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BASIC SCIENCE
Vascular biology

Interplay among H3K9-editing enzymes SUV39H1, JMJD2C and SRC-1 drives p66^{Shc} transcription and vascular oxidative stress in obesity

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Aims Accumulation of reactive oxygen species (ROS) promotes vascular disease in obesity, but the underlying molecular mechanisms remain poorly understood. The adaptor p66^{Shc} is emerging as a key molecule responsible for ROS generation and vascular damage. This study investigates whether epigenetic regulation of p66^{Shc} contributes to obesity-related vascular disease.

Methods and results ROS-driven endothelial dysfunction was observed in visceral fat arteries (VFAs) isolated from obese subjects when compared with normal weight controls. Gene profiling of chromatin-modifying enzymes in VFA revealed a significant dysregulation of methyltransferase SUV39H1 (fold change, -6.9, $P < 0.01$), demethylase JMJD2C (fold change, 3.2, $P < 0.01$), and acetyltransferase SRC-1 (fold change, 5.8, $P < 0.01$) in obese vs. control VFA. These changes were associated with reduced di-(H3K9me2) and trimethylation (H3K9me3) as well as acetylation (H3K9ac) of histone 3 lysine 9 (H3K9) on p66^{Shc} promoter. Reprogramming SUV39H1, JMJD2C, and SRC-1 in isolated endothelial cells as well as in aortas from obese mice (*Lep*$^{Ob/Ob}$) suppressed p66^{Shc}-derived ROS, restored nitric oxide levels, and rescued endothelial dysfunction. Consistently, *in vivo* editing of chromatin remodellers blunted obesity-related vascular p66^{Shc} expression. We show that SUV39H1 is the upstream effector orchestrating JMJD2C/SRC-1 recruitment to p66^{Shc} promoter. Indeed, SUV39H1 overexpression in obese mice erased H3K9-related changes on p66^{Shc} promoter, while SUV39H1 genetic deletion in lean mice recapitulated obesity-induced H3K9 remodelling and p66^{Shc} transcription.

Conclusion These results uncover a novel epigenetic mechanism underlying endothelial dysfunction in obesity. Targeting SUV39H1 may attenuate oxidative transcriptional programmes and thus prevent vascular disease in obese individuals.

Keywords Epigenetics • Chromatin remodelling • Vascular disease • Oxidative stress • Endothelial dysfunction • Obesity

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Translational perspective

Current knowledge of the molecular pathways underlying vascular disease in patients with obesity is limited, and novel breakthrough therapies are in high demand. In this work, we unveil new epigenetic connections underpinning oxidative stress and vascular dysfunction in experimental and human obesity. We show that a dynamic interplay among histone 3 lysine 9 (H3K9)-editing enzymes SUV39H1, SRC-1, and JMJD2C drives transcription of the mitochondrial adaptor $p66^{Shc}$, a pivotal modulator of vascular oxidative stress. These translational findings open perspectives for pharmacological targeting of epigenetic networks to correct deregulated gene expression as a strategy to alleviate the clinical burden of obesity-driven cardiovascular disease.

Introduction

Worldwide at least 2.8 million people die each year as a result of being overweight or obese.[1,2] Among the constellation of weight-related co-morbidities, cardiovascular disease (CVD) carries the largest burden for obese patients.[3,4] Indeed, the risk of coronary heart disease and ischaemic stroke rises steadily with increasing body mass index.[5] A large body of evidence supports the notion that endothelial dysfunction contributes to the pathogenesis of obesity-related CVD.[6] Seminal work has revealed that generation of reactive oxygen species (ROS) is a key event preceding the development of endothelial dysfunction and vascular atherosclerotic disease in this setting.[6,7] Furthermore, the expression of several pro-oxidant genes is derailed in obese subjects and may participate to adverse vascular phenotypes.[8,9] However, the molecular orchestrators of vascular redox signalling remain to be fully elucidated. Plastic modifications of chromatin architecture have shown to repress or activate gene expression, thus representing a novel underpinning of disease states.[10–12] Eukaryotic chromatin is structured into highly organized complexes—nucleosomes—which include chromosomal DNA packaged around histone proteins.[13] A key mode of regulation that controls chromatin organization is the covalent modification (i.e. acetylation and methylation) of specific aminoresidues on histone tails.[10] These modifications may cluster in different patterns to regulate chromatin accessibility.[14] For example, acetylation of histone 3 at lysine 9 (H3K9ac) residue is associated with an open chromatin and active gene transcription, whereas H3K9 methylation favours the shift towards heterochromatin, characterized by condensed and repressed genomic regions.[15]

Understanding the regulation of ROS-generating pathways is one of the most important challenges in cardiovascular research.[16] Reactive oxygen species, besides irreversibly modifying organelles and macromolecules, also trigger important events such as senescence, inflammation, and multiple cell death pathways.[16] In this regard, the adaptor $p66^{Shc}$ protein is part of a complex mitochondrial system regulating endogenous production of free radicals and apoptosis.[17,18] In the mitochondrial intermembrane space, $p66^{Shc}$ oxidizes cytochrome *c* shifting electrons towards the production of ROS and subsequent opening of permeability transition pore (PTP).[19,20] This latter event increases mitochondrial membrane permeability to ions, solutes, and water triggering swelling and disruption of the organelle with release of proapoptotic factors.[21] By this background, we have postulated that chromatin modifications may regulate vascular ROS by altering the transcription of $p66^{Shc}$.[19,22] Intracellular free radicals are reduced in cells lacking the $p66^{Shc}$ gene as well as in $p66^{Shc-/-}$ mouse models exposed to high oxidative stress.[17,23] Moreover, reduced production of ROS resulting from inhibition of $p66^{Shc}$ pathway is associated with preservation of mitochondrial integrity and resistance to apoptosis induced by a variety of different mediators such as hydrogen peroxide, growth factor deprivation, and ultraviolet radiation.[6,19] In this study, we show that a complex interplay among chromatin remodellers SUV39H1, JMJD2C, and SRC-1 drives the transcription of the adaptor $p66^{Shc}$ and subsequent endothelial dysfunction in the vasculature of obese subjects and mice. These mechanistic insights offer novel molecular targets pointing towards potential epigenetic drugs for the prevention of oxidative burst and vascular dysfunction in obesity.

Methods

A detailed description of the Methods is reported in Supplementary Material online.

Study population

Obese patients were recruited among 220 consecutive patients from January 2010 to January 2014 for screening in view of laparoscopic bariatric surgery. Control subjects were recruited among patients hospitalized in the Surgery Unit (University of Pisa, Italy) to undergo laparoscopic surgery for cholecystectomy. The study protocol was approved by local ethics committee, and, in accordance with institutional guidelines, all participants were aware of the investigational nature of the study and signed a written consent for their participation.

Preparation of small visceral fat arteries

Small visceral fat arteries (150–300 μm, approximately 2 mm long) were isolated from visceral fat immediately after biopsy sample procurement, performed during laparoscopic surgery, and mounted on 2-glass microcannulae in a pressurized myograph, as previously described.[24]

Mice

C57BL/6 $Lep^{Ob/Ob}$ and $Lep^{Ob/Ob}/p66^{-/-}$ double-mutant mice were kindly provided by Dr M. Giorgio (European Institute of Oncology, Milan, Italy). Homozygous SUV39H1-mutant mice ($SUV39H1^{-/-}$) on a pure C57BL/6 background and SUV39H1 wild-type (WT) littermates were provided by Dr Thomas Jenuwein and Dr Andrew J. Pospisilik (Department of Epigenetics, Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany). Animal experiments were conducted in accordance with the guidelines approved by the Institutional Animal Care Committee of the University of

Table 1 Demographics and laboratory parameters in the study population

	Controls ($n = 20$)	Obese ($n = 21$)	*P*-value
Demographics			
Age (years)	49.5 (4.6)	48.4 (7.0)	0.551[a]
Gender (F:M)	11:9	13:8	0.690[b]
Body mass index (kg/m^2)	24.0 (1.9)	42.2 (5.0)	<0.001[a]
Waist circumference (cm)	92.9 (5.7)	135.3 (13.3)	<0.001[a]
Systolic blood pressure (mmHg)	125.5 (7.2)	129.9 (10.0)	0.116[a]
Diastolic blood pressure (mmHg)	80.0 (76.5–82.0)	84.0 (80.0–87.0)	0.048[c]
Heart rate (bpm)	71.2 (6.2)	78.9 (7.1)	0.001[a]
Laboratory parameters			
Triglycerides (mg/dL)	119.5 (20.9)	141.4 (38.6)	0.031[a]
HDL cholesterol (mg/dL)	50.5 (44.5–63.0)	42.0 (37.5–50.0)	0.001[c]
LDL cholesterol (mg/dL)	124.6 (20.3)	138.7 (17.6)	0.022[a]
Total cholesterol (mg/dL)	202.4 (18.0)	210.7 (18.7)	0.158[a]
FPG (mg/dL)	84.3 (7.7)	103.1 (7.9)	<0.001[a]
$Hb1_{Ac}$ (%)	5.2 (0.2)	5.9 (0.1)	<0.001[a]
Fasting insulin (μU/mL)	7.0 (0.5)	13.4 (1.3)	<0.001[a]
HOMA-IR	1.5 (0.2)	3.7 (0.5)	<0.001[a]
Creatinine (mg/dL)	0.9 (0.2)	0.8 (0.2)	0.115[a]

Values are expressed as mean (SD) or median (interquartile range). *P*-value refers to Student's *t*-test and χ^2-tests.
BMI, body mass index; DBP, diastolic blood pressure; FPG, fasting plasma glucose; $Hb1_{Ac}$, glycated haemoglobin; HDL-C, high density lipoprotein cholesterol; HOMA-IR, homeostasis model assessment; HR, heart rate; LDL-C, low density lipoprotein cholesterol; SBP, systolic blood pressure; TG, triglycerides.
[a]Reported *P*-values are from unpaired two-sample *t*-test, unless otherwise stated.
[b]From χ^2 test.
[c]From Mann–Whitney *U* test.

Rome 'Sapienza' (Rome, Italy), University of Zürich (Zurich, Switzerland), and Max Planck Institute (Freiburg, Germany).

Statistical analysis

The normality of continuous variables was assessed by the Kolmogorov–Smirnov test. All normally distributed variables are expressed as mean (standard deviation), unless otherwise stated. Data not normally distributed are shown as median (interquartile range). Comparisons of continuous variables were performed using unpaired two-sample *t*-test and Mann–Whitney *U* test, as appropriate. Categorical variables were compared by using the χ^2 test. All multiple comparisons between normally distributed variables were performed by one-way analysis of variance (ANOVA), followed by Bonferroni correction. Between variable correlations were assessed by Spearman's test. A multiple *t*-test using the Benjamini–Hochberg false discovery rate procedure was employed for the analysis of gene expression data [real-time polymerase chain reaction (PCR) array]. Probability values <0.05 were considered statistically significant. All analyses were performed with GraphPad Prism Software (version 7.03).

Results

p66^{Shc} up-regulation and endothelial dysfunction in obese subjects

We first investigated the link between p66^{Shc} gene expression, oxidative stress, and endothelial dysfunction in visceral fat arteries (VFAs) isolated from 21 obese and 20 control subjects. Characteristics of study population are shown in *Table 1*. Isometric tension studies in VFA showed an impairment of acetylcholine-induced vasorelaxation in obese subjects when compared with the controls (*Figure 1A*), whereas response to sodium nitroprusside (SNP) did not differ between the two groups (see Supplementary material online, *Figure S1*). Pretreatment with the antioxidant ascorbic acid restored relaxation to acetylcholine, suggesting that ROS contribute to endothelial dysfunction in this setting (*Figure 1A*). Gene expression of p66^{Shc} was significantly higher in obese when compared with the control VFA (*Figure 1B*). Moreover, messenger RNA levels of p66^{Shc} negatively correlated with endothelium-dependent vasorelaxation in VFA from obese patients (*Figure 1C*).

Obesity-induced epigenetic remodelling of p66^{Shc} promoter

To investigate epigenetic regulation of p66^{Shc} in obesity, real-time PCR array for chromatin-modifying enzymes was performed in VFA isolated from obese and normal weight individuals. A dysregulation of 27 of 84 total genes was observed in obese when compared with control VFA. Of these, 21 were up-regulated and 6 down-regulated by more than two-fold (*Figure 2A*, see Supplementary material online, *Table S1*). Since epigenetic regulation of p66^{Shc} occurs on histone 3 (H3)-binding human promoter region, chromatin immunoprecipitation (ChIP) was performed to unveil the H3 epigenetic modifiers interacting with p66^{Shc} promoter (*Figure 2B*). ChiP assays showed that only methyltransferase SUV39H1, demethylase JMJD2C, and

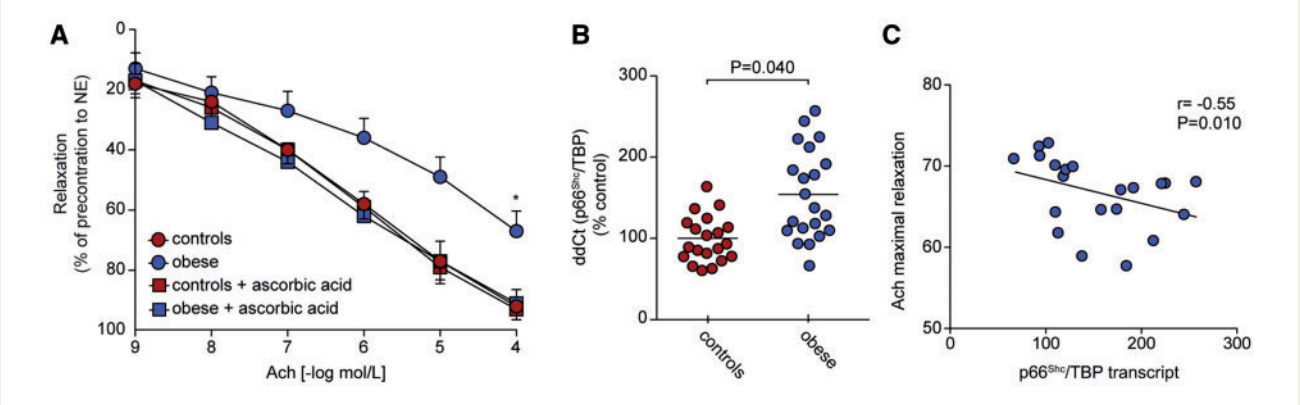

Figure 1 p66^{Shc} up-regulation, oxidative stress, and endothelial dysfunction in obese subjects. (*A*) Endothelium-dependent relaxations to acetylcholine in small visceral fat arteries isolated from obese subjects and age-matched normal weight controls, in the presence or in the absence of antioxidant ascorbic acid; (*B*) Gene expression of the mitochondrial adaptor p66^{Shc} in isolated vessels from the two groups; (*C*) Spearman correlations of p66^{Shc} gene expression with maximal relaxations to acetylcholine in obese patients. Data are expressed as means ± standard deviation, $n = 20–21$ per group. Ach, acetylcholine; AA, ascorbic acid.

acetyltransferase SRC-1, involved in epigenetic remodelling of H3K9, were bound to p66^{Shc} promoter (*Figures 2C* and see Supplementary material online, *Figure S2*). Di- (H3K9me2) and tri-methylation (H3K9me3) of H3K9 on p66^{Shc} promoter were reduced, whereas H3K9 acetylation (H3K9ac) was increased in obese when compared with the control VFA (*Figure 2D*). These findings suggest that deregulation of H3K9-modifying enzymes SUV39H1, JMJD2C, and SRC-1 may significantly contribute to p66^{Shc} transcription in the vasculature of obese subjects.

p66^{Shc} contributes to reactive oxygen species-dependent vascular dysfunction in obese mice

To delineate a causal role of p66^{Shc} in obesity-driven vascular dysfunction, genetically obese mice (leptin deficient, *Lep*$^{Ob/Ob}$) were crossed with p66^{Shc} knockout mice (*p66*$^{-/-}$) to generate double-mutant animals (*Lep*$^{Ob/Ob}$*p66*$^{-/-}$). *Lep*$^{Ob/Ob}$ and *Lep*$^{Ob/Ob}$*p66*$^{-/-}$ showed similarly increased body weight and dysglycaemia when compared with WT littermates (see Supplementary material online, *Figure S3*). In line with human findings, p66^{Shc} gene expression was significantly higher in the vasculature of obese mice (*Figure 3A*). Endothelium-dependent relaxation to acetylcholine was impaired in *Lep*$^{Ob/Ob}$ when compared with WT littermates, whereas double-mutant mice were protected against endothelial dysfunction (*Figure 3B*). In contrast, relaxation to SNP did not change across the different groups (see Supplementary material online, *Figure S4*). The O_2^- scavenger polyethylene glycol–superoxide dismutase (PEG–SOD) rescued the response to acetylcholine in obese *Lep*$^{Ob/Ob}$ mice, thus confirming that endothelial dysfunction is driven by ROS (*Figure 3C*).

Reprogramming H3K9-editing enzymes suppresses obesity-induced p66^{Shc} transcription and endothelial dysfunction

H3K9-editing enzymes SUV39H1, JMJD2C, and SRC-1 were also deregulated in *Lep*$^{Ob/Ob}$ aorta (*Figure 3D* and see Supplementary material online, *Figures S5* and *S6*). ChIP assays showed interaction of these chromatin-modifying enzymes with the murine p66^{Shc} promoter (*Figure 3E*). Moreover, H3K9me2 and H3K9me3 levels were reduced, whereas H3K9ac was increased on p66^{Shc} promoter of obese mice when compared with lean controls (*Figure 3F*). To define the relative contribution of SUV39H1, JMJD2C, and SRC-1 to obesity-related endothelial dysfunction, their expression was selectively reprogrammed in freshly isolated endothelial cells as well as in aortas from *Lep*$^{Ob/Ob}$. In *Lep*$^{Ob/Ob}$ endothelial cells, either overexpression of SUV39H1 or gene silencing of JMJD2C and SRC-1 blunted p66^{Shc} up-regulation and O_2^- generation while restoring nitric oxide levels (*Figure 3G–I*). Interestingly, *ex vivo* reprogramming of SUV39H1, JMJD2C, and SRC-1 rescued endothelial dysfunction in aortas from *Lep*$^{Ob/Ob}$ mice (*Figure 3J*). Of note, *in vivo* editing of H3K9-chromatin modifiers by chronic i.v. administration of SUV39H1 overexpressing vector or small-interfering RNAs (siRNAs) for JMJD2C and SRC-1 significantly attenuated vascular p66^{Shc} expression in obese mice (*Figure 4A* and *B*).

Methyltransferase SUV39H1 triggers p66^{Shc} expression by orchestrating chromatin accessibility

Although SUV39H1, JMJD2C, and SRC-1 are all critically involved in epigenetic regulation of *p66*^{Shc}, it was crucial to unmask a putative molecular hierarchy among them. By ChIP experiments, we found that recruitment of acetyltransferase SRC-1 to p66^{Shc} promoter is significantly reduced in the vasculature of obese mice treated with either SUV39H1 overexpressing vector or JMJD2C siRNA (*Figure 4C*). In contrast, knockdown of SRC-1 did not affect the interaction of SUV39H1 and JMJD2C with p66^{Shc} promoter. Interestingly, only SUV39H1 overexpression hampered the recruitment of both JMJD2C and SRC-1 leading to repression of p66^{Shc} transcription (*Figure 4C*). Our findings are strengthened by the observation of p66^{Shc} up-regulation in the aorta of lean *SUV39H1*$^{-/-}$ mice (*Figure 4D*). Accordingly, we found that genetic deletion of SUV39H1

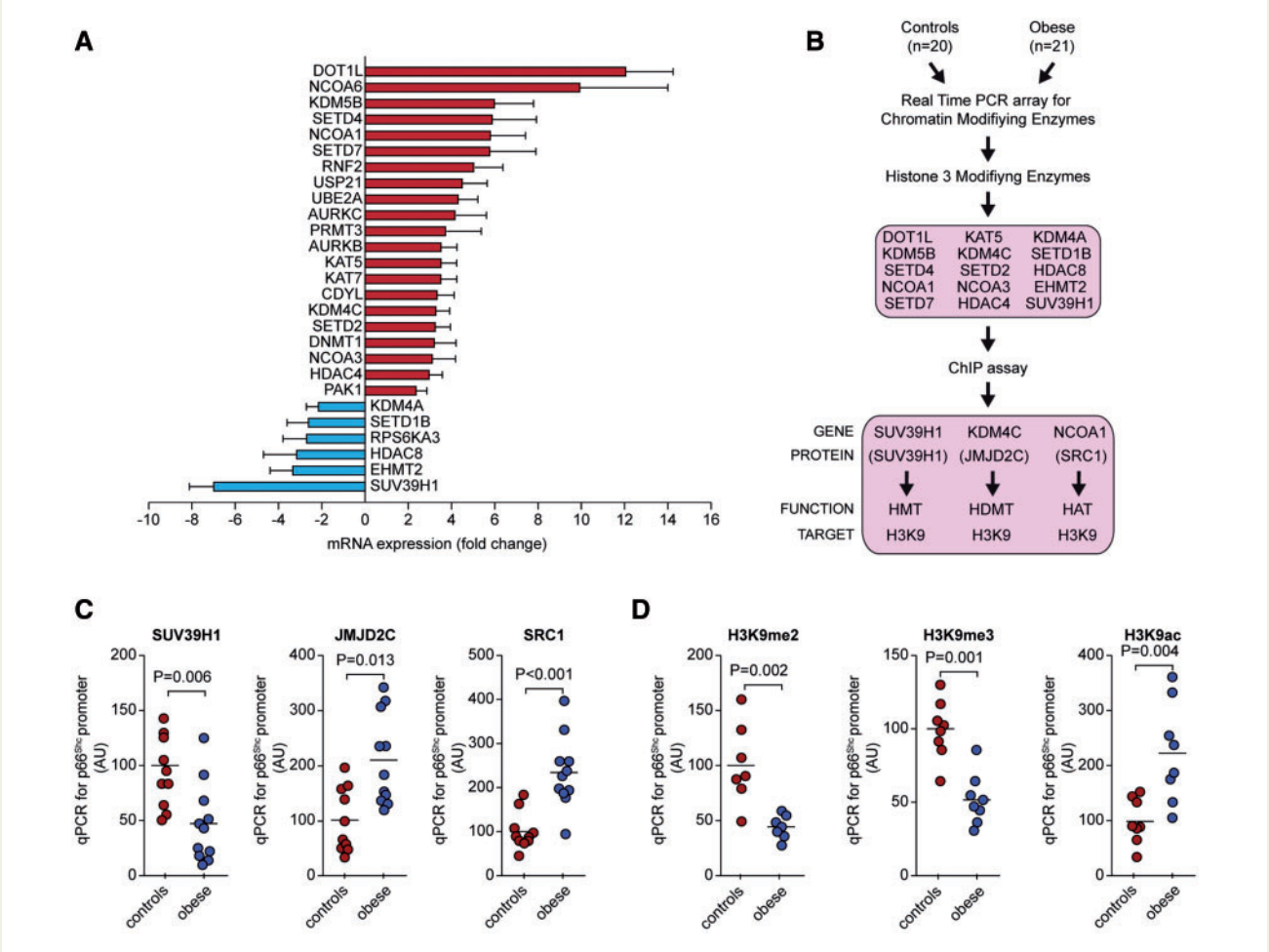

Figure 2 Adverse epigenetic remodelling of H3K9 on human p66^{Shc} promoter. (*A*) Real-time polymerase chain reaction array showing deregulated chromatin modifying enzymes in obese vs. control visceral fat artery. (*A*) change of at least two-fold (>2 or < -2) with $P < 0.05$ was considered significant; (*B*) Schematic showing ChIP-based selection of chromatin-modifying enzymes-binding p66^{Shc} promoter; (*C*) Interaction of chromatin-modifying enzymes SUV39H1, JMJD2C, and SRC-1 with human p66^{Shc} promoter assessed by ChIP assays; (*D*) Methylation and acetylation of H3K9 on p66^{Shc} promoter. Chromatin was immunoprecipitated with specific antibodies against H3K9m2, H3K9m3, and H3K9ac, and real-time polymerase chain reaction for p66^{Shc} promoter was performed. Data are expressed as means ± standard deviation, $n = 10$ per group. H3K9, histone 3 lysine 9; AU, arbitrary units.

triggers recruitment of JMJD2C and SRC-1 on p66^{Shc} promoter (*Figure 4E*). In conclusion, obesity-induced down-regulation of SUV39H1 fosters JMJD2C and SRC-1 cooperativity on p66^{Shc} promoter. These events are associated with active p66^{Shc} transcription via derangement of H3K9 methylation/acetylation and, hence, vascular oxidative stress (*Figure 5*).

Discussion

In this study, we demonstrate in a translational approach that reversible epigenetic signatures drive up-regulation of the mitochondrial adaptor p66^{Shc}, leading to oxidative stress and endothelial dysfunction in human and experimental obesity. Several lines of evidence support our conclusions. First, increased expression of p66^{Shc} is associated with epigenetic changes of its promoter, namely demethylation and acetylation of H3K9. Second, these results are elicited by derangement of H3K9-editing enzymes SUV39H1, JMJD2C, and SRC-1. Third, deletion of p66^{Shc} protects obese mice against ROS generation and endothelial dysfunction. Fourth, *in vitro* and *in vivo* reprogramming of SUV39H1, JMJD2C, and SRC-1 blunted vascular p66^{Shc} overexpression and oxidative stress. Finally, down-regulation of SUV39H1 was found to be the upstream molecular event responsible for recruitment of JMJD2C/SRC-1 on p66^{Shc} promoter and, thus, gene transcription of the adaptor protein.

Current knowledge of the molecular pathways underlying vascular disease in patients with obesity is limited. The interaction between gene and environment as a putative mechanism for obesity-related vascular complications remains poorly understood.[25–27] Previous work has shown that epigenetic editing of H3K9 is associated with deregulation of metabolic genes, appetite changes, insulin signalling defects, and altered expression profile of inflammatory genes.[28–32]

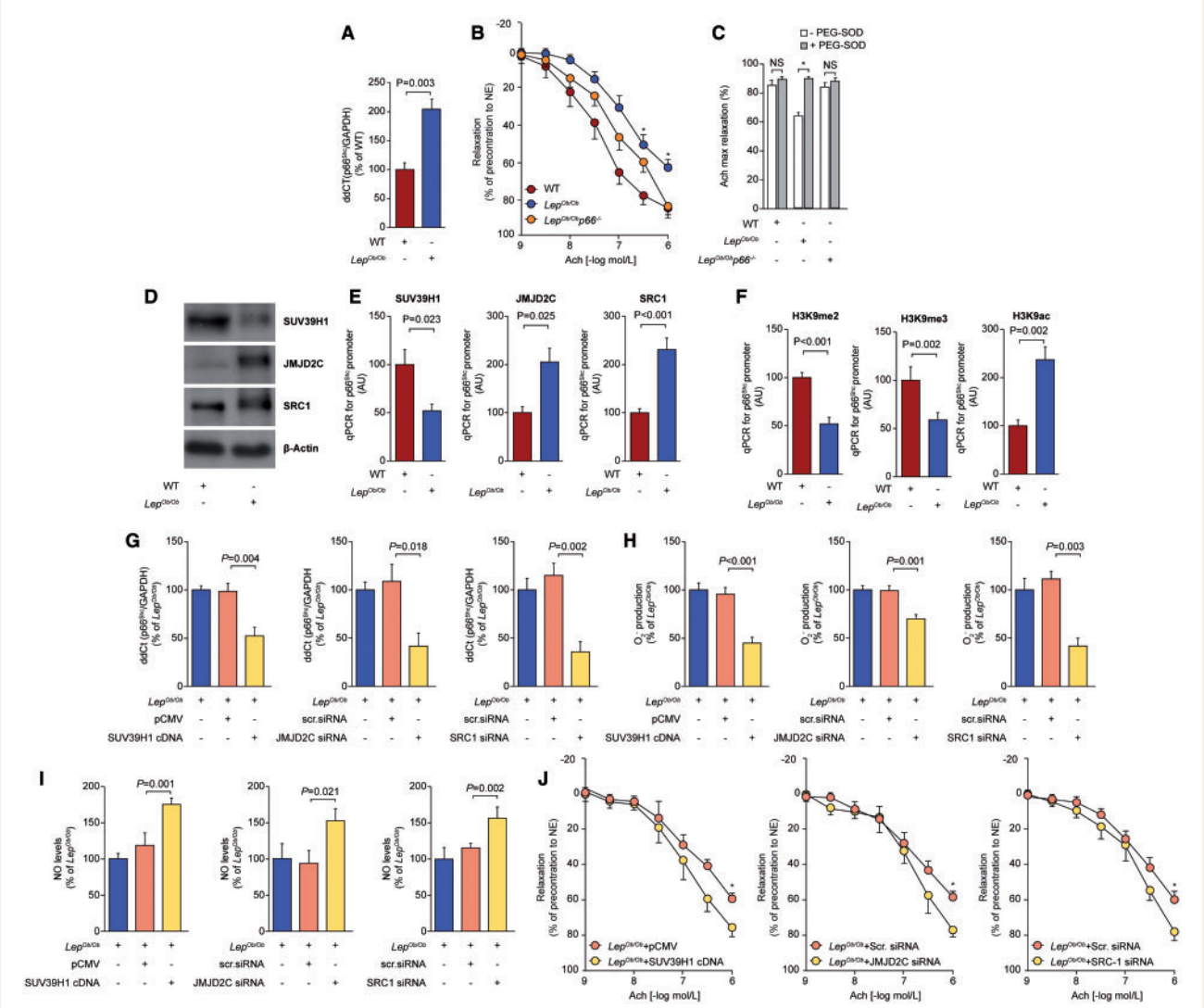

Figure 3 Chromatin-modifying enzymes regulate p66^{Shc} transcription and vascular oxidative stress in obese mice. (*A*) Real-time polymerase chain reaction showing gene expression of p66^{Shc} in the aorta of *Lep*$^{Ob/Ob}$ and lean WT mice; (*B*) Endothelium-dependent relaxations to Ach in WT, *Lep*$^{Ob/Ob}$, and *Lep*$^{Ob/Ob}$/*p66*$^{-/-}$ mice. *Lep*$^{Ob/Ob}$ displayed an impairment of vasorelaxation when compared with WT, whereas endothelial function was preserved in double-mutant mice (n = 9–12 mice/group); (*C*) Ach-induced vasorelaxation in the presence or in the absence of PEG–SOD (150 U/mL; n = 6–8 mice/group); (*D*) Representative Western blots of chromatin modifiers SUV39H1, JMJD2C, and SRC-1 protein expression in the aorta of obese *Lep*$^{Ob/Ob}$ when compared with WT mice. (*E*) ChIP assays showing interaction of SUV39H1, JMJD2C, and SRC-1 with murine p66^{Shc} promoter; (*F*) ChIP assay showing methylation and acetylation of H3K9-binding p66^{Shc} promoter. Chromatin was immunoprecipitated with specific antibodies against H3K9m2, H3K9m3, H3K9ac (n = 6/group, respectively). (*G–I*) p66^{Shc} gene expression, O_2^- and NO levels following reprogramming of chromatin-modifying enzymes SUV39H1, JMJD2C, and SRC-1 in endothelial cells isolated from obese mice. pCMV and scramble siRNA were used as controls for vector-based overexpression and siRNA-mediated knockdown, respectively (n = 4–6/group). (*J*) Endothelium-dependent vasorelaxation in aortic rings isolated from Lep$^{Ob/Ob}$ mice, in the presence or in the absence of epigenetic reprogramming of SUV39H1, JMJD2C, and SRC-1. *P < 0.01, n = 5/group. Data are expressed as mean ± standard deviation. Ach, acetylcholine; AU, arbitrary units; NO, nitric oxide; O_2^-, superoxide anion; PEG–SOD, polyethylene glycol–superoxide dismutase; WT, wild-type.

Most importantly, H3K9-related marks significantly contribute to intergenerational metabolic reprogramming with a potentially important impact on phenotypic variation and evolution.[33] Whether variations of H3K9 landscape are associated with obesity-related oxidative signals and vascular phenotypes remains largely unknown. Here, we showed that human and experimental obesity are associated with a specific remodelling of H3K9, characterized by reduced H3K9me2 and H3K9me3 as well as increased H3K9ac. The observed changes on H3K9 arise from deregulation of the chromatin modifiers SUV39H1, JMJD2C, and SRC-1 interacting with p66^{Shc} promoter in obese VFA. As assessed by gene profiling, SUV39H1 was down-regulated, whereas JMJD2C and SRC-1 were increased. These

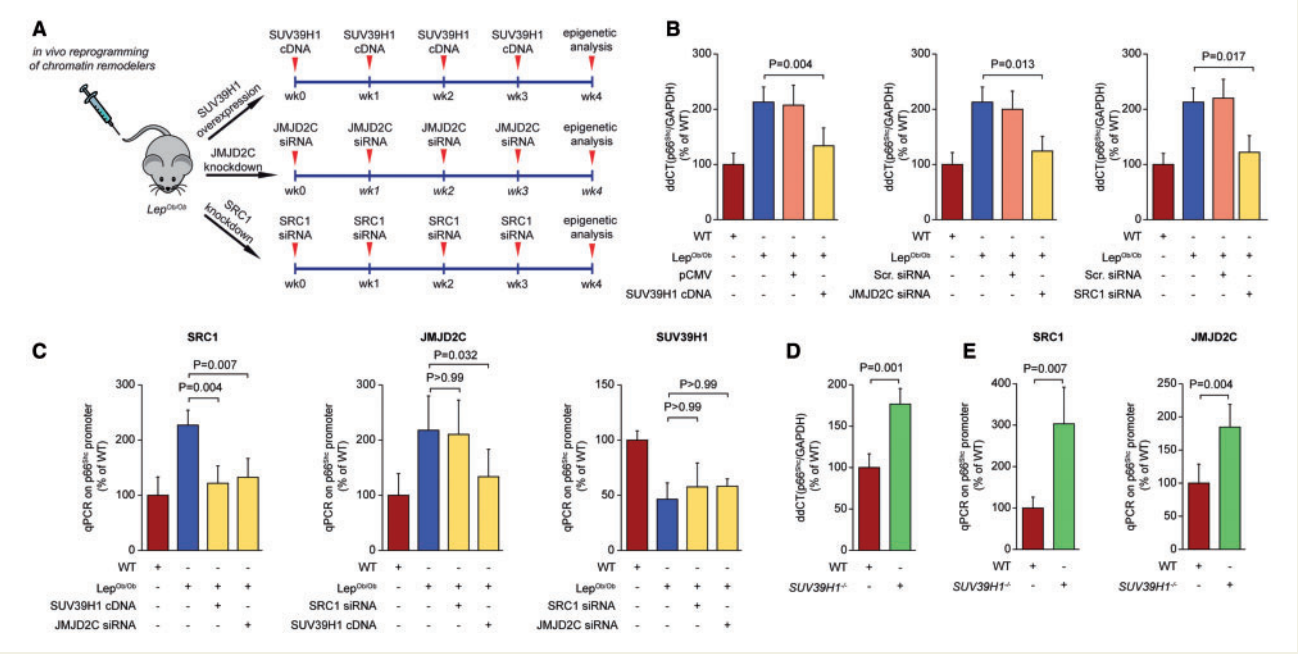

Figure 4 SUV39H1 orchestrates JMJD2C/SRC-1 recruitment on p66^{Shc} promoter. (*A*) Schematic showing 4-week *in vivo* reprogramming of chromatin-editing enzymes in obese mice. (*B*) Gene expression of p66^{Shc} in the aorta of obese mice chronically treated with SUV39H1 overexpressing vector as well as JMJD2C or SRC-1 siRNAs assessed by real-time polymerase chain reaction. (*C*) ChIP experiments suggesting molecular hierarchy among deranged chromatin-modifying enzymes. Recruitment of acetyltransferase SRC-1 to p66^{Shc} promoter is significantly reduced in the vasculature of obese mice treated either with SUV39H1 overexpressing vector or JMJD2C siRNA. In contrast, knockdown of SRC-1 did not exert any effect on the interaction of SUV39H1 and JMJD2C with p66^{Shc} promoter. Only SUV39H1 overexpression prevented the recruitment of both JMJD2C and SRC-1 enabling repression of p66^{Shc} transcription. (*D*) p66^{Shc} gene up-regulation in the aorta of *SUV39H1*$^{-/-}$ when compared with WT mice. (*E*) ChIP experiments show enhanced recruitment of SRC-1 and JMJD2C to p66^{Shc} promoter in *SUV39H1*$^{-/-}$ mice. pCMV and scramble siRNA were used as controls for vector-based overexpression and siRNA-mediated knockdown, respectively. Data are expressed as mean ± standard deviation, *n* = 4–6/group. Scr. siRNA, scrambled siRNA; WT, wild type.

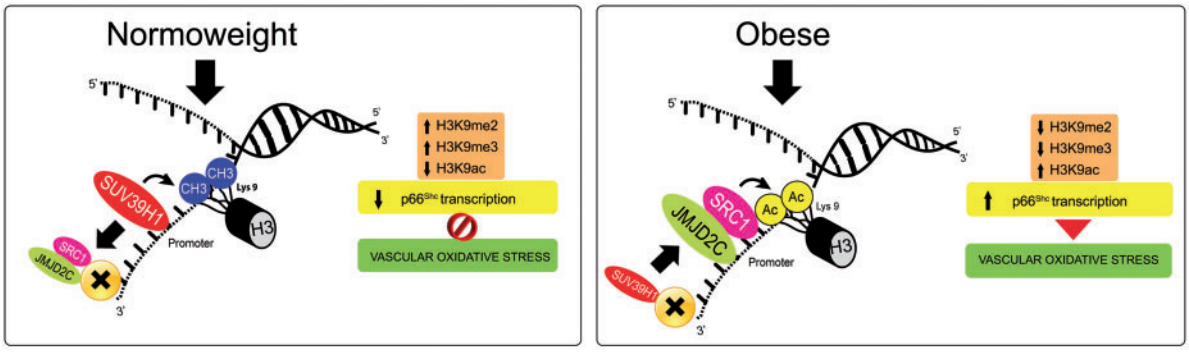

Figure 5 Role of SUV39H1 in obesity-induced vascular oxidative stress. In normal weight, SUV39H1 expression maintains H3K9 methylation levels preventing the binding of chromatin remodellers SRC-1 and JMJD2C to p66^{Shc} promoter. In the presence of obesity, down-regulation of SUV39H1 facilitates recruitment of SRC-1/JMJD2C with reduced di-/trimethylation and acetylation of H3K9 on p66^{Shc} promoter. This chain of events fosters gene transcription of mitochondrial p66^{Shc}, oxidative stress and endothelial dysfunction. H3K9me2, histone 3 lysine 9 dimethylation; H3K9me3, histone 3 lysine 9 trimethylation; H3K9ac, histone-3 acetylation.

alterations led to decreased H3K9 methylation by SUV39H1, increased removal of methyl groups by JMJD2C, and enhanced H3K9 acetylation by SRC-1, respectively. Overexpression of SUV39H1 or gene silencing of JMJD2C and SRC-1 in endothelial cells isolated from obese mice were able to repress p66^{Shc} transcription by editing the H3K9 landscape. To further strengthen our *in vitro* findings, reprogramming of chromatin remodellers was performed *in vivo* by i.v. injection of SUV39H1 overexpressing vector as well as JMJD2C and SRC-1 siRNAs. Consistently, this approach yielded to a significant reduction of p66^{Shc} expression in obese mice, highlighting the importance of these epigenetic players in vascular oxidative stress. Our ChIP experiments revealed that SUV39H1 triggers the recruitment of JMJD2C/SRC-1 to p66^{Shc} promoter, whereas JMJD2C and SRC-1 failed to affect SUV39H1 activity. Therefore, SUV39H1 downregulation is the initial effector responsible for increased activity of JMJD2C and SRC-1 on p66^{Shc} promoter. This notion is supported by the observation that genetic disruption of SUV39H1 in lean mice phenocopied obesity-related epigenetic marks on H3K9. Taken together, these results have unmasked a novel epigenetic mechanism underpinning mitochondrial oxidative stress in the vasculature.

We demonstrate for the first time that obesity-induced adverse epigenetic remodelling primes p66^{Shc} transcription, ROS generation, and endothelial dysfunction. Targeting H3K9-editing enzymes blunts p66^{Shc} expression and oxidative stress. These results deserve attention since effective approaches to reduce oxidative stress in humans are yet to be identified. The analysis of recent clinical trials has shown that targeting oxidative stress by ROS scavengers is an inefficacious and, sometimes, harmful therapeutic strategy.[16] Accordingly, antioxidants only partially scavenge cellular ROS without any impact on mitochondrial redox signalling.[34] In contrast, modulation of SUV39H1 expression may represent a valid therapeutic approach to revert adverse H3K9-epigenetic remodelling and p66^{Shc}-driven vascular oxidative stress in obesity. Growing understanding of chromatin architecture and metabolism has led to the design of specific molecules capable of modulating chromatin accessibility via enhancement/repression of epigenetic marks on DNA/histone complexes.[35] Noteworthy, some of these drugs have been already approved for the treatment of several conditions including cancer, neurological, and cardiovascular disease.[36] Although pharmacological activators of SUV39H1 are not yet available, ongoing high-throughput screenings of chemical libraries will be instrumental to identify selective compounds to be tested in experimental studies.[37]

This study has some limitations. Although we have successfully translated our experimental findings to the human setting, we could not fully prove a causal relation between p66^{Shc} and endothelial dysfunction in obese patients. In this regard, pharmacological modulation of p66^{Shc} would have been the best approach—however, p66^{Shc} inhibitors are not yet available. An important aspect which deserves further investigation is whether epigenetic regulation of p66^{Shc} may counteract the atherosclerotic phenotype in this setting. Finally, ChIP experiments were performed in mouse and human vascular homogenates containing a variety of cell types. Therefore, we cannot fully rule out that epigenetic regulation of smooth muscle cells, macrophages, or other vascular cells may participate to obesity-related vascular phenotype.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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