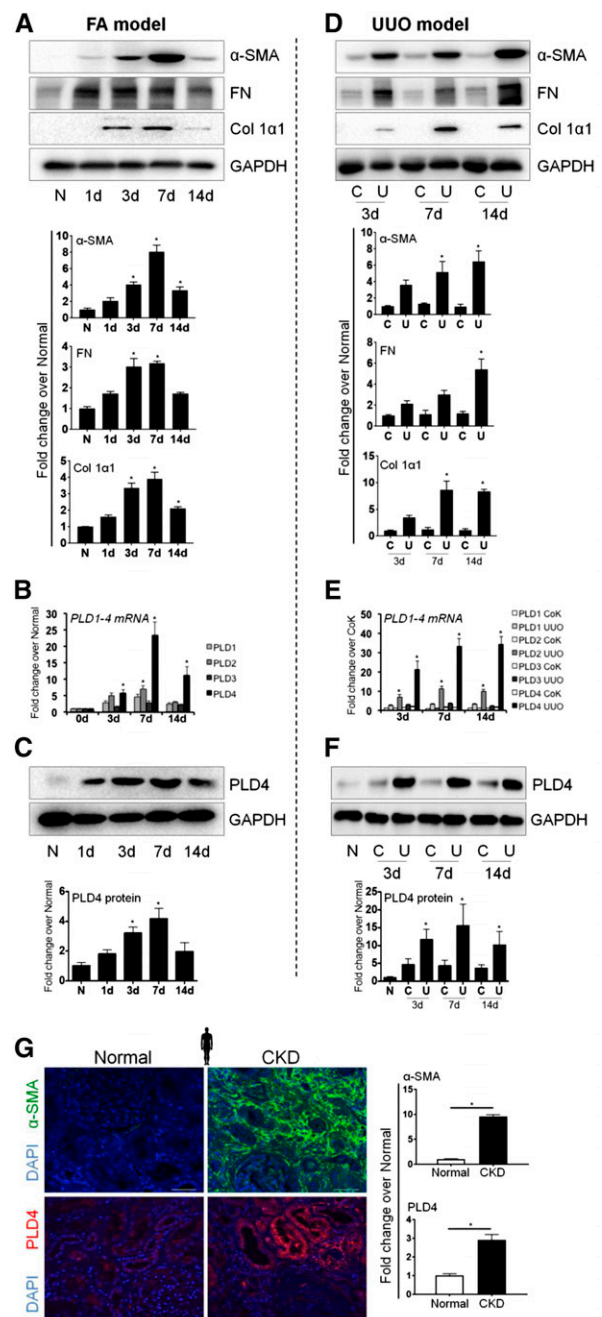


Figure 1. Expression of fibrotic markers as well as PLD4 increases in FA- and UUO-induced kidney fibrosis. Protein expression of the fibrotic markers α -SMA, fibronectin (FN), and collagen 1 α 1 (Col 1 α 1) in the kidneys of mice (A) injected with FA and (D) subjected to UUO ($n=5$ /group). (B and E) mRNA levels of PLD1, PLD2, PLD3, and PLD4 and (C and F) protein level of PLD4 in the kidneys of mice injected with FA and subjected to UUO, respectively. Data were normalized to GAPDH and are presented as mean \pm SEM ($n=5$ /group) of the fold change over normal. $*P < 0.05$. (G) Immunostaining and the relative quantitation of α -SMA and PLD4 in the kidney sections from patients with biopsy-proven tubulointerstitial fibrosis versus patients with no evidence of fibrosis ($n=5$ /group). Scale bars, 50 μ m. Data are presented as mean \pm SEM of the fold change over normal. $*P < 0.05$. C, contralateral; CoK, contralateral kidney; N, normal; U, UUO.

significant attenuation ($P < 0.05$) in the expression of fibrotic markers α -smooth muscle actin (α -SMA), fibronectin, and collagen 1 α 1 (Figure 2, A–C, Supplemental Figure 3, A–D) in the PLD4^{-/-} mice compared with the wild-type (PLD4^{+/+}) mice subjected to either FA administration or surgical UUO. The protection from fibrosis was further confirmed pathologically by observing a significant decrease in collagen accumulation and scar tissue formation as evidenced by Picrosirius Red and Masson's trichrome staining in both the models (Figure 2C).

Proximal Tubule Epithelial Cell-Specific PLD4 Knockdown Confers Protection against Kidney Fibrosis

Because PLD4 is also expressed in leukocytes, the global knock-out mouse model could not distinguish whether PLD4 expressed in leukocytes or epithelial cells mediates fibrosis. Therefore, we generated conditional mice lacking PLD4 in the kidney proximal tubular epithelial cells. We generated these mice by crossing PLD4^{fl/fl} mice with mice expressing Cre recombinase under the control of the tamoxifen-inducible SLC34a1 promoter, a transport protein expressed only in the proximal tubule cells.¹⁹ We compared SLC34a1^{GCE/+} PLD4^{fl/fl} (GCE stands for eGFP-CreER^{T2}) with SLC34a1^{GCE/+} PLD4^{wt/wt} mice; Cre was present in both the experimental groups, and all mice were injected with tamoxifen. In SLC34a1^{GCE/+} PLD4^{wt/wt} mouse kidneys (baseline), PLD4 was broadly expressed in proximal tubule cells as evident from the costaining of PLD4 and Lotus tetragonolobus lectin (LTL, proximal tubule marker). In SLC34a1^{GCE/+} PLD4^{fl/fl} mouse kidneys (baseline), PLD4 was specifically deleted only in LTL-positive proximal tubule cells (thick arrows) but not in distal tubular segments (thin arrows) (Figure 2E). Tamoxifen was injected into SLC34a1^{GCE/+} PLD4^{wt/wt} and SLC34a1^{GCE/+} PLD4^{fl/fl} mice on days 1, 3, and 5 after FA injection and mice were euthanized 7 days after FA injection (Figure 2D). SLC34a1^{GCE/+} PLD4^{fl/fl} mice showed significantly decreased expression of α -SMA, fibronectin, and collagen 1 α 1 compared with the SLC34a1^{GCE/+} PLD4^{wt/wt} mice (Figure 2F, Supplemental Figure 3E). Although leukocyte-associated PLD4 might still play a role in fibrogenesis, our data demonstrates that PLD4 expressed in proximal tubular epithelial cells play a predominant role in propagating fibrosis in the kidneys.

PLD4 Modulates the Immune Responses and Upregulates α 1-Antitrypsin (a Serine Protease Inhibitor) in Kidney Fibrosis

To investigate the mechanisms of protection from fibrosis, we performed RNA sequencing (Supplemental Figure 4) in the kidneys of global PLD4^{-/-} and PLD4^{+/+} mice at baseline and after UUO. We observed differential expression of genes involved in the innate and adaptive immune responses between the PLD4^{-/-} and PLD4^{+/+} mice at baseline and after UUO (Figure 3, A and B, Supplemental Figure 4F). Experimentally, we confirmed a significant reduction ($P < 0.05$) of neutrophils (CD11b⁺Ly6G⁺) and monocytes (CD11b⁺Ly6C⁺) in the PLD4^{-/-} mice at day 5 after UUO (Figure 3C, Supplemental Figure 5A). On the other hand, CD8⁺ and MHC-II⁺ cells were significantly higher

($P < 0.05$) at baseline and after UUO, whereas CD4⁺ cells were significantly higher ($P < 0.05$) only after UUO in the PLD4^{-/-} mice (Figure 3C, Supplemental Figure 5A). There were no differences in the levels of natural killer cells (NK1.1⁺ cells) or B cells (CD19⁺ cells) between the PLD4^{-/-} and PLD4^{+/+} mice (Supplemental Figure 5B). We also measured cytokines in the kidney (Figure 3D, Supplemental Figure 5C) and found that mRNA levels of INF- γ , TNF- α , IL-1 β , IL-6, IL-17, and IL-10 were significantly higher ($P < 0.05$) in the PLD4^{-/-} mice after UUO (Figure 3D). Also, there was a significant decrease in Arg1 (a M2 macrophage marker), but no difference in iNOS (a M1 macrophage marker), between the PLD4^{-/-} and PLD4^{+/+} mice after UUO (Figure 3D, Supplemental Figure 5C). INF- γ and TNF- α are known to be antifibrotic cytokines whereas M2 macrophages are known to be profibrotic because of their ability to inhibit and induce TGF- β secretion, respectively.^{20–22} INF- γ and TNF- α also exert their antifibrotic effect by suppressing the synthesis of TGF- β -induced collagen and connective tissue growth factor, respectively.²³ We observed downregulation of TGF- β signaling in the PLD4^{-/-} mice after UUO as evidenced by a significant ($P < 0.05$) decrease in the expression of pSmad 2, Smad2, pSmad3, and Smad3 (Figure 3E, Supplemental Figure 5D).

RNA sequencing (Supplemental Figure 4) also revealed downregulation of serpin family genes in the PLD4^{-/-} mice (Figure 3A, Supplemental Figure 4F). Among serpin family genes, serpin1d was the most downregulated gene, and serpin1d translates to α 1-antitrypsin (AAT), which is a serine protease inhibitor. Downregulation of AAT was confirmed by observing a significantly decreased protein expression of AAT in the PLD4^{-/-} mice after fibrosis (Figure 3F, Supplemental Figure 5E). The association between PLD4 and AAT was further established by observing a significant increase in the AAT expression ($P < 0.05$) in PLD4-overexpressed HEK293T cells (Supplemental Figure 6). AAT specifically inhibits neutrophil elastase (NE), a serine protease known to degrade the ECM proteins such as fibronectin and collagen.²⁴ Significantly increased ($P < 0.05$) expression of NE was observed in the PLD4^{-/-} mice (Figure 3F, Supplemental Figure 5E), suggesting that degradation of the ECM proteins by NE was partly playing a role in protecting these mice from fibrosis.

The role of NE in decreasing the expression of fibrotic markers was further confirmed *in vitro* using a coculture system of primary human kidney fibroblasts and HEK293T cells (transfected with pCMV or pCMV-PLD4). NE treatment (50 nM) for 48 hours significantly decreased the levels of α -SMA and fibronectin compared with the untreated cells. TGF- β treatment (10 ng/ml) for 48 hours led to overexpression of the fibrotic markers in the cocultured cells. TGF- β treatment in the PLD4-overexpressed HEK293T cells cocultured with the primary human kidney fibroblasts led to further increase in the expression of α -SMA and fibronectin. Cotreatment of cells with NE (50 nM) and TGF- β for 48 hours significantly decreased the level of fibrotic markers compared with TGF- β -only treatment. The NE-mediated decrease in TGF- β -induced fibrotic markers was significantly lower in the coculture system with pCMV-PLD4-transfected HEK293T cells compared with the

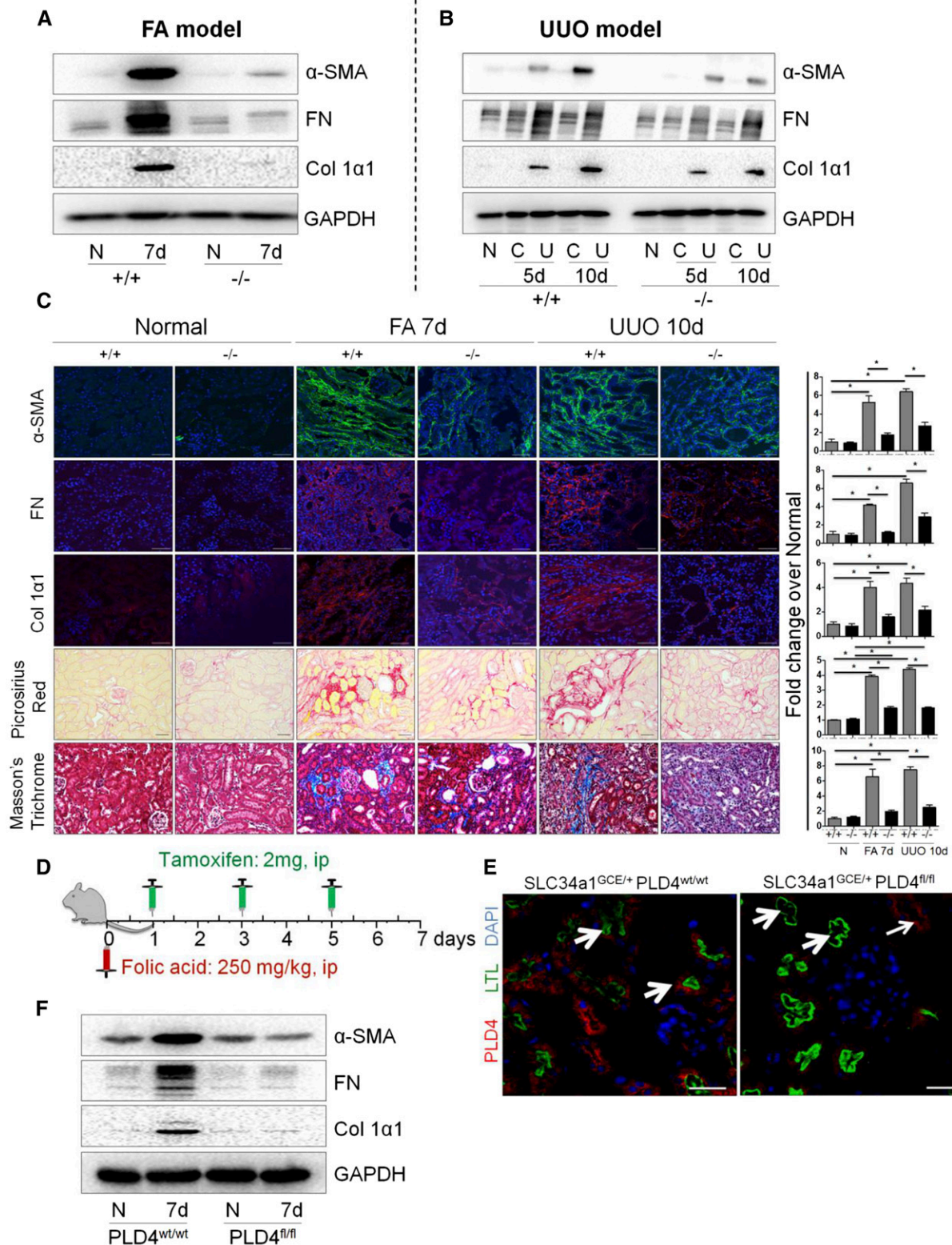


Figure 2. PLD4^{-/-} mice are protected from FA- and UUO-induced kidney fibrosis. Protein expression of the fibrotic markers α -SMA, fibronectin (FN), and collagen 1 α 1 (Col 1 α 1) in the kidneys of PLD4^{+/+} and PLD4^{-/-} mice injected with (A) FA and (B) subjected to UUO ($n=5-7$ /group). (C) Representative photomicrographs depicting the expression of fibrotic markers (α -SMA, FN, and Col 1 α 1) in mouse kidneys ($n=5$ /group), and Picrosirius Red and Masson's trichrome staining depicting collagen deposition in mouse kidneys at baseline and after fibrosis ($n=3$ /group). Scale bars, 50 μ m. Relative quantitation of immunostaining

pCMV-transfected cells, which might be because of PLD4-induced AAT expression. This observation strengthens our *in vivo* findings reporting the role of PLD4 in inducing fibrogenesis, at least in part, by downregulating NE expression (Supplemental Figure 7). Expression of the fibrotic markers in cells treated with TGF- β (10 ng/ml) for 48 hours followed by NE treatment (50 nM) for 6 hours was not significantly different compared with the TGF- β alone-treated cells (Supplemental Figure 7). This indicates that NE treatment alone might not be effective in reversing fibrosis at a later time point.

Targeting PLD4 Using Small Interfering RNA Protects against Kidney Fibrosis

We next investigated whether silencing PLD4 in mice using a small interfering RNA (siRNA) strategy protects from kidney fibrosis (Figure 4A). PLD4 siRNA (validated *in vitro* using mouse inner medullary collecting duct [mIMCD3] cells, Supplemental Figure 8A) was delivered to the kidney (Figure 4B, Supplemental Figure 8, B and C) and protected mice from kidney fibrosis as evident from the decreased expression of fibrotic markers (Figure 4, B and C, Supplemental Figure 8C). The therapeutic effect of PLD4 siRNA was mediated *via* the same mechanisms, *i.e.*, downregulation of TGF- β signaling (Figure 4D, Supplemental Figure 8D), decreased expression of AAT, and increased expression of NE (Figure 4E, Supplemental Figure 8E). PLD4 silencing decreased kidney fibrosis without significantly reducing the initiation of kidney injury, as evident from the levels of plasma creatinine, BUN, and kidney injury molecule 1 (KIM1) expression in the kidney on day 2 after FA injection (Figure 4, F and G).

PLD4 Interacts with Calmegin, Lectin, Mannose Binding 2, and Suppressor of Lin-12-Like Protein 1 and Upregulates MAPK Signaling *via* Tropomyosin-Related Kinase A Pathway to Mediate Fibrogenesis

To further delineate PLD4-mediated signaling pathways in promoting fibrogenesis, we next took an immunoprecipitation/mass spectrometry-based interactome proteomics approach²⁵ (Supplemental Figure 9) and found several proteins to be the binding partners of PLD4 (Figure 5A), most of which are endoplasmic reticulum proteins, although some are localized in the Golgi apparatus, mitochondria, and plasma membrane (Figure 5B). The subcellular localization of PLD4 was assessed using

HeLa cells (Figure 5C) as well as HEK293 cells (Figure 5D), and we found PLD4 to be localized in the endoplasmic reticulum, Golgi apparatus, and mitochondria. We experimentally confirmed the interaction between PLD4 and each of the top three proteins—calmegin (CLGN), lectin, mannose binding 2 (LMAN2), and suppressor of lin-12-like protein 1 (SEL1L)—(on the basis of the weighted and normalized D score [WDN score]) using coimmunoprecipitation/western blot (Co-IP/WB) (Figure 5E). Interestingly, CLGN, LMAN2, and SEL1L are known to have a common interactor, neurotrophic receptor tyrosine kinase 1 (NTRK1), also known as tropomyosin-related kinase A (TrkA) (Supplemental Figure 10, <https://thebiogrid.org>), which in turn upregulates MAPK *via* fibroblast growth factor receptor substrate 2 (FRS2 α). PLD4 inhibition (genetic knockdown as well as PLD4 siRNA treatment) significantly decreased the protein expression of CLGN and SEL1L in mouse kidneys; however, LMAN2 expression was not affected. There was a significant decrease in the expression of TrkA, and the downstream signaling proteins, FRS2 α , pERK, and ERK, with PLD4 inhibition, suggesting that PLD4 mediates fibrogenesis by upregulating MAPK signaling *via* TrkA pathway (Figure 5, F and G, Supplemental Figure 11).

DISCUSSION

Collectively, our results demonstrate that PLD4 expressed on the kidney proximal tubular epithelial cells is a novel target against kidney fibrosis. We show that PLD4 facilitates fibrogenesis by modulating innate and adaptive immune responses and promoting the TGF- β signaling pathway. Moreover, PLD4 induces the expression of AAT and the subsequent downregulation of NE expression, thereby, at least in part, leading to the accumulation of ECM proteins (Figure 4H).

Although inflammation is well known to drive fibrogenesis,²⁶ here we observed that PLD4^{-/-} mice were protected from fibrosis in spite of elevated proinflammatory cytokines and T cells. Consistent with our findings, proinflammatory cytokines, IFN- γ , and TNF- α , have been shown to be antifibrotic using *in vitro* and *in vivo* models of kidney, liver, and lung fibrosis.^{27–29} The proposed antifibrotic mechanisms of IFN- γ are antagonism toward TGF- β -induced signaling and collagen deposition by signal transducer and activator of transcription (STAT)-1 activation in lung, as well as inhibition of

and Picrosirius Red and Masson's trichrome staining. Data are presented as mean \pm SEM of the fold change over PLD4^{+/+} normal. * $P < 0.05$. (D) Schematic representation of the timeline of tamoxifen and FA administration in mice. Mice were injected with FA (250 mg/kg, ip) followed by treatment with tamoxifen (2 mg, diluted in corn oil and 3% ethanol, ip) at 1, 3, and 5 days after FA injection. Mice were euthanized on day 7 after FA injection ($n = 5$ /group). (E) Specificity of PLD4 knockdown in kidney proximal tubular epithelial cells was confirmed by coimmunostaining PLD4 with LTL, a proximal tubular marker (thick arrows, LTL-positive tubules; thin arrows, LTL-negative tubules), using the kidneys obtained from SLC34a1^{GCE/+} PLD4^{fl/fl} (PLD4^{fl/fl}) and wild-type mice SLC34a1^{GCE/+} PLD4^{wt/wt} (PLD4^{wt/wt}) ($n = 5$ /group). Scale bars, 50 μ m. (F) Protein levels of α -SMA, FN, and Col 1 α 1 in the kidneys of PLD4^{wt/wt} and PLD4^{fl/fl} mice injected with FA ($n = 5$ /group). C, contralateral; ip, intraperitoneally; N, normal; U, UUO; +/+, PLD4 wild-type; -/-, PLD4 knockout.

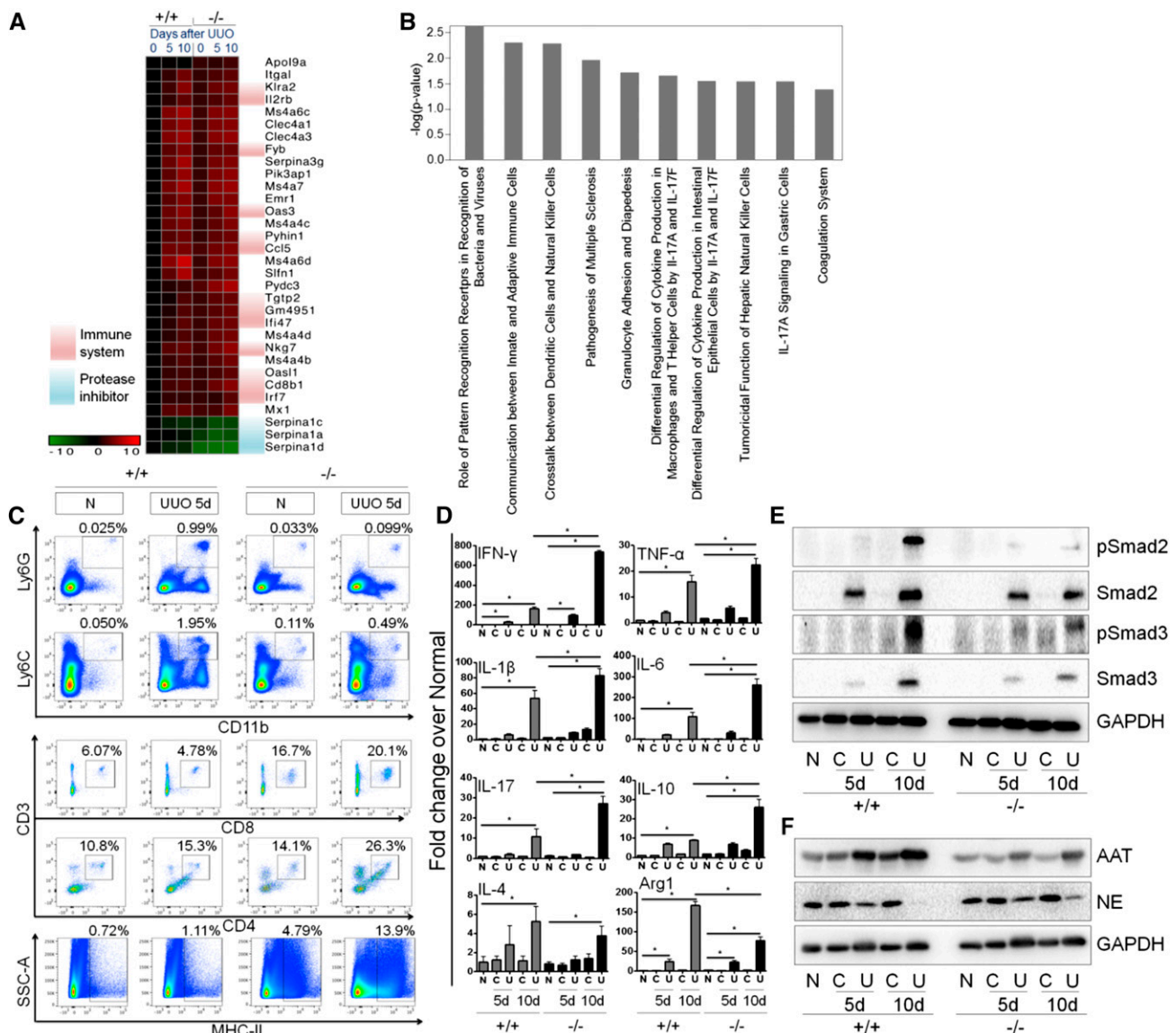


Figure 3. PLD4^{-/-} mouse kidneys have differentially expressed innate and adaptive immune genes and decreased level of a protease inhibitor, AAT, compared with the PLD4^{+/+} mice. (A) Heat map depicting differentially expressed genes between PLD4^{+/+} and PLD4^{-/-} mouse kidneys at baseline and after UUO (days 5 and 10) ($n=4$ /group). (B) Pathway analysis of differentially expressed genes between PLD4^{+/+} and PLD4^{-/-} mouse kidneys at baseline. (C) Flow cytometric analysis of innate and adaptive immune system components in the kidneys of mice at baseline and after UUO (day 5) ($n=3$ /group). (D) mRNA levels of cytokines in the kidneys of mice at baseline and after UUO (days 5 and 10). Protein expression of (E) TGF- β signaling molecules, pSmad2, Smad2, pSmad3, and Smad3; and (F) AAT and NE in mouse kidneys. Data were normalized to GAPDH and are presented as mean \pm SEM ($n=5-7$ /group) of the fold change over PLD4^{+/+} normal. * $P<0.05$. C, contralateral; N, normal; U, UUO; +/+, PLD4 wild-type; -/-, PLD4 knockout.

hepatic stellate cell activation.^{20,30} Similarly, TNF- α exerts an antifibrotic effect by inhibiting TGF- β /Smad signaling through the activation of AP-1 components, activating the inhibitory Smad7 by NF- κ B/RelA and suppressing TGF- β 1-induced myofibroblast activation.^{22,31,32} Furthermore, treatment with recombinant IL-2 and TNF- α significantly reduced the appearance of fibrocytes and the severity of fibrosis in the UUO model.³³ Increased proinflammatory cytokines in PLD4^{-/-} mice could, at least in part, be because of a decreased level of AAT, an endogenous inhibitor of proinflammatory cytokine

production in whole blood.³⁴ Renal AAT gene induction was observed in experimental and clinical kidney damage,³⁵ which corroborates our finding.

Another finding from our study is that significantly less fibrosis in global PLD4^{-/-} mice was associated with an increased level of NE, indicating a protective role of NE in kidney fibrosis. Although NE is known to damage the lungs,³⁶ we observed that, in kidneys, increased expression of NE mirrors the decreased extent of fibrosis. Interestingly, the histologic examination of the lungs of PLD4^{-/-} mice did not show any

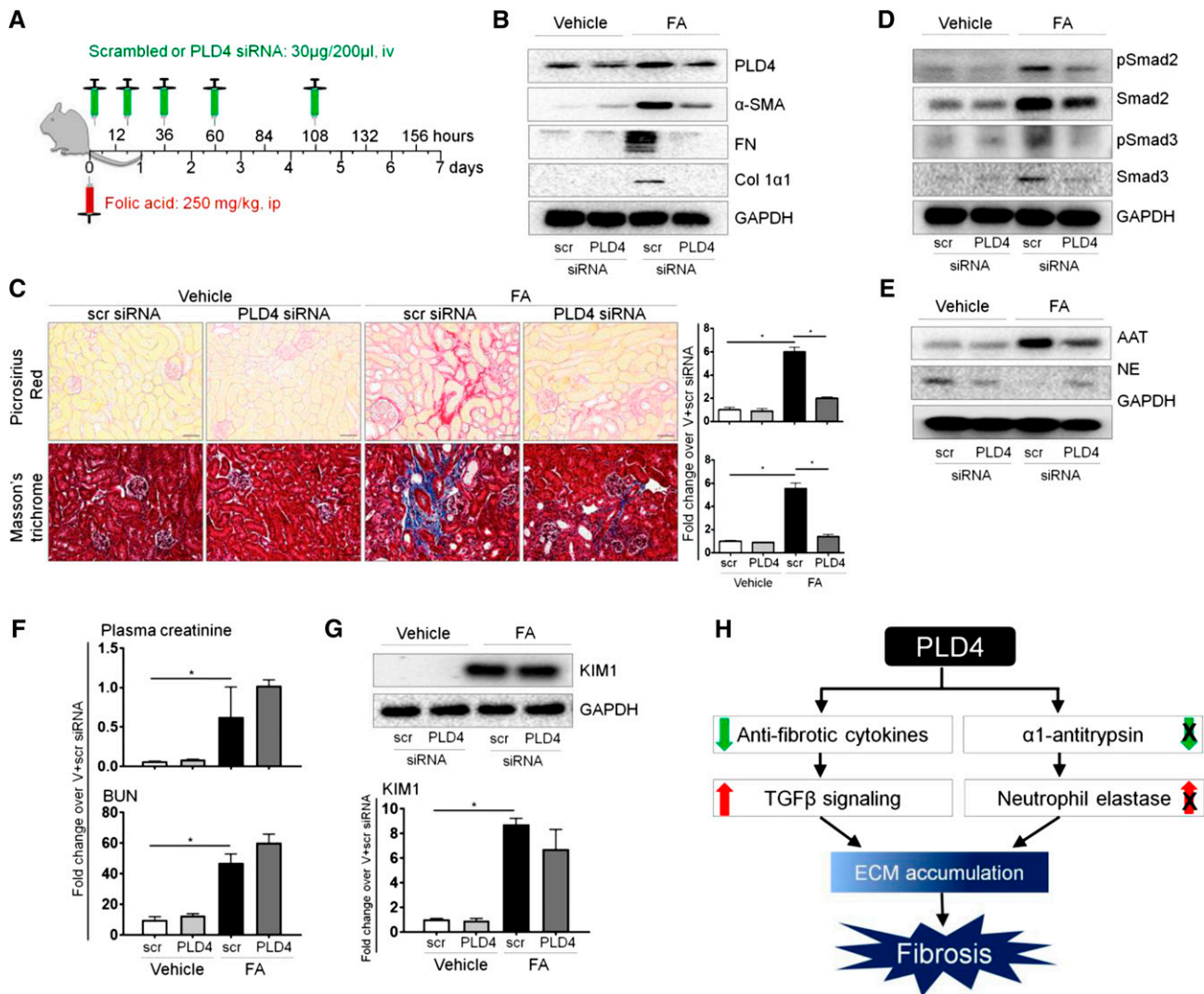


Figure 4. Therapeutic targeting of PLD4 by specific siRNA protects mice from kidney fibrosis. (A) Schematic representation of the treatment protocol with FA and siRNA (scrambled or PLD4). Mice were injected with FA (250 mg/kg, ip) followed by treatment with scrambled or PLD4 siRNA (30 µg/200 µl, iv) at 2, 20, 38, 62, and 110 hours after FA injection. Mice were euthanized on day 7 after FA injection (n=5/group). (B) Protein levels of PLD4 and the fibrotic markers α-SMA, fibronectin (FN), and collagen 1α1 (Col 1α1) in mouse kidneys (n=5/group). (C) Picrosirius Red and Masson's trichrome staining depicting collagen deposition in mouse kidneys (n=3/group). Scale bars, 50 µm. Data are presented as mean ± SEM (n=5/group) of the fold change over vehicle+scrambled siRNA. *P<0.05. Protein expression of (D) TGF-β signaling molecules, pSmad2, Smad2, pSmad3, and Smad3; and (E) AAT and NE in mouse kidneys (n=5/group). (F) Level of plasma creatinine and BUN in mice and (G) protein level of KIM1 in mouse kidneys (n=4/group). Data are presented as mean ± SEM (n=4–5/group) of the fold change over vehicle+scrambled siRNA. *P<0.05. (H) Schematic representation depicting how PLD4 drives fibrosis. PLD4 leads to a decrease in anti-fibrotic cytokines, upregulation of TGFβ signaling and downregulation of neutrophil elastase expression by preventing a decrease in α1-antitrypsin expression. All these result in increased ECM accumulation that progresses to fibrosis. ip, intraperitoneally; iv, intravenously; Scr, scrambled siRNA; V+scr, vehicle+scrambled siRNA.

abnormality. However, given the destructive effect of NE in lungs, the role of NE in kidney fibrosis needs to be definitively confirmed by performing additional studies such as reversing the protective effect of PLD4-siRNA against kidney fibrosis with either (1) reconstitution of AAT level in PLD4-siRNA-treated mice similar to that in scrambled siRNA-treated mice and/or (2) using blocking antibodies to NE. We speculate that increased NE might be exerting an antifibrotic effect in

conjunction with the proinflammatory cytokines. NE degrades collagen in three-dimensional culture by acting synergistically with proinflammatory cytokines.³⁷ Decreased level of elastase due to increased expression of monocyte/neutrophil elastase inhibitor was associated with accumulation of elastin, an ECM protein, in kidneys of mice with diabetic nephropathy.³⁸ Interestingly, although the neutrophils were significantly less in the PLD4^{-/-} mice after UUO, these mice had higher

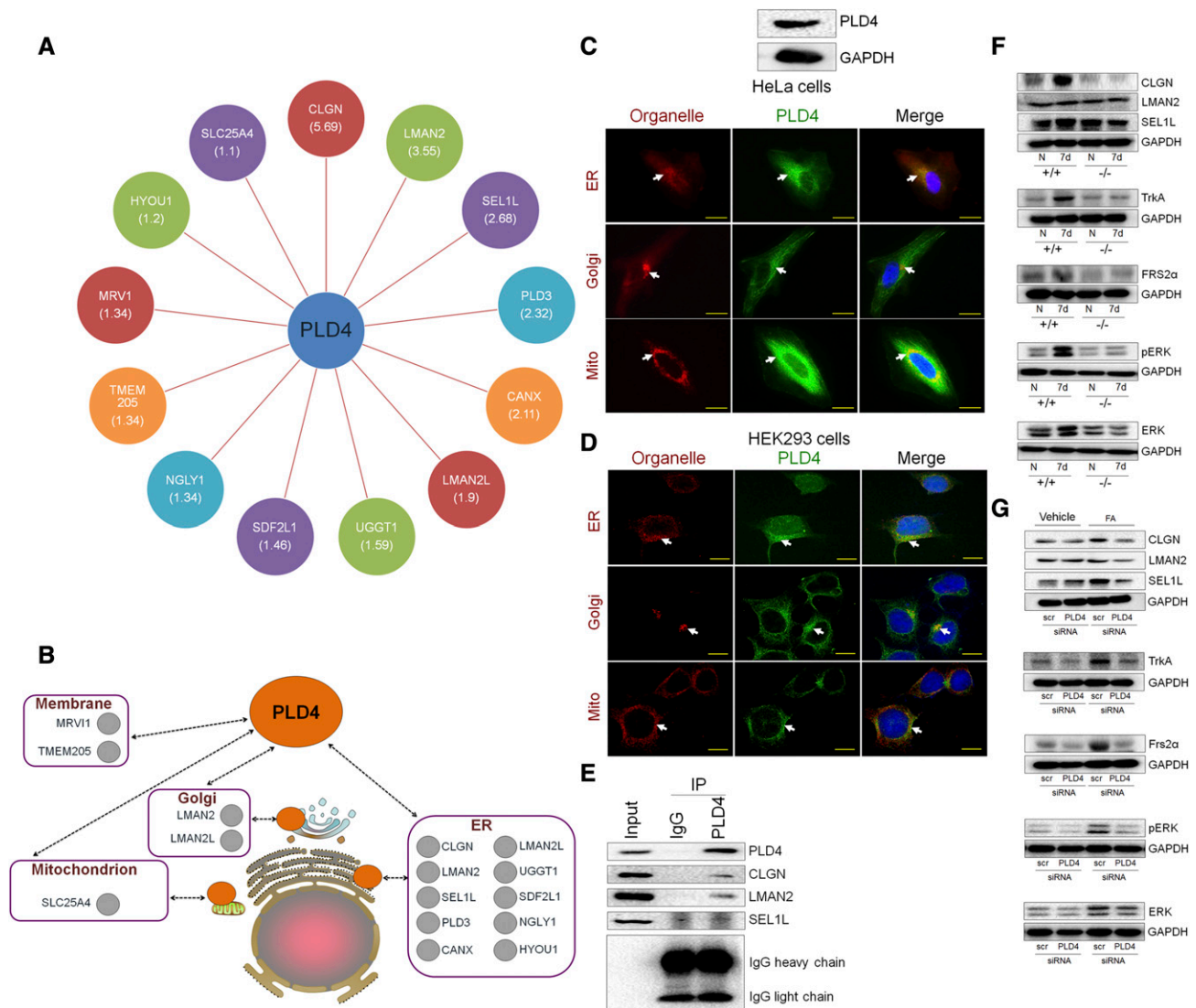


Figure 5. PLD4 upregulates MAPK signaling via TrkA pathway to mediate fibrogenesis. (A) Overview of the PLD4 interaction network on the basis of the immunoprecipitation/mass spectrometry (IP/MS) analysis (respective WDN scores indicated in parentheses). (B) Diagrammatic representation of the localization of interacting partners of PLD4. Orange circles represent PLD4 and gray circles represent the binding partners of PLD4. Immunofluorescence analysis of the subcellular localization of PLD4 by costaining PLD4 with ER (Calnexin)-, Golgi (Golga1)-, and mitochondria (Tom20)-specific proteins in (C) HeLa cells and (D) HEK293 cells. Scale bars, 20 μ m. (E) Coimmunoprecipitation of the top three interacting partners of PLD4 (CLGN, LMAN2, and SEL1L, selected on the basis of the WDN score) using PLD4-overexpressed HEK293T cells. Protein expression of CLGN, LMAN2, SEL1L, TrkA, FRS2 α , pERK, and ERK in the kidneys of (F) PLD4^{+/+} and PLD4^{-/-} mice and (G) siRNA (scr and PLD4)-treated mice injected with FA ($n=5$ /group). ER, endoplasmic reticulum; IP, immunoprecipitation; Mito, mitochondria; scr, scrambled; +/+, PLD4 wild-type; -/-, PLD4 knockout.

expression of NE, which potentially could be because of a significantly lower expression of AAT in the kidneys.

PLD4 interaction network and Co-IP/WB confirm that PLD4 binds with CLGN, LMAN2, and SEL1L and induces fibrosis via TrkA-mediated MAPK signaling pathway. TrkA, a common interactor of the binding partners of PLD4 (CLGN, LMAN2, and SEL1L), activates members of the MAPK pathway via FRS2 α , a signaling adapter that directly binds to the phosphotyrosine residue of TrkA.^{39,40} Moreover, TrkA expression is elevated in several nephropathies,

including diabetic nephropathy.⁴¹ The MAPK pathway plays a vital role in ECM synthesis *in vitro*⁴² and in progressing bleomycin-induced pulmonary fibrosis,⁴³ and UUO-induced kidney fibrosis *in vivo*.^{44,45} Downregulation of TrkA-mediated MAPK signaling with PLD4 inhibition depicts the role of PLD4 in inducing this pathway. However, which among CLGN, LMAN2, and SEL1L plays a critical role in inducing TrkA needs to be further explored. PLD4-induced AAT expression also might, at least in part, be playing a role in upregulating MAPK signaling, because AAT is known to stimulate fibroblast

proliferation and procollagen production *via* MAPK signaling pathway.⁴⁶ Together, our findings provide several novel insights to conclude that PLD4 regulates fibrogenesis in kidney. Moreover, overexpression of α -SMA, FN, Col 1 α 1, and PLD4 in CCl₄-induced fibrotic liver suggests that PLD4 could be playing a causal role in liver fibrosis as well making PLD4 a potential pan-fibrotic target.

In summary, these findings implicate that abrogation of PLD4 led to (1) increased antifibrotic cytokines, (2) downregulated TGF- β signaling, (3) increased NE due to decreased AAT level, and (4) downregulated MAPK signaling, and these effects rescued the mice from scar tissue formation. Our work not only establishes the functional significance of PLD4 overexpression in kidney fibrosis but also highlights the therapeutic potential of blocking PLD4 as a new approach to treat kidney fibrosis, an unmet medical need.

CONCISE METHODS

Animals

All animal maintenance and treatment protocols were approved by the Harvard Medical School Animal Care and Use Committees (Institutional Animal Care and Use Committees), and complied with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Experiments were performed on male C57BL/6J mice (8–10 weeks old) purchased from Charles River Laboratories. Global PLD4^{-/-} mice and PLD4^{fl/fl} with C57BL/6J background were obtained from Dr. David Nemazee at the Scripps Research Institute, La Jolla, California, and were then bred and maintained in our animal facility. SLC34a1-GFPCreERT2 mice were obtained from Dr. Benjamin D. Humphreys, Washington University School of Medicine, St. Louis, Missouri. Detailed methods are described in the Supplemental Methods.

Western Blot Analysis, Quantitative Real-Time PCR, and Histology

All of the techniques were performed using standard protocols established in the laboratory.^{47–49} Primary antibodies used are indicated in the Supplemental Material, and primer pairs used are listed in Supplemental Table 1.

Immunoprecipitation and Proteomic Analysis

Immunoprecipitation and proteomic analysis were performed as described previously^{25,50,51} (Supplemental Figure 9). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for the plasmid transfection of human embryonic kidney 293T (HEK293T) cells according to manufacturer's specifications. The cells were harvested 48 hours post-transfection for further analysis. Viral particles were generated in HEK293T cells through transfection with a pHAGE lentiviral vector, containing a C-terminal HA-Flag-tagged gene of interest and four helper vectors (VSVG, Tat1b, Mgpm2, and CMV-Rev). Virus-containing supernatants were used to infect HEK293T cells. Cells stably expressing the tagged proteins were selected with Puromycin (Invitrogen, Carlsbad, CA) for a week. For IP, cells (approximately 10⁷) were lysed in lysis buffer and rotated for 30 minutes at 4°C to obtain

whole-cell extracts. Lysates were cleared by centrifugation at 4°C for 15 minutes at maximum speed. Lysates were incubated with anti-Flag beads, and proteins were eluted with a Flag peptide. Proteins were then subjected to TCA precipitation and trypsinized before passage through StageTips. Samples were run in technical duplicate on a Thermo LTQ mass spectrometer, and spectra search was conducted with Sequest before target-decoy peptide filtering and linear discriminant analysis. Protein Assembler was used to convert spectral counts to average peptide spectral matches, which were uploaded into the CompPASS algorithm housed within the Core facility. For CompPASS analysis of HEK293T cells, a database of 48 baits was used. The CompPASS system identifies high-confidence candidate interacting proteins (HCIPs) on the basis of the WDN score, and proteins with WDN score >1.0 are considered HCIPs.

Library Preparation and RNA Sequencing

RNA samples ($n=4$ mice/group) were checked for quantity and quality (RIN value >8.0) using Agilent 2200 TapeStation instrument and by SYBR qPCR assay. Libraries were prepared using Illumina TruSeq Stranded mRNA sample preparation kits (San Diego, CA) from 1 μ g of purified total RNA according to the manufacturer's protocol. The finished dsDNA libraries were quantified by Qubit fluorometer, Agilent TapeStation 2200, and RT-qPCR was performed using the Kapa Biosystems library quantification kit (Wilmington, MA) according to the manufacturer's protocols. Uniquely indexed libraries were pooled in equimolar ratios and sequenced on a single Illumina NextSeq500 run with single-end 75-bp reads by the Dana-Farber Cancer Institute Molecular Biology Core Facilities. STAR aligner was used to map sequenced reads to the mm9 genome assembly and to quantify gene level expression. Testing for differential gene expression was performed using DESeq2. The full dataset is available in the National Center for Biotechnology Information Gene Expression Omnibus database with the accession number GSE87212.

Flow Cytometry

Mouse kidneys were digested as described previously,⁵² washed with FACS buffer, FC blocked, and stained for surface expression of CD11b (BV711; BD Biosciences, CA), Ly6C (FITC; Biolegend, CA), Ly6G (PE; Biolegend), CD3 (eFluor 450; eBioscience, CA), CD8 (APC-eFluor 780; eBioscience), CD4 (FITC; eBioscience), NK1.1 (PerCP-Cy 5.5; BD Biosciences), CD19 (PerCP-Cy 5.5; BD Biosciences), and MHC-II (I-A/I-E PE; Biolegend). Antibody concentrations used for staining were according to manufacturers' specifications. Gating for live cells was on the basis of staining the cells with DAPI. Data were acquired using a BD LSRII flow cytometer at the Harvard Medical School Systems Biology FACS Core Facility and were analyzed with FlowJo software (TreeStar).

Statistical Analyses

Data are expressed as mean \pm SEM. Statistical significance was calculated by nonparametric *t* test. For multiple comparisons, statistical difference was calculated by one-way ANOVA. *Post hoc* analysis was performed with Tukey's test when ANOVA showed significant differences. $P<0.05$ was considered statistically significant. Statistical

analysis was performed using GraphPad Prism (GraphPad, Inc., La Jolla, CA).

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DISCLOSURES

None.

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