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Original Article

H3K4me3 aberrantly regulates transcription of Synla in superior cervical ganglion neurons in diabetic rats

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Abstract: Diabetic cardiac autonomic neuropathy (DCAN) is one of the severe syndromes in diabetics. The neuropathy at superior cervical ganglia (SCG) is one of the important causes for leading to cardiac dysfunction upon diabetes. Previous studies reveal the deficiency of synapsin I (Syn1) in sciatic, retinal and hippocampal neurons in diabetes rodent model. However, the expression pattern of Syn1 in SCG of diabetic rat is poorly understood. In our study, we observed the presence of differential expression of two transcript isoforms of Syn1 in SCG. Isoform a (the longer one) was lower expressed while isoform b (the shorter one) was normally expressed in diabetic rats compared to control. Furthermore, we also explored the epigenetic landscape of H3K4me3, H3K9me3 and H3K36me3 on Syn1 and found that the enrichment of H3K4me3 and RNA pol II at the terminal exons was significantly different in SCG of diabetic and normal rats. In addition, the genomic H3K4me3 level of SCG abnormally increases in diabetic rats. Thus, we concluded that diabetes may impact on the distribution of H3K4me3, which can further regulate the transcriptional preference of Syn1 variants.

Keywords: Diabetes, SCG, Syn1, isoform, H3K4me3

Introduction

Diabetes is a common world-wide chronic disease and a severe public health problem that has involves in 285 million patients according to the statistics of international diabetes federation in 2010 [1]. The syndromes of diabetes have attracted more and more attention, including diabetic cardiac autonomic neuropathy (DCAN), Diabetic Nephropathy Diabetes Gastroparesis, and nervous bladder. DCAN is one of the most serious syndromes of diabetes, presenting heart rate variability, Static tachycardia, orthostatic hypotension, declining ability of physique and blood pressure regulation.

Most of patients with diabetics can develop the cardiac autonomic nervous system disorder. The nerve impulses transmit to peripheral sympathetic nerves and then transfer to superior cervical ganglia (SCG) for regulating the cardiac function. The damage to peripheral or central nervous ganglia such as sciatic, retinal, SCG and hippocampal neurons driven by diabetes had been reported [2-5].

Synapsin I (Syn1) is a neural specific synapse membrane protein, which mainly distributes in presynaptic membrane of nerve terminal of central and peripheral nervous system [6], and modulates synaptic vesicle movement towards nerve terminal for neurotransmitter release and regulation [7]. Previous studies revealed that diabetes can affect Syn1 down-regulation in retinal [8] and hippocampal [9] neurons and thereby further disturb the synaptic connection and signaling transmission. However, how Syn1 is transcriptionally regulated and impacted by diabetes in SCG is still poorly understood.

In our study, we focused on alternative splicing of Syn1 transcript and found the abnormal Syn1 transcriptional variants in SCG cells of diabetic mice model. And we further explored the epigenetic pattern on Syn1 genomic region and observed the connection between alternative splicing and H3K4me3 modification on Syn1. Our study revealed the underlying mechanism of Syn1 transcriptional regulation and provided the potential therapeutic targets against DCAN.

Materials and methods

Animal study

All the procedures were approved by the Institutional Animal Care and Use Committee of Shanghai, China. Total forty 40-week-old outbred male Sprague-Dawley (SD) rats were used in this study. Animals were fed with food and water ad libitum freely. Twenty rats (weight 200 g) received intraperitoneal injection with 50 mg/kg streptozotocin (Sigma, USA), followed by adaptive breeding for 5 months. The rats whose index of blood glucose stably more than 16.7 mmol/L and body weight significantly less than normal control (n=20) were considered as diabetic model (Tables S1 and S2).

Rats were anesthetized by 50 mg/kg ketamine and dissected the skin and subcutaneous connective tissue at the middle line of neck, and then bluntly separated the muscle around the weasand following the arteria carotis communis until at the crossing between internal and external carotid artery. The arterial sheath were removed and the white oval substantial, namely superior cervical ganglia (SCG) were isolated. SCG tissues were cleaned the fibrous and connective tissue on surface and grinded by Dounce tissue grinders (Kimble, USA) into cell suspension for next studies.

Reverse transcription PCR assay

Total RNA was extracted from 20 arteries of individual from experimental and control group respectively using Trizol methods. cDNA was produced from 1 mg of total RNA by iScript cDNA Synthesis kit (Bio-Rad, CA, USA) according to the manufacturer's protocol. The level of different isoforms was amplified using appropriate primers (Table S3) and identified by 1% agarose gel electrophoresis. The gray intensity of bands on agarose gel was analyzed by imaging system (Tanon, China).

Immunoblotting and immunofluorescence assay

In brief, for immunoblotting, total proteins were transferred onto nitrocellulose membrane and blocked using 5% nonfat milk, followed by incubation with appropriate primary antibodies of Syn1, Actin (Santa Cruz, USA) and secondary antibodies. ECL western blotting kit was used to develop the membrane for protein detection.

For immunofluorescence assay, tissue fixed in 2% paraformaldehyde were sliced (25 μm depth) and permeabilized using 0.3% Trion X-100 and blocked using 5% horse serum, followed by incubation with appropriate primary antibodies of Syn1 and Actin 4°C overnight. The secondary antibodies with anti-mouse, anti-rabbit antibodies at a 1:5000 dilution and 600 mM 2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) were added together for 1 hour incubation at lucifugal place with room temperature.

Chromatin immunoprecipitaion assay

For ChIP assay as previously described [10], extracted DNA was sonicated and incubated with appropriate ChIP grade antibodies (H3K4me3, H3K9me3 and H3K36me3, RNA pol II, CHD1, HP1γ, MRG15, PTB, SF1, ESRP1 purchased from CST, USA) or IgG overnight, then harvested and detected the those proteins enrichment at genomic regions of Syn1, where the primers setting for qPCR were designed to encompass about 200 bp (Table S3). Ct value was analyzed to calculate enrichment using delta-delta methods.

Statistical analysis

All the analysis were processed by SPSS 20.0 software. Data are presented as means ± standard deviations for three independent experiments. The difference of values was analyzed using Student's t-test. *P* values less than 0.05 were considered as statistical significance (*, $P<0.05$; **, $P<0.01$).

Results

The differential expression of Syn1 variants in SCG of diabetic rats

Diabetic rat model was prepared via intraperitoneal injection with 50 mg/kg streptozotocin,

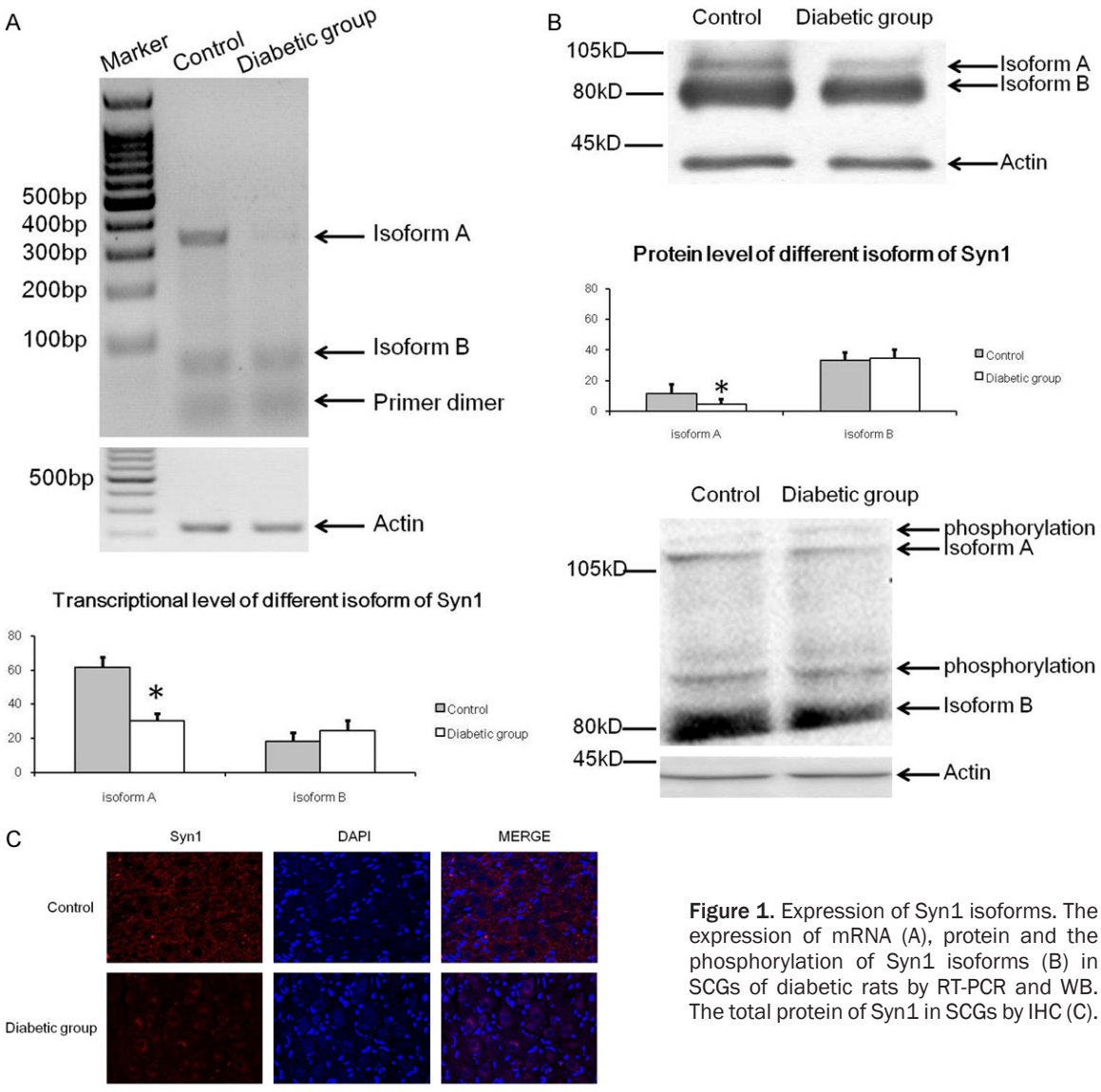

Figure 1. Expression of Syn1 isoforms. The expression of mRNA (A), protein and the phosphorylation of Syn1 isoforms (B) in SCGs of diabetic rats by RT-PCR and WB. The total protein of Syn1 in SCGs by IHC (C).

followed by acclimation breeding for 5 months. The rats whose index of blood glucose stably more than 16.7 mmol/L were deemed to be qualified for the next study. SCG were isolated and used for investigating the expression of Syn1 by PCR, WB and IHC. The presence of differential expression of Syn1 variants in SCG was observed. Isoform a (Syn1a, the longer one) was declined while isoform b (Syn1b, the shorter one) was normally expressed in diabetic group compared to control (**Figure 1A**). The protein pattern showed the similar tendency that Syn1a expressed lower in diabetic compared to control (**Figure 1B**). We tested the phosphorylation of Syn1 to validate the correct variants of Syn1 in immunoblotting assay used in our study and also found no difference of phosphorylation level between control and diabetic group. Furthermore, the results of IHC displayed that Syn1 was down-regulated in diabetic group compared to that in the control (**Figure 1C**). Taken together, these data reflected that the alternative splicing of Syn1 is abnormally regulated in diabetes.

Differential H3K4me3 pattern on final intron and exon of Syn1

To explore the cause of the transcriptional regulation of Syn1a upon diabetes, we investigated the H3K4me3, H3K9me3 and H3K36me3 patterns on the genomic region of Syn1 by ChIP-qPCR and found that only H3K4me3 had an apparent difference on 3' terminal regions

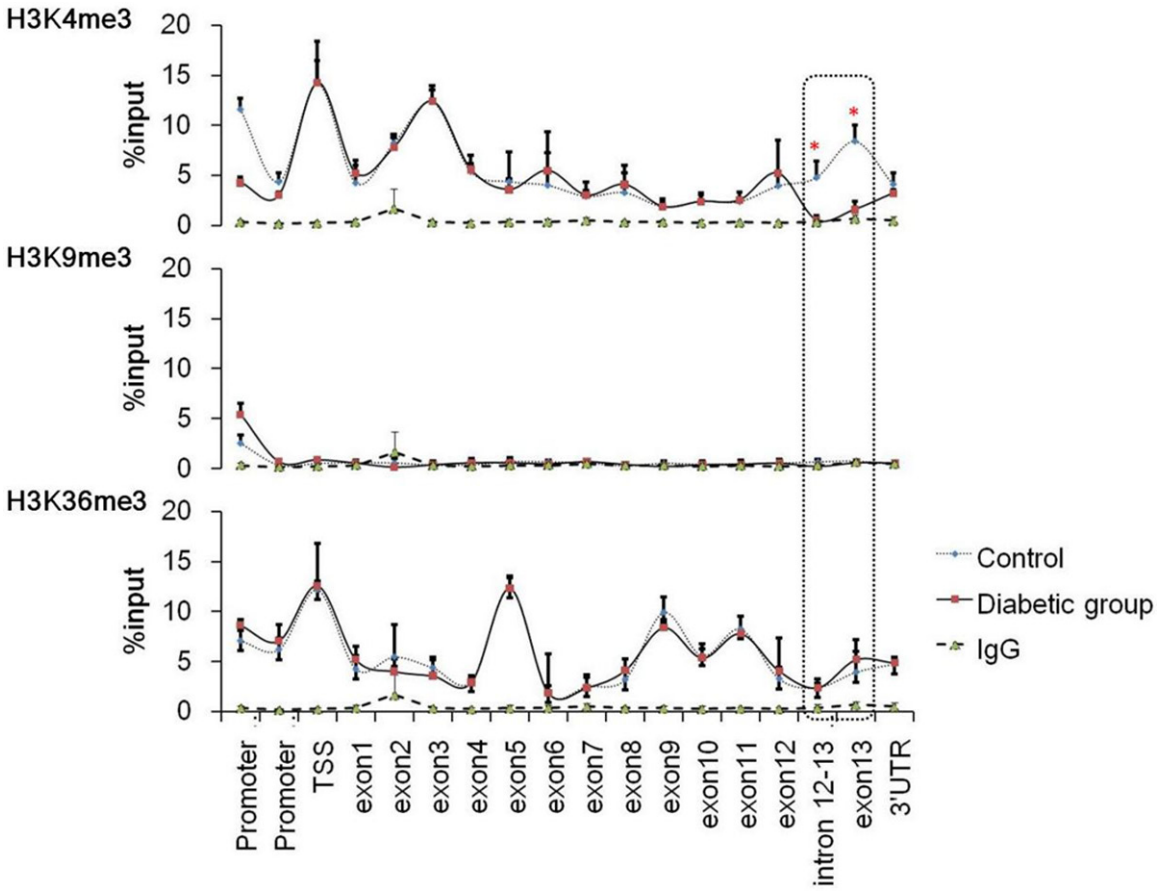

Figure 2. Histone modification on Syn1 genomic regions. The significantly statistical difference marked by red asterisk on final exon and intron were highlighted by gray frame.

(**Figure 2**). The enrichment of H3K4me3 in final intron and exon in diabetic SCG was observed lower than control group, while H3K9me3 was failed to be detected in most of regions in both two groups, and H3K36me3 level displayed in negligible difference. Interestingly, the proximal front intron as well as the splicing region on final exon, which produces the difference between two isoforms were both H3K4me3 highly enriched (**Figure 2**). Collectively, we observed that H3K4me3 differentially assembled on the alternative splicing site of Syn1 in SCG of diabetes.

Enrichment of RNA pol II, splicing factors and chromosome remodeling proteins on Syn1

Previous studies reported some important chromosome remodeling associated proteins such as CHD1, HP1γ and MRG15 recruited by H3K4me3, H3K9me3 and H3K36me3 respectively could impact on RNA pol II elongation and alternative splicing process. And the important splicing factors such as PTB, SF1 and ESRP1 might also participate in mRNA alternative splicing pathway. To verify our results, we further explored the enrichment of RNA pol II, MRG15, CHD1 and HP1γ on Syn1 by ChIP-qPCR (**Figure 3**). Consistent with H3K4me3, we observed PTB and CHD1 also less assembled on final exon of Syn1, and RNA pol II was also weakly enriched on promoter, exons and 3'UTR of Syn1 in diabetic SCG cells.

In all, our results concluded that H3K4me3 at the final exon of Syn1 down-regulated in SCG cells of diabetic mice model, then blocked RNA pol II and the important splicing factors for isoform a of Syn1 transcriptional preference.

Discussion

Two Syn1 isoforms resulting from the use of different splice acceptors for the final exon (exon

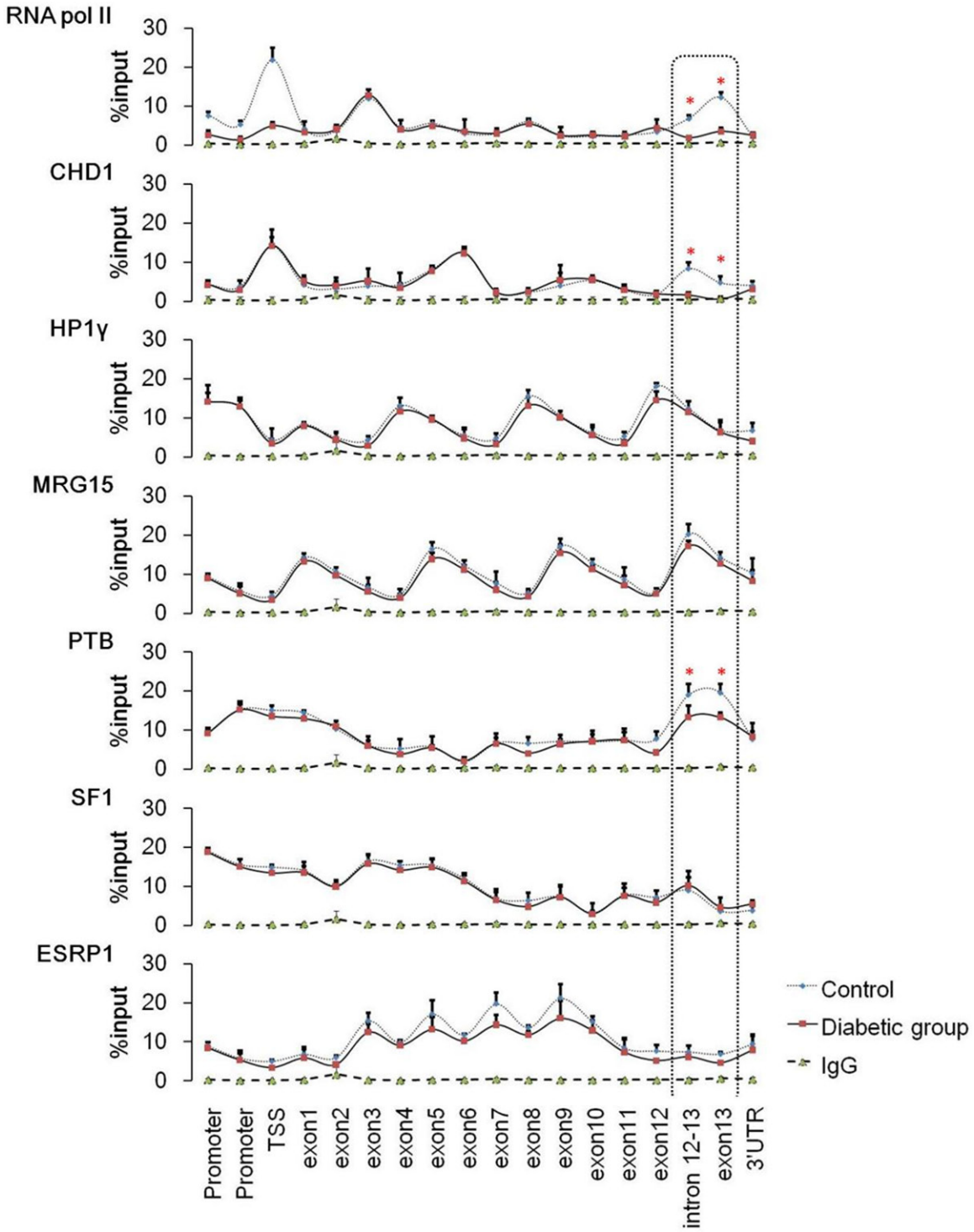

Figure 3. RNA pol II, splicing factors and chromosome remodeling proteins on Syn1. The significantly statistical difference marked by red asterisk on final exon and intron were highlighted by gray frame.

13) and encoding proteins of 669 and 705 amino acids [11]. The family members and their isoforms are differentially expressed in subsets of neurones, but the functional significance of this is unknown [12]. In our study, we explored the Syn1 expression and unexpectedly observed the differential mRNA and protein expression landscape of two Syn1 isoforms in

diabetic rats. We concluded that Syn1 was reduced in SCG which consistent with other previous retinal and hippocampal studies on Syn1 in diabetic model. Nevertheless, our study further indicated that the silencing of isoform a not isoform b of Syn1 is the mainly cause for Syn1 down-regulation. And in our study, we mainly focus on the underlying regulatory mechanism of Syn1, but not the function of these two isoforms in physiological and pathological states in diabetic SCG.

Recent studies reported that chromatin structure and nucleosome positioning could affect splicing complex assembly to impact RNA splicing [13, 14]. Some splicing factors can recognized the nucleosomes and form the complex to regulate RNA splicing. Moreover, nucleosomes can also impact the movement and elongation of RNA pol II. For histone modification, H3K4me3, H3K9me2 and H3K36me3 and their corresponding protein factors have been studied to associate with RNA splicing [15]. Therefore, we investigated these histone patterns on Syn1 and tried to illustrate the connection between epigenetic effect and pre-mRNA alternative splicing. Our results revealed that only the enrichment of H3K4me3 in final intron and exon in diabetic SCG was observed lower than control group, while H3K9me3 was failed to be detected both in two groups, and H3K36me3 level displayed in negligible difference. H3K4me3 usually represents transcriptional activation and distributes on gene promoters and enhancers. Consistently, we also observed PTB and CHD1 also less assembled on final exon of Syn1, and RNA pol II was also weakly enriched on promoter, exons and 3'UTR of Syn1 in diabetic SCG cells. Here we revealed the loss of H3K4me3 enriched on introns and exons that might enhance the tightness of nucleosome and obstruct the RNA pol II recruitment in diabetic rats. However, we failed to observe any difference of H3K9me2 or H3K36me3. We speculated that diabetes might affect SCG cells via preferential histone markers, which needs to be further investigated. And our study provides the potential therapeutic targets against DCAN.

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Disclosure of conflict of interest

None.

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Table S1. The blood glucose level in diabetic model

	Diabetic group	Control
1st month	20.62±5.79*	5.62±0.73
2nd month	18.74±7.48*	5.84±0.51
3rd month	17.58±9.06*	5.02±0.0.64
4th month	17.80±9.3*	5.40±0.92
5th month	17.44±9.50*	5.50±1.14

"*" represents the significant statistical analysis (*p* value less than 0.01).

Table S2. The body weight of diabetic model

	Diabetic group	Control
1st month	320.67±32.29*	390.33±28.47
2nd month	303.11±19.19**	438.67±27.99
3rd month	314.22±36.26**	500.16±27.47
4th month	317.78±52.32**	524.83±30.58
5th month	320.22±70.51**	546.83±35.16

"*" and "**" represents the significant statistical analysis (*p* value less than 0.05 and 0.01).

Table S3. All the primers used in this study

Gene symbol	Sequence	Tm (°C)
Syn1	CATTCTGGTGTGCACA	58
	AAGTGCCACCATTCGA	
Actin	TACAACCTCCTTGCATCTCC	56
	GGATCTTCATGAGGTAGTCAGTC	
Promoter for ChIP	ATCCGGGTGTGTGTCAC	55
	TCCCTCCAGGTGCAC	
Promoter for ChIP	CTGCATGCTGCAAACG	58
	CATTGGGCACACATT	
TSS for ChIP	AATTGGCCGCAGCAT	58
	AAGGGCTGCTGACA	
Exon 1 for ChIP	GGTTGACACAGGTG	60
	AAGGTCGCTGCTAC	
Exon 2 for ChIP	ACTTGTGTGCACCAGCA	60
	AGAGTTTGTTTTCCT	
Exon 3 for ChIP	TTCTCGGGTCTACA	60
	CCCGGCGCTGTAAAT	
Exon 4 for ChIP	CCCTGTGCCCAGCTGC	60
	GGCGTGCTCGCCATT	
Exon 5 for ChIP	CCGAATTTAGCTTGCAC	58
	CCTGCATGCTAGCAGCGAC	
Exon 6 for ChIP	AGAGTGCTGACCA	58
	TTGCTAGTCACAGGAC	
Exon 7 for ChIP	AGTTGGACCAGTG	60
	AGGTTCCCACTGCC	
Exon 8 for ChIP	CACAAAATTGGGCTGCT	58
	ACACGGGTGGCCAC	
Exon 9 for ChIP	TTCGCTCACACGGCA	55
	TTTGGCACCCATG	
Exon 10 for ChIP	CCCAAGTTGGTGCA	58
	GGCGCCACACTTGCA	
Exon 11 for ChIP	CACGGTGGCACA	58
	ATTGTTTCATCTAC	
Exon 12 for ChIP	TTATTCTGGCTCAG	60
	CCATTGGTTAACCA	
Exon 13 for ChIP	TTATTATGGCTCC	60
	TTATTCTGTGTCAC	
Intron 1 for ChIP	CTTTGGTCGTACAC	60
	CACATTTGGCTGCTTGGCA	
Intron 2 for ChIP	ATGGGTAAGCCTGA	60
	CACAGTGGCCACAGG	