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ARTICLE

Glucose-induced expression of the homeotic transcription factor *Prep1* is associated with histone post-translational modifications in skeletal muscle

Marco Ciccarelli[1,2] · Viviana Vastolo[1,2] · Luigi Albano[1,2] · Manuela Lecce[1,2] · Serena Cabaro[1,2] · Antonietta Liotti[1,2] · Michele Longo[1,2] · Francesco Oriente[1,2] · Gian Luigi Russo[3] · Paolo Emidio Macchia[4] · Pietro Formisano[1,2] · Francesco Beguinot[1,2] · Paola Ungaro[1,2]

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Abstract

Aims/hypothesis Chronic hyperglycaemia worsens insulin resistance in individuals with type 2 diabetes. Whether this effect is contributed by epigenetic dysregulation and which genes are involved remain unclear. *Prep1* (also known as *Pknox1*) is a gene exerting major effects on the sensitivity of the glucose transport machinery to insulin. Here, we show that dysregulation of *Prep1* expression by high glucose levels is associated with histone modifications at its 5′ regulatory region.

Methods We used mouse and cell models to investigate *Prep1* transcriptional regulation by glucose.

Results Differentiated L6 skeletal muscle cells were grown in the presence of either 5.5 or 25 mmol/l glucose (normal [NG] and high glucose [HG], respectively). The HG exposure increased nuclear factor κ light chain enhancer of activated B cells (NF-κB) p65 binding and recruitment of the su(var)3-9, enhancer-of-zeste, trithorax domain-containing lysine methyltransferase 7 (SET7) histone methyltransferase and p300 acetyltransferase to the 5′ region of *Prep1*, leading to enhanced transcription. In addition, chromatin immunoprecipitation assays revealed concomitantly increased histone H3 mono- and dimethylation and acetylation at Lys4 and Lys9/14, respectively. Skeletal muscle tissue from streptozotocin-treated diabetic mice also showed *Prep1* overexpression accompanied by similarly increased recruitment of NF-κB p65 and histone modifications at the 5′ region of *Prep1*. In these same mice, as well as in *Prep1*-overexpressing L6 cells, *Prep1*-induced recruitment of the repressor complex myocyte enhancer factor 2 (MEF2)/histone deacetylase 5 (HDAC5) at the *Glut4* promoter was also increased, leading to reduced *Glut4* expression.

Conclusions/interpretation These studies indicate that HG exposure induces NF-κB recruitment and histone modification at the *Prep1* 5′ region, thereby enhancing the transcription of *Prep1* and repressing that of *Glut4*. Histone changes at the *Prep1* gene may contribute to insulin resistance in individuals with type 2 diabetes.

Keywords Chromatin remodelling · GLUT4 · Histone-modification enzymes · NF-κB · Prep1 · Type 2 diabetes

Abbreviations

2-DG	2-Deoxyglucose
AcH3	Histone H3 acetylation
(p)AMPK	(phosphorylated) 5′ AMP-activated protein kinase
ChIP	Chromatin immunoprecipitation
ECL	Enhanced chemiluminescence

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✉ Paola Ungaro
pungaro@ieos.cnr.it

[1] Dipartimento di Scienze Mediche Traslazionali, Università degli Studi di Napoli, 'Federico II', Naples, Italy

[2] URT 'Genomica Funzionale' Istituto di Endocrinologia ed Oncologia Sperimentale, 'G. Salvatore', Consiglio Nazionale delle Ricerche, Via Sergio Pansini, 5, 80131 Naples, Italy

[3] Istituto di Scienze dell'Alimentazione, Consiglio Nazionale delle Ricerche, Avellino, Italy

[4] Dipartimento di Medicina Clinica e Chirurgia, Università degli Studi di Napoli, 'Federico II', Naples, Italy

HDAC5	Histone deacetylase 5
HG	High glucose
H3K4Me1	Histone H3 Lys4 monomethylation
H3K4Me2	Histone H3 Lys4 dimethylation
MEF2	Myocyte enhancer factor 2
NF-κB	Nuclear factor κ light chain enhancer of activated B cells
NG	Normal glucose
PREP1	Pbx-regulating protein 1
Re-ChIP	Double Chromatin Immunoprecipitation
SET7	Su(var)3-9, enhancer-of-zeste, trithorax domain-containing lysine methyltransferase 7
sh	Small hairpin
STZ	Streptozotocin

Introduction

Epigenetics refers to modifications that amend the genotype without changing the DNA sequence [1]. Important epigenetic processes are based on chemical modifications to DNA, such as methylation, or to DNA-associated proteins, such as histones [2, 3]. Among post-translational modifications of histones, acetylation/deacetylation and methylation/demethylation have been extensively investigated because of their major functional consequences on the genome [4–8]. Recently, the possibility that epigenetic mechanisms mediate the effects of the environment on progression to type 2 diabetes, and to other chronic disorders, has attracted increasing attention [9–11]. This interest is due, in part, to the limited understanding of the molecular events involved in the remarkable impact of the environment on type 2 diabetes risk [12, 13] and in the mechanisms that are responsible for the familial risk of this disorder [14].

Glucose levels represent a major feature of the cellular environment as changes evoke metabolic responses and adaptation mechanisms [15]. In vivo, hyperglycaemia worsens insulin resistance in type 2 diabetes [16] and provides a major contribution to the long-term complications of type 2 diabetes [17, 18]. Whether these effects are determined by epigenetic dysregulation induced by glucose, and which genes are affected, have been only partially elucidated. Current evidence shows that high glucose (HG) concentrations induce in vivo chromatin-remodelling events. These converge on inflammatory genes, which exert negative effects on glucose tolerance and lead to diabetes complications [19, 20]. For example, exposure of THP-1 (human leukemia monocytic cell line) monocytes to HG induces recruitment of the CBP (CREB-binding protein) and p/CAF (p300/CBP-associated factor) histone acetyltransferase co-activators to the promoter of inflammatory genes, leading to acetylation of histones H3 and H4 [21]. These effects are accompanied by increased activity of the nuclear factor κ light chain enhancer of activated B cells (NF-κB) p65 transcription factor at the promoter of the TNF-α and cyclooxygenase (COX)-2 inflammatory cytokines [21, 22]. Further studies have recently revealed that the su(var)3-9, enhancer-of-zeste, trithorax domain containing lysine methyltransferase 7 (SET7) histone methyltransferase interacts with NF-κB and regulates the expression of several key NF-κB-downstream genes by determining chromatin remodelling at their promoters [23].

Evidence generated in our laboratory revealed that the Pbx-regulating protein 1 (PREP1) homeodomain transcription factor has a physiological role in the control of glucose tolerance. Indeed, *Prep1* (also known as *Pknox1*) hypomorphic mice (*Prep1*$^{i/i}$), which express about 3% of *Prep1* mRNA and up to 10% of PREP1 protein, feature enhanced insulin sensitivity for glucose disposal and are protected from streptozotocin (STZ)-induced diabetes.

This effect on glucose tolerance results from different tissue-specific mechanisms. In the skeletal muscle tissue, *Prep1* action on glucose disposal involves the control of GLUT4 expression. The details of *Glut4* transcriptional control by PREP1 have been only partially elucidated. Despite the important role of PREP1 in the physiological control of glucose tolerance, *Prep1* gene variants associated with type 2 diabetes have never been identified and *Prep1* transcriptional dysregulation in type 2 diabetes remains mechanistically unexplained.

In this study, we have examined the hypothesis that environmentally induced histone modifications affect *Prep1* expression and insulin sensitivity. Our work identifies *Prep1* as a previously unrecognised gene downstream of NF-κB that is capable of inducing insulin resistance in response to hyperglycaemia and inflammatory hits, and focuses on the epigenetic state of *Prep1* at the level of the histones.

Methods

Materials See the ESM Methods for details of the materials used.

Cell culture and treatments L6 rat skeletal muscle cells (ATCC, Manassas, VA, USA) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution at 37°C in a humidified 95% air and 5% CO_2 atmosphere (all vol./vol.). The differentiation protocols are specified in the ESM Methods. Mycoplasma contamination was not detected in L6 cells. Trichostatin A treatment and 2-deoxyglucose (2-DG) uptake were performed as previously reported [24, 25].

RNA interference Transient transfection of *Prep1*-small hairpin (sh)RNA constructs was performed using the Lipofectamine method according to the manufacturer's

instructions. For further details, please refer to the ESM Methods.

Real-time PCR analysis Total RNA extraction, cDNA synthesis and real-time PCR were performed as described in Ungaro et al [26]. Relative quantification of gene expression was calculated by the $\Delta\Delta C_t$ method [27]. For copy number analysis, calibration curves were made from serial tenfold dilutions of plasmid DNA, as previously described [28]. The equations drawn from the graph of the standard curves were used to calculate the precise number of specific cDNA molecules present in the samples (copy molecules). The SYBR Green primer sequences are listed in ESM Table 1.

Western blot and nuclear extract preparation Cell lysis, nuclear extract preparation and western blot for protein expression analysis were performed as described in Ungaro et al [26] and Dignam et al [29]. Immunoblots were performed using primary antibodies for PREP1 (1:1000; # sc-25282 Santa Cruz Biotechnology, Dallas, TX, USA) and NF-κB p65 (1:1000; # sc-8008) and for phosphorylated 5′ AMP-activated protein kinase (pAMPK) (1:1000; # 2531 Cell Signaling Technology, Danvers, MA, USA) and AMPK (1:1000; # 2532 Cell Signaling Technology), with β-actin (1:1000; # sc-1616) or lamin A/C (1:1000; # sc-20681) as loading control. Immunodetected proteins were visualised using an enhanced chemiluminescence (ECL) kit (BioRad, Hercules, CA, USA).

Chromatin immunoprecipitation and re-chromatin immunoprecipitation assay procedures The chromatin immunoprecipitation (ChIP) assay was performed as described previously [24]. DNA fragments were recovered and subjected to PCR using the primer sequences described in ESM Table 1. For the double ChIP (re-ChIP) assay, immunoprecipitates with the first antibody were eluted in 50 μl of dithiothreitol 10 mmol/l and immunoprecipitated with the second antibody. Following immunoprecipitation, samples were processed as described for the ChIP assay; [30] eluted DNA was amplified by PCR or real-time PCR with the specific primers described in ESM Table 1.

Animal studies The 4-week-old male C57BL/6J mice (Charles River Laboratories, Lecco, Italy) were hosted at the common facility of the University of Naples 'Federico II'. Mice were randomly assigned to receive streptozotocin (50 mg/kg body weight) or vehicle (0.05 mol/l sodium citrate, pH 4.5) for five consecutive days, and glycaemia and body weight were measured during the following 3 weeks. Blood glucose levels were measured with Accu-CheK glucometers (Roche Diagnostic, Monza, Italy). Samples of skeletal muscle tissue (gastrocnemius) were collected rapidly after the mice were killed; tissues were snap frozen in liquid nitrogen and stored at −80°C for subsequent analysis. The *Prep1*-hypomorphic mice (*Prep1*$^{i/+}$) have been previously described [31]. All the experiments involving animals were approved by the local ethics committee and conducted in accordance with the principles of laboratory care.

Statistical procedures Statistical analysis was performed with a software package (StatView 5.0; Abacus Concepts, Berkeley, CA, USA) using the Student's *t* test. Values of $p<0.05$ were considered statistically significant.

Results

***Prep1* upregulation by high glucose levels** We have tested the hypothesis that the exposure of L6 skeletal muscle cells to HG concentrations upregulates *Prep1* transcription, thereby impairing glucose metabolism. Total RNA and protein extracts were obtained from L6 myotubes maintained in medium supplemented with either 5.5 mmol/l (normal glucose [NG]) or 25 mmol/l glucose (HG) for 72 h.

Time-course experiments showed that there were already significant increases in *Prep1* expression (both mRNA and protein) at 4 h, while the maximum effect was achieved after 48 and 72 h, respectively, of cell exposure to HG (Fig. 1a, b). The levels of *Prep1* mRNA were specifically increased by glucose, as treatment with equimolar concentrations of either xylose or 2-DG for up to 48 h elicited no effect (Fig. 1c). In parallel, *Prep1* produced a maximal repression of *Glut4* after 4 h of HG exposure, indicating that submaximal upregulation of *Prep1* is sufficient to exert almost complete GLUT4 downregulation (Fig. 1d). The L6 myotubes transiently transfected with *Prep1*-specific shRNA clones showed an increase in 2-DG uptake consistent with the role of *Prep1* on glucose-uptake modulation (Fig. 1e, f).

NF-κB-dependent activation of *Prep1* gene by HG exposure In silico analysis of the rat *Prep1* promoter revealed the presence of an NF-κB binding site at −255 bp from the ATG, and nucleotide alignment of rat and human 5′-flanking regions showed that this region is highly conserved, suggesting an important role in regulating *Prep1* transcription. We therefore used ChIP assays to analyse the NF-κB transcription complex at the *Prep1* promoter of HG-exposed L6 cells. On immunoprecipitation with specific NF-κB p65 antibody, the *Prep1* promoter region was PCR amplified using specific primers for the NF-κB binding site. After 48 h of HG exposure, a >2.5-fold selective increase in occupancy of the *Prep1* promoter by NF-κB p65 was found, compared with barely detectable occupancy in the NG condition (Fig. 2a). In addition, L6 cells transfected with an expression vector encoding NF-κB p65 showed *Prep1* expression increased almost two-fold, supporting the possibility that HG-induced NF-κB

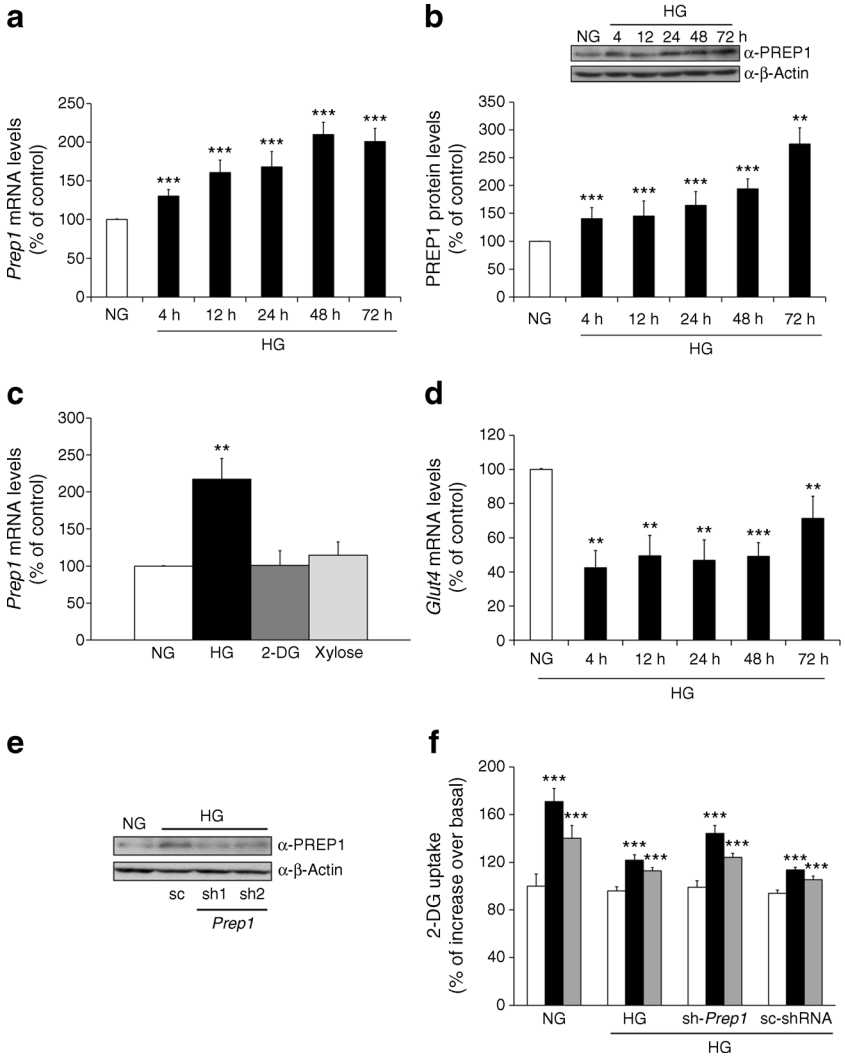

Fig. 1 Effect of HG on *Prep1* expression. L6 cells were cultured under NG or HG conditions for 48 h (**c**, **e**, **f**) or various time periods (**a**, **b**, **d**), or 5.5 mmol/l glucose plus 20 mmol/l 2-DG (dark grey), or 5.5 mmol/l glucose plus 20 mmol/l xylose (light grey) for 48 h (**c**). *Prep1* (**a**, **c**), *Glut4* (**d**) and *Gapdh* mRNA levels were measured by quantitative RT-PCR. Data were normalised for *Gapdh* and are presented as fold increase or decrease relative to the control (NG) (**a**, **c**, **d**). Cells were solubilised, and lysates were analysed by SDS-PAGE and subjected to western blotting with anti PREP1 or β-actin antibodies (α-PREP1, α-β-Actin), as indicated. All blots were analysed by ECL, film detection and quantification of films by densitometry. The blots shown are representative of two additional experiments with very similar results (**b**, **e**). (**f**) L6 myotubes transiently transfected with the *Prep1*-shRNA clones (sh1 and sh2) or with a scrambled shRNA (see **e**) were treated with insulin (10 and 100 nmol/l, grey and black bars, respectively) or without (white bars), and 2-DG uptake was assayed as described in the Methods. Bars represent the mean ± SD of at least three independent experiments, each performed in triplicate. $^{**}p<0.01$ and $^{***}p<0.001$ vs NG; in (**f**) $^{***}p<0.001$ vs untreated cells. sc-shRNA, scrambled shRNA

occupancy of the *Prep1* promoter upregulates *Prep1* transcription (Fig. 2b).

Western blot analysis further showed that NF-κB p65 was upregulated on exposure to HG (Fig. 2c). Activated NF-κB translocates to the nucleus to induce target genes [32]. Accordingly, we have further investigated whether NF-κB delocalises on HG exposure. Indeed, western blot experiments revealed that nuclear levels of NF-κB p65 progressively increased on HG exposure (Fig. 2d).

Two NF-κB inhibitors, JSH-23 and SN-50, which selectively block the nuclear translocation of NF-κB, were used to further assess the involvement of the NF-κB pathway in the glucose-induced effect on *Prep1* transcription. Relevant to our hypothesis, both of these agents attenuated the effect of HG exposure on *Prep1* expression (Fig. 2e, f).

Recruitment of SET7 and p300 at the *Prep1* gene by HG exposure NF-κB interaction with different co-activators, including the p300 histone acetyltransferase and SET7 histone methyltransferase [1, 33, 34], induces NF-κB-dependent gene transactivation and expression. We first demonstrated that, in parallel with the effect of NF-κB, exposure to HG led to a significant increase in the presence of p300 histone acetyltransferase and SET7 histone methyltransferase at the *Prep1*

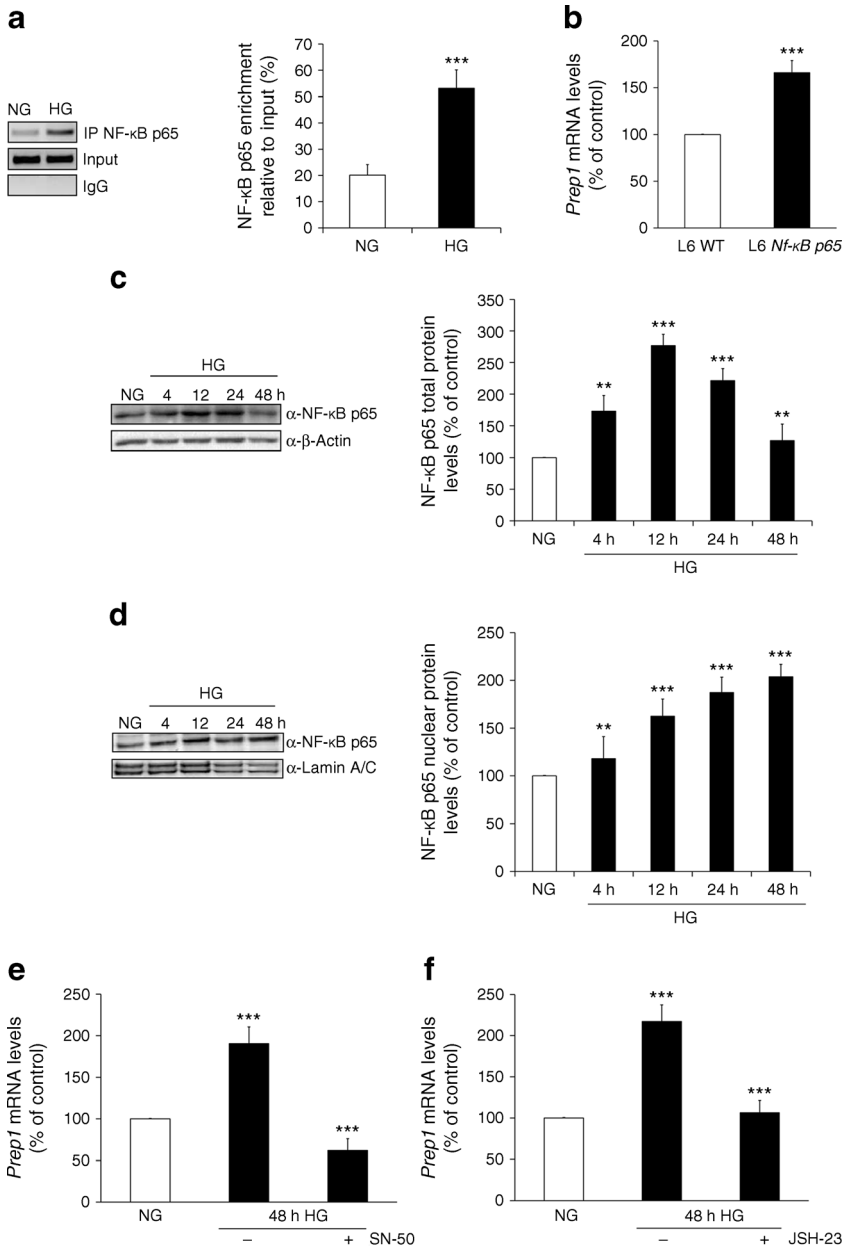

Fig. 2 The effect of HG on NF-κB binding at the *Prep1* promoter. L6 myotubes were cultured in NG or HG for 48 h (**a**) or for various time periods (**c**, **d**). (**a**) ChIP experiments were performed using antibody against NF-κB p65. Results are expressed as enrichment relative to input (%) and corrected for IgG control levels. (**b**) L6 myotubes were transfected with a vector containing the *Nf-κB p65* (also known as *Rela*) cDNA (black bar). At 48 h after transfection, *Prep1* mRNA levels were quantified by RT-PCR. Data were normalised to *Gapdh* mRNA and are expressed as per cent increase vs cells transfected with the empty vector (L6 WT, white bar). Total protein (**c**) and nuclear extracts (**d**) were separated by SDS-PAGE followed by immunoblotting with NF-κB p65, β-actin (**c**) or lamin A/C (**d**) antibodies (α-NF-κB p65, α-β-Actin, α-Lamin A/C), as indicated. All blots were analysed by ECL, film detection and quantification of films by densitometry. Representative blots are shown. (**e**, **f**) L6 myotubes cultured in HG were pretreated with SN-50 (18 μmol/l) or JSH-23 (5 μmol/l) for 48 h. RT-PCR was performed on total RNA to estimate *Prep1* mRNA expression using *Gapdh* as internal control. Bars represent the mean ± SD of three independent experiments, each performed in triplicate. ***$p<0.001$ and **$p<0.01$ vs NG. IP, immunoprecipitate

5′ region (Fig. 3a, b). This event was coincident with the appearance of increased histone H3 acetylation (AcH3) of Lys9 and Lys14 and Lys4 mono- and dimethylation (H3K4me1 and H3K4dime), markers of active transcription (Fig. 3c, d). Time-course experiments showed that AcH3 levels were specifically increased by glucose, as treatment with equimolar concentrations of 2-DG had no effect (ESM Fig. 1).

We next tested the possibility that, in L6 cells, the HG-induced recruitment of p300 and SET7 depends on NF-κB using re-ChIP experiments. We sequentially performed ChIP assays with NF-κB p65 and then with either p300 or SET7 antibody. As shown in Fig. 3e, f, these experiments showed the co-recruitment of NF-κB p65 with both p300 and SET7 on HG exposure of the cells, suggesting they might depend on NF-κB presence at the *Prep1* gene.

To explore this hypothesis in greater detail, we analysed whether NF-κB inhibition affects the occurrence of these chromatin events at the *Prep1* promoter in response to HG. The ChIP-enriched DNA generated using SET7 and p300 antibodies was subjected to PCR using primers spanning the NF-κB binding site. Interestingly, on JSH-23 treatment of the cells, the increased recruitment of SET7 and p300, as well as the increased levels of H3K4me1, H3K4me2 and AcH3, induced by HG at the *Prep1* 5′ region, were attenuated (Fig. 3g), indicating that they depend on NF-κB recruitment.

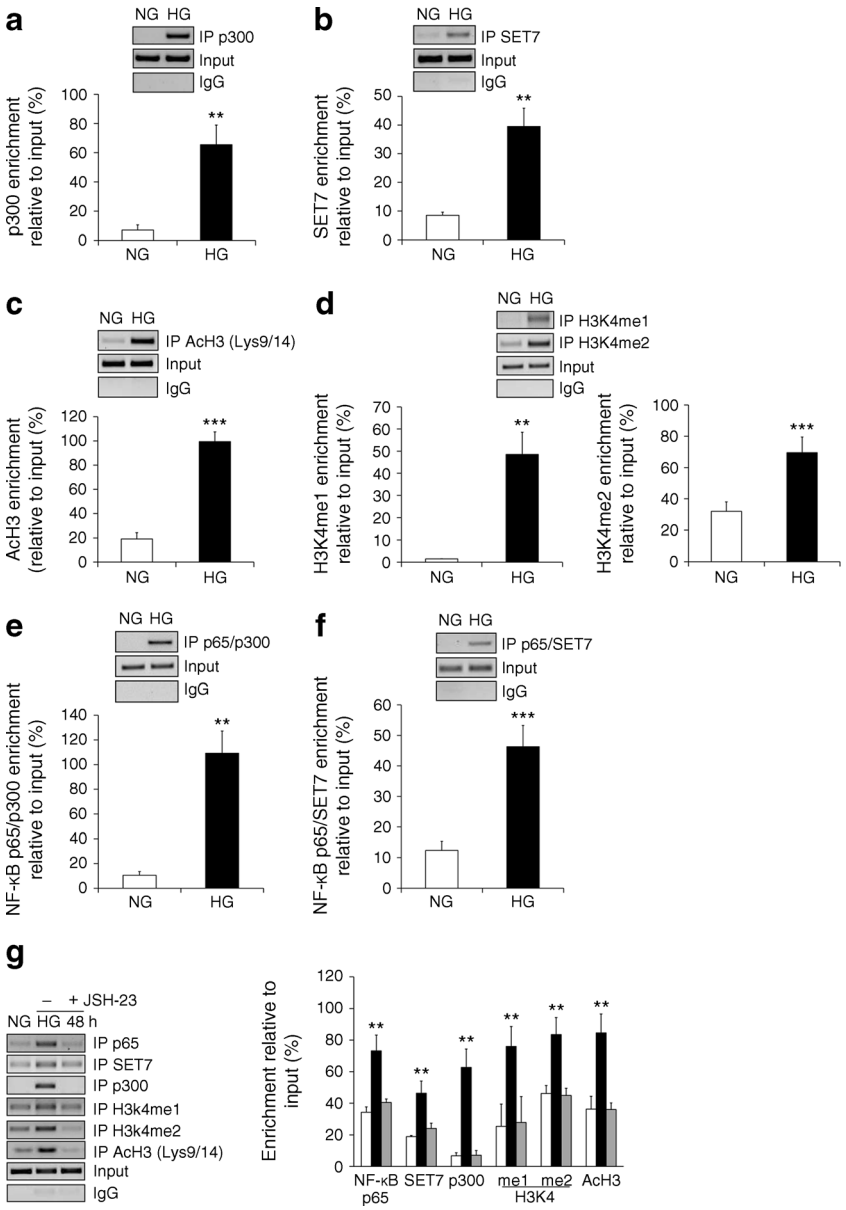

Fig. 3 Recruitment of CBP/p300 and SET7 at the *Prep1* gene is NF-κB p65 dependent. L6 myotubes were cultured in NG and HG (white and black bars, respectively, **a**–**f**) or in NG and HG and in the presence of 5 μmol/l JSH-23 (grey bars) for 48 h (**g**). ChIPs were performed using antibodies against CBP/p300 (**a**, **g**), SET7 (**b**, **g**), AcH3 (**c**, **g**), H3K4me1 and H3K4me2 (**d**, **g**) and NF-κB p65 (**e**–**g**). ChIP was followed by PCR amplification with primers for the NF-κB binding site at the rat *Prep1* promoter. Re-ChIPs were performed using antibodies against NF-κB p65 (first ChIP) and CBP/p300 (**e**) and SET7 (**f**) (second ChIPs) to identify protein complexes at the NF-κB binding site on the *Prep1* promoter. The amount of precipitated DNA from the first ChIP was used as input. Amplification products were separated on agarose gels and revealed by ethidium bromide. Representative gels are shown. Results are expressed as enrichment relative to input (%) and corrected for IgG control levels. Bars represent the mean ± SD of three independent measurements each in triplicate. ***$p<0.001$ and **$p<0.01$ vs NG. IP, immunoprecipitate

***Prep1* expression in STZ-treated mice** To further address the in vivo role of glucose on *Prep1* expression, we impaired beta cell function in C57BL/6J mice by low-dose STZ administration (50 mg/kg body weight). Plasma glucose levels significantly increased in these animals, while body weight did not change during treatment (Fig. 4a, b). Interestingly, increased *Prep1* expression (both mRNA and protein) was also observed in skeletal muscle tissue from STZ-treated mice (Fig. 4c, d). ChIP assays performed with DNA from these tissues exhibited enhanced NF-κB recruitment at the *Prep1* promoter accompanied by increased histone H3 acetylation at Lys9 and Lys14 and histone H3 mono- and dimethylation at Lys4 (Fig. 4e). Thus, hyperglycaemia is associated with histone modifications at the *Prep1* gene in vivo as well as in vitro. As PREP1 attenuates insulin signal transduction and reduces glucose storage in liver [35], we investigated the effect of hyperglycaemia on histone H3 Lys9 acetylation and Lys4 mono and dimethylation on the *Prep1* gene in liver from STZ-treated mice. No differences were found between the two groups of animals (ESM Fig. 2), suggesting that different mechanisms underlie *Prep1* regulation by hyperglycaemia in muscle tissue and liver.

Myocyte enhancer factor 2/histone deacetylase 5 interactions on *Glut4* gene in *Prep1*-overexpressing cells and in STZ-treated mice To mechanistically clarify the functional consequences of the increased expression of *Prep1* in skeletal muscle, we stably transfected L6 myotubes with a vector driving the expression of the human *Prep1* cDNA($L6_{Prep1}$) [36]; two clones, $L6_{Prep1Cl2}$, and $L6_{Prep1Cl4}$, showing increased

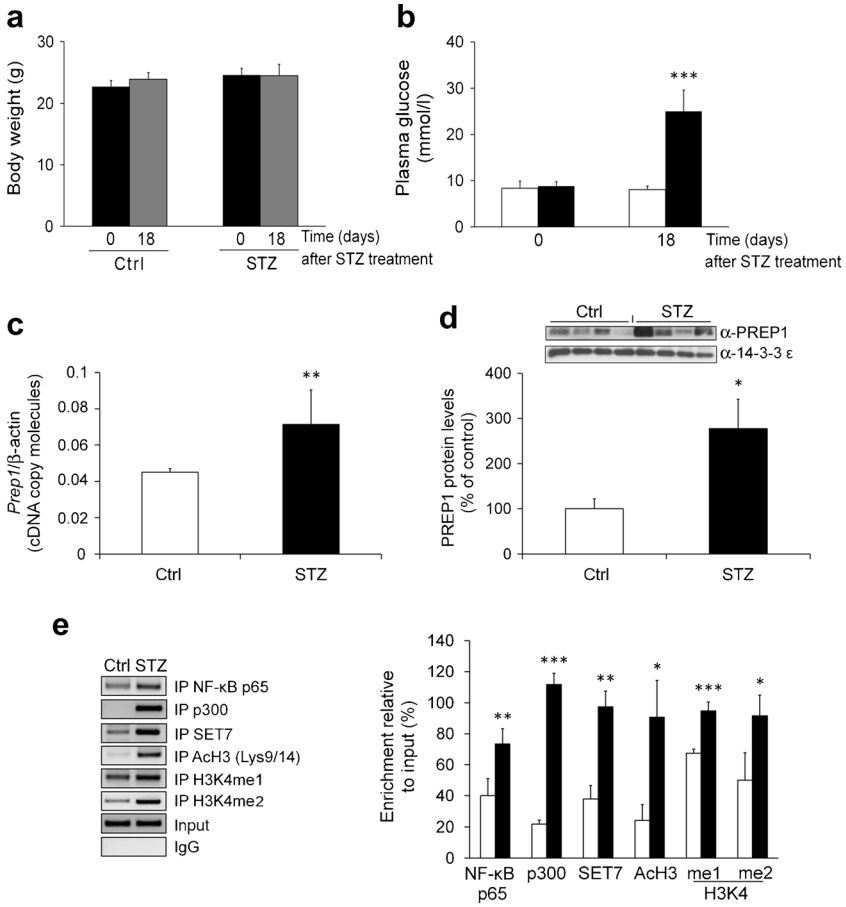

Fig. 4 *Prep1* expression in STZ-induced hyperglycaemic mice. Male C57BL/6J mice were subjected to daily i.p. administration of either STZ (50 mg/kg body weight) or citrate buffer, as control, for five consecutive days as described in the Methods. Body weight (**a**) and plasma glucose levels (**b**) were assessed both before and during the next 18 days after STZ treatment (seven animals per treatment group). (**c**) Analysis of *Prep1* mRNA levels by absolute real-time PCR quantification method in skeletal muscle tissue from STZ-treated and control mice. Results are expressed as *Prep1* cDNA copy molecules normalised to β-actin cDNA molecules. (**d**) Total protein extracts were separated by SDS-PAGE followed by immunoblotting with PREP1 or 14-3-3ε antibodies, as indicated (α-PREP1, α-14-3-3ε). All blots were analysed by ECL, film detection and quantification of films by densitometry. (**e**) ChIP was performed using antibodies against NF-κB p65, SET7, p300, AcH3 and H3K4me1 and H3K4me2 on the *Prep1* gene both in STZ-treated and control mice. Representative gels are shown. Results are expressed as enrichment relative to input (%) and corrected for IgG control levels. Bars represent the mean ± SD of three separate experiments $*p<0.05$, $**p<0.01$ and $***p<0.001$ STZ-treated vs control mice. White bars, control; black bars, STZ. Ctrl, control; IP, immunoprecipitate

Prep1 expression (Fig. 5a, b) were studied in detail. ChIP assays performed in these cell clones revealed increased association of the monocyte enhancer factor 2 (MEF2) transcription factor with histone deacetylase 5 (HDAC5) at the *Glut4* promoter (Fig. 5c), indicating a positive effect of PREP1 on promoter recruitment of the MEF2/HDAC5 transcriptional repressor. Incubation of the $L6_{Prep1}$ cell clones with the deacetylase inhibitor trichostatin A reverted the *Glut4* repression occurring in these cells (Fig. 5d). Consistent with these findings, Re-ChIP experiments performed with DNA from the skeletal muscle tissue of STZ-treated mice showed that the increased *Prep1* expression was accompanied by a significantly greater occupancy of the *Glut4* promoter by MEF2/HDAC5 complexes (Fig. 5e). It has been reported that AMPK is a potential HDAC5 kinase; increased AMPK activity is associated with HDAC5 dissociation from MEF2A and subsequently increased GLUT4 expression [37]. As shown in Fig. 5g, active AMPK was significantly decreased in STZ-treated mice presenting a major MEF2/HDAC5 association on the *Glut4* promoter (see Fig. 5e). In contrast, active AMPK was increased in skeletal muscle of *Prep1* hypomorphic mice ($Prep1^{i/+}$) (Fig. 5h) where the level of *Glut4* promoter occupancy by the MEF2/HDAC5 transcriptional repressor was reduced (Fig. 5f). In conclusion, these results indicate that, in vivo, epigenetic dysregulation of *Prep1* in response to HG deregulates the main *Glut4* insulin-dependent transporter, probably through a mechanism involving AMPK.

Discussion

How the environment, both the external and internal milieu, impacts on the evolution towards type 2 diabetes and its co-

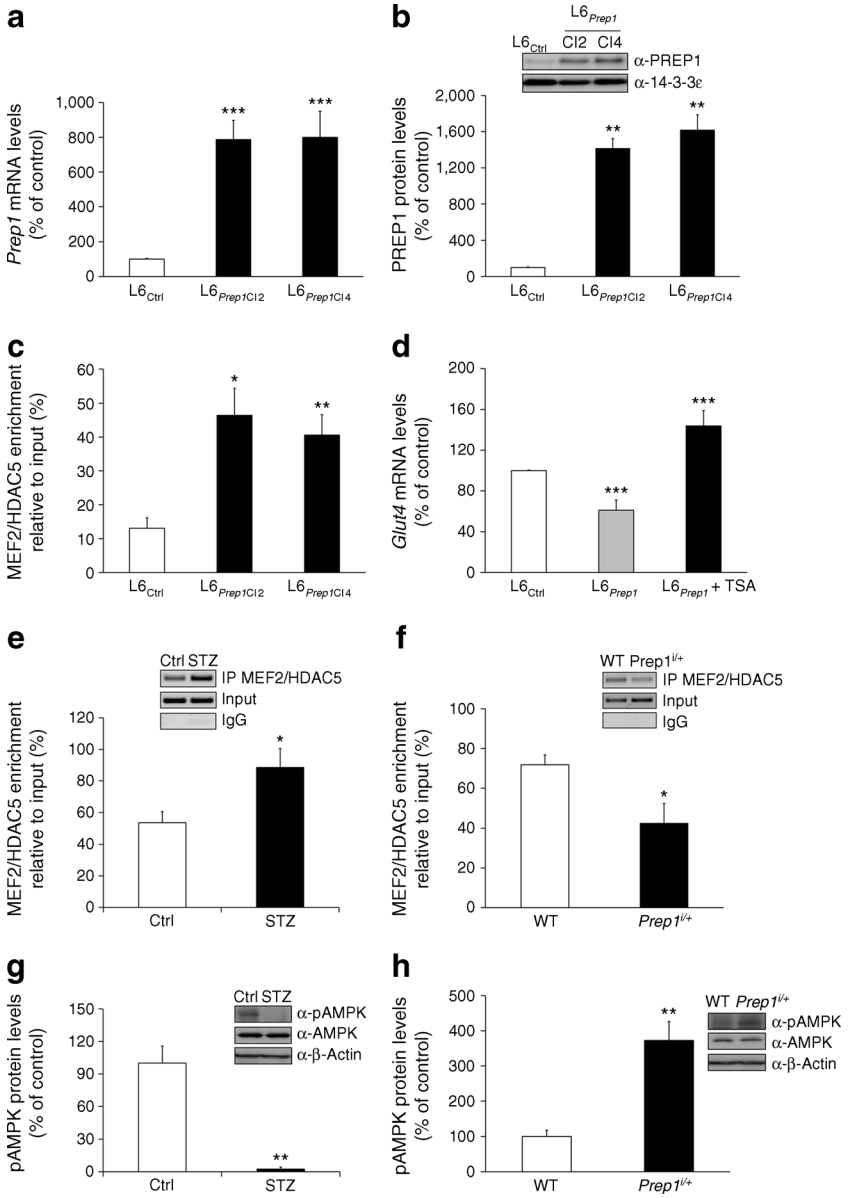

Fig. 5 Effect of *Prep1* overexpression on the *Glut4* gene. L6 myotubes were stably transfected with the pRc/CMV-*Prep1* expression vector. Total RNA was obtained from *Prep1*-overexpressing ($L6_{Prep1Cl2}$, $L6_{Prep1Cl4}$) and control $L6_{Ctrl}$ cells (cells transfected with the empty plasmid) (**a**) or from $L6_{Prep1}$ cells treated with 330 nmol/l trichostatin A (black bar) and $L6_{Ctrl}$ and $L6_{Prep1}$ cells treated with vehicle (white and grey bar, respectively) for 24 h (**d**). *Prep1* (**a**), *Glut4* (**d**) and *Gapdh* mRNA levels were measured by quantitative RT-PCR. Data were normalised for *Gapdh* and presented as fold increase or decrease vs control ($L6_{Ctrl}$). (**b**) Total protein extracts were separated by SDS-PAGE followed by immunoblotting with PREP1 or 14-3-3ε antibody. (α-PREP1, α-14-3-3ε) (**c**, **e**, **f**) Re-ChIP experiments were performed using antibodies against MEF2 (first ChIP) and HDAC5 (second ChIP) to identify protein complexes at the MEF2 binding site on the *Glut4* promoter. The amount of precipitated DNA from the first ChIP was used as input. Results by qPCR are expressed as enrichment relative to input (%) and corrected for IgG control levels (**c**, **e**, **f**). Representative gels are shown (**e**, **f**). Protein samples from skeletal muscle tissue from STZ-treated (black bars) and control mice (white bars) (**g**) and from $Prep1^{i/+}$ (black bars) and wild-type mice (white bars) (**h**) were analysed by western blot with pAMPK and AMPK antibodies, and with actin antibody for normalisation (α-pAMPK, α-AMPK, α-β-Actin). All blots were analysed by ECL, film detection and quantification of films by densitometry. The autoradiograph shown is representative of four independent experiments. Bars represent the mean ± SD from each group of animals (control mice vs STZ-treated mice or wild-type mice vs $Prep1^{i/+}$ mice). In a–d ***$p<0.001$, **$p<0.01$ and *$p<0.05$ vs $L6_{Ctrl}$; in e–h **$p<0.01$ and *$p<0.05$ vs control or wild type. Ctrl, control; IP, immunoprecipitate; TSA, trichostatin A; WT, wild-type

morbidities has been only partially investigated from a mechanistic perspective. However, the role of the dynamic epigenome in contributing the major traits of type 2 diabetes is receiving progressively greater attention because this information may expand present understanding of the pathogenesis of type 2 diabetes [38–40].

In the present work, we focused on *Prep1*, a gene exerting major effects on the insulin sensitivity of glucose transport, as

its overexpression causes insulin resistance [36]. We have shown that L6 skeletal muscle cells exposed to elevated glucose concentrations exhibit increased *Prep1* expression. This effect was preceded by HG-induced recruitment of NF-κB p65 at the *Prep1* 5′-flanking region, along with recruitment of the p300 histone acetyltransferase and SET7 histone methyltransferase. Indeed, HG exposure was associated with increased histone H3 Lys 9/14 acetylation and Lys4 mono- and dimethylation at the promoter. All of these events were significantly attenuated by different NF-κB pharmacologic inhibitors, indicating the priming effect of NF-κB recruitment in driving the action of epigenetic enzymes at the *Prep1* 5′ regulatory region.

In addition, we have obtained positive evidence that these events are functionally relevant in vivo as well as in cultured cells. Consistent with this possibility, we have demonstrated increased *Prep1* expression in skeletal muscle tissue of STZ-treated and hyperglycaemic mice compared with normoglycaemic mice. Very much like the HG-exposed L6 cells, muscle from the STZ-treated mice featured enhanced NF-κB p65 occupancy associated with increased Lys9/14 acetylation and Lys4 mono- and dimethylation of the *Prep1* 5′ regulatory region.

Hyperglycaemia is known to promote inflammatory processes through different mechanisms, including oxidative stress and abnormally elevated protein *O*-linked *N*-acetylglucosaminylation (*O*-GlcNAcylation) [22]. Chronic low-grade inflammation has a major role in the development of insulin resistance, with many initiating events converging on NF-κB signalling [41–44]. The present work has now identified *Prep1*, a previously unrecognised gene downstream of NF-κB, as capable of inducing insulin resistance in response to inflammatory stimuli. Consistent with this proposal, exposure of cultured muscle cells to the oxidative-stress-generating agent hydrogen peroxide also enhances *Prep1* transcription (data not shown).

As to the functional consequences of *Prep1* upregulation, previous work by our group demonstrated that the overexpression of *Prep1* in L6 skeletal muscle cells reduces the amount of GLUT4 protein [36]. Total GLUT4 levels in muscle tissue are largely regulated at the transcriptional level [45], with the MEF2 transcription factor playing an essential role in fine-tuning the process [46]. Indeed, reduced MEF2 expression is accompanied by suppression of *Glut4* transcription [46]. In many cell types, MEF2 associates with the histone deacetylase transcriptional repressor HDAC5, which suppresses transcription by recruiting factors with HDAC activity and compacting chromatin structure [47]. In the present report, we show that *Prep1* overexpression in L6 myotubes augments the presence of MEF2/HDAC5 complex at the *Glut4* promoter, suggesting that *Prep1* itself induces HDAC recruitment at the *Glut4* gene. Although it is not clear whether HDAC5 directly regulates the *Glut4* gene, it has been previously observed that HDAC5 dissociation from MEF2 and subsequent HDAC5 nuclear export is associated with increased *Glut4* gene expression in human skeletal muscle [48]. The enzyme AMPK is an HDAC5 kinase; indeed, phosphorylation of HDAC5 on Ser259 and Ser498 by AMPK causes HDAC5 dissociation from MEF2 and concomitantly increases *Glut4* gene expression [37]. Here, we propose a mechanism in which PREP1, by modulating AMPK activation, regulates the association of MEF2/HDAC5 on the *Glut4* promoter. This possibility is corroborated by our in vivo findings. In fact, we have shown that in the STZ-treated mouse model showing elevated *Prep1* expression: (1) AMPK activation decreased while the presence of the MEF2/HDAC5 repressor complex at the *Glut4* promoter increased; and (2) in *Prep1*$^{i/+}$ hypomorphic mice, expressing low *Prep1* mRNA levels, the abundance of MEF2/HDAC5 repressor complex is significantly reduced, while AMPK activation is enhanced.

In contrast to expression in adipocytes, skeletal muscle GLUT4 expression is not compromised in diabetes and obesity [49]. However, overexpression of GLUT4 in skeletal muscle ameliorates the insulin resistance associated with these diseases [50–52]. Therefore, clarifying the molecular mechanism underlying GLUT4 expression in skeletal muscle might enable the identification of therapeutic targets for the treatment of insulin resistance associated with type 2 diabetes and obesity.

Despite lacking evidence for PREP1 expression in skeletal muscle tissue from patients with type 2 diabetes, our findings made *Prep1* an attractive gene through which to investigate how the environment impinges on individual genomes affecting the evolution towards diabetes. The effect of glucose, a major component of the internal milieu, on *Prep1* histone modification and transcriptional activity led us to propose that other environmental factors, including lifestyle-related factors that facilitate the development of low-grade chronic inflammation, may also induce *Prep1* to cause insulin resistance through epigenetic mechanisms, including DNA methylation, as well as histone modifications, and facilitate type 2 diabetes progression. Lifestyle factors are commonly shared in family groups, and whether the environment induces changes in *Prep1* in humans is an important issue presently under investigation in the laboratory.

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Contribution statement MC was the main contributor in terms of conception, design, acquisition and interpretation of data, and drafting the article. VV, LA, ML, SC, AL and ML contributed to the conceptual design and the acquisition of data. FO, GLR, PEM, PF and FB contributed to the conceptual design, analysis and interpretation of data and discussion of the results. PU contributed to the conceptual design, interpretation and discussion of the results and the supervision of the overall work. All of the authors critically revised the article and approved the final version. PU is responsible for the integrity of the work as a whole.

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