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## Methylation determines fibroblast activation and fibrogenesis in the kidney

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### Abstract

Fibrogenesis is a pathological wound repair process that fails to cease, even when the initial insult has been removed. Fibroblasts are principal mediators of fibrosis, and fibroblasts from fibrotic tissues fail to return to their quiescent stage, including when cultured *in vitro*. In a search for underlying molecular mechanisms, we hypothesized that this perpetuation of fibrogenesis is caused by epigenetic modifications. We demonstrate here that hypermethylation of *RASAL1*, encoding an inhibitor of the Ras oncoprotein, is associated with the perpetuation of fibroblast activation and fibrogenesis in the kidney. *RASAL1* hypermethylation is mediated by the methyltransferase Dnmt1 in renal fibrogenesis, and kidney fibrosis is ameliorated in *Dnmt1*<sup>+/-</sup> heterozygous mice. These studies demonstrate that epigenetic modifications may provide a molecular basis for perpetuated fibroblast activation and fibrogenesis in the kidney.

Renal fibrosis is a pathologic scarring process<sup>1</sup>. A hallmark of fibrosis, as opposed to physiological wound healing, is that the active scarring does not cease once the initial insult has been contained and instead becomes a continuous process<sup>1,2</sup>. What perpetuates scarring in the setting of fibrosis is not yet known<sup>3</sup>. Activated fibroblasts are commonly considered main mediators of renal fibrosis<sup>3</sup>. The term ‘activated’ refers to the observation that fibroblasts associated with physiological repair or fibrosis show an increased proliferative activity, increased production of extracellular matrix constituents and expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)<sup>3,4</sup>. In the setting of fibrosis, such activation is so robust that primary fibroblasts isolated from fibrotic kidneys maintain their activated state even when

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cultured *in vitro*<sup>5,6</sup>. It has been hypothesized that such maintenance of the activated stage reflects the failure of the fibroblasts in the fibrotic kidney to return to their resting state as they do in physiological wound healing<sup>1,3</sup>. This phenomenon is observed not only in fibrotic kidney fibroblasts but also in activated fibroblasts isolated from any given fibrotic organ<sup>4,7-9</sup>. Here we hypothesized that epigenetic modifications are a molecular cause for fibrotic fibroblast activation and fibrosis.

## Results

### Identification of *RASAL1* hypermethylation in fibrotic fibroblasts

To test our hypothesis that an epigenetic modification, in particular hypermethylation, is involved in renal fibrogenesis, we treated CD1 mice that had been challenged with folic acid to induce fibrosis with the demethylating agent 5'-azacytidine<sup>10,11</sup>. Progressive fibrosis and kidney failure was significantly inhibited in the mice that received 5'-azacytidine from day 3 to day 28 after folic acid injection (Fig. 1a-c). Treatment with 5'-azacytidine significantly ameliorated accumulation of activated fibroblasts, as it decreased accumulation of fibroblast-specific protein-1 (FSP-1)-positive fibroblasts (Fig. 1d,e),  $\alpha$ -SMA+ myofibroblasts (Fig. 1d,f) and type I collagen in the folic acid-challenged kidneys (Fig. 1d,g). We next assessed the impact of 5'-azacytidine on fibrotic human kidney fibroblasts *in vitro*. In cell culture experiments, exposure to 5'-azacytidine normalized proliferative activity, type I collagen expression and  $\alpha$ -SMA expression of the fibrotic fibroblasts (Supplementary Fig. 1). These observations prompted us to study whether hypermethylation of specific genes in fibroblasts could be involved in both perpetuation of fibroblast activation and renal fibrogenesis.

Methylation of DNA occurs on cytosine residues that precede a guanosine in the DNA sequence (the CpG dinucleotide)<sup>12</sup>. Abnormal methylation of CpG clusters (CpG islands) in the promoter region (referred to as 'hypermethylation') often results in silencing of the gene<sup>13</sup>. In addition to the functional implications of gene inactivation in tumor development, these aberrant hypermethylation patterns also represent excellent genomic targets for diagnostic approaches<sup>13-17</sup>.

To identify candidate genes that are hypermethylated in fibrotic fibroblasts, we compared primary human fibroblasts from fibrotic kidneys with fibroblasts from nonfibrotic kidneys with a genome-wide methylation screen (sample characteristics are specified in Supplementary Table 1). This screen revealed 12 genes that were methylated in all seven tested fibrotic fibroblast samples and nonmethylated in all tested nonfibrotic fibroblast samples (Supplementary Table 2).

Among the identified genes, *RASAL1* stood out as a candidate to facilitate fibroblast activation and progression of renal fibrosis. *RASAL1* was one of three genes (out of the 12) that had similar CpG islands in both humans and mice (enabling us to study its role in mice) and that consistently showed decreased expression in fibrotic mouse kidneys (Supplementary Table 2).

Ras proteins in general are signal switch molecules that regulate cell fate by coupling receptor activation to downstream effector pathways that control diverse cellular responses, including proliferation<sup>18,19</sup>. *RASAL1* is a member of the RAS-GAP family, which catalyzes Ras inactivation by binding to GTP-Ras and catalyzing hydrolysis to GDP-Ras<sup>20</sup>. In cancer cells, growth factor-independent Ras hyperactivity causes stimulation of autonomous cell proliferation<sup>21,22</sup>. Such Ras hyperactivity is most often caused by gain of function mutations of Ras genes<sup>21,22</sup> but can also be caused by loss of Ras-GAPs, including *RASAL1* (refs. 23-25). We hypothesized that epigenetic silencing of *RASAL1* could have a mechanistic role in

fibrotic fibroblast activation. We confirmed by methylation-specific PCR (MSP) and bisulfite genomic sequencing (BGS) that *RASAL1* was hypermethylated in all tested fibrotic fibroblasts as compared to nonfibrotic fibroblasts (Fig. 2a,b and Supplementary Table 1). *In vitro* methylation significantly inhibited *RASAL1* promoter activity (Fig. 2c), and, in fibrotic fibroblasts, *RASAL1* methylation was associated with substantial repression of *RASAL1* expression (Fig. 2d).

### Renal fibrosis is associated with *Rasal1* methylation in mice

To gain insights into the role of *Rasal1* hypermethylation on the progression of renal fibrosis, we next investigated *Rasal1* methylation and *Rasal1* expression in fibrotic and nonfibrotic mouse kidneys. *Rasal1* expression was decreased in fibrotic kidneys of mice which had been challenged with folic acid (Fig. 2e–g). Using a strategy that combines immunoprecipitation of methylated DNA followed by analysis of the captured methylated DNA by *Rasal1*-specific PCR (MeDIP)<sup>26</sup>, we analyzed *Rasal1* methylation in total DNA samples from nonfibrotic control kidneys and from fibrotic kidneys. *Rasal1* was hypermethylated in all fibrotic kidneys but not in control kidneys (Fig. 2h), confirming that *Rasal1* methylation correlates with decreased *Rasal1* expression. We next performed immunofluorescence double-labeling experiments with antibodies to *Rasal1* and FSP-1 to assess *Rasal1* expression specifically by fibroblasts in the kidney (Fig. 2i,j). Whereas ~76% of FSP-1<sup>+</sup> fibroblasts in control kidneys were also positive for *Rasal1*, the percentage of FSP-1<sup>+</sup>*Rasal1*<sup>+</sup> fibroblasts among all FSP-1<sup>+</sup> fibroblasts was significantly lower in fibrotic kidneys (~12%), suggesting that fibroblast accumulation in renal fibrogenesis is associated with *Rasal1* silencing (Fig. 2i,j). To rule out the possibility that *Rasal1* hypermethylation is only relevant in the folic acid-induced nephropathy model, we also assessed *Rasal1* methylation in the model of nephrotoxic serum nephritis in C57BL/6 mice<sup>27,28</sup>. We established that fibrosis in this second model was similarly associated with *Rasal1* methylation and decreased *Rasal1* expression (Supplementary Fig. 2).

Given that fibrogenesis upon kidney injury correlates with *Rasal1* hypermethylation, we next elucidated whether absence of fibrosis upon kidney injury also correlates with absence of *Rasal1* methylation. First, we analyzed *Rasal1* methylation in the mouse model of ischemia-reperfusion injury, a model of physiological repair similar to wound healing<sup>29,30</sup>. Our rationale for using this model was that in this model an acute kidney injury is induced (by brief clamping of the renal pedicle), is spontaneously regenerated and, unlike the folic acid nephropathy and nephrotoxic serum nephritis models, does not lead to substantial long-term fibrotic lesions (Fig. 3a,b). MeDIP analysis revealed that *Rasal1* was not hypermethylated upon ischemia-reperfusion injury (Fig. 3c). Additionally, we performed secondary analysis on kidney samples with folic acid nephropathy and 5'-azacytidine treatment. Whereas *Rasal1* was hypermethylated in fibrotic kidneys of mice that had been challenged with folic acid, *Rasal1* was not hypermethylated in the kidneys with ameliorated fibrosis of mice who had received 5'-azacytidine in addition to folic acid (Fig. 3d).

### Hyperactive Ras contributes to fibroblast activation and fibrosis

We next explored the role of *Rasal1* in fibroblast activation *in vitro*. *Rasal1* was hypermethylated and *Rasal1* expression was lower in fibrotic mouse kidney fibroblasts compared to nonfibrotic fibroblasts (Fig. 4a,b), as we had observed in human renal fibroblasts. *Rasal1* knockdown lowered *Rasal1* expression in nonfibrotic fibroblasts to the level observed in fibrotic fibroblasts (Fig. 4b), correlating inversely with Ras activity (Fig. 4c). Such *Rasal1* knockdown phenocopied the intrinsic proliferative activity in serum-free medium that we observed in fibrotic fibroblasts (Fig. 4d), as well as in the increased type I collagen expression (Fig. 4e) and  $\alpha$ -SMA expression of fibrotic fibroblasts (Fig. 4f). We obtained similar results when we analyzed the role of *RASAL1* in the activation of human

fibroblasts (Supplementary Fig. 3). In summary, our data suggest that *RASAL1* methylation causes increased intrinsic proliferative activity, type I collagen expression and  $\alpha$ -SMA expression of fibrotic fibroblasts via Ras hyperactivity (Supplementary Fig. 4).

We next explored whether Ras hyperactivity also links *Rasal1* silencing to renal fibrogenesis *in vivo*. We induced fibrosis in CD1 mice with folic acid and gave them either DMSO (vehicle control) or the pan-Ras inhibitor *S*-trans, trans-farnesylthiosalicylic acid (FTS)<sup>18</sup> from day 90 (when fibrotic lesions are already established in this model) to day 150, when we killed the mice. Kidneys of FTS-treated mice showed significantly less fibrosis as compared to the untreated mice (Fig. 4g). FTS had a protective effect on kidney function (Fig. 4h), despite similar *Rasal1* methylation in both groups (Fig. 4i). Similarly, FTS administration substantially ameliorated fibrosis and preserved excretory renal function in mice that had been challenged with nephrotoxic serum (Supplementary Fig. 5)<sup>31</sup>.

### Dnmt1 is involved in *Rasal1* hypermethylation in fibrogenesis

We next aimed to gain insights into the mechanisms that underlie hypermethylation in renal fibrogenesis. A crucial step in DNA methylation involves DNA methyltransferases (Dnmts) that catalyze methylation of CpG dinucleotides in genomic DNA<sup>32,33</sup>. There are three known biologically active Dnmts: Dnmt1, Dnmt3a and Dnmt3b (no function has yet been found for Dnmt2)<sup>32,33</sup>. To gain insights into the possible involvement of Dnmts in renal fibrogenesis, we performed quantitative real-time PCR (qRT-PCR) on total RNA samples isolated from control mouse kidneys and from kidneys that were challenged with folic acid. In the folic acid-challenged kidneys, expression of Dnmt1, but not of Dnmt3a and Dnmt3b, was increased (Fig. 5a). Immunofluorescence double-labeling revealed more nuclear Dnmt1 labeling in kidneys of mice that had received folic acid as compared to control kidneys (Fig. 5b). Whereas most FSP-1<sup>+</sup> fibroblasts had Dnmt1<sup>+</sup> nuclei, Dnmt1 staining was not restricted to fibroblasts (Fig. 5b). Given these findings, we hypothesized that Dnmt1 has a role in hypermethylation of *RASAL1* and in renal fibrogenesis. We next performed chromatin immunoprecipitation (ChIP) assays to establish a link between increased Dnmt1 expression and *Rasal1* methylation. ChIP assay showed that Dnmt1 was bound to *Rasal1* DNA in folic acid-challenged kidneys, but not in control kidneys (Fig. 5c), suggesting that Dnmt1 is involved in methylation of *Rasal1*.

To gain further insights into the contribution of Dnmt1 to *Rasal1* methylation and progression of renal fibrogenesis, we challenged *Dnmt1*<sup>+/-</sup> heterozygous mice (in which Dnmt1 expression is decreased by 70% (ref. 34)) and wild-type control mice with folic acid (all on a C57BL/6 background). Renal fibrosis was reduced in *Dnmt1*<sup>+/-</sup> mice as compared to wild-type mice (Fig. 5d–f and Supplementary Fig. 6), correlating with ameliorated *Rasal1* methylation (Fig. 5g). Both fibrosis and *Rasal1* methylation were similarly reduced in *Dnmt1*<sup>+/-</sup> mice upon challenge with nephrotoxic serum (Supplementary Fig. 7).

### Two distinct mechanisms of transcriptional silencing

To gain additional insights into *Rasal1* expression and *Rasal1* methylation, we exposed primary mouse kidney fibroblasts to transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), which is known to induce fibroblast activation *in vitro*<sup>35–37</sup>. TGF- $\beta$ 1 caused decreased *Rasal1* mRNA expression within 8 h (Fig. 6a), whereas *Rasal1* methylation could be detected after 5 d of TGF- $\beta$ 1 incubation but not yet after 24 h (Fig. 6b). This suggested that TGF- $\beta$ 1 silences *Rasal1* expression via a dual mechanism—first via direct (and potentially reversible) transcriptional repression and then via methylation (potentially irreversible). To investigate this hypothesis, we next evaluated the return of *Rasal1* expression upon TGF- $\beta$ 1 removal after TGF- $\beta$ 1 incubation for 24 h (when *Rasal1* expression is suppressed but the *Rasal1* promoter is not yet methylated) and after 5 d (when the *Rasal1* promoter is methylated).

After 24-h TGF- $\beta$ 1 incubation, *Rasal1* expression returned almost to baseline levels when the TGF- $\beta$ 1 was removed, whereas *Rasal1* expression remained suppressed when TGF- $\beta$ 1 was removed after a 5-d TGF- $\beta$ 1 incubation (Fig. 6a). Thus, short-term exposure to TGF- $\beta$ 1 caused reversible suppression of *Rasal1* without methylation, whereas long-term exposure caused methylation of the *Rasal1* promoter, causing its irreversible transcriptional suppression, as is observed in fibrotic fibroblasts (Supplementary Fig. 8). These findings suggest that *RASAL1* suppression is a constituent of physiological (and reversible) TGF- $\beta$ 1-induced fibroblast activation and that methylation of the *RASAL1* promoter is a mechanism to perpetuate silencing of this common pathway, resulting in sustained fibroblast activation, which is typical of fibrogenesis. In this regard, we found that the transient fibroblast activation, which occurs during physiological repair after ischemia-reperfusion injury in the kidney (which involves short-term TGF- $\beta$ 1 increase), was associated with transient suppression of *Rasal1* expression without *Rasal1* methylation (Supplementary Fig. 9)<sup>38–40</sup>.

### Dnmt1 mediates *Rasal1* methylation in kidney fibroblasts

After establishing that TGF- $\beta$ 1 can induce *Rasal1* methylation within 5 d, we next used this system to elucidate a possible link between TGF- $\beta$ 1, Dnmt1 activation and *Rasal1* methylation. Comparison of serum-starved and TGF- $\beta$ 1-stimulated primary mouse kidney fibroblasts revealed that TGF- $\beta$ 1 induced Dnmt1 expression and caused nuclear translocation of Dnmt1 after 72 h, whereas we could not yet detect increased Dnmt1 expression after 24 h (Fig. 6c,d). To study the role of Dnmt1 in TGF- $\beta$ 1-induced *Rasal1* methylation, we isolated primary fibroblasts from kidneys of *Dnmt1*<sup>+/-</sup> heterozygous mice and from littermate control mice and exposed them to TGF- $\beta$ 1. Whereas exposure to TGF- $\beta$ 1 caused *Rasal1* methylation in wild-type fibroblasts, *Rasal1* methylation was ameliorated in TGF- $\beta$ 1-stimulated *Dnmt1*<sup>+/-</sup> fibroblasts (Fig. 6e). Dnmt1 knockdown with small interfering RNA similarly ameliorated *Rasal1* methylation in response to TGF- $\beta$ 1 (Fig. 6f), suggesting that *Rasal1* hypermethylation in response to the profibrotic growth factor TGF- $\beta$ 1 is dependent on Dnmt1.

To correlate the cell culture studies with progression of kidney fibrosis, we next explored whether active TGF- $\beta$ 1 signaling in injured kidneys is associated with increased Dnmt1 expression. We performed immunofluorescence double-labeling of nonfibrotic kidneys from control mice and kidneys of mice that had been challenged with folic acid using antibodies to phosphorylated Smad2 and Smad3, which transmit signals from TGF- $\beta$  receptors to the nucleus, and antibodies to Dnmt1. Whereas in control kidneys we did not detect phosphorylated Smad2 and Smad3 or Dnmt1, nuclear Dnmt1 immunolabeling in the fibrotic kidneys correlated with nuclear phosphorylated Smad2 and Smad3 staining (indicative of active TGF- $\beta$  signaling in these cells) (Fig. 6g), suggesting that TGF- $\beta$  induces Dnmt1 in the injured kidney similar to its effect *in vitro*.

## Discussion

Activated fibroblasts are considered principal mediators of fibrogenesis. Unlike in physiological wound repair, where fibroblast activation is spontaneously reversible, the fibroblast activation associated with fibrogenesis is perpetuated, and fibroblasts maintain their activated phenotype even when cultured *in vitro*. Here we found that the demethylating agent 5'-azacytidine normalizes the phenotype of fibrotic fibroblasts *in vitro* and ameliorates experimental kidney fibrosis in mice. Even though we are aware that the effect of 5'-azacytidine is not limited to fibroblasts, this led us to hypothesize that hypermethylation of specific genes in fibroblasts is pivotal in perpetuating fibrogenesis. To gain insights into possible genes that are affected by hypermethylation in fibrotic fibroblasts, we performed a genome-wide methylation screen and identified 12 genes that are selectively

hypermethylated in primary human fibrotic fibroblasts. Among these genes, we focused on *RASAL1*, an inhibitor of Ras. Our studies highlight commonalities between cancer progression and perpetuated fibrogenesis<sup>41</sup>. The capacity of fibrotic fibroblasts to proliferate in serum-free media until they reach senescence resembles the autonomous growth of cancer cells. Because, to our knowledge, mutated oncogenes cannot yet be linked to fibrogenesis, it is possible that commonalities between cancer and fibrosis are based on common epigenetic modifications. We provide evidence that epigenetic *RASAL1* silencing causes fibroblast activation and fibrogenesis, similar to cancer cells that are enabled to proliferate in a growth factor-independent manner upon *RASAL1* silencing<sup>24,25</sup>. Furthermore, it remains to be determined whether the activated phenotype observed in fibrotic fibroblasts and in cancer-associated fibroblasts share common underlying mechanisms. Our studies further highlight differences between fibrogenesis and physiological repair. Fibroblast activation is associated with transcriptional *RASAL1* repression in both the settings of physiological kidney repair and pathological fibrogenesis. However, whereas reversible fibroblast activation (typical of physiological repair) is associated with reversible *RASAL1* suppression without its hypermethylation, sustained fibroblast activation typical of fibrotic kidneys is associated with irreversible *RASAL1* expression due to hypermethylation of the *RASAL1* promoter. This suggests that hypermethylation perpetuates fibroblast activation and ultimately fibrogenesis by imprinting pathways of fibroblast activation that are engaged during the transient fibroblast activation typical of physiological repair. We further reveal that hypermethylation associated with fibrogenesis is mediated by the methyltransferase Dnmt1 and that pathological hypermethylation by Dnmt1 can be induced by long-term exposure to the profibrotic growth factor TGF- $\beta$ 1. Additional studies are required to gain further insights into the mechanisms that cause Dnmt1 to hypermethylate specific genes.

Although our studies demonstrated that the *RASAL1* hypermethylation correlates strongly with fibrogenesis and absence of *RASAL1* methylation correlates with physiological repair upon kidney injury, future studies manipulating *RASAL1* *in vivo* are needed to elucidate whether *RASAL1* silencing alone is sufficient to induce fibrogenesis in the kidney or whether it modulates the rate at which induced fibrosis progresses.

Our studies emphasize Ras hyperactivity as a potential therapeutic target for kidney fibrosis, confirming previous studies that suggested a prominent role of Ras signaling in renal fibrogenesis<sup>31</sup>. Previous studies have shown that increased Ras signaling within the fibrotic microenvironment is due to autocrine and paracrine growth factor stimulation. Our results show a new mechanism of epigenetic *RASAL1* silencing causing increased intrinsic Ras-GTPase activity in fibroblasts. These results suggest that the antifibrotic capacity of Ras inhibitors should be further explored.

To our knowledge, this is the first report of hypermethylation as a mechanism of perpetuating fibroblast activation and fibrogenesis. In summary, hypermethylation of specific genes contributes to fibrogenesis. Future studies are needed to elucidate the utility of methylated genes as diagnostic markers to predict fibrosis and the possible therapeutic efficacy of methylation inhibitors in progression of fibrogenesis.

## Methods

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

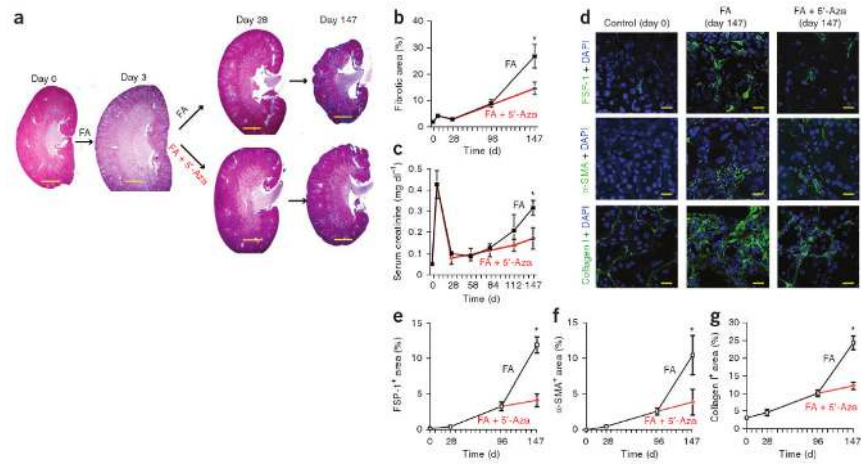
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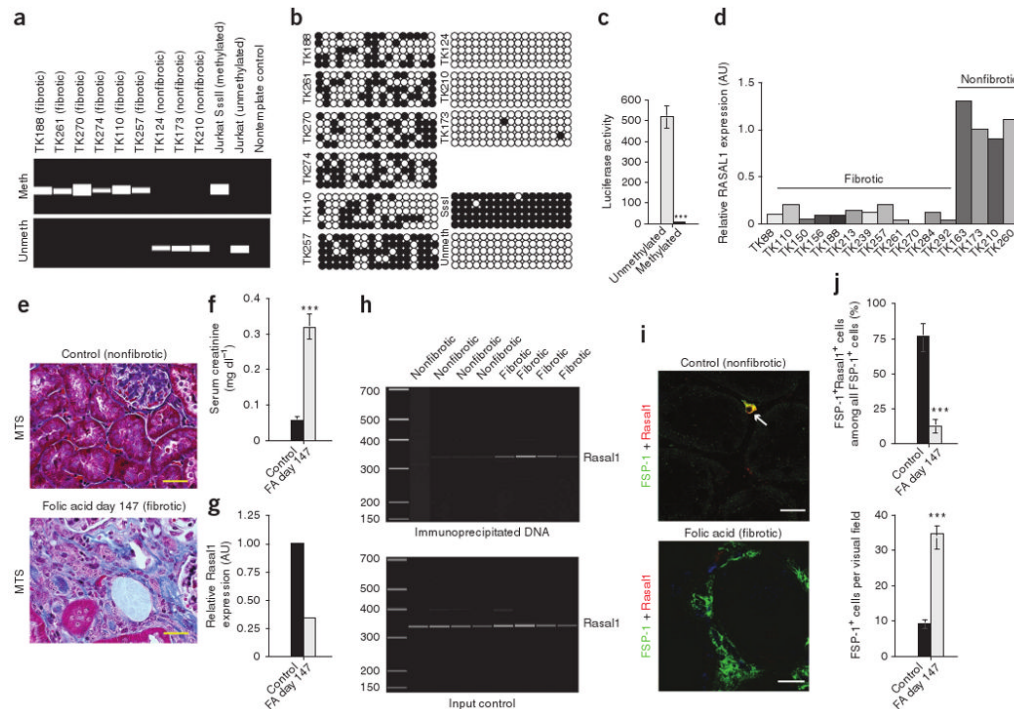
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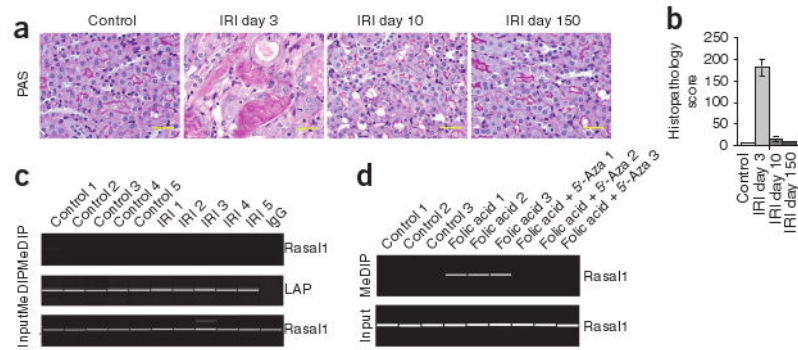
**Figure 1.**

5'-azacytidine ameliorates experimental renal fibrosis. **(a)** Representative photomicrographs of Masson's trichrome-stained kidney sections from mice that received folic acid (FA) (control) or folic acid and 5'-azacytidine (5'-Aza). Mice were analyzed at 0 d, 3 d, 28 d or 147 d after folic acid injection. Scale bar, 200  $\mu\text{m}$ . **(b)** Interstitial fibrotic area in Masson's trichrome-stained sections. The graph summarizes average values at the indicated time points of each group. **(c)** Average serum creatinine concentrations at the indicated time points. **(d)** Representative confocal photomicrographs of immunofluorescence labeling for FSP-1 (top),  $\alpha$ -SMA (middle) or type I collagen (bottom) on kidney sections of control CD1 mice (left column), kidneys of mice after folic acid injection (middle column) and from kidneys of mice that had received both folic acid and 5'-azacytidine (right column). Scale bar, 20  $\mu\text{m}$ . **(e-g)** Relative stained area in frozen sections that were labeled with antibodies to FSP-1 **(e)**,  $\alpha$ -SMA **(f)** or type I collagen **(g)**. Data are presented as means  $\pm$  s.e.m. \* $P < 0.05$ .



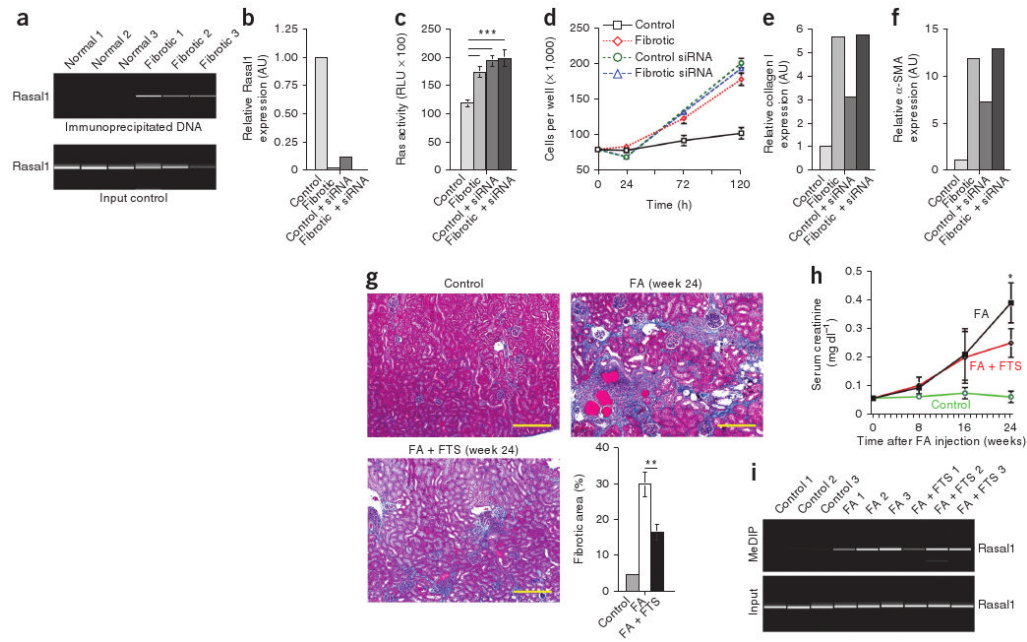
**Figure 2.**

*RASAL1* hypermethylation in fibrotic kidney fibroblasts. **(a)** Electrophoresis of PCR products using primers for methylated *RASAL1* (top) and unmethylated *RASAL1* (bottom). Primary fibroblasts TK188, TK261, TK270, TK274, TK110 and TK257 were isolated from fibrotic human kidneys; TK124, TK173 and TK210 were derived from nonfibrotic human kidneys. **(b)** BGS of the indicated cells. Each box is representative of the indicated cell culture; each row of dots in the boxes is representative of the *RASAL1* CpG island; each dot is representative of a single CpG. Empty dots indicate unmethylated CpGs; black dots indicate methylated CpGs. Each row represents a single sequenced clone (five for each cell line). We methylated DNA with the bacterial methyltransferase SssI as positive control. **(c)** Relative activity of pGL4-luciferase plasmid containing unmethylated or methylated *RASAL1* promoter. **(d)** Average *RASAL1* expression (by qRT-PCR) of each fibroblast culture (normalized to TK173 nonfibrotic fibroblasts set arbitrarily to 1). AU, arbitrary units. **(e)** Representative photomicrographs of Masson's trichrome–stained kidneys of control CD1 mice and of CD1 mice 5 months after folic acid injection. Scale bars, 20  $\mu$ m. **(f)** Average serum creatinine concentrations of control CD1 mice and of mice with renal fibrosis. **(g)** Relative *Rasal1* expression (qRT-PCR) in kidneys of control CD1 mice and of mice that had received folic acid. Expression in control mice was set to an arbitrary value of 1. **(h)** *Rasal1* methylation, as analyzed by MeDIP. The top picture shows a virtual gel of *Rasal1* PCR products of captured (methylated) DNA; the bottom picture shows *Rasal1* PCR products of input DNA (to control for equal loading in immunoprecipitation). **(i)** Immunofluorescence double-labeling with antibodies to *Rasal1* (red) and FSP-1 (green). The arrow highlights an FSP-1<sup>+</sup>*Rasal1*<sup>+</sup> fibroblast. Scale bars, 20  $\mu$ m. **(j)** The average number of FSP-1<sup>+</sup>*Rasal1*<sup>+</sup> cells among all FSP-1<sup>+</sup> fibroblasts per group (top) and the number of FSP-1<sup>+</sup> fibroblasts per visual field in each group (bottom). Data are presented as means  $\pm$  s.e.m. \*\*\**P* < 0.001.

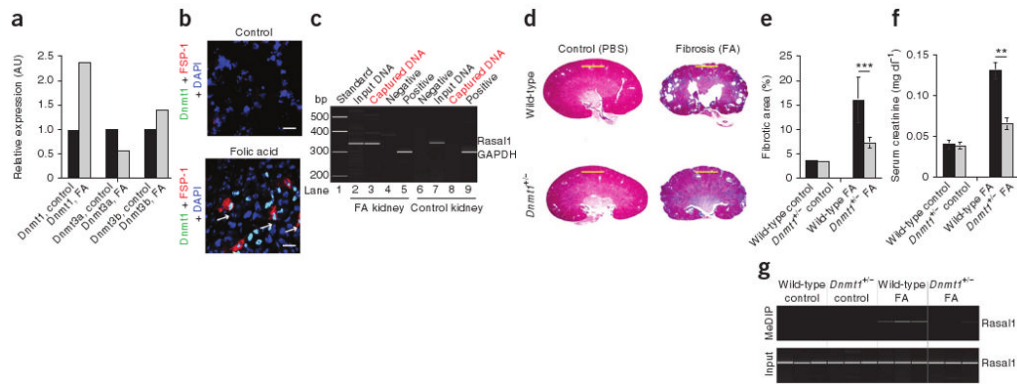


**Figure 3.**

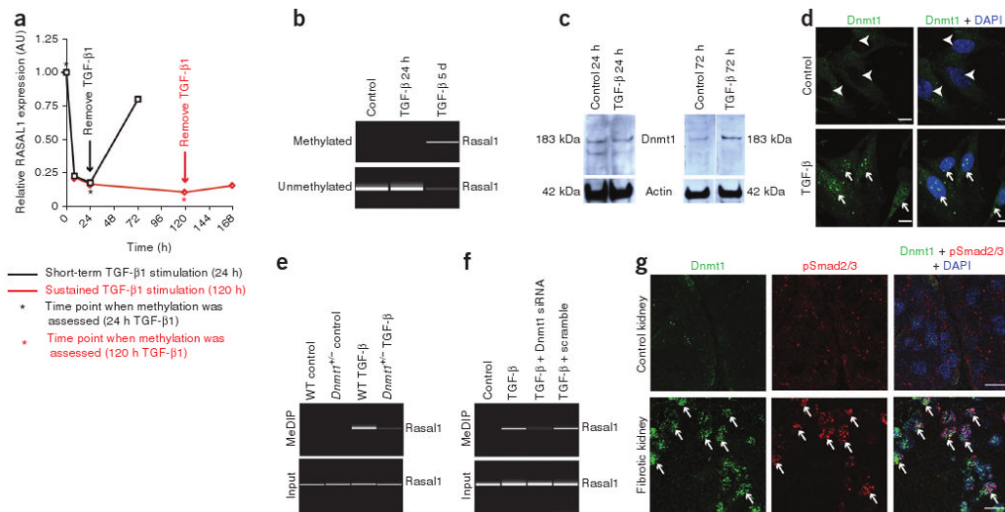
Absence of *Rasal1* hypermethylation in kidneys that do not become fibrotic upon injury. **(a)** Representative photomicrographs of periodic acid–Schiff–stained kidney before and 3, 10 and 150 d after initiation of ischemia-reperfusion injury (IRI). Scale bars, 20  $\mu$ m. **(b)** Average histopathological degree of tubular injury over time. **(c)** *Rasal1* methylation upon ischemia-reperfusion injury, as analyzed by MeDIP. Top, PCR analysis of immunoprecipitated methylated DNA with primers specific to *Rasal1*. Middle, leucine aminopeptidase (LAP) positive control. Bottom, input DNA control. **(d)** *Rasal1* methylation, as analyzed in control kidneys, in fibrotic kidneys of mice that had been challenged with folic acid and in kidneys of mice that had received folic acid and had been also treated with 5'-azacytidine. The picture shows a virtual gel of methylated DNA immunoprecipitation analysis. Data are presented as means  $\pm$  s.e.m.

**Figure 4.**

Rasal1 silencing causes renal fibroblast activation via Ras hyperactivity. **(a)** Methylation of *Rasal1* in primary fibroblasts isolated from fibrotic and nonfibrotic mouse kidneys, as analyzed by MeDIP. Top, sample analysis; bottom, input DNA control reaction. **(b)** Relative Rasal1 expression in control fibroblasts, fibrotic fibroblasts, control fibroblasts transfected with Rasal1-specific siRNA and fibrotic fibroblasts transfected with Rasal1-specific siRNA. **(c)** Ras activity of nonfibrotic control fibroblasts, nonfibrotic control fibroblasts transfected with Rasal1-specific siRNA, fibrotic fibroblasts and fibrotic fibroblasts transfected with Rasal1-specific siRNA after 3 d of culture in serum-free medium. **(d)** Average cell counts for nonfibrotic control fibroblasts, nonfibrotic control fibroblasts transfected with Rasal1-specific siRNA, fibrotic fibroblasts and fibrotic fibroblasts transfected with Rasal1-specific siRNA at the indicated time points. **(e,f)** Relative type I collagen and  $\alpha$ -SMA expression, respectively, in each group listed. **(g)** Representative photomicrographs of Masson's trichrome–stained kidneys of healthy CD1 mice (control), mice who had received a single injection of folic acid (24 weeks after folic acid injection) and mice who had received treatment with the Ras inhibitor FTS in addition to receiving folic acid. Scale bars, 100  $\mu$ m. The bar graph summarizes the relative fibrotic area in each group. **(h)** Average serum creatinine concentrations at the indicated time points in the indicated groups. **(i)** Kidneys of three representative mice in each group were analyzed by MeDIP; the top picture shows amplified methylated *Rasal1* DNA, and the bottom picture shows the control input DNA. For **b**, **e** and **f**, control values were arbitrarily set to 1. Data are presented as means  $\pm$  s.e.m. \*  $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**Figure 5.**

*Dnmts* in the mouse model of folic acid–induced nephropathy. **(a)** Relative expression of *Dnmt1*, *DNmt3a* and *Dnmt3b* in nonfibrotic and fibrotic mouse kidneys. Expression in FA-treated kidneys was normalized to kidneys from uninjected control mice, with each control value set arbitrarily to 1. **(b)** Representative photomicrographs of immunofluorescence labeling with antibodies specific to *Dnmt1* (green) and FSP-1 (red). The arrows highlight nuclear DNMT1 staining in FSP-1<sup>+</sup> fibroblasts in fibrotic kidneys (bottom). Scale bars, 20  $\mu$ m. **(c)** *Dnmt1* protein–*Rasaf1* promoter complexes were captured with *Dnmt1*-specific antibodies, detected by *Rasaf1* PCR and analyzed by electrophoresis. **(d)** Representative photomicrographs of Masson's trichrome–stained kidneys from wild-type control mice (top left), wild-type mice that had received folic acid (top right), *Dnmt1*<sup>+/-</sup> control mice (bottom left) and *Dnmt1*<sup>+/-</sup> mice that had received folic acid (bottom right). Scale bars, 200  $\mu$ m. **(e)** Average fibrotic area in each of the indicated groups. **(f)** Average serum creatinine concentrations in each of the indicated groups. **(g)** MeDIP analysis for *Rasaf1* (top) and the input control DNA (bottom) of DNA isolated from kidneys of each indicated group. Data are presented as means  $\pm$  s.e.m. \*\**P* < 0.01, \*\*\**P* < 0.001.

**Figure 6.**

TGF- $\beta$ 1–induced methylation of *Rasal1* in kidney fibroblasts is mediated by Dnmt1. (a) Relative Rasal1 expression over time. (b) *Rasal1* methylation in primary mouse kidney fibroblasts in response to TGF- $\beta$ 1 exposure for 24 h and 5 d, as indicated by the asterisks in panel a. We analyzed methylation by methylation-specific PCR. Top, a virtual gel of the PCR with primers specific for methylated Rasal1; bottom, the corresponding result when primers specific for nonmethylated Rasal1. (c) Immunoblot with antibodies specific for Dnmt1. (d) Representative photomicrographs of fibroblasts labeled with antibodies to Dnmt1 (green). Fibroblasts were maintained in serum-free medium (control) or exposed to TGF- $\beta$ 1 for 72 h. Arrowheads highlight Dnmt1<sup>-</sup> nuclei in control fibroblasts, and arrows point to Dnmt1<sup>+</sup> nuclei in TGF- $\beta$ 1–treated fibroblasts. (e) *Rasal1* methylation in response to exposure to TGF- $\beta$ 1 (for 5 d) in primary fibroblasts isolated from kidneys of wild-type (WT) C57BL/6 mice and from littermate *Dnmt1*<sup>+/-</sup> mice. Top, MeDIP sample analysis; bottom, input DNA control reaction. (f) *Rasal1* methylation in control fibroblasts, fibroblasts transfected with Dnmt1-specific siRNA, TGF- $\beta$ 1–stimulated fibroblasts and fibroblasts transfected with Dnmt1-specific siRNA and stimulated with TGF- $\beta$ 1 for 5 d. (g) Double-immunofluorescence labeling on control mouse kidneys (top) and on kidneys that were made fibrotic by a single injection of folic acid (bottom) using antibodies to Dnmt1 (green) and phosphorylated Smad2 and Smad3 (pSmad2/3, red). Arrows highlight nuclear pSmad2/3 colocalized with nuclear Dnmt1. Scale bars, 200  $\mu$ m.