Sirt1- A Guardian of the Development of Diabetic Retinopathy

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ABSTRACT

Diabetic retinopathy is a multifactorial disease, and the exact mechanism of its pathogenesis remains obscure. A multifunctional deacetylase Sirtuin 1 (Sirt1), is implicated in regulation of many cellular functions and transcription of genes, and retinal Sirt1 is inhibited in diabetes. Our aim is to determine the role of Sirt1 in the development of diabetic retinopathy, and elucidate the molecular mechanism of its downregulation. Using mice overexpressing Sirt1, diabetic for 8 month, structural, functional and metabolic abnormalities were investigated in vascular and neuronal retina. The role of epigenetics in Sirt1 transcriptional suppression was investigated in the retinal microvessels. Compared to wildtype diabetic mice, retinal vasculature from Sirt1 diabetic mice did not present any increase in the number of apoptotic cells, degenerative capillaries and decrease in vascular density. Sirt1 diabetic mice were also protected from mitochondrial damage, and they had normal ERG responses and ganglion cell layer thickness. Wildtype diabetic mice had Sirt1 promoter DNA hypermethylated, which was alleviated in Sirt1 diabetic mice, suggesting the role of epigenetics in its transcriptional suppression. Thus, strategies targeting amelioration of Sirt1 inhibition have potential to maintain retinal vascular and neuronal homeostasis, providing opportunities to retard the development of diabetic retinopathy in its early stages.
INTRODUCTION

Diabetic retinopathy remains the major cause of acquired blindness in working age adults, and high circulating glucose is considered as the major instigator of the deleterious functional, structural and metabolic changes (1-3). Chronic hyperglycemia increases oxidative stress, activates protein kinases and polyol pathways, and results in neuronal and vascular damage including loss of ganglion cells and formation of degenerative capillaries (1; 2; 4; 5), but the exact molecular mechanism of the development of diabetic retinopathy remains to be established.

Sirtuin 1 (Sirt1), a member of the silent information regulator 2 family, is a Class III histone deacetylase, which interacts with target proteins, and regulates many cellular functions including cell proliferation, apoptosis and inflammatory responses (6-8). Sirt1 is mainly a nuclear protein, and its activity depends on cellular NAD availability (9). It is expressed throughout the retina, and upregulation of Sirt1 is protective against various ocular diseases including retinal degeneration, cataract and optic neuritis (10). Our previous work has shown that its expression and activity are decreased in the retina and its capillary cells in diabetes (11). However, the direct role of Sirt1 in the development of diabetic retinopathy remains elusive.

Sirt1 also regulates gene transcription, and this is mediated either by altering the acetylation status of the transcription factor, or by regulating the epigenetic modifications at the transcriptional factor binding site of a gene (12). In the pathogenesis of diabetic retinopathy, inhibition of Sirt1 is implicated in the hyperacetylation and activation of nuclear transcription factor kB (NF-kB), and NF-kB plays a major role in the transcriptional activation of mitochondria-damaging matrix metalloproteinase-9 (MMP-9) (11; 13; 14). Sirt1 is also a redox-sensitive enzyme (15), and oxidative stress, besides regulating NAD levels, affect its activity by...
regulating posttranslational modifications and protein-protein interactions (16); in diabetes, regulation of oxidative stress prevents decrease in Sirt1 activity in the retinal vasculature (11). How diabetes regulates Sirt1 is, however, not clear.

The expression of a gene, along with its DNA sequence, is also regulated by epigenetic modifications (17; 18). Diabetes alters the epigenetic machinery (activated/inhibited) in the retina, and many genes considered to play important role in mitochondrial homeostasis are epigenetically modified (2; 3; 11; 19-21). We have shown that dynamic activation of DNA methylating-hydroxymethylating enzymes, DNA methyltransferases (Dnmts) and Ten eleven translocation enzymes (Tets) respectively, maintains DNA methylation status of retinal MMP-9 promoter to keep it transcriptionally active (19). The role of epigenetics in the regulation of Sirt1 in the pathogenesis of diabetic retinopathy, however, remains to be explored.

Sirt1 is a multifunctional protein implicated in a wide range of molecular/epigenetic pathways (6-8), and diabetic retinopathy is a multifactorial disease (1; 2); the goal of this study is to determine if regulation of Sirt1 ameliorates the development of diabetic retinopathy, and also, to elucidate the mechanism responsible for Sirt1 regulation. Using mice overexpressing Sirt1, we investigated its role in structural, functional and metabolic abnormalities that are critically associated with the development of diabetic retinopathy. The possible mechanism of Sirt1 transcriptional suppression is elucidated by investigating DNA methylation status of its promoter.
METHODS

Mice: Wild-type C57BL/6J (WT) and Sirt1 overexpressing (St, C57BL/6-Actbtm3.1 (Sirt1) Npa/J; Sirt1) mice (Jackson Laboratory, Bar Harbor, ME), ~20g BW (either sex), were made diabetic by streptozotocin injection (55 mg/kg BW for 4 consecutive days), and mice presenting >250 mg/dl blood glucose two days after the last injection were considered diabetic (19; 22). Age-matched normal WT and Sirt1 mice were used as their respective controls. Compared to WT normal mice, although Sirt1 expression was significantly increased in the retina of Sirt1 mice, kidney from same animals did not show any increase (Supplement Figure 1S). Mice were sacrificed ~8 months after induction of diabetes, and one eye was fixed in 10% buffered formalin and retina from the other eye was removed immediately for biochemical measurements. Glycated hemoglobin (GHb) was measured ~6 months of diabetes using a kit from Helena Laboratories (Beaumont, TX), and serum high-density lipoprotein (HDL) was quantified as described previously (23). Sirt1 and WT normal mice had similar glucose and HDL levels, and the severity of hyperglycemia (blood glucose and glycated hemoglobin) was also similar in WT and Sirt1 diabetic mice (Supplement Table 1S). The treatment of animals conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research, and Institutional Guidelines.

Retinal microvessels were prepared by hypotonic shock method by incubating the retina at 37°C in 5-6 ml de-ionized water for 60 minutes in a shaking water bath. The nonvascular tissue was gently removed under the microscope (22).

Gene expression was quantified in the cDNA synthesized from the retinal microvessels by SYBR green based qPCR using their gene specific primers (Table I). Ribosomal 18S RNA was used as a housekeeping gene, and the fold change was calculated using ddCt method (19; 22).
Histopathology and apoptosis in retinal microvessels: The whole retina isolated from the formalin-fixed eyes was rinsed overnight in running water, followed by incubation at 37°C in 3% crude trypsin (Invitrogen-Gibco, Grand Island, NY) containing 200 moles/L sodium fluoride for 45 to 70 minutes. After gently brushing away the neuroretinal tissue under a microscope, the vasculature was stained with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling stain (TUNEL; In Situ Cell Death kit; Roche Molecular Biochemicals, Indianapolis, IN). As a control, retinal vasculature, treated with DNAse was also TUNEL stained. The TUNEL-positive capillary cells were counted under a microscope, and the slides were then stained with periodic acid-Schiff-hematoxylin to count acellular capillaries-pericyte ghosts by light microscopy (23).

Immunofluorescence staining: Using specific primary antibodies, retinal cryosections (8µm) were stained for MMP-9, Sirt1 (Abcam, Cambridge, MA) and Dnmt1 (Sigma-Aldrich) following the method reported previously (29). The slides were then incubated with fluorescence labelled secondary antibodies; DyLight 488 (Green) for Sirt1 and Texas Red (Red) for Dnmt1 and MMP-9. The sections were mounted with DAPI (blue) containing medium and photographed under ZEISS ApoTome fluorescence microscope at 40X magnification (Carl Zeiss, Chicago, IL).

Vascular permeability was quantified by fluorescein angiography ~2 weeks before termination of the experiment using a Micron IV retinal imaging microscope (Phoenix Research Laboratories, Pleasanton, CA). Animals were anesthetized with a mixture of Ketamine 67mg/kg and Xylazine 10mg/kg (i.p.), their pupil was dilated with 0.1% tropicamide ophthalmic solution, and the cornea was lubricated with Goniovisc (hypromellose 2.5%). The fundus was photographed using fundus camera for small animals. AK-FLUOR (0.5% solution, 0.01ml/g BW) was then injected
intraperitoneally, and the photographs were taken after 10 minutes of fluorescein injection using a barrier filter for fluorescein angiography (24).

Vascular permeability was confirmed by quantifying albumin leakage into the retina by Evans blue technique (25). Evans blue (30 mg/kg) was injected in the tail vein, and after maintaining mice on a heating pad for 2 hours, paraformaldehyde was perfused and the eye globes were enucleated immediately. The dye was extracted from the retina using formamide, and absorbance was measured at 620nm.

**Vascular density** was measured in fluorescein angiograms after converting them to grayscale (to eliminate fluorescein background) using Angio-tool software from National Cancer Institute (26), and also by isolectin staining of retinal flatmount (27) using FITC-conjugated Isolectin B4 (Alexa Fluor 488, 1:100 dilution; Life Technologies, Carlsbad, CA). The flatmounts were visualizing under a confocal microscope at 10X magnification (Leica SP5; Wetzlar, Germany), and the images were converted to grayscale for analysis by Angio-tool.

**Mitochondrial DNA damage** was determined by analyzing the sequence variants in the regulatory region of the mtDNA (Displacement loop, D-loop), the region which experiences more damage in diabetes than other regions of the mtDNA (28), using Surveyor Mutation Detection kit (IDT Inc. Coralville, IA). Sequence variants were determined by digesting the amplicons with a surveyor nuclease, a mismatch-specific endonuclease with high specificity for the sites of base substitution sequence variants. The digested products were electrophoresed on a 2% agarose gel and analyzed by visualizing under a UV transilluminator, as reported previously (28).

**Mitochondrial DNA copy numbers** were quantified in retinal microvessels using primers for *Cytochrome B (CytB)* as a marker for mtDNA and *β-Actin* for nuclear DNA (Table I). SYBR
green based real time qPCR was carried out by amplifying at 95°C for 10 minutes, followed by 40 cycles of 15 second at 95°C and 60 second at 60°C. The ratio of mtDNA to nuclear DNA (CytB:β-Actin) within each sample was used to calculate mtDNA copy numbers (28).

**DNA methylation** of *Sirt1* promoter was determined by quantifying 5 methyl cytosine (5mC) using methylated DNA immunoprecipitation kit (Epigentek, Farmingdale, NY) (19).

**Acetylation of Dnmt1** promoter was performed by quantifying acetylated H3K9 using chromatin immunoprecipitation technique, as reported previously (21). Dnmt1 protein acetylation was analyzed by immunoprecipitating acetyl lysine (Abcam), followed by western blotting for Dnmt1 (22).

**Electroretinogram (ERG)** was performed in dark adapted mice, anesthetized with Ketamine-Xylazine. After dilating the pupil with tropicamide ophthalmic solution and lubricating the cornea with Goniovisc, ERG responses were measured using Ocuscience HMsERG system by placing a silver embedded thread electrode over the cornea above the lubricant solution 2.5% hypromellose. A contact lens was used to keep the electrode in place. ERG responses were recorded using a series of Ganzfeld flashes with intensities ranging from 100-25,000 mcd.s/m^2. The amplitudes and the implicit times of both a-wave and b-wave were measured using ERGview software (23).

**Retinal thickness** was quantified by optical coherence tomography (OCT) using an OCT module, customized for retinal imaging of small animals, of the Micron IV. The anesthetized animals were positioned in front of the camera, and high-resolution b-scan of the retinal cross-sections was obtained from both the eyes by averaging and spatially aligning 50 individual b-scans along the same horizontal axis through the optic disc (30). Thickness of the ganglion cell layer (GCL)
+ inner plexiform layer (IPL), and of the inner nuclear layer (INL) was measured at 200-400µm distance on either side of the optic disc using the caliper tool available in the InSight software.

To confirm changes in the retinal layers, retinal cryosections were stained with hematoxylin-eosin (31), and the images were analyzed at three random places using ImageJ software.

Statistical analysis was performed using SigmaStat software (Systat, San Jose, CA). Comparison among groups was analyzed using one-way ANOVA followed by Student-Newman-Keuls test for the data with normal distribution. For the data, where normality test failed, one-way ANOVA, followed by Dunn’s test was performed. A p value <0.05 was considered statistically significant.
RESULTS

Retinal vasculature from WT diabetic mice, as expected (13), had significantly higher number of acellular capillaries compared to their age-matched WT normal mice; ~8 acellular capillaries in normal compared to ~20 in diabetic mice (Figure 1A). Similarly, the number of pericytes and TUNEL-positive cells was increased from 5-8 in normal to 16-25 in diabetic mice. However, both Sirt1 diabetic and normal mice had ~9 acellular capillaries and 6-8 TUNEL positive capillary cells. Figure 1B-D is included to show significant decrease in Sirt1 expression (protein and mRNA) in retinal vasculature in WT-diabetic mice, and its prevention in Sirt1 mice.

Since vascular leakage is one of the hallmarks of diabetic retinopathy (1), the effect of Sirt1 overexpression on vascular health was determined by fluorescein angiography. Although some vascular leakage was observed in WT diabetic mice, consistent with WT and Sirt1 normal mice, no leakage was observed in Sirt1 diabetic mice (Figure 2A). Similarly, protection of diabetes-induced retinal vascular leakage by Sirt1 was also confirmed by Evans blue method, (Figure 2B). The effect of Sirt1 on retinal vascular health was further evaluated by quantifying capillary density. Compared to WT normal mice, both fluorescein angiography and isolectin staining showed significant decrease in the retinal vascular density in WT diabetic mice (Figure 2C and D), however diabetes had no effect on retinal vascular density in Sirt1 mice, and the density was similar to that seen in WT normal mice.

To investigate the effect of Sirt1 on mitochondrial damage, mtDNA damage was analyzed in the retinal microvasculature. Consistent with our previous results (28), WT diabetic mice had increased number of sequence variants in the D-loop and decreased parent amplicon band intensity (Figure 3A and B), which was prevented in Sirt1 diabetic mice. Sirt1 also plays a significant role in mtDNA biogenesis (32), and in diabetes, mtDNA copy numbers are decreased
As shown in figure 3C, mtDNA copy numbers were significantly lower in the retinal microvessels of WT diabetic mice, but Sirt1 diabetic mice had similar copy numbers as obtained from normal mice (WT or Sirt1).

Since upregulation of MMP-9 in diabetes is implicated in the mitochondrial damage seen in the retina and its vasculature (11; 13), the effect of Sirt1 on MMP-9 expression was evaluated. The retinal microvasculature of Sirt1 diabetic mice was also protected from increase in MMP-9 and the values obtained from WT and Sirt1 normal and Sirt1 diabetic were not different from each other (Figure 4A). Figures 4B further confirm decreased immunostaining of MMP-9 in the retinal cryosections from Sirt1 diabetic mice compared to WT diabetic mice.

Cytosine methylation results in transcriptional repression (17; 18; 34), and in diabetes DNA methylation machinery is activated (19; 20). To understand the mechanism responsible for Sirt1 suppression, its promoter DNA methylation was investigated. Compared to WT normal mice, retinal microvasculature from WT diabetic mice had over 3 fold higher 5mC at the Sirt1 promoter, and ~2.5 fold increased Dnmt1 expression (Figure 5A). However, in Sirt1 diabetic mice, although 5mC levels were higher compared to normal mice, these values were significantly lower compared to those obtained in WT diabetic mice, suggesting a role of DNA methylation in diabetes-induced Sirt1 suppression. Similarly, diabetes-induced increase in Dnmt1 expression was also ameliorated in Sirt1 diabetic mice (Figure 5B). Immuno-histochemical data confirmed protective effect of Sirt1 in preventing increase in Dnmt1 staining (Figure 5C and 5D). Sirt1 can deacetylate both histones and protein, and deacetylation of Dnmt1 by Sirt1 deactivates it (35). To investigate the role of Sirt1 in diabetes-induced increase in Dnmt1, acetylation of histone at Dnmt1 promoter was investigated. As shown in figure 5E,
although diabetes increased acetylated H3K9 levels, which was ameliorated in Sirt1-diabetic mice, it had no effect on Dnmt1 protein acetylation (Supplement Figure 2S).

Nonvascular cells of the retina are also affected in diabetes, resulting in abnormal electrical responses (5). To investigate the effect of Sirt1 on functional changes in nonvascular cells, ERG was performed. Compared to WT normal mice, WT diabetic mice had ~20% decreased a-wave and b-wave amplitudes, and these changes were prevented in Sirt1 diabetic mice; the values were similar to those obtained from age-matched WT normal mice (Figure 6A-C). Similarly, the implicit times of a- and b- waves were also increased and 15-20% increased. Consistent changes in a- and b- waves were also observed at 3000 and 10,000 med.s/m^2.

Due to accelerated loss of retinal cells in diabetes, thinning of the retinal layers is also commonly observed (36); the effect of Sirt1 overexpression on diabetes-induced changes in retinal layer thickness was determined. At 200 µm away from the optic disc, WT diabetic mice had significant decrease in the thickness of GCL+IPL (40.43±1.10 µm) compared to WT normal mice (44.00±1.26 µm, p<0.0001). Similar decreases were seen in the INL (22.83±1.72 µm vs 20.29±1.3 µm; p<0.013). However, in Sirt1 diabetic mice, thickness of both GCL+IPL and INL layers were significantly different from those observed in WT diabetic mice (43.83±0.82 µm and 23.00±0.60 µm respectively; p<0.0001). Similar differences in the thickness in WT diabetic and Sirt1 diabetic animals were also observed at 300 µm away from the optic disc (Figure 7A). Retinal cryosections, stained by hematoxylin-eosin also presented similar protection of thinning of retinal layers in Sirt1 diabetic mice (Figure 7B).
DISCUSSION

Diabetic retinopathy is a multi-factorial disease with many structural, biochemical, molecular and functional abnormalities implicated in its development (1; 2). Sirt1 is a cellular energy sensor, which plays a critical role in linking metabolic stress with cellular response (6; 7), and in diabetes, it is downregulated in the retina and its vasculature (11). Downregulation of Sirt1 hyperacetylates many regulatory proteins including transcription factors associated with oxidative stress and apoptosis (6; 8; 11). Here, we show that Sirt1 is also intimately associated with the development of diabetic retinopathy and related retinal vascular and neuronal damages. Our novel data clearly demonstrate that diabetic mice overexpressing Sirt1 are protected from the development of degenerative capillaries, a histopathology characteristic of retinopathy, and their retina has normal vascular density and is not leaky. Sirt1 also protects mitochondria from diabetes-induced increase in mtDNA damage and decrease in copy numbers, and obliterates activation of mitochondrial damaging MMP-9. Sirt1 diabetic mice are spared from retinal neuronal damage; ERG responses are normal and thickness of GCL+IPL, and that of INL layers are not different than seen in WT or Sirt1 normal mice, suggesting a major role of Sirt1 in preventing diabetes-induced damage to the overall health of the retina. Furthermore, we elucidate the role of DNA methylation in diabetes-induced Sirt1 transcriptional suppression, and show that Sirt1, via modulating Dnmt1 promoter histone acetylation, on the contrary, regulates its transcription. Together, these results imply a significant role of Sirt1 in the development of diabetic retinopathy, and the mechanism of Sirt1 suppression appears to be the epigenetic modification of its promoter.

Sirtuin family of proteins are highly conserved NAD-dependent deacetylases; humans encode seven different sirtuins, Sirt1-Sirt7, and among them Sirt1 is the most extensively studied
member (6; 8). Sirt1 is linked to cellular energy metabolism and the redox state through multiple signaling and survival pathways, and is also implicated in the pathophysiology of many chronic diseases including diabetes, neurodegenerative disorders and cardiovascular disease (37). Sirt1 controls the redox environment and counteracts oxidative damage by converting NAD to its reduced form, NADH (8); in coronary heart and cerebrovascular diseases, activation of Sirt1 protects against oxidative stress at the cellular level, and also increases survival at the systemic level to further limit these diseases (38). Sirt1-transgenic mice are protected from obesity (both diet and genetically)- induced diabetes (39). However, although Sirt1 is a core systemic regulator of cellular metabolism, mice overexpressing Sirt1 have a normal lifespan, and the protection is restricted to the specific tissues (40). Here, we provide convincing data showing a direct role of Sirt1 in diabetic retinopathy, the retinal vasculature of diabetic mice overexpressing Sirt1 is protected from the development of histopathology, and also from accelerated apoptosis, a phenomenon which precedes the development of histopathology (41), implying that the activators of Sirt1 could have potential to impede the development of retinopathy in diabetic patients. Consistent with this, decreased Sirt1 (mRNA and activity) is also seen in the retinal microvasculature and peripheral blood mononuclear cells from human donors with diabetic retinopathy (11; 42). Decrease in Sirt1, via regulating acetylation of liver X receptor, is shown to increase both hyperglycemia and dyslipidemia in type 2 diabetes, contributing to the progression of diabetic retinopathy (43). Furthermore, administration of Sirt1 activator resveratrol ameliorates age-related increased retinal degeneration and cell apoptosis in rats (44).

Clinical and experimental studies have documented many alterations in the retinal vasculature in diabetic retinopathy including increased vascular permeability, leukostasis and capillary degeneration (1; 2), and the vascular density is decreased (45). Damage to the blood-
retinal barrier is considered as an early clinical sign of the development of diabetic retinopathy (1). We show that, whereas the retina of WT diabetic mice have decreased vascular density and increased vascular leakage compared to WT normal mice, Sirt1 diabetic mice have normal retinal vascular density with no vascular leakage, suggesting that, in addition to preventing increased capillary cell apoptosis and formation of degenerative capillaries, Sirt1 overexpression also helps in maintaining the overall health of the retinal vasculature.

Mitochondrial damage plays a critical role in the pathogenesis of diabetic retinopathy; in diabetic rodents, increased mitochondrial functional and structural damage is observed before increased capillary cell apoptosis and degenerative capillaries in the retinal vasculature (13; 28). Compromised mtDNA biogenesis and reduced mtDNA copy numbers are observed in retinal microvasculature, and damaged mtDNA, via compromising the electron transport chain system, continues to fuel into the vicious cycle of free radicals (2; 3). Here, we show that the retinal microvasculature of these Sirt1 diabetic mice are also protected from mtDNA damage and their mtDNA copy numbers are normal. In agreement, Sirt1 is critically involved in mitochondrial quality maintenance; it regulates mtDNA biogenesis and turnover of the damaged mitochondria (32), and manipulation of Sirt1 is shown to regulate hyperglycemia-induced mitochondrial dysfunction and apoptosis in human umbilical cord vascular cells (46). Furthermore, retinal poly(ADP-ribose) polymerase (PARP) is also activated in diabetes (22), and chronic activation of PARP-1, via depleting NAD, inactivates Sirt1, results in mitochondrial dysfunction (47), further fueling into mitochondrial damage.

Activation of proteinases, especially MMP-2 and MMP-9, is implicated in the retinal mitochondrial damage seen in diabetes (13; 29). MMP-9 promoter has multiple transcriptional factor binding sites, and many transcriptional factors are involved in its transcription (48). We
have shown that due to hyperacetylation of transcriptional factors NF-κB and activator protein-1 (AP-1), their binding at the MMP-9 promoter is increased, resulting in MMP-9 transcriptional activation (11; 14). Furthermore, Sirt1 also regulates the binding of PARP-1 at the MMP-9 promoter, which, via manipulating the binding of NF-κB/AP-1, regulates MMP-9 expression (22). The results presented here clearly show that the retinal vasculature of the same Sirt1 diabetic mice that are protected from the development of diabetic retinopathy, also have normal MMP-9 expression, further confirming the role of Sirt1 in mitochondrial damage seen in diabetes.

Diabetes downregulates Sirt1 in many tissues including retina and kidney (11; 49), and one of the mechanisms implicated in its inactivation is increased oxidative stress (8). However, in diseased state, epigenetic modifications also regulate gene transcription, and diabetes favors many of these epigenetic modifications, including DNA methylation and histone modifications (2; 19). DNA methylation is considered as a gene suppressor (34), and the machinery responsible for maintaining DNA methylation is activated in the retina in diabetes (19). Our results demonstrating hypermethylation of Sirt1 promoter in the retinal microvessels from diabetic mice are supported by reports from others showing increased Sirt1 promoter methylation in the peripheral blood from Alzheimer’s patients (50). Consistent with the regulation of DNA methylation of Sirt1 promoter, in the same Sirt1 diabetic mice, the promoter of Dnmt1 (the only member of the Dnmt family which is upregulated in the retina in diabetes (19), is protected from increased acetylation of H3K9, which results in regulation of Dnmt1 transcription. These results suggest that Sirt1, via preventing acetylation of H3K9 at the Dnmt1 promoter, prevents its activation, which, in turn, impedes DNA methylation of Sirt1 promoter, regulating its expression.
Although the histopathology of diabetic retinopathy is observed in the retinal vasculature, neuronal cells also undergo many structural/functional and metabolic changes (5; 36). Neuronal cell apoptosis and thinning of the GCL are seen before vascular cell apoptosis, and diabetic patients (and rodents) present functional neurogenic changes with prolonged implicit time and decreased amplitudes before histopathology characteristic of diabetic retinopathy can be observed (5; 23). Here, our exciting data clearly show that Sirt1 diabetic mice are also protected from neuronal abnormalities, their GCL does not show any thinning, and the ERG signals are not different from those observed in normal mice. Although the mechanism via which Sirt1 overexpression could be preventing neuronal damage remains to be investigated, our results clearly suggest that Sirt1 has an important protective role in both retinal neuronal damage and vascular damage associated with the development of diabetic retinopathy.

In summary, our convincing structural, functional and molecular data demonstrate that Sirt1, a multifunctional protein which deacetylates proteins (including transcriptional regulators) and histones, has a protective role in the development of diabetic retinopathy. Amelioration of its inhibition maintains retinal vascular homeostasis by preventing mitochondrial damage, and protects the vasculature from undergoing accelerated apoptosis and becoming leaky. Regulation of Sirt1 inhibition also prevents neuronal structural and functional damage, the damage which is observed before vascular pathology, further providing opportunities to ameliorate the development of diabetic retinopathy during its early stages. The mechanistic insight into Sirt1 regulation in diabetes suggests the role of epigenetics in its transcriptional suppression. Thus, our study raises therapeutic potential of strategies targeting Sirt1 activation in maintaining retinal vascular/neuronal health, and preventing the continuation
of the vicious cycle of mitochondrial damage, ultimately, ameliorating the development of this blinding disease.

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**Conflict of Interest.** MM, AJD and RAK do not have any conflict of interest.

**Author Contributions.** MM: researched data, literature search and manuscript editing, AJD: researched data and literature search, RAK: experimental plan, literature search, manuscript writing/editing. RAK is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Table I

Primer sequences

| Genes           | Sequence                                                                 |
|-----------------|--------------------------------------------------------------------------|
| Sirt1           | 5’-GGGGTTTGCCCCATGGAAT-3’<br>5’-GAGCCCATCCCCACACTGTA-3’                  |
| MMP-9           | 5’-GGGGTTTGCCCCATGGAAT-3’<br>5’-GAGCCCATCCCCACACTGTA-3’                  |
| Dnmt1           | 5’-CCTAGTTCCGTGGCTACGAGGAGAA-3’<br>5’-TCTCTCTCCTCTGCAGCCGACTCA-3’        |
| 18S             | 5’-GCCCTGTAAATTGGAATGAGTCCACTT-3’<br>5’-CTCCCAAGATCCAACTACGAGCTTT-3’     |
| Dnmt1 promoter  | 5’-TCCTCTGCAAGAGGCAGCACTA-3’<br>5’-ATGTACCACACAGGGCAAGA-3’               |
| Sirt1 promoter  | 5’-GGCAGCACACACCTTTTACA-3’<br>5’-AGATTTGCTGCTCATCTGCCT-3’               |
| D-Loop          | 5’-AGCACCCAAAGCTGATTCTTCT-3’<br>5’-CCAGGACCCAAACCTTTGTGTTT-3’            |
| CytB            | 5’-AGACAAAGCCACCTTGAACCGAT-3’<br>5’-ACGATTGCTAGGGCCGCGAT-3’             |
| β-actin         | 5’-AAAGGAAGCGCAGACCCGCCG-3’<br>5’-GCGCAGTGTAGGCGAGCTT-3’                |
FIGURE LEGENDS

Figure 1: Effect of Sirt1 overexpression on retinal capillary cell damage in diabetes: Trypsin-digested retinal microvessels from C57BL/6J WT and Sirt1 overexpressing mice, diabetic for ~8 months, were TUNEL-stained, and TUNEL+ cells were counted in the entire retinal vasculature. The microvasculature was then stained with periodic acid Schiff-hematoxylin, and (A) representative periodic acid Schiff-hematoxylin-stained microvasculature with the arrow head showing an acellular capillary and arrow a pericyte ghost. (B) Sirt1 expression was quantified in the retinal cryosections by immunofluorescence staining using DyLight 488 (Green) labelled secondary antibodies. Retinal microvessels prepared by hypotonic shock method were analyzed for Sirt1 (C) gene expression by SYBR green-based q-PCR and (D) protein expression by western blot technique. WT-N and WT-D = C57BL/6J wildtype normal and diabetic mice respectively; Sirt-N and Sirt-D = Sirt1 overexpressing normal and diabetic mice respectively. Values are represented as mean ± SD from 5-7 mice/group; *p<0.05 compared with age-matched WT-N and #p<0.05 with WT-D.

Figure 2: Effect of Sirt1 overexpression on vascular leakage and capillary density. (A) Fluorescein angiography was performed using a Micron IV retinal imaging microscope containing a barrier filter for fluorescein angiography. Representative angiograms from mice in each group; the arrow in the inset indicates vascular leakage. (B) Tail vein injected Evans blue dye in the retinal extract was quantified spectrophotometrically at 620nm. Vascular density was determined (C) in fluorescein angiograms, and (D) by isolectin staining of retinal flatmounts using FITC-conjugated Isolectin B4 under a confocal microscope. Analysis was performed in the grayscale converted images by Angio-tool software, and the accompanying graph represents vessel area. The values obtained from WT normal mice are considered as 100%. WT-N and WT-
D = wildtype normal and diabetic mice respectively, and Sirt-N and Sirt-D = Sirt1 overexpressing normal and diabetic mice respectively. Each group had 5-6 mice. *p<0.05 compared to WT-N and #p<0.05 compared to WT-D.

Figure 3: Effect of Sirt1 upregulation on mtDNA damage and biogenesis. Retinal microvessels were analyzed for (A) DNA damage by measuring sequence variants in the amplified D-Loop region using a mismatch-specific surveyor endonuclease, followed by analysis on a 2% agarose gel, and (B) the parent band amplicon intensity was quantified and intensity of the amplicons from WT-N mice was considered 100%. (C) Mitochondrial copy numbers were quantified in the total DNA isolated from retinal microvessels by qPCR using CytB as mtDNA marker and β-Actin as a nuclear DNA marker. The results are representative of 5-6 microvessel preparations/group. WT-N and WT-D = wildtype normal and diabetic respectively, and Sirt-N and Sirt-D = Sirt1 overexpressing normal and diabetic mice respectively.*p and #p <0.05 compared to WT-N and WT-D respectively.

Figure 4: Effect of Sirt1 overexpression on the diabetes induced retinal MMP-9. (A) MMP-9 gene transcript was quantified in retinal microvasculature by SYBR green-based q-PCR using 18S as the housekeeping gene. Values are mean ± SD from 4-6 samples per group. *p<0.05 compared to WT-N and #p<0.05 compared to WT-D. (B) Expression of MMP-9 in retinal cryosections was performed by immunofluorescence using DyLight 488 (green) and Texas Red (red) conjugated secondary antibodies for Sirt1 and MMP-9 respectively, and mounting by DAPI (blue) mounting medium. The insets show magnified areas. Values are mean ± SD from 4-6 samples per group. *p<0.05 compared to WT-N and #p<0.05 compared to WT-D.

Figure 5: DNA methylation of retinal Sirt1. Retinal microvessels were used to quantify (A) 5mC levels by methylated DNA immunoprecipitation technique, and (B) Dnmt1 gene transcripts by q-
PCR using 18S as the housekeeping gene. (C) Dnmt1 expression in the cryosections was determined by immunofluorescence technique using secondary antibodies conjugated with DyLight 488 (green) for Sirt1 and with Texas Red (red) for Dnmt1. DAPI (blue)-based mounting medium was used for mounting the sections and (D) represents the mean fluorescence intensity. (E) Acetylated H3K9 levels at Dnmt1 promoter were quantified in retinal microvessels by immunoprecipitating genomic DNA with H3K9Ac antibody, followed by qPCR using primers for Dnmt1 promoter. WT-N and WT-D = wildtype normal and diabetic mice respectively, and Sirt-N and Sirt-D = Sirt1 overexpressing normal and diabetic mice respectively. Values are represented as mean ± SD from 4-6 retinal microvessel preparations/group. *p<0.05 vs WT-N and #p<0.05 vs WT-D.

Figure 6: Effect of Sirt1 on retinal neuronal function: ERG was performed in dark adapted mice using Ocuscience HMseERG system. The electrical response was recorded using a series of Ganzfeld flashes with intensities ranging from 100-25,000 mcd.s/m^2. (A) A representative ERG response at 1000 mcd.s/m^2 from one mouse in each group. (B) a-wave amplitude, and (C) b-wave amplitude are represented as %normal, with the values obtained from WT-N being 100%. *p and #p <0.05 compared to WT-N and WT-D respectively, and Sirt-N and Sirt-D = Sirt1 overexpressing normal and diabetic mice respectively.

Figure 7: Effect of Sirt1 overexpression on retinal layer thickness. (A) OCT was performed using an OCT module, customized for retinal imaging of small animals, of the Micron IV retinal imaging system. An average high-resolution b-scan of retinal cross-sections was obtained by spatially aligning 50 individual b-scans along the same horizontal axis through the optic disc, marked with a green arrow on the fundus images. The right panel shows representative b-scans from each group with the layer thickness measurement points marked. Thickness of GCL+IPL,
and that of INL at 200µm, 300µm and 400µm on either side of the optic disc was measured using the InSight software; measurements on the left side of optic disc are represented as negative and that on the right side as positive integers. (B) Retinal cryosections were stained with hematoxylin-eosin, and thickness of the layers was analyzed at three random places using ImageJ software. WT-N and WT-D = wildtype normal and diabetic mice respectively and Sirt-N and Sirt-D = Sirt1 overexpressing normal and diabetic mice respectively.
Figure 1

279x361mm (300 x 300 DPI)
Figure 2

279x361mm (300 x 300 DPI)
Figure 4

A

B

279x361mm (300 x 300 DPI)
Figure 5

279x361mm (300 x 300 DPI)
Figure 6

A

B

C

279x361mm (300 x 300 DPI)
Figure 7

279x361mm (300 x 300 DPI)
Supplementary data

Table IS

Metabolic data

| Group    | BW (g)   | Glucose (mg/dl) | GHb (%) | HDL (mg/dl) |
|----------|----------|-----------------|---------|-------------|
| WT-N     | 22.2±1.9 | 106.4±7.8       | 7.3±0.6 | 126.3±27.4  |
| WT-D     | 24.6±2.2 | 410.8±112.6 *   | 11.9±0.9 * | 87.4±20.4 * |
| Sirt1-N  | 27.9±3.9 | 104.8±8.1       | 8.2±0.6 | 140.8±46.9  |
| Sirt1-D  | 26.3±2.7 | 502.8±108.3 *   | 11.5±0.8 * | 110.8±17.5  |

*p<0.05 vs WT-N
Figure 1S: Sirt1 expression in the retina and kidney was quantified by western blot technique using β-actin as a loading control. WT-N and WT-D = C57BL/6J wildtype normal and diabetic mice respectively; Sirt-N and Sirt-D = Sirt1 overexpressing normal and diabetic mice respectively. Values are represented as mean ± SD from 4-6 mice/group, with each measurement made in duplicate; *p<0.05 compared with age-matched WT-N and #p<0.05 with WT-D.
Figure 2S: Effect of diabetes on acetylation of Dnmt1. Dnmt1 acetylation was quantified by immunoprecipitating acetylated proteins from the retinal microvessels, followed by western blotting for Dnmt1. WT-N and WT-D = C57BL/6J wildtype normal and diabetic mice respectively; Sirt-N and Sirt-D = Sirt1 overexpressing normal and diabetic mice respectively. Values are represented as mean ± SD from 4 or more mice/group with each measurement made in duplicate.