miR-93 regulates Msk2-mediated chromatin remodelling in diabetic nephropathy

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How the kidney responds to the metabolic cues from the environment remains a central question in kidney research. This question is particularly relevant to the pathogenesis of diabetic nephropathy (DN) in which evidence suggests that metabolic events in podocytes regulate chromatin structure. Here, we show that miR-93 is a critical metabolic/epigenetic switch in the diabetic milieu linking the metabolic state to chromatin remodelling. Mice with inducible overexpression of a miR-93 transgene exclusively in podocytes exhibit significant improvements in key features of DN. We identify miR-93 as a regulator of nucleosomal dynamics in podocytes. miR-93 has a critical role in chromatin reorganization and progression of DN by modulating its target Msk2, a histone kinase, and its substrate H3S10. These findings implicate a central role for miR-93 in high glucose-induced chromatin remodelling in the kidney, and provide evidence for a previously unrecognized role for Msk2 as a target for DN therapy.
Diabetic nephropathy (DN) is a major microvascular complication of both type 1 and 2 diabetes, and the most common cause of end-stage kidney disease in the United States. Prolonged hyperglycaemia leads to chronic metabolic and haemodynamic changes that result in a myriad of genetic and epigenetic changes, which ultimately set the stage for the progression of DN. However, how metabolic responses in the cytoplasm lead to transcriptional and epigenetic changes in the nucleus in DN is not clear.

MicroRNAs (miRNAs) are short noncoding RNAs that generally function through suppression of their complementary target messenger RNAs (mRNAs) via formation of the effector ribonucleoprotein complex RNA-induced silencing complex (RISC). miRNAs are involved in numerous biological processes in the cell, and have emerged as potential targets in the treatment of a wide variety of disease states, including heart failure, cancer and diabetes. Studies have linked miRNAs to several kidney diseases, and an important kinase for Histone H3 Ser10 phosphorylation (H3S10P) is Msk2 (mitogen and stress-activated kinase-2; Rps6ka4). Msk2 is directly involved in nucleosomal remodelling by a select group of kinases, and its phosphorylation by serine/threonine kinases facilitates chromatin remodelling. The impact and influence of Msk2-mediated H3S10P in podocytes is in current study, we investigate the effect of miR-93 in DN using both genetic and pharmacological approaches, and explore possible mechanisms of how miR-93 can influence progression of DN. Importantly, we define a unique mechanistic role of miR-93 in DN, whereby metabolically regulated miR-93 serves as a metabolic/epigenetic switch in the regulation of chromatin states in podocytes in the diabetic milieu. In addition, we identify Msk2 (mitogen and stress-activated kinase-2; Rps6ka4) as a target of miR-93 and a novel target for DN therapy.

Msk2 is a member of the RSK (Ribosomal S6 Kinase) family of serine/threonine kinases, and an important kinase for Histone H3 Ser10 phosphorylation (H3S10P). H3S10 is phosphorylated by a select group of kinases, and its phosphorylation by Msk2 is directly involved in nucleosomal remodelling and global transcriptional activation upon exposure to mitogens and stress signals. Although H3S10P facilitates chromatin remodelling, the impact and influence of Msk2-mediated H3S10 phosphorylation on chromatin remodelling in podocytes, and whether Msk2/H3S10P contribute to the pathogenesis of DN, is mostly unexplored.

In the current study, we find that changes in miR-93 expression, through modulation of Msk2-dependent H3S10P, can lead to widespread changes in chromatin organization and gene transcription. Furthermore, we demonstrate that targeting Msk2 in vivo could provide a target for prevention of DN progression. Our results support a model in which miR-93 by targeting Msk2, a chromatin modifier, regulates a group of seemingly unrelated as well as functionally related genes, greatly amplifying its downstream effect in DN.

**Results**

**Generation of a podocyte-specific inducible miR-93 mouse model.** miR-93 is a signature miRNA under high glucose (HG) conditions, whose expression is reduced in several experimental models of DN. To elucidate the consequences of restoring miR-93 levels in in vivo, we generated a conditional and inducible mouse model of miR-93 overexpression (floxed miR-93Tg) (Fig. 1a; Supplementary Fig. 2a–c). To induce podocyte-specific overexpression of miR-93, we employed mice carrying a tamoxifen-inducible improved pharmacological delivery of miR-93 attenuates key features of DN. Pharmacological delivery of miR-93 mimics for in vivo use were injected intraperitoneally (i.p.) twice a week to diabetic db/db mice (Fig. 2k). Using confocal microscopy, we first tracked the presence of exogenously administered Dy547-labelled miR-93 mimics in vivo, where miR-93 mimics appeared to be predominantly localized in the tubules, and to some extent in glomeruli (Fig. 2l). We observed no significant changes in kidney histology or podocyte morphology compared to controls as assessed by Periodic-Acid Schiff’s (PAS) staining and transmission electron microscopy (TEM). (Fig. 1g). These mice displayed no functionally significant differences in albumin to creatinine ratio (ACR), body weight, blood pressure or fasting blood glucose compared to non-induced controls (Fig. 1b–k). These findings suggest that conditional overexpression of miR-93 in podocytes does not significantly alter kidney homeostasis or function in non-diabetic mice.

Pod-Cre-ERT2 mice were crossed with mTomato/mGFP reporter mice to further validate the podocyte-specific activity of our Cre mice. Treatment with tamoxifen-induced efficient recombination with a robust induction of miGFP expression in podocytes in Pod-Cre-ERT2 mice (Fig. 1b,c; Supplementary Fig. 2d). Crossing floxed miR-93Tg mice with Pod-Cre-ERT2 mice allowed for generation of the desired podocyte-specific miR-93 bigenic mice (Pod-Cre-ERT2/miR-93Tg mice, hereafter referred to as miR-93PodTg mice) (Fig. 1d,e). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of podocytes isolated from miR-93PodTg mice following tamoxifen induction revealed a physiologically relevant increase (~twofold) in miR-93 expression levels (Fig. 1f; Supplementary Fig. 2e,f).
increase in miR-93 levels in kidneys from miR-93 mimetic-treated mice as compared to kidneys from non-targeting (NT) controls (Fig. 2m). Several other organs (for example, liver and spleen) also exhibited robust increases in miR-93 levels compared with mice receiving NT mimics (Fig. 2m). To assess the therapeutic potential of miR-93 in DN, we injected miR-93 mimics or NT mimics to 8-week-old db/db mice twice per week for 8 weeks (Fig. 2k; Supplementary Fig. 3f–g). We found that pharmacological administration of miR-93 reduced proteinuria in diabetic mice (Fig. 2n). We observed significant rescue in several podocyte parameters, including a significant reduction in glomerular desmin staining, attenuated mesangial matrix expansion, increased WT1+ cells and increased synaptopodin, and nephrin levels in miR-93 mimic-treated mice compared to controls (Fig. 2o, p; Supplementary Fig. 3h–j). Thus, our findings suggest that pharmacological replacement of miR-93 may have therapeutic benefits in DN.

**miR-93 mediates chromatin dynamics in podocytes.** miR-93 does not have its own promoter and is localized in an intron of the *MCM7* gene (Fig. 3a). Since the levels of miR-93 and its host
gene, MCM7, in patients with DN are not known, we initially used publicly available datasets from Nephroseq\textsuperscript{19} to interrogate the expression level of MCM7 in patients with DN. We found that MCM7 mRNA was significantly reduced in patients with DN compared to control subjects (Fig. 3b). We also assessed miR-93 expression levels in human kidney biopsy samples from control subjects and in patients with DN (Supplementary Table 1), and found miR-93 expression was significantly reduced in patients with DN (∼50%) compared to control subjects (Fig. 3c).

We next interrogated the molecular mechanisms by which miR-93 exerts its protective effects in podocytes. Using standard miRNA target prediction tools, we had previously identified vascular endothelial growth factor (VEGF) as a target of miR-93 \textit{in vitro}\textsuperscript{10}, and thus we initially validated VEGF as a miR-93 target.

| miR-93 Levels | Nondiabetic | Diabetic |
|---------------|-------------|----------|
| db/db-miR-93<sup>35</sup>Ftg | ** | ns |
| db/db+miR-93 mimic | ** | ns |

![Image](image-url)

**Figure 3:** (a) Representative PAS images showing mesangial matrix expansion and GBM thickness in diabetic db/db mice. (b) Body weight and blood glucose levels in diabetic db/db mice treated with tamoxifen or vehicle. (c) Kidney function parameters in diabetic db/db mice treated with tamoxifen or vehicle. (d) Relative miR-93 levels in nondiabetic and diabetic db/db mice treated with tamoxifen or vehicle. (e) Glomerular sclerosis in diabetic db/db mice treated with tamoxifen or vehicle. (f) Inset image showing mesangial matrix expansion in diabetic db/db mice treated with tamoxifen or vehicle. (g) Representative immunohistochemical staining for Desmin in diabetic db/db mice treated with tamoxifen or vehicle. (h) Body weight and blood glucose levels in nondiabetic and diabetic db/db mice treated with tamoxifen or vehicle. (i) Kidney function parameters in nondiabetic and diabetic db/db mice treated with tamoxifen or vehicle. (j) Relative miR-93 levels in nondiabetic and diabetic db/db mice treated with tamoxifen or vehicle. (k) Glomerular sclerosis in nondiabetic and diabetic db/db mice treated with tamoxifen or vehicle. (l) Representative immunohistochemical staining for Desmin in nondiabetic and diabetic db/db mice treated with tamoxifen or vehicle. (m) Aorta and kidney function parameters in nondiabetic and diabetic db/db mice treated with tamoxifen or vehicle. (n) Relative miR-93 levels in nondiabetic and diabetic db/db mice treated with tamoxifen or vehicle. (o) Representative immunohistochemical staining for Desmin in nondiabetic and diabetic db/db mice treated with tamoxifen or vehicle. (p) Mesangial matrix expansion in nondiabetic and diabetic db/db mice treated with tamoxifen or vehicle.
in vivo (Supplementary Fig. 4). However, we were interested to use an unbiased approach to identify functionally important miR-93 targets that may be the key to unravelling its biological function in podocytes. Using RNA-Seq analysis, we explored genes differentially regulated by miR-93 overexpression in podocytes (Fig. 3d). The list of significantly downregulated genes was analyzed for the over-representation of any miRNA seed sequence motifs. This analysis confirmed that the miR-93 seed motif was enriched within the 3′-UTRs of significantly downregulated genes (Supplementary Fig. 5a–c). We then searched for differences in the expression of gene sets that may be altered by miR-93 overexpression using the gene set enrichment analysis (GSEA) database26. Unexpectedly, GSEA analysis revealed that miR-93 could mediate the expression of genes from several target gene sets implicated in chromatin remodelling (Fig. 3e,f, gene sets highlighted in red).

To gain insight into this previously unrecognized chromatin-modifying function of miR-93 in cultured podocytes in an unbiased manner, we utilized DNaseI hypersensitivity assays coupled with high-throughput sequencing (DNase-Seq) and ChIP-seq21–25. DNase-Seq has evolved into a powerful technique for identifying genome-wide DNase hypersensitive sites (DHS), which typically occur in nucleosome-free regions and near gene regulatory elements. We therefore assessed miR-93-modulated DNase1 hypersensitivity changes in podocytes cultured in HG conditions (Fig. 4a). Importantly, we identified a number of HG-related DHS sites, which were reversed upon miR-93 overexpression (Fig. 4b). In parallel, our DNase-Seq data revealed that miR-93 overexpression reversed HG-induced changes in hypersensitive sites at the transcription start sites (TSS) of several genes critical in the development of DN, including Fln1 (fibronectin), Ctgf (connective tissue growth factor), Serpine1 (serpin peptide inhibitor member 1), Rock1 (rho-associated, coiled-coil containing protein kinase 1) and Wt1 (Wilms’ tumour protein 1) (Fig. 4c–g).26,27 Gain or loss of hypersensitivity upon miR-93 overexpression at these genes coincided with differential patterns of mRNA expression, suggesting changes in chromatin structure were functionally related to changes in gene expression (Fig. 4h; Supplementary Fig. 6). While we observed a number of specific changes in DHS scores at several loci, the location of these sites, as measured by read distribution, was not significantly altered (Supplementary Fig. 7a). This suggests that miR-93 could alter the state of existing DHS sites, but did not necessarily lead to the creation of new sites. Finally, to test whether genes near DHS sites that experienced changes in hypersensitivity were functionally related, we performed GSEA analysis and found miR-93-induced changes in hypersensitive sites significantly affected pathways important for global regulation of transcription, consistent with the predicted role of miR-93 in chromatin remodelling (Supplementary Fig. 7b). Taken together, these data suggest that one salient but unexpected feature of miR-93 overexpression is its potential to reverse HG-induced changes in chromatin structure and global gene transcription.

In order to tease out the specific downstream target of miR-93 that could mediate this effect, we analyzed the RNA-Seq results and assessed several potential targets of miR-93 with the ability to mediate chromatin remodelling. Among several potential candidates, we chose to test the contribution of the mitogen and stress-activated kinase 2 (Msk2) gene as a differentially regulated target gene (Fig. 4i). Msk2 has previously been shown to regulate serine 10-phosphorylation of histone H3 (H3S10P), an epigenetic mark with an important role in global gene regulation and chromatin dynamics.11,28 To assess the relevance of Msk2 in DN, we interrogated public datasets in patients with DN, which revealed a significant increase in MSK2 mRNA expression in DN patients compared to controls (Fig. 4k). Importantly, elevated MSK2 expression was correlated significantly with reduced glomerular filtration rate in the same patients (Fig. 4l). Consistent with our selection, we observed a reduction in RNA tag density at Msk2 in miR-93 mimic transfected podocytes compared to controls (Fig. 4i; Supplementary Fig. 5d), suggesting that high levels of miR-93 are indeed linked to lower Msk2 mRNA levels (Fig. 4j). In addition, podocytes from 24-week-old db/db mice exhibited a significant increase in Msk2 protein expression compared with podocytes obtained from db/m mice (Fig. 4m). Similarly, HG-cultured podocytes demonstrated increased Msk2 expression, compared to NG controls (Fig. 4n,o). Taken together, these findings strongly suggest that Msk2 expression is elevated in experimental models of DN.

miR-93 mediates Msk2-regulated chromatin remodelling. We identified a single highly evolutionarily conserved miR-93 binding site within the 3′-UTR of the mouse Msk2 gene, suggesting Msk2 was a bona fide target of miR-93 (Fig. 4p; Supplementary Fig. 5f, left panel). To verify this, we executed several experiments in parallel: first, we performed RNA-IP against endogenous argonaute in cultured podocytes transfected with miR-93 mimics, anti-miR-93 mimics or respective NT controls. Msk2 mRNA was significantly enriched in RISC complex upon overexpression of miR-93 compared with controls. Inhibition of miR-93 attenuated the incorporation of Msk2 mRNA into RISC complexes (Fig. 5a). Second, we introduced the fulllength Msk2 3′-UTR into a luciferase expression vector (Supplementary Fig. 5f), and found that overexpression of miR-93 attenuated reporter activity compared to NT controls. Mutation of the putative miR-93 binding site in the 3′-UTR of Msk2 resulted in decreased luciferase activity, indicating that this site is indeed targeted by miR-93.

Figure 2 | miR-93 overexpression attenuates features associated with DN. (a) Representative images of db/db-miR-93PodTg mice and kidneys from control and tamoxifen-injected groups at 24 weeks of age. (b–d) Phenotyping of non-diabetic miR-93PodTg from control (n = 7) and tamoxifen-injected mice (n = 6) and diabetic miR-93PodTg mice from control (n = 11) and tamoxifen-injected (n = 14) (b) body weight, (c) blood glucose and (d) kidney weight/body weight (KW/BW) ratio. (e) ACR of non-diabetic miR-93PodTg from control (n = 7) and tamoxifen-injected mice (n = 6) and diabetic miR-93PodTg mice from control (n = 11) and tamoxifen-injected (n = 14). (f) The 24-h albumin excretion rate (AER) from mice as in (e). (g) Representative images of PAS staining (first row); desmin staining (second row); TEM micrographs (third and fourth rows with magnified inset) and SEM micrographs (fifth row). Red asterisks on TEM and SEM micrographs denote effaced podocyte foot processes. Scale bars denote 50 μM (first row); 200 μM (second row); 0.5 μM (third row); and 1 μM (fourth row). (h) Quantification of mesangial matrix expansion. (i) Glomerular area positive for desmin staining, and (j) GBM thickness in non-diabetic and diabetic miR-93PodTg mice from control and tamoxifen-injected groups. (n = 4–7 mice/group). (k) miRNA mimics injection protocol. (l) Fluorescent micrographs of kidney sections from vehicle-injected and 3′-Dy547-miR-93 mimic-injected mice. White arrows specify tubular localization and yellow arrows specify glomerular localization. Scale bars, 50 μM. (m) qRT-PCR analysis of liver, spleen and kidney RNAs from NT mimic and miR-93 mimic-injected mice. All values were normalized to U6 snRNA internal controls (n = 3 mice/group). (n) ACR measurements at 16 weeks of age in listed groups (n = 8 mice/group). (o) Top, representative PAS staining of kidney sections and bottom, representative desmin staining, respectively of kidney sections from each group. Scale bars denote 50 μM and 200 μM, respectively. (p) Quantification of mesangial matrix index and glomerular area positive for desmin staining for each group (n = 8 mice/group). Data expressed as mean ± s.e.m. ns: no significance, *P<0.05, **P<0.01. one-way analysis of variance with Tukey’s post test for multiple comparisons was used for groups of three or more.
Figure 3 | RNA-seq analysis implicates miR-93 in chromatin remodelling. (a) Top, human miR-93 localized to intron 13 of the MCM7 gene on chromosome 7. Bottom, sequence conservation of the mature miR-93 sequence in several species. Hsa: Homo Sapiens, Mmu: Mus musculus, Rno: Rattus norvegicus, Dre: Danio rerio, Bta: Bos taurus, Rhe: Rhesus (b) MCM7 expression values in patients with DN for two independent gene array probes obtained from publicly available datasets from Nephroseq (www.nephroseq.org)19. Data expressed as log2 median-centered intensity values. (c) miR-93 expression level in biopsy samples from control (n = 8) and DN patients (n = 7). (d) Left, experimental approach employed to generate transcriptome-wide RNA-Seq data. Right, heatmap of differentially regulated genes in NT and miR-93 mimic transfected podocytes. Heatmaps represents hierarchical clustering of the genes, which were significantly differentially regulated (log2 FC \( \pm 1 \)) (e, f) GSEA analysis of (e) significantly upregulated and (f) significantly downregulated genes upon miR-93 overexpression in mouse podocytes (k/k indicates the number of miR-93 responsive genes over the number of genes in a given gene set). Red highlighted gene sets suggest that miR-93 may play a role in modulating expression of genes regulated by various chromatin remodelers. Data expressed as mean \( \pm \) s.e.m. ns: no significance, *P < 0.05, **P < 0.01, ***P < 0.001. Student’s t-test was employed for comparisons between two groups. Cuffdiff2 was used to identify features differentially expressed between conditions for RNA-Seq analysis. A 0.05 false discovery rate was used in selecting significant genes. To further define differentially regulated genes, a cutoff of \(- \log_{10} (P \text{ value}) > 1\) and a log2 fold change (FC) of \(< 0.5\) or \(> 0.5\) was employed.
Knockdown of Msk2 expression was sufficient to recapitulate the effect of miR-93 on H3S10P. We transfected podocytes with siRNA against Msk2 or a scramble siRNA control. Western blot of HG-treated podocytes revealed that siRNA knockdown of Msk2 expression resulted in reduced H3S10P in HG conditions, whereas no striking differences were observed in HG-treated podocytes transfected with a scramble siRNA (Fig. 5g). To assess a link between miR-93 expression and Msk2 in vivo, we used our db/db; miR-93PodTg mice. We observed that both Msk2 and H3S10P protein levels in glomeruli were reduced in tamoxifen-induced diabetic mice compared to non-tamoxifen-induced diabetic mice (Fig. 5h, I; Supplementary Fig. 5h). In addition, we observed that db/db mice treated with miR-93-mimics exhibited a significant reduction in glomerular Msk2 and H3S10P levels compared to controls (Fig. 5j, k).

Targeting of Msk2 in vivo prevents progression of DN. While we have established that Msk2 mediates chromatin remodelling.
downstream of miR-93, whether targeting Msk2 would be sufficient to prevent the progression of DN in vivo remained unknown. To address this, we utilized shRNA plasmids directed against Msk2 coupled with a recently described kidney-specific nanoparticle-based liposomal reagent delivery system. We confirmed knockdown of Msk2 in the podocytes of diabetic mice (Fig. 6a). While we observed no differences in body weight or blood glucose, shMsk2 treatment led to significantly attenuated albuminuria in diabetic (db/db) mice compared to control littermates (Fig. 6b; Supplementary Fig. 9a,b). TEM analysis revealed a significant reduction in GBM thickening and podocyte foot process effacement in shMsk2-treated diabetic mice compared to controls (Fig. 6c–f). shMsk2 treatment also prevented podocyte loss (Fig. 6c,e), and resulted in a modest, but significant reduction in mesangial matrix expansion (Fig. 6c,d), that confirmed that H3S10P was reduced in podocytes from shMsk2-treated mice (Fig. 6i). We also found that podocin, nephrin and Wt1 mRNA levels were restored in shMsk2-treated diabetic mice (Fig. 6j; Supplementary Fig. 9c,d). To test if Msk2 depletion by a second method was similarly renoprotective, we employed locked nucleic acids (LNA)-Gapmers directed against Msk2 mRNA (Fig. 6k). We tested the Msk2 gapmer in vitro and observed reduced Msk2 protein expression in podocytes, with no effect on Msk1 expression (Fig. 6l, top panel). To further test the effect of Msk2 gapmer in DN in vivo, we employed a similar protocol to our miR-93 mimic injection experiments, wherein 8-week-old db/db mice were treated i.p. with either Msk2 or a scrambled sequence control gapmer twice a week for 8 weeks (Fig. 6l, bottom panel). Immunoblot analysis of kidney cortices from Msk2 gapmer administered mice confirmed reduced Msk2 protein expression compared to mice administered scramble gapmer (Fig. 6m). Mice receiving either Msk2 or control gapmer did not exhibit differences in blood glucose (Supplementary Fig. 9c–h), however we observed significantly reduced albuminuria in db/db mice receiving the Msk2 gapmer compared with db/db mice receiving the scramble gapmer (Fig. 6n). Histological analysis revealed significantly attenuated mesangial matrix expansion and restored podocyte numbers in Msk2 gapmer-treated mice (Supplementary Fig. 9i–k). Ultrastructure analysis revealed that Msk2 inhibition led to reduced podocyte foot process effacement, reduced foot process width, reduced GBM thickening and increased foot process density (Supplementary Fig. 9l–n). Msk2 gapmer inhibition led to a modest reversal in the expression of several transcript levels related to DN (Supplementary Fig. 9o). Last, while Msk2 knockout mice do not exhibit significant phenotypic changes (Supplementary Fig. 9p–r), diabetic Msk2 knockout mice showed a significant reduction in albuminuria compared to controls (Fig. 6o). Taken together, these data uncover a potential novel contribution of the histone kinase, Msk2, to the pathogenesis of DN.

Discussion

The molecular mechanisms linking metabolic changes in the cytoplasm to chromatin reorganization in the kidney remain mostly unexplored. In the current study, we elucidated a signalling pathway, whereby metabolically regulated miR-93 has an important role in translating metabolic cues into chromatin reorganization in DN. We first showed that conditional over-expression of miR-93 leads to significant improvements in biochemical and histological features of DN, and pharmacological restoration of miR-93 may have therapeutic implications in DN. Mechanistically, we showed that miR-93 exerts an unexpected role on nucleosomal remodelling via Msk2 and H3S10P in the diabetic milieu. We suggest a model, in which miR-93 serves as a critical metabolic-epigenetic switch in the diabetic environment, linking the metabolic state in the cytoplasm to chromatin reorganization in the nucleus though its target Msk2. In addition, we identified Msk2 as a novel target of miR-93 in DN. Using LNA-modified gapmer oligonucleotides and a kidney-targeted shRNA system, we found that Msk2 mRNA knockdown leads to significant improvements in key features of DN. Taken together, this study not only unravelled a previously unrecognized miR-93/Msk2/H3S10P signalling pathway, but also helps to introduce and lay the groundwork for future investigation into the role of Msk2 as a novel target for further drug development in patients with DN.

Links between metabolism and nucleosomal remodelling are not unexpected. However, less is known regarding the role of miRNAs in relaying metabolic signalling to alterations in chromatin. In this study, we report a central role of miR-93 in remodelling of the chromatin structure using an unbiased approach. DNase-Seq has evolved into a powerful tool to interrogate global chromatin dynamics in steady state or pathological conditions. In this study, we employed DNase-Seq to assess the impact of miR-93 overexpression on DNase accessibility, and asked whether miR-93 overexpression could alter the chromatin state observed in HG conditions. Alterations of DNase-Seq hypersensitivity were identified at several candidate gene loci provided further evidence that levels of miR-93 are directly linked to the gene expression profiles of several genes critical for the pathogenesis of DN.

Our findings link levels of miR-93 to changes in chromatin structure via H3S10P. This regulatory effect seems to be achieved

Figure 4 | HG-induced chromatin remodelling is reversed by miR-93 overexpression. (a) Experimental approach employed for DNase-Seq. (b) Hierarchical clustering analysis of ∆DHS scores for NG, HG + NT mimic, and HG + miR-93 mimics. Dashed red lines surround regions that demonstrate a reversal in HG-induced changes in hypersensitivity, following miR-93 overexpression. P value < 0.05 for all comparisons. (c–g) Examples of chromatin accessibility that highlight differences in DNase hypersensitivity at the (c) Ctgf, (d) Fn1, (e) Serpine1 (f) Rock1 and (g) Wt1 loci. As reference, DHS signal tracks from the Encode project are depicted in pink. The experimental approach employed for DNase-Seq. The data track shows tag density from DNase-Seq assays from podocytes treated with NG, HG + NT mimics or HG + miR-93 mimics. (h) Gene expression analysis of the aforementioned genes. All values normalized to internal Gapdh controls. (i) Volcano plot highlighting differentially regulated genes by miR-93. (j) mRNA tag density at exons -13 to 13 and 3'UTR of murine Msk2 in podocytes transfected with miR-93 mimics or NT controls. (k) Msk2 expression values from patients with DN obtained from publicly available datasets from Nephroseq (nephrosseq.org). (l) Data expressed as log2, median-centered intensity values. (m) Linear regression analysis of relative Msk2 mRNA levels from patients in (k) correlated with estimated glomerular filtration rate values obtained from Nephroseq. (m) Top, western blot and densitometric quantification of total Msk2 expression levels in podocytes isolated from 24-week-old db/db and db/mice, (n = 5 mice/group). (n) Top, western blot of total Msk2 expression levels from whole cell lysates in podocytes cultured under NG or HG conditions. (o) Quantification of nuclear fluorescent intensity of Msk2 expression in podocytes cultured under NG or HG conditions. Scale bar, 10 μm. (p) Evolutionary conservation of the miR-93 seed site within the 3'UTR of Msk2. Mmu: Mus musculus, Hsa: Homo sapiens, Pte: Pan troglodytes, Mml: Macaca mulatta, Str: Spermophilus tridecemlineatus, Rno: Rattus norvegicus, Ssc: Sus scrofa, Bta: Bos taurus. Data expressed as mean ± s.e.m. ns: no significance, *P < 0.05, **P < 0.01, ***P < 0.001. Student’s t-test was employed for comparisons between two groups, one-way analysis of variance with Tukey’s post test for multiple comparisons was used for groups of three or more.
Figure 5 | miR-93 targets Msk2. (a) Msk2 RNA in RISC complexes from RNA-immunoprecipitation experiments in mouse podocytes transfected with miR-93 mimics or anti-miR-93. (b) Luciferase activity in HEK293T cells co-transfected with either 3.1-luc-Msk2 wild-type 3’-UTR (WT-UTR) or a 3.1-luc-Msk2 mutant 3’ UTR (Msk2 mut) and NT or miR-93 mimics, respectively. Luciferase activity levels were normalized to β-gal activities. (c) Western blot and corresponding densitometric analysis for total Msk2 expression in podocytes transfected with miR-93 mimics or anti-miR-93 inhibitors. (d) Western blot analysis from HG-cultured podocytes transfected with miR-93 mimic or a NT mimic with antibodies directed against Msk2, H3S10P, total H3 and actin. (e) Immunofluorescence against H3S10P and Msk2 in HG-cultured podocytes transfected with miR-93 mimic or a NT mimic. Scale bar, 50 μm. (f) Western blot analysis of cultured podocytes exposed to HG and transfected with miR-93 mimic or a NT mimic with bivalent antibodies against H3S10K14-P-Ac and total H3. (g) Top panel, western blot analysis of Msk2 protein levels in podocytes transfected with a NT siRNA or siRNA specific to Msk2. Bottom panel, western blot analysis of H3S10P and total H3 protein levels in podocytes transfected with siMsk2 or siScr in HG conditions. (h,i) Representative immunofluorescence micrographs of kidney sections from db/m (n = 3), control db/db.miR-93<sup>3’UTR</sup> (n = 4) and tamoxifen-induced db/db.miR-93<sup>3’UTR</sup> mice (n = 4) stained with anti-Synaptopodin and anti-Msk2 or anti-H3S10P antibodies. Sections were counterstained with DAPI. Sections were counterstained with DAPI (j,k) Representative immunofluorescence micrographs of kidney sections from db/m control (n = 2) and db/db mice allocated to miR-93 mimic (n = 3) or NT mimic (n = 3) injections with anti-synaptopodin, anti-Msk2 and/or anti-H3S10P antibodies. Merged images are meant to highlight podocyte-specific staining and insets are meant to highlight podocyte-specific localization of Msk2 or H3S10. Sections were counterstained with DAPI. h–k Scale bars, 50 μm. Data expressed as mean ± s.e.m. ns: no significance, *P < 0.05, **P < 0.01, ***P < 0.001. Student’s t-test was employed for comparisons between two groups; one-way analysis of variance with Tukey’s post test for multiple comparisons was used for groups of three or more.
through Msk2, which serves as a kinase to phosphorylate H3 at Ser10, among other targets. H3S10P has been shown to have an important role in gene activation following exposure to growth factors or stress signals. Importantly, Msk2 is the preferential kinase for H3S10 that integrates upstream signals from growth factor and stress signalling to elicit the gene activation response. One puzzling observation has been the discovery that H3S10P is also associated with both chromosome condensation and gene activation. One possible explanation for this dichotomy could be that the outcome of H3S10 phosphorylation depends on the type of stimulus and the cellular context.

This study provides additional insights into how miRNAs, by modifying chromatin structure, may greatly amplify their gene expression effects.
regulatory networks. A prevailing view based largely on *in vitro* studies is that miRNAs exert their effects through 'fine tuning' of numerous functionally related targets35. However, our findings provide a view of miRNAs wherein manipulation of a single miRNA, by modifying the chromatin signature, may have a dramatic impact on a disease-related chromatin signature via a broadly acting mechanism. Evidence from our podocyte-specific model of miR-93 overexpression in diabetic mice suggests the miR-93/Msk2/H3S10P cascade in podocytes is critical for progression of DN. However, we recognize that some of the renoprotective effects of targeting this pathway in DN may stem from modulation of chromatin dynamics in other cell types of the kidney, especially since miR-93 and Msk2 expression are both broadly distributed throughout the kidney10,34. These assumptions are not entirely surprising as there is considerable cross talk among different cell types of the kidney, and improvements in one cell type might positively impact neighbouring cell types as well45,46.

Understanding how miRNAs contribute to disease progression is important since miRNA-based therapies for several diseases are currently progressing through early-stage clinical trials37. Furthermore, identification of novel miRNA targets may provide unique therapeutic opportunities. Recent preclinical studies have established the role of targeting Msk2 in a variety of diseases38,48,49. In this current study, our findings linking global and kidney-specific targeting of Msk2 expression, establish Msk2 as a novel target in DN. Additional work is underway in our laboratory to elucidate the tissue-specific impact of targeting Msk2 in DN.

In conclusion, we propose a previously unrecognized role for miR-93 and Msk2/H3S10P as regulators of nucleosomal remodelling in the diabetic milieu. Furthermore, the findings of this study shed further light on the therapeutic promise of using miR-93 mimics in experimental models of DN, as well as highlight a novel target for therapy by identifying Msk2 as a central mediator of DN progression.

**Methods**

**Tissue culture.** Conditionally immortalized mouse podocytes were cultured on BD BioCoat Collagen I plates (BD Biosciences, NJ, USA) at 33 °C in the presence of 20 U ml⁻¹ mouse recombinant IFN-γ (Sigma, St Louis, MO, USA) to enhance expression of a thermosensitive antigen52. Mouse podocyte cell lines are routinely characterized in the lab based on morphology and gene expression patterns. Cells for all experiments reported herein were between passage 4–12. Cells were confirmed to be free of mycoplasma contamination. To induce differentiation, podocytes were maintained at 37 °C without IFN-γ for 10–12 days. Podocytes prepared for experiments involving high glucose (HG, 25 mM) conditions were serum deprived for 24h before addition of HG. Likewise, control cells were serum deprived and cultured with normal glucose, NG, (5 mM). Cell culture experiments were repeated at least three independent times.

**Animal work.** All animal studies were conducted according to the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and approval was obtained for all animal studies under the guidelines of the IACUC of The University of Texas MD Anderson Cancer Center. Transgenic, Pod-iCreER12 mice were previously generated in our laboratory16. To induce Cre recombination, 8-week-old mice with the appropriate genotypes were administered 80 mg kg⁻¹ tamoxifen via intraperitoneal injection for 10 days16. miR-93 premiRNA transgenic mice that express the membrane-targeted tandem dimer tomato (mTdTomato) before Cre-mediated excision and membrane-targeted GFP (mGFP) after excision were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Diabetic db/db mice and their control littermates, db/m, were also obtained from Jackson Laboratories (strain: BKS.Cg-Dock7mT/+ o Lep+/−, Bar Harbor, ME, USA). Genotyping for mice harbouring the db mutation in the Lepr gene was carried out using primers specified by Jackson Labs, followed by restriction enzyme digestion with Rsa1. Vegfhi10 and VegfΔ12/+ were kind gifts from Dr András Nagy (Lunenfeld Research Institute, Toronto, ON, Canada). Msk2 Knockout (KO) mice on the C57BL/6J background were a kind gift from Dr Simon Arthur (University of Dundee, Dundee, UK). Homozygous Msk2 KO mice were bred with heterozygous C57BLKsldb6mT/+ to eventually obtain db/mMsk2−/−. These mice were intercrossed with each other to obtain db/dbMsk2−/−. Tail DNA was used to genotype mice at the time of weaning using the REDExtract-n-Amp genotyping kit (Sigma Aldrich, St Louis, MO). All mice used in experiments were male. Ages of mice are reported in figure legends or denoted in figure panels. For experiments investigating kidney histology and function mice were 16–24 weeks old. No animals were excluded from the studies performed. All animals were maintained on a normal chow diet and housed in a room with a 12:12-h light/dark cycle and an ambient temperature of 22 °C.

**Transgenic mice overexpressing miR-93.** The 309-bp miR-93 precursor was PCR-amplified from genomic DNA isolated from mouse podocytes and introduced downstream of the GFP coding region using Xho1 to Not1 restriction sites into the LB2-FLIP-Ubic luridins vector. We cloned the expression cassette in an inverse, anti-sense orientation between two different pairs of loxP sites. The construct was designed so that Cre induction could be used to mediate the inversion of the GFP and pre-miR-93 cassette into a sense orientation39. Cre induction mediates an initial flipping, followed by deletion of selection markers, Puruvycin and Thy1.1, based on orientation and location of two sets of mutant loxP sites. The expression cassette is under the control of the Ubiquitin C (ubiC) promoter. Lentiviral particles for microinjection were generated by the Diabetes and Endocrinology Research Center (DERC) Vector Core at BCM (http://www.bcm.edu/research/advanced-technology-core-labs/lab-listing-genetically-engineered-mouse/diabetes-research/core-labs/gene-vector-core-site). Transgenic miR-93 (miR-93Tg) mice were generated by lentiviral transduction of preimplantation C57/B6l blastocysts. Floxed miR-93Tg mice bred in expected Mendelian frequencies and proportional male/female ratios. Transgenic mice were generated by the GeneCore at BCM (http: //www.bcm.edu/research/advanced-technology-core-labs/lab-listing-genetically-engineered-mouse/home.htm) by injection of preimplantation C57/B6l blastocysts with the LB2-FLIP-miR-93Tg lentivirus. Founder tail DNA was genotyped with primers to detect GFP, Puruvycin and Thy1.1 found in Supplementary Table 2. All progeny thereafter were genotyped using primers amplifying the GFP transgene region.

**Mimics, siRNA and target site reporter reagents.** Pre-miRNA precursor molecules and anti-miR oligos, ON-Target plus siRNA against mouse Msk2 and

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**Figure 6 | Targeting Msk2 in vivo prevents progression of DN.** (a) Left, representative western blot analysis for Msk2 levels in podocytes isolated from non-diabetic (db/m) (n = 4), db/db + shCtrl (n = 3), and db/db + shMsk2 (n = 5) mice treated with shMsk2. Right, densitometric quantification of western blots to quantify Msk2 levels. (b) ACR of non-diabetic (db/m), db/db + shCtrl (n = 3) and db/db + shMsk2 (n = 5). (c) Representative images of TEM micrographs, PAS staining and WT1 staining from shMsk2-treated mice. Scale bars, 0.5 μm; Msk2 or more. (d-f) Quantification of (d) mesangial matrix expansion, (e) WT1 + cells, (f) GBM thickness, (g) Foot process density and (h) Foot process width in shMsk2-treated mice and controls. (i) Representative immunofluorescence micrographs of kidney sections from shMsk2-treated mice and controls from a stained with anti-synaptopodin and anti-H3S10P. Sections were counterstained with DAPI. (j) qPCR analysis of candidate specific genes, WT1, podcin and nephrin from podocytes isolated from shMsk2-treated mice and controls. All expression values were normalized to Gapdh internal controls.

(k) Schematic describing mode of action and general design of LNA-modified long RNA gapmers (l) Top, western blot analysis of podocytes transfected with Msk2 or Scramble, negative control gapmers with antibodies directed against Msk2, Msk2 and actin. Bottom, study design used for the administration of Msk2 or Scramble, negative control gapmers in db/dm mice. (m) Western blot analysis of kidney cortices from db/m and db/db mice administered gapmers directed against Msk2 or Scramble, negative control gapmers, all values normalized to actin (n = 5 mice/group). (n) ACR of non-diabetic (db/m), db/dm mice administered gapmers directed against Msk2 or Scramble, negative control gapmers, all values normalized to actin (n = 5 mice/group). (o) ACR of 24-week old db/ m;Msk2−/+ (n = 3), db/m;Msk2−/− (n = 5), db/db;Msk2−/+ (n = 6), and db/db;Msk2−/− (n = 6) mice. Data expressed as mean ± s.e.m. ns: no significance, *P < 0.05, **P < 0.01, ***P < 0.001. One-way analysis of variance with Tukey’s post test for multiple comparisons was used for groups of three or more.
miR mimic and LNA-Gapmer injections. The mmu-miR-93 and non-targeting (Cel-67) in vivo miR mimics were obtained from Thermo Scientific. Lyophilized mimics were resuspended in sterile saline and were stored as aliquots in −80°C, before use and mixing with TurboFect in vivo transfection reagent (Life Technologies, Grand Island, NY, USA) for i.p. injections according to manufacturer’s instructions. Diabetic db/db mice were allocated in a randomized and blinded manner into miR-93 mimic or NT control-treated groups. The 8-week-old db/db mice were administered 100 μg of mature miR-93 mimic or a NT mimic via i.p. injection twice a week for 10 weeks. The mmu-Msk2 and scramble Gapmer oligos were custom designed by Exiqon Inc. Gapmer oligos were LNA modified with a phosphothioate backbone. Lyophilized gapmers were resuspended in sterile saline and were stored as aliquots in −80°C, before use for i.p. injections according to manufacturer’s instructions. db/m and db/db mice were administered from Jackson Labs, db/db mice were allocated in a randomized and blinded manner into Msk2 gapper or scramble Gapmer-treated groups. 8-week-old mice were administered 100 μg of Msk2 gapper or scramble gapmer via i.p. injection, twice a week for 8 weeks.

In vivo Msk2 knockdown. Kidney-specific knockdown of Msk2 was achieved by delivering Msk2 shRNA (mouse Rps6ka4, 29mer shRNA constructs in an untagged retrovector) or a scramble control shRNA construct purchased from OriGene (Rockville, MD) and followed by the kidney’s in vivo transfection reagent (Life Technologies, Grand Island, NY, USA). Overall, 50 μg of endotoxin-free plasmid DNA/mouse was co-injected to the reagent according to manufacturer’s guidelines immediately before injections. Eight-week-old diabetic (db/db) mice were divided into two groups, Msk2 shRNA or scramble control, and received injections once a week i.p., until week 16. Urine was collected at the end of the 16 weeks and mice were sacrificed for further histological and biochemical analysis.

Human kidney biopsy analysis. Clinical data from patients with DN at the time of renal biopsy are summarized in Supplementary Table 1. Human kidney biopsy samples were obtained from Dr Katalin Susztak (BioBank at the University of Pennsylvania, Perelman School of Medicine)27–30. Total cortex RNA was isolated formalin fixed, paraaffin embedded kidney sections were deparaffinized and dehydrated using Histo-Clear and a series of increasingly concentrated ethanol washes. Before deparaffinization, sections were further adhered to glass slides by incubating in a hybridization oven at 60 °C for 1 h. Sodium citrate (10 mM, pH 6.4) antigen retrieval using a microwave oven was performed (3 min high power, 17 min 20% power) then cooled in ice. Immunohistochemical staining was performed overnight at 4 °C. Sections were stained using appropriate antibodies overnight at 4 °C followed by secondary antibody incubation (1:100) for 1 h. ATP was performed using CASAVA 1.8 with no mismatches.
kindly provided by Dr Ralph Nicholas (Dartmouth Medical School, Hanover, NH, USA). The putative miR-93 binding site (CACUUU) in mouse Msk2 was mutated by oligonucleotide-directed PCR with primers listed in Supplementary Table 2. For experiments using 3.1-luc luciferase reporter constructs in vitro, 1.5 × 10^5 HEK293T cells were plated in 12-well plates. Overall, 1 μg of either 3.1-luc empty vector, 3.1-luc-Msk2-W3-3UTR or 3.1-luc-Msk2-Mut-3UTR and 50 ng of pSV β-gal control vector (Promega) and 50 nM of miR-93 mimics were transfected using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA). And 48 h after transfection, luciferase activity was measured using Steady-Glo Luciferase Assay System (Promega, Madison, WI, USA) on a FLUOstar Omega luminometer (BMG Labtech, Cary, NC, USA) as previously reported. Levels were normalized to β-gal internal controls.

**RNA immunoprecipitation.** Mouse podocytes were transfected with miR-93 mimics, anti-miR-93 mimics and non-targeting non-targeting controls. Cells were ultraviolet crosslinked 48 h after transfection and subjected to Argonaute immunoprecipitation with a Pan-Agonante antibody (2A8, 5 μg/Ip, Milipore) and RNA isolation was performed as described previously. Briefly, kidneys from VEGF-LacZ mice were collected under sterile conditions. Kidney capsules were removed and kidney cortices dissected and cut into small pieces (~1 mm). Kidney cortex pieces were collected using a roller bottle incubator (model 1,000; Robbins Scientific, Sunnyvale, CA, USA) at 37 °C, 5% CO2, 20% O2, 75% N2 for 72 h in Dulbecco’s modified Eagle’s medium, containing 10% fetal bovine serum, 1× anti-anti (Gibco-LifeTech), under either normal glucose (5 mM β-glucose) or high glucose conditions (25 mM β-glucose). Overall, 10 μM of a VEGF target site protector or NT target site protector were included into the cultured kidney pieces using 4 mM of the Endo-Porter delivery system (Gene Tools). After 72 h, kidney pieces were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde. X-gal staining (Millipore) was performed at 37 °C in 0.2% glutaraldehyde, 5 mM K3Fe[CN]6, 5 mM K4Fe[CN]6, and 2 mM MgCl2 as described previously. Kidney pieces were then post fixed in 4% paraformaldehyde followed by paraffin embedding. Paraffin sections were dewaxed and mounted onto glass slides for image acquisition. Images were obtained on Nikon Eclipse 50i Light microscope (Nikon Inc., Tokyo, Japan).

**Scanning electron microscopy.** Samples were treated with a fixative containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.5, overnight at 4 °C. The samples were then washed with 0.1 M cacodylate buffer, pH 7.3 for 3 × 10 min. The samples were then post fixed with 1% cacodylate buffered osmium tetroxide for 1 h, washed with 0.1 M cacodylate buffer for 3 × 10 min, then in distilled water, 2 × 5 min. The samples were then dehydrated with a graded series of increasing concentrations of ethanol for 5 min each. The samples were then transferred to graded series of increasing concentrations of hexamethyldisilazane (HMDS) for 5 min each and air dried overnight. Samples were mounted on to double-stick carbon tabs (Ted Pella, Inc., Redding, CA, USA), which have been previously mounted on to aluminum specimen mounts (Electron Microscopy Sciences, Ft. Washington, PA, USA). The samples were then coated under vacuum using a Balzer MED 010 evacuator (Technofraude, NH, Manchester, USA) with platinum alloy for a thickness of 25 mm, then immediately flash carbon coated under vacuum. The samples were transferred to a dericisor for examination at a later date. Samples were examined in a JSM-5910 scanning electron microscope (JEOL, USA, Inc., Peabody, MA, USA) at an accelerating voltage of 5 kV.

**Transmission electron microscopy.** Fixed samples were washed in 0.1 M cacodylate buffer, post fixed with 1% buffered osmium tetroxide for 1 h, and en bln etc with aqueous 1% Millipore-filtered uranyl acetate. The samples were washed several times in water, then dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in LX-112 medium. The samples were polymerized in a 60 °C oven for about 3 days. Ultrathin sections were cut in a Leica LKB ultratome III; Leica, Deerfield, IL. Sections were stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1011 transmission electron microscope (JEOL, USA, Inc., Peabody, MA, USA) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA, USA).

**Immunofluorescence.** Podocytes were grown on 35 mm BioCoat Collagen I coated coverslips (BD, San Jose, CA, USA). After differentiation and treatment as indicated, cells were washed twice with PBS and fixed in 4% paraformaldehyde (PFA) for 10 min at room temperature. Cells were washed three times with PBS and permeabilized with 0.2% Triton X-100. Cells were washed twice with PBS and blocked in 10% donkey serum for 30 min at room temperature. The following primary antibodies Msk2 (R&D Systems, MAB2310, 1/200), H3S10P (Cell Signaling, 9701), 1/200, H3Ip (Sigma), 1/200, Lamin (Santa Cruz Biotechnology, sc-7273), Nephrin (Fitzgerald, 20R-NP002, 1/100), WTI (Santa Cruz Biotechnology, sc-192, 1/200). Podocytes were incubated in primary antibody, overnight at 4 °C, and then washed three times with 1 × PBS. Fluorophore conjugated secondary antibodies to rat (1/200) or rabbit (1/200) were applied to their respective primary antibodies and incubated at room temperature for 1 h in the dark. Cells were washed three times with PBS and nuclei were counterstained with DAPI (Thermo Scientific) and mounted with ProLong Gold Antifade mounting reagent (LifeTech, Garland Island, NY, USA). Images were obtained using the Deltavision Deconvolution microscope (Applied Precision, LLC, Issaquah, WA, USA). For quantification purposes, NIH ImageJ version 1.62 was used, fluorescent intensities were measured on nuclei of cells with built in software. Kidneys for immunofluorescence analyses were drop-fixed in 4% PFA overnight at 4 °C. Kidneys were embedded in Tissue-Tek O.C.T. compound (Sakura FineTek, Torrance, CA, USA) and frozen on dry ice and OCT mountant. Sections from Podocin-mTom/mGFP mice were imaged directly. Sections for immunofluorescence were processed as described above. All kidney sections were counterstained with DAPI.

**RNA-Seq analysis.** The raw data quality was assessed using FastQC software. Adaptor presence was tested using Trimomatic. Reads from each sample were aligned to the NCBI mouse reference genome build 37.2 using TopHat2 v2.0.4. Transcript quantification, normalization and assembly were carried out using Cufflinks. Cuffdiff was used to identify features differentially expressed between conditions. A 0.05 false discovery rate was used in selecting significant genes. To further define differentially regulated genes, a cutoff of log2 (P value) > 1 and a log2 fold change (FC) < -0.5 or > 0.5 was employed. TargetScan Mouse Release 5.2 was then run on input lists to find predicted miR-93 binding sites. Distributions of the relative expression levels of all genes containing 7-mer and/or 8-mer seeds were evaluated to the effect of miR-93 overexpression on gene expression. Significantly differentially regulated genes were analyzed for motif enrichment using miRvestigator. miRNAs that exhibit the functional signatures of the significant miRNAs were further defined as miRNA targets.

**DNPase-Seq analysis.** DNPase-seq reads underwent strict quality control processing with the TrimGalore! package. After removing low-quality bases as well as adapter sequences. Reads that passed quality control were mapped to the mm10 version of the mouse genome using the Bowtie short read aligner. Duplicate reads were then filtered. Reads were shifted 50 nt downstream of their mapped location and the number of complete reads that mapped within 500 nt of each unique transcript start site as defined by ENSEMBL vGRCh38.73. The mouse transcriptome was used for hypersensitivity analysis. TSSs that had at least the equivalent of 10 reads in a single condition were considered to the conditions of interest. A cutoff of 10 reads was set for retained mapping. The number of DNPase-seq reads per TSSs were normalized across conditions to eliminate systematic differences by the DESeq algorithm. A pooled dispersion estimation was then done in DESeq2, followed by a negative binomial test to identify differences in enrichment of DNPase-seq reads across each of the three conditions. This analysis was carried out on the top 200 most differentially enriched TSSs in each of the conditions using the GSEA resources as before. Peaks were called using the MACS2 peak-calling algorithm using the parameters recommended by for DNPase-seq data. The input DNA was used as a control for each of the three samples. Differential DNPase-seq scores were calculated as described previously. Sites with positive value scores were considered more hypersensitive, sites with negative value scores were considered less hypersensitive.

**Nephroseq analysis.** Human MSK2 and MCM7 expression data were downloaded from the Woronecka et al. and Ju et al. datasets using the Nephroseq data mining platform (www.nephroseq.org). Statistical analysis. Group data are all expressed as mean ± s.e. Comparisons of multiple groups were performed using one-way analysis of variance (one-way ANOVA) followed by Tukey’s multiple comparisons test. Comparisons between two groups were performed using the student’s t test. All tests were two-tailed, with P values considered to be statistically significant. Tests were performed with GraphPad version 6.0b (GraphPad Software Inc. La Jolla, CA, USA). We employed algebraic expression data from db/db mice from previous experiments and published data to estimate the number of mice required to achieve 80% power, with a 0.05 significance level.

**Data availability.** High-throughput sequencing data that support the findings of this study have been deposited in GEO with primary accession code GSE68081. The data that support the findings of this study are available from the corresponding author upon request.

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Acknowledgements
This work was supported by NIH Grants RO1DK091310 and RO1DK078900 (F.R.D.), and T32GM088129 (S.S.B.). Work performed by the UT-MDACC high-resolution microscopy facility was supported by Institutional funds (Core Grant CA16672). High-throughput sequencing data was generated by the UT-MDACC Sequencing and Microarray Facility (Core Grant CA016672 SMF). Analysis of RNA-Seq data was performed by the UT-MDACC Bioinformatics and Computational Biology Shared Resource (Mary K. Chapman Foundation, Support Grant CA016672). Analysis of DNase-Seq data was performed by the Duke University Integrative Genomic Shared Resource (http://www.genome.duke.edu/cores/analysis). Blood pressure measurements were performed by the Baylor College of Medicine Mouse Phenotyping Core. Lentivirus for transgenic mice was generated by the Diabetes and Endocrinology Research Center (P30DK079638) at Baylor College of Medicine. Mouse embryo infections were performed by the Genetically Engineered Mouse Core at Baylor College of Medicine (http://www.bcm.edu/research/advanced-technology-core-labs/lab-listing/genetically-engineered-mouse/home.htm).

Author contributions
S.S.B., B.H.C., D.L.C., P.O. and F.R.D. designed experiments and analyzed data. S.S.B. and F.R.D wrote the manuscript. S.S.B., Y.W. and J.L. performed experiments and analyzed the data.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Badal, S.S. et al. miR-93 regulates Msk2-mediated chromatin remodelling in diabetic nephropathy. Nat. Commun. 7:12076 doi: 10.1038/ncomms12076 (2016).

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