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GENE ENVIRONMENT-INTERACTION AND CARDIOVASCULAR PHENOTYPE IN OBESITY AND DIABETES

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To my family

ABSTRACT

Although a large body of evidence supports the notion that genes determine cardio-metabolic traits and outcomes, the non-genetic regulation of these events has recently gained increasing attention. Plastic chemical modifications of DNA-histone complexes defined epigenetic changes regulate gene expression by modifying chromatin accessibility to transcription factors. In the present thesis, we have investigated the emerging role of epigenetic modifications as fine-tuning regulators of gene expression in diabetic cardiomyopathy, as well as in obesity and diabetes-driven endothelial dysfunction.

Study I: The objective was to investigate whether mitochondrial adaptor $p66^{Shc}$ contributes to obesity-related vascular dysfunction. Oxidative stress and vascular expression of chromatin modifying enzymes were investigated in visceral fat arteries (VFA) from obese and age-matched healthy subjects. VFA from obese patients displayed enhanced mitochondrial reactive oxygen species (ROS) and endothelial dysfunction as well as a significant dysregulation of chromatin modifier enzymes methyltransferase SUV39H1, demethylase JMJD2C and acetyltransferase SRC-1 as compared to control VFA. These changes were associated with reduced methylation and acetylation of histone 3 lysine 9 (H3K9) on $p66^{Shc}$ promoter. Specifically, we demonstrated that obesity-induced downregulation of SUV39H1 orchestrates JMJD2C/SRC-1 recruitment to $p66^{Shc}$ promoter, fostering adverse H3K9 remodeling and $p66^{Shc}$ upregulation.

Study II: We sought to investigate whether epigenetic regulation of pro-oxidant adaptor $p66^{Shc}$ contributes to persistent myocardial dysfunction despite intensive glycemic control (IGC). $p66^{Shc}$ expression was increased in the heart of diabetic mice, and IGC did not revert this phenomenon. Dysregulation of methyltransferase DNMT3b and deacetylase SIRT1 linked to upregulation of miRNAs (miR-218 and miR-34a) drive persistent transcription of the adaptor $p66^{Shc}$, thereby leading to mitochondrial oxidative stress, myocardial inflammation and left ventricular dysfunction. Our findings showed that adverse epigenetic signatures on $p66^{Shc}$ promoter contribute to left ventricular (LV) dysfunction in the setting of diabetes.

Study III: Here we demonstrate for the first time a protective role of activated protein-1 (AP-1) transcription factor JunD against derangement of ROS homeostasis, inflammation and myocardial impairment in the setting of diabetes-induced hyperglycemia. JunD transcriptional activity was reduced in the heart of wild-type mice with streptozotocin-induced diabetes and was associated with downregulation of free radical scavengers, increased expression of ROS-generating NADPH oxidase and marked increase in myocardial superoxide anion generation. These redox changes were paralleled by activation of NF-κB-dependent inflammatory pathways and left ventricular dysfunction. Interestingly enough, such detrimental changes did not occur in diabetic mice with cardiac-specific overexpression of JunD (α-MHC-$JunD^{tg}$) and LV function was not impaired, indicating the relevant role of JunD in counteracting hyperglycemia-induced redox changes and cardiac damage in diabetes.

Study IV: Enhancer of zeste homologue 2 (EZH2), a member of the family of SET1 methyltransferase and a catalytic component in the polycomb repressive complex 2, is associated with transcriptional repression through histone H3K27me3 modification. Therefore, we hypothesize that its pharmacological modulation could have an impact on hyperglycemia-driven endothelial dysfunction. We demonstrated that pharmacological inhibition of EZH2 by GSK126 might prevent key hallmarks of diabetic vascular dysfunction, such as oxidative stress and inflammation. Experiments in human aortic endothelial cells showed that GSK126 protects against hyperglycemia-induced oxidative stress and inflammation via restoration of JunD, SOD1 and SOD2 expression and inhibition of Nox4 upregulation. Moreover, GSK126 was able to prevent activation of transcription factor NF-kB and subsequent upregulation of inflammatory adhesion molecules IL-6 and MCP-1.

Altogether, our studies provide novel molecular insights on the regulation of redox and inflammatory pathways implicated in the impairment of obesity and diabetes-induced endothelial and cardiac function. Moreover, by targeting epigenetic changes responsible of derailed pro-oxidant and pro-inflammatory transcriptional programmes, we shed some light on putative pharmacological strategies to reduce the burden of cardiovascular disease in this setting.

LIST OF SCIENTIFIC PAPERS

I. Sarah Costantino, Francesco Paneni , Agostino Virdis, SHAFAAT HUSSAIN, Shafeeq Ahmed Mohammed, Giuliana Capretti, Alexander Akhmedov, Kevin Dalgaard, Sergio Chiandotto, J Andrew Pospisilik, Thomas Jenuwein, Marco Giorgio, Massimo Volpe, Stefano Taddei, Thomas F Lüscher, Francesco Cosentino (# Equally contributed)
Interplay among H3K9-editing enzymes SUV39H1, JMJD2C and SRC-1 drives p66Shc transcription and vascular oxidative stress in obesity
European Heart Journal (2019); 40 (4):383-391

II. Sarah Costantino , Francesco Paneni , Katharyn Mitchell, Shafeeq A.Mohammed, SHAFAAT HUSSAIN, Christos, Gkolfos, Liberato Berrino, MassimoVolpe, Colin Schwarzwald, Thomas Felix Lüscher, Francesco Cosentino (# Equally contributed)
Hyperglycaemia-induced epigenetic changes drive persistent cardiac dysfunction via the adaptor p66Shc
International Journal of Cardiology (2018); 268: 179-186.

III. SHAFAAT HUSSAIN, Abdul Waheed Khan, Alexander Akhmedov, Sarah Costantino, Francesco Paneni, Kenneth Caidahl, Shafeeq A. Mohammed, Rosa Suades, Camilla Hage, Christos Gkolfos, Hanna Björck, John Pernow, Lars H. Lund1, Thomas F. Luscher, Francesco Cosentino
Protective role of AP-1 transcription factor JunD in the diabetic heart
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IV. SHAFAAT HUSSAIN, Abdul Waheed Khan, John Pernow, Francesco Cosentino
EZH2 inhibition via GSK126 attenuates high glucose induced oxidative stress and inflammation in human aortic endothelial cells
Manuscript to be submitted

Publications by the author, which are not included in the thesis

Abdul Waheed Khan, Lukas Streese, Arne Deiseroth, SHAFAAT HUSSAIN, Rosa Suades Soler, Andre Tiaden, Diego Kyburz, Henner Hansen, Francesco Cosentino
High-intensity interval training modulates retinal microvascular phenotype and DNA methylation of $p66^{Shc}$ gene: a randomized controlled trial (EXAMIN AGE)
European Heart Journal (2019) pending revision

Rosa Suades, SHAFAAT HUSSAIN, Abdul Waheed Khan, Francesco Cosentino
AP-1 transcription factor JunD protects against cardiac microRNA derangement in diabetes
Data collection

CONTENTS

LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme inhibitors
Ach	Acetylcholine
AGEs	Advance glycation end products
ALDH2	Aldehyde dehydrogenase 2
ARB	Angiotensin receptor blocker
ASAP	Advanced study of aortic pathology
BMI	Body mass index
BSA	Bovine serum albumin
ChIP	Chromatin Immunoprecipitation
CMH	1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine
CP	Paramagnetic 3-carboxy-proxyl
CPH	1-hydroxy-3-carboxy-pyrrolidine
CVD	Cardiovascular disease
D-HAECs	Diabetic human aortic endothelial cells
DBP	Diastolic blood pressure
DNMTs	DNA methyltransferases
EF	Ejection fraction
eNOS	Endothelial nitric oxide synthase
ESR	Electron spin resonance
EZH2	Enhancer of zeste homolog 2
FOXO1	Forked head box protein-1
FPG	Fasting plasma glucose
FS	Fractional shortening
H3	Histone 3
H3K27me3	Histone 3 lysine 27 trimethylation
H3K4me1	Histone 3 lysine 4 monomethylation
H3K4me3	Histone 3 lysine 4 trimethylation
H3K9ac	Histone 3 lysine 9 acetylation
H3K9me1	Histone 3 lysine 9 monomethylation
H3K9me3	Histone 3 lysine 9 monomethylation
HAECs	Human aortic endothelial cells
Hb1Ac	Glycated hemoglobin
HDL-C	High density lipoprotein cholesterol
HF	Heart failure
HG	High Glucose
HOMA-IR	Homeostasis model assessment
HR	Heart rate
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
Ik-Bα	Inhibitory subunit of nuclear factor-kappa B
LDL-C	Low density lipoprotein cholesterol
$Lep^{Ob/Ob}$	Leptin deficient mice
LVEDD	Left ventricle end diastolic diameter
LVEF	Left ventricle ejection fraction
MBD	Methyl-CpG-binding protein

MRA	Mineralocorticoid receptor antagonists
NE	Norepinephrine
NF-kB	Nuclear factor-kappa B
NG	Normal glucose
NOS	Nitric oxide
PCR	Polymerase chain reaction
PKC	Protein kinase *c*
ROS	Reactive oxygen species
RT-PCR	Real time-polymerase chain reaction
SBP	Systolic blood pressure
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
STZ	Streptozotocin
T2D	Type 2 diabetes
TG	Triglycerides
VFAs	Visceral fat arteries
WT	Wild type
T1D	Type 1 diabetes
AP-1	Activator ptotein-1
ICAM-1	Intracellular adhesion molecule-1
MCP-1	Monocyte chemoattractant protein-1
VCAM-1	Vascular adhesion molecule-1
3 –NT	3-Nitrotyrosine

1 INTRODUCTION

1.1 GLOBAL BURDEN OF METABOLIC DISEASES

The prevalence of obesity and type 2 diabetes (T2D) is alarmingly increasing worldwide at an unprecedented rate.[1, 2] The main determinants behind this process are modifiable (environment, overnutrition, sedentary habits, smoking, etc.) and non-modifiable factors such as genetic susceptibility and aging.[3] Environmental changes and physical inactivity are capable to alter gene expression and thereby cellular processes, and these derangements can be transmitted to offspring, hence, anticipating metabolic traits even in young normal weight individuals.[4, 5] Accordingly, obesity and pre-diabetes are dramatically increasing in young adolescents and represent a severe public health problem.[1, 6] The International Diabetes Federation currently estimates that worldwide almost 1.1 billion people will be overweight and 500 million people obese by the year 2040.[7] Increase in body weight and visceral fat accumulation associate with several cardiovascular risk factors, such as insulin resistance, low-grade inflammation, arterial hypertension and dyslipidemia, which contribute to increase morbidity and mortality in these individuals.[3, 8] Noteworthy, overweight and obesity are powerful predictors of T2D.[9] The development of T2D from pre-diabetes occurs along a "continuum", not necessarily linear with time, that includes different cellular mechanisms such as changes in glucose transport, tissue-specific alterations of insulin signaling, beta cell dysfunction as well as deregulation of important genes involved in oxidative stress and inflammation.[10-12] High glucose and insulin levels in obese patients associate with increased cardiovascular risk, even without diabetes.[13] Among the constellation of weight-related comorbidities, cardiovascular disease (CVD) carries the largest burden.[11] Indeed, risks of coronary heart disease and ischemic stroke rise steadily with increasing BMI, a measure of weight relative to height. [12] Previous studies have shown that the expression of oxidant genes is also derailed in obese subjects.[14, 15] However, the underlying mechanisms remain poorly understood.

Currently, 425 million people have been diagnosed with diabetes worldwide and anticipate an increase up to 629 million by the year 2045.[1] Furthermore, over the last decade, the prevalence of diabetes is increasing more in middle low-income countries than high-income countries. The most part of people living in these countries are unaware of the disease and remain undiagnosed for many years, leading to clear delays in the implementation of prevention and treatment strategies.[1] Diabetes is a complex disease characterized by an array of different mechanisms ultimately resulting in elevated blood glucose levels.[16] The disease

occurs when either the pancreatic beta cells are not able to produce enough insulin (a hormone that promotes the glucose absorption from the blood into adipose tissue, skeletal muscle and liver cells) or when the body fails to use the insulin effectively. This results in elevated blood glucose levels. Diabetes is associated with high morbidity and mortality, due to serious complications occurring primarily in the cardiovascular system (heart failure, coronary heart disease, peripheral artery disease, and stroke). Diabetes also affects the kidneys (diabetic nephropathy), the eye (retinopathy), the peripheral nervous system (neuropathy).[17] Apart from them, diabetes also increases susceptibility to cognitive decline, cancer, infections and gastrointestinal disease.[18, 19] Only 5% of people have type 1 diabetes (T1D).[20] Patients who develop T2D are generally sedentary and obese. The progression from impaired glucose tolerance, a condition defined as pre-diabetes, to T2D may take many years to occur, leading to different intermediate disease phenotypes with continuous changes in glucose parameters and shifts in glucose tolerance category.[3, 21] Hence, understanding the factors predisposing to T2D is a major challenge. The annual reported rate of T2D varies from 2-11 % in individuals with impaired fasting glucose (IGT) and 1-10 % in individuals with impaired fasting glucose (IFG), depending on the risk profile of different study populations.[22-25]

1.2 ASSOCIATION OF DIABETES AND CARDIOVASCULAR DISEASE

There is a strong biological link between T2D and cardiovascular disease (CVD).[26] Cardiovascular disease, including stroke, coronary heart disease, heart failure, are common causes of morbidity and mortality among patients with T2D.[27] In these patients, metabolic alterations (insulin resistance, reduce insulin secretion or both) are responsible for endothelial dysfunction, inflammation and platelet reactivity. These conditions trigger and accelerate atherosclerotic vascular disease.[28, 29] The deleterious effect of diabetes on cardiovascular system is highlighted by the fact that 75% of deaths in diabetic subjects are due to CVD.[30] A seminal Finish study revealed that diabetes raises the 7-year risk of myocardial infarction and death in aged subjects.[31] In addition, patients with diabetes also have an increased rate (three-to-six-fold) of ischemic cerebrovascular complications.[32, 33] Certainly, T2D was a potent predictor of stroke in subjects enrolled in a prospective Finnish study.[34] In the Euro Heart Survey, one-year follow-up survival was significantly higher in pre-diabetic patients than in patients with T2D.[35] However, in the long-term survival curves tend to overlap, hence strengthening the idea that all stages of impaired glucose regulation are linked with increased cardiovascular risk.[36, 37]

1.3 MULTIFACTORIAL INTERVENTION AND CARDIOVASCULAR RISK

Advances in therapy have reduced morbidity and mortality in patients with diabetes. However, cardiovascular risk is far from being eliminated, and mechanism-based therapeutic strategies are in high demand.[29] High glucose levels trigger endothelial inflammation, mitochondrial oxidative stress, and reduced availability of nitric oxide in patients with diabetes.[28] This chain of events favors the development of macro- and microvascular disease.[28] Although the link between obesity/diabetes and atherosclerosis is well established, a better comprehension of the underlying mechanisms is of utmost importance to identify novel molecular targets.

1.4 REDOX CHANGES AND VASCULAR DYSFUNCTION

In the last two decades, basic and translational research have unmasked a strong biological relation between high glucose, impaired insulin signaling and CVD in the setting of T2D.[38-41] However, despite these investigations provided important mechanistic insights, the detrimental effects of hyperglycemia and insulin resistance on the heart and vessels remain to be fully elucidated.[42] Recent studies performed in endothelial cells isolated from patients with T2D have shown activation of detrimental pathways favoring mitochondrial disruption and apoptosis.[43] It was demonstrated that reactive oxygen species (ROS) are upstream regulators of complex molecular networks leading to endothelial dysfunction and, hence, vascular diabetic complications.[29, 39, 44] In patients with diabetes, hyperglycemia leads to the generation of excessive mitochondrial ROS and subsequent activation of advanced glycation end products (AGEs), protein kinase C (PKC), nuclear factor-kB (NF-κB), polyol and hexosamine flux (**Figure 1**).[45] This hyperglycemic environment induces a chronic elevation of diacylglycerol levels in endothelial cells with subsequent membrane translocation of conventional (α, β_1, β_2) and non-conventional (δ) PKC isoforms. Specifically, PKCβ_2 isoform is highly activated in the diabetic endothelium and correlates with oxidative stress, impaired insulin signaling and, most importantly, endothelial dysfunction.[46] One mechanism by which PKCβ_2 elicits its deleterious effect is through the mitochondrial adaptor $p66^{Shc}$.[28, 47] Indeed, glucose-induced activation of PKCβ_2 isoform phosphorylates the adaptor $p66^{Shc}$ at serine 36, favoring its localization to the mitochondria, oxidation of cytochrome c and subsequent ROS generation[48, 49]. Adaptor $p66^{Shc}$ functions as a redox enzyme implicated not only in mitochondrial ROS generation but also in the translation of oxidative signals into apoptosis.[50, 51] In this regard, diabetic *p66$^{Shc-/-}$* mice are protected against hyperglycemia-induced endothelial dysfunction and oxidative stress.[52] Besides, the relevance of $p66^{Shc}$ in the clinical setting of diabetes is further supported by the notion that $p66^{Shc}$ gene expression is increased

in peripheral blood mononuclear cells obtained from patients with T2D and correlates with plasma isoprostane levels, a reliable *in vivo* marker of oxidative stress.[53] In addition, we have demonstrated that hyperglycemia-induced $p66^{Shc}$ upregulation is not reverted by intensive glycemic control in diabetic mice and contributes to persistent oxidative damage and vascular dysfunction via a complex vicious cycle involving ROS, epigenetic changes and PKC activation.[54] Interestingly enough, *in vivo* gene silencing of $p66^{Shc}$, performed at the time of normoglycemia restoration with insulin, was able to blunt persistent endothelial dysfunction, showing that $p66^{Shc}$ is an important source of free radicals involved in the "metabolic memory" phenomenon.[54, 55] Altogether these findings indicate that knocking down $p66^{Shc}$ gene may be a promising option to rescue vascular dysfunction in diabetes. Another major source of ROS by PKC is via activation of NADPH oxidase subunit p47(phox). Indeed, treatment with a PKC inhibitor blunts NADPH-dependent ROS generation.[39, 56, 57] A previous study from our group showed that PKCβ_2 is also a regulator of NF-kB signaling in hyperglycemic conditions. PKCβ_2 activation reduces protein levels of the inhibitory subunit NF-κB (Ik-Bα) thus enabling NF-kB-driven transcription of VCAM-1.[47] Selective PKCβ_2 inhibition abolished Ik-Bα degradation thereby preventing hyperglycemia-induced endothelial inflammation. Taken together, PKC could be regarded as a key upstream regulator of hyperglycemic damage as well as impaired insulin signaling.[48] Although the complete understanding of hyperglycemia-driven oxidative and inflammatory regulatory pathways remains challenging, targeting specific molecular mechanisms may represent a promising strategy to reduce cardiovascular burden in patients with diabetes.

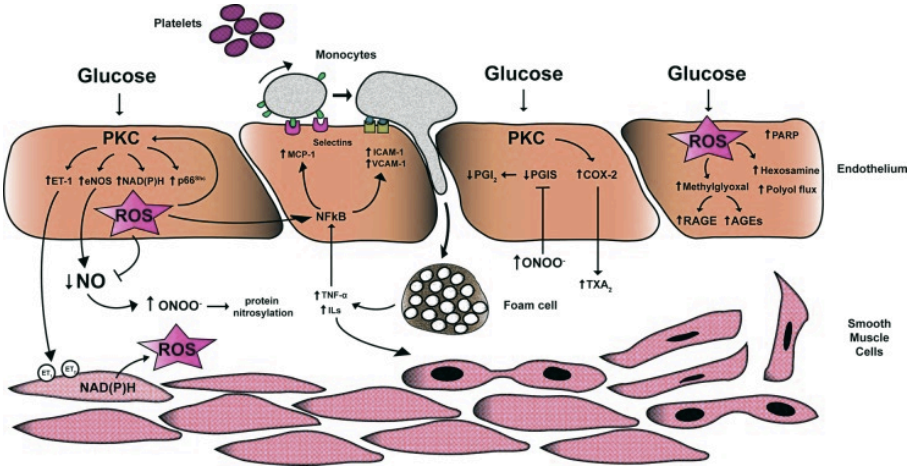

Figure 1. Mechanisms of glucose-induced vascular damage. *PKC* protein kinase C, *eNOS* endothelial nitric oxide synthase, *ET-1* endothelin 1, *ROS* reactive oxygen species, *NO* nitric oxide, *MCP-1* monocyte chemoattractant protein-1, *VCAM-1* vascular cell adhesion molecule-1, *ICAM-1* intracellular cell adhesion molecule-1, *AGEs* advanced glycation end products (modified from Paneni et al., 2013).

1.5 HEART FAILURE IN DIABETES

It is well established that patients with diabetes have a high risk of left ventricular dysfunction and heart failure (HF) as compared with non-diabetic subjects.[58-60] Epidemiological studies have shown that poor glycaemic control is associated with an increased risk of HF.[61] Recent studies clearly indicate that hyperglycaemic environment contributes to myocardial damage. Overproduction of ROS in mitochondria alters myocyte functionality leading to myocardial dysfunction, inflammation and fibrosis.[62, 63] Therefore, understanding the molecular mechanisms of cardiac redox signalling may have important implications to counteract maladaptive changes occurring in the diabetic heart.[64]

1.6 PATHWAYS LINKING AGING AND METABOLISM

Over the last few years, molecular investigations have unveiled common signaling networks linking the aging process with deterioration of cardiovascular homeostasis and metabolic disturbances. Aging is not only able to impair pathways leading to adverse metabolic profile, but also conditions, such as obesity, diabetes and insulin resistance, anticipate vascular and cardiac senescence. It is emerging that a dynamic interplay between $p66^{Shc}$, NF-kB and activator protein-1 (AP-1) transcription factor JunD may favor adverse vascular and cardiac phenotypes in this setting.

Recent studies have demonstrated that the adaptor $p66^{Shc}$ is an important molecular effector that may explain how aging is connected with metabolic and cardiovascular disease. $p66^{Shc-/-}$ mice exposed to oxidative stimuli have shown reduced levels of free radicals[50, 65], an observation explained by the well-known concept that $p66^{Shc}$ is a major source of ROS.[66] In line with these data, we observed that aging-induced impairment of endothelium-dependent relaxation to acetylcholine was not present in $p66^{Shc-/-}$,[67] due to preservation of nitric oxide availability in mice lacking $p66^{Shc}$ gene.[67] Further work has demonstrated that $p66^{Shc}$ activation is critically involved in different processes including adipogenesis, insulin resistance and diabetes-related cardiovascular complications.[52, 68] More recently, we found that $p66^{Shc}$ levels are increased in genetically obese mice and participate in endothelial insulin resistance.[69] Activation of $p66^{Shc}$ also predispose to the acquisition of the heart senescent phenotype and development of heart failure in diabetic mice [70]. Gene expression of $p66^{Shc}$ is increased in mononuclear cells obtained from patients with T2D and coronary artery disease.[53, 71] Based on this background, it is possible to conclude that $p66^{Shc}$ fosters ROS accumulation, with subsequent deregulation of pathways implicated in mitochondrial dysfunction, fat accumulation, insulin resistance and diabetes.

Activation of NF-kB mediates vascular and myocardial inflammation in metabolic and age-related diseases. A recent study clearly demonstrated that endothelial suppression of NF-kB prolongs lifespan in mice and improves obesity-induced endothelial insulin resistance. Interestingly, transgenic mice with endothelium-specific overexpression of the inhibitory NF-kB subunit were protected against insulin resistance in adipose tissue and skeletal muscle.[72] Impaired insulin signaling is indeed an important hallmark linking metabolic disease with premature aging.[73]

An emerging player in adverse cardiovascular remodeling is JunD, a member of the activated protein 1 (AP-1) family of transcription factors that is a major gatekeeper against oxidative stress. AP-1 is a heterodimeric complex which is composed of several proteins belonging to the c-Fos, c-Jun, ATF and CREB families.[74] AP-1 modulates gene expression in response to cellular environment (infections, stress, cytokines, growth factors) [74]. JunD, which is the most recently known gene of the Jun family, regulates cell growth and survival and protects against oxidative stress by modulating genes involved in antioxidant defense and ROS production.[75] Accordingly, *JunD*$^{-/-}$ mice exhibit features of premature aging, shortened life span and increased incidence of aggressive cancers.[76-78] Recent evidence demonstrated an accumulation of ROS in *JunD*$^{-/-}$ murine embryonic fibroblasts which was reduced by treatment with ascorbate.[75] By contrast, overexpression of JunD abolished ROS production, blunted redox signaling and apoptosis.[75, 78] Gene expression profiling of *JunD*$^{-/-}$ murine embryonic fibroblasts showed downregulation of several free radical scavenging enzymes associated with an increase in expression of ROS-producing NADPH oxidase.[75] In this regard, we have recently reported that JunD is highly relevant for cardiovascular homeostasis.[78] We observed that vascular JunD expression declines with aging, thus altering the balance between pro-oxidant (NADPH oxidase) and antioxidant enzymes (manganese-superoxide dismutase and aldehyde dehydrogenase-2), with subsequent accumulation of free radicals. Indeed, genetic deletion of JunD in young mice was associated with premature disturbances of redox signaling, mitochondrial disruption and endothelial dysfunction.[78] Moreover, young *JunD*$^{-/-}$ mice displayed premature features of vascular senescence, which were comparable with those observed in aged animals. We found that age-dependent downregulation of JunD was the result of epigenetic changes occurring on its promoter.[78] This finding is in agreement with the notion that epigenetics may significantly alter the expression of genes involved in senescence, metabolic disorders and cardiovascular damage.[78] Interestingly enough, JunD expression was reduced in peripheral blood monocytes isolated from aged compared to young healthy subjects. Taken together, the results of this work imply that JunD can be considered as an attractive molecular target to prevent or delay

age-driven cardiovascular diseases. In agreement with our observations, genetic manipulation of JunD (disruption or overexpression) promotes pressure overload-induced apoptosis, hypertrophic growth and angiogenesis in the heart[79] and blunts phenylephrine-mediated cardiomyocyte hypertrophy.[80] Notably, JunD protein levels are decreased in patients with end-stage heart failure.[81] Reduced JunD levels may also affect longevity by controlling pathways relevant to angiogenesis and insulin signaling. It has been reported that insulin-IGF-1 signaling is constitutively stimulated in mice lacking JunD, leading to inactivation of FOXO1, a positive regulator of longevity.[76] Similarly, *JunD*$^{-/-}$ mice display hyperinsulinemia, most probably resulting from enhanced pancreatic islet vascularization due to chronic oxidative stress.[76] Interestingly, long-term treatment with an antioxidant rescued the metabolic disturbances observed in *JunD*$^{-/-}$ mice.[76] These data clearly indicate that JunD can be regarded as a major effector in the interplay between aging, metabolism and cardiovascular disease.

1.7 THE ROLE OF EPIGENETICS IN CARDIOMETABOLIC DISEASES

Eukaryotic chromosome is composed of histone-DNA complexes forming the chromatin that are organized into subunits called nucleosomes. In nucleosomes, chromosomal DNA is packaged around histone proteins.[15] Epigenetic changes refer to plastic and dynamic chemical changes of DNA/histone complexes that can modify gene regulation without changing the DNA sequence.[82] Epigenetic alterations can be classified into three main types: 1) DNA methylation; 2) post-translational histone modifications; 3) non-coding RNA (ncRNA).[83] DNA methylation is the process by which methyl group is covalently added to the carbon 5 position of cytosine and thereby represses gene transcription, either by preventing transcription factor binding to the promoter region or by fostering the recruitment of chromatin remodeling enzymes.[84] DNA methylation is catalyzed by a family of DNA methyltransferases (DNMTs), which include DNMT1, DNMT3a, DNMT3b. DNMT1 maintains methylation status during replication whereas DNMT3a/DNMT3b are involved in de novo methylation.[85]

DNA-related changes together with posttranslational modifications of histones such as acetylation, ubiquitination and phosphorylation may arrange in different patterns to regulate chromatin structure.[86] In contrast to DNA methylation, impact of histone modifications on gene expression may differ depending on the particular chemical modification.[87] For instance, lysine mono-methylation of histones generally activate gene transcription, but di- or trimethylation can either activate gene (e.g. H3K4me3) or repress gene transcription (e.g. H3K9me3).[88] Non-coding RNAs do not affect chromatin structure directly but a play a

pivotal role in post-transcriptional regulations of genes.[89] Of interest, non-coding RNAs strictly cooperate with both acetyl- or methyl writing and erasing enzymes to edit chromatin conformation and gene expression.[90] It has been shown that microRNAs regulate the expression of both chromatin modifying enzymes as well as DNA methyl transferases (i.e. DNMT3a and DNMT3b). On the other side, chromatin modifications may affect the transcription of non-coding RNAs.[91] Thus, the distinct epigenetic processes are not independent, instead they can interact and influence each other. This complex regulation of gene transcription is also tissue and cell-specific.[92]

Although great advancements in the field of epigenetic and cancer, the environmental regulation of gene expression in the setting of cardiometabolic diseases including diabetes remains poorly understood.[93] Despite an established body of evidence supports the notion that genes influence the cardiometabolic features and outcomes, the epigenetic modifications occurring in this setting are gaining more attention.[82] Given that epigenetic regulation is reversible in nature, novel avenues for therapeutic intervention in cardiometabolic diseases, including diabetes, are warranted.[94] Although epigenetic drugs are well-known for their antineoplastic properties, they are currently investigated for non-oncological applications.[94] Among new emerging epigenetic drug target candidates, EZH2 (catalytic component Enhancer of zeste homolog 2) is a catalytic component of PRC2 complex, which methylates lysine 27 of histone H3. Trimethylation of lysine 27 of histone 3 (H3K27me3) is a repressive histone mark associated with suppression of gene expression.[95] EZH2 regulation effects are involved in different physiological and pathological processes, including cancer.[96-99] Several studies have reported EZH2 plays important role in ROS generation, cardiac hypertrophy and inflammation.[100-102] Indeed, increased EZH2 expression blunts SOD-2 expression leading to increase ROS generation that contributes to progression of pulmonary artery hypertension.[100] A recent study demonstrated that targeting EZH2 in erythroid cells exposed to ferric nitrilotriacetate and cobalt-60 radiation protects against oxidative stress.[103] Moreover, it has been shown that high glucose induces oxidative stress via increased expression of EZH2 in human mesenchymal stem cells.[104] Similar to these findings, elevated levels of EZH2 expression and activity in retinal tissues from diabetic animals and endothelial cells exposed to high glucose are linked to dysregulation of miR-200b and the development of diabetic retinopathy.[105] Furthermore, several lines of evidence have implicated EZH2 upregulation in the development and progression of atherosclerosis. For instance, recent studies have shown increased H3K27me3 in endothelial cells isolated from early and advanced human atherosclerotic plaques.[106] In addition, specific inhibition of EZH2 by a small molecule inhibitor protects against neuro-inflammation in microglial cells.[102] Indeed, EZH2-dependent

conformational changes promote accumulation of free radicals and inflammation.[100, 107] Lastly, EZH2 is required for NF-κB signaling in cancer.[108] Currently, strong efforts are made to selectively target EZH2. In this respect, GSK126 is a small-molecule inhibitor of EZH2 that is more than 1000-fold selective for EZH2 over other methyltransferases and 150-fold as compared to EZH1.[109] Thus, studies evaluating the effects of GSK126 deserve further investigation.

2 AIMS OF THE THESIS

We hypothesize that obesity/diabetes-induced epigenetic changes may regulate ROS-driven vascular and cardiac dysfunction by altering the transcription of key genes implicated in redox balance and inflammatory-related pathways. Therefore, the overall aim of the present thesis was to investigate the molecular and cellular mechanisms underpinning obesity/diabetes-mediated vascular and cardiac dysfunction.

Specific Aims

- To study the role of epigenetic regulation of $p66^{Shc}$ in obesity-induced vascular dysfunction **(Study I).**
- To investigate whether epigenetic regulation of $p66^{Shc}$ is responsible for persistent myocardial oxidative stress and inflammation despite intensive glycemic control **(Study II).**
- To assess the protective effect of AP-1 transcription factor JunD in the diabetic heart **(Study III).**
- To determine the role of histone methyltransferase EZH2 in high glucose-induced oxidative stress and inflammation (**Study IV).**

3 STUDY DESIGN AND METHODS

The studies included in this thesis are based on the use of different *in vitro* and *in vivo* methods to address our specific research questions. A detailed description of the methods employed is reported in individual papers.

3.1 PARTICIPANTS (STUDIES I AND III)

In Study I, the clinical study population consisted on the inclusion of 21 subjects with severe abdominal obesity and 20 non-obese age-matched control subjects (**Table 1**). Obese subjects were recruited among 220 consecutive patients referred to the Department of Endocrinology (University of Pisa, Italy) from January 2010 to January 2014 for screening in view of laparoscopic bariatric surgery. The exclusion criteria were as follows: (a) history or clinical evidence of hypertension (blood pressure >140/90 mmHg), (b) smoking history, (c) ethanol consumption (more than 60 g or one-half liter of wine/day), (d) hypercholesterolemia, (e) diabetes mellitus, (e) overt cardiovascular disease, and (f) renal dysfunction or menopause. In addition, patients taking cardiovascular or metabolic drugs were also excluded. Control subjects were recruited among patients hospitalized in the Surgery Unit (University of Pisa, Italy) to undergo laparoscopic surgery for cholecystectomy. Ethical approval for the study was obtained from the Local Ethical Committee and all participants provided written informed consent for their participation.

In study III, left ventricle specimens were obtained from cardiac biopsies performed in 5 patients with diabetes and heart failure (New York Heart Association [NYHA] class III) and 6 control subjects (**Table 2**) from the Advanced Study of Aortic Pathology (ASAP) biobank undergoing selective open-heart surgery for ascending aortic aneurysm at Karolinska University Hospital, Stockholm. The biopsies were immediately snap-frozen in liquid nitrogen and stored at -80°C.

Table 1. Demographics and laboratory parameters in the study I population

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

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-insulin resistance; HR, heart rate; LDL-C, low density lipoprotein cholesterol; SBP, systolic blood pressure; TG, triglycerides.

Table 2. Demographics and laboratory parameters in the study III population.

Characteristics	Patients with DM (n=5)	Control subjects (n=6)	*p*-value
Age	64 ± 4.38	60 ± 11.10	0.51
Gender (M/F)	4/1	6/0	
Weight (Kg)	86.42±12.18	87.33 ± 9.62	0.90
BMI	29.76 ± 4.14	27.96 ± 2.73	0.45
Blood pressure			
Systolic (mmHg)	110 (82;87)	135 ± 21.7	0.08
Diastolic (mmHg)	65 ± 12.98	84.6 ± 17.77	0.09
Laboratory			
Creatinine (umol/L)	116 ± 43.23	85.16 ± 29.43	0.23
Glucose (mmol/L)	10.05 ± 3.15	6 ± 0	<0.05
Medications	n (%)	n (%)	
ACE-Inhibitors	4 (80)	2 (33)	
ARBs	2 (40)	2 (33)	
β-blockers	5 (100)	5 (83)	
MRAs	4 (80)	--	
Loop diuretics	5 (100)	1 (17)	
Anticoagulants	3 (33)	1 (17)	
Statins	5 (100)	5 (83)	
Hypoglycemic agents	5 (100)	--	
ECHO parameters			
LVEF (%)	20.8 ± 8.65	60.83 ± 4.48	<0.001
LVEDD (mm)	67.2 ± 5.87	49.5 ± 3.77	<0.001

Data are mean ± standard deviation unless otherwise indicated. BMI, body mass index; ACE, angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers; MRA, mineralocorticoid receptor antagonists; LVEF, left ventricle ejection fraction; LVEDD left ventricle end diastolic diameter.

3.2 INDUCTION OF DIABETES (STUDIES II AND III)

Diabetes was induced in 4-months old C57BL/6 wild type (WT) and α-MHC-*JunD*^{fg} male mice by a single high dose of streptozotocin (STZ, 180 mg/KG, intraperitoneal), dissolved in sterile citrate buffer (pH 4.5) and injected within 15 minutes (min). Both control groups received citrate buffer alone. STZ-treated animals with 3 random blood glucose levels >13.9 mmol/l defined as diabetic. Animals were housed under standard laboratory conditions with free access to water and laboratory chow diet. Animal experiments were followed in accordance with the guidelines approved by Institutional Animal Care Committee of Karolinska Institutet. All animals were euthanized after 4 weeks of follow-up.

3.3 *IN VIVO* EDITING OF CHROMATIN MODIFIERS (STUDY I)

For overexpression of SUV39H1, obese (*Lep*$^{Ob/Ob}$) mice were either injected 40 μg of predesigned mouse SUV39H1 cDNA clone (MC200652) or cloning vector (PCMV6-Kan/Neo, PCMV6KN) together with the cationic transfection reagent *in vivo*-jetPEI, according to instructions provided by manufacturer. For *in vivo* knockdown of JMJD2C and SRC-1, predesigned siRNAs were injected, as previously reported [110]. Based on time-dependent studies, SUV39H1 cDNA clone as well as JMJD2C and SRC-1 siRNAs were injected intravenously for four weeks after every five days. A scrambled-siRNA was used as a negative control.

3.4 CONVENTIONAL ECHOCARDIOGRAPHIC MEASUREMENTS (STUDIES II AND III)

Echocardiographic parameters were assessed before and four weeks after the diabetes induction. Mice were anesthetized with 2-5% of isoflurane mixed with oxygen. Echocardiography was performed to evaluate left ventricular functions by using high resolution Micro-Ultrasound System (Vevo 2100, Visualsonics) equipped with a 22-55 MHz (MS550D) linear array transducer. A rectal temperature probe was inserted to monitor and maintain mice body temperature at ~37°C using heating pad and heating lamp. The chest was shaved and pre-warmed ultrasonic gel was applied to the shaved site. M-mode and B-mode images were obtained at the mid-papillary level in the parasternal short-axis and long-axis views.

3.5 SPECKLE-TRACKING BASED STRAIN MEASURES OF MYOCARDIAL DEFORMATION (STUDIES II AND III)

It has been found that parasternal long-axis views are the most reproducible myocardial views for longitudinal strain analyses in mice. Parasternal short-axis views at the mid-papillary level

were acquired for circumferential and radial strain analyses, and all images were acquired at a frame rate >200 frames/second and at an average depth of 11 mm. Strain analyses were conducted by a single investigator on all groups of mice by using a speckle-tracking algorithm (VevoStrain, VisualSonics).

3.6 ORGAN CHAMBER EXPERIMENTS (STUDY I)

Mice were sacrificed by intraperitoneal injection of sodium pentobarbital (50 mg/Kg). The aorta was completely excised from the heart to the iliac bifurcation and put it immediately in cold modified Krebs-Ringer bicarbonate solution (pH 7.4, 37°C, 95% O_2; 5% CO_2) composed of the following constituents (in mmol/L): NaCl (118.6), KCl (4.7), $CaCl_2$ (2.5), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $NaHCO_3$ (25.1), glucose (11.1), and calcium EDTA (0.026). The aorta was then cleaned of fat and connective tissues and used immediately in isometric tension studies. Endothelium-dependent and endothelium-independent relaxations were determined in a pressurized myograph by measuring responses to cumulative concentrations of acetylcholine (Ach, 1 nmol/l to 100 μmol/l) and sodium nitroprusside (0.01 to 100 μmol/l), respectively. Vessels were pre-contracted with norepinephrine (NE, 1 μmol/l).

3.7 ASSESSMENT OF SUPEROXIDE ANION GENERATION BY ESR SPECTROSCOPY (STUDIES I, III AND IV)

Superoxide anion generation (O_2^-) was assessed in endothelial cells and mouse heart tissues by electron spin resonance (ESR) spectroscopy using the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine (CMH). O_2^- production was determined by the oxidation of 1-hydroxy-3-carboxy-pyrrolidine (CP-H) to paramagnetic 3-carboxy-proxyl (CP). Signals were quantified by measuring the total amplitude after correction of baseline and subtraction of background.

3.8 MEASUREMENTS OF 3-NITROTYROSINE LEVELS (STUDY II)

Levels of oxidative stress marker 3-nitrotyrosine (3-NT) in heart homogenates and cell lysates were measured using OxySelect^{TM} Nitrotyrosine ELISA kit ((ab116691; Abcam) following the manufacturer`s instructions. Briefly, 50 μL of nitrated BSA standard or samples were added into nitrated BSA pre-absorbed enzyme-linked immunosorbent assay (EISA) plate and incubated for 2 hours. To each well, an anti-nitrotyrosine antibody was added and incubated for 1 hour followed by addition of HRP-conjugated secondary antibody. Development solution was added to each well following addition of stop solution. Absorbance was immediately read at 450 nm using spectrophotometer. Nitrotyrosine in

samples was determined by comparing with a standard curve prepared from predetermined nitrated BSA standards.

3.9 NF-KB BINDING ACTIVITY (STUDIES II-IV)

NF-κB p65 binding reaction was performed with 20 μg of nuclear lysate or 40 μg of whole cell lysate in a 96-well plate immobilized with consensus sequences for NF-κB (GGGACTTTCC) for 1 hour at RT. Then, washing (with washing buffer) and incubation (with anti- NF-κB p65 antibody for 1 hour at RT and, thereafter, with horseradish peroxidase-conjugated secondary antibody) steps were performed. Finally, the degree of NF-κB p65 DNA binding was assessed by spectrophotometry at 450 nm.

3.10 ISOLATION OF MITOCHONDRIAL AND CYTOSOLIC FRACTION (STUDY II)

Heart tissues were suspended in the mitochondrial buffer (10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 0.25 M sucrose, pH 7.2). The tissues were gently homogenized with 30 strokes in a Dounce homogenizer. The homogenate was centrifuged at 750 xg for 10 min at 4°C to pellet nuclei and unbroken cells and the supernatant was collected and centrifuged at 10,000 xg for 15 min. The resulting mitochondrial-enrich pellet was collected by resuspending it in lysis buffer (named mitochondrial fraction) whereas the supernatant was referred as the cytosolic fraction.

3.11 MITOCHONDRIAL SWELLING ASSAY (STUDY II)

40 μg of freshly isolated mitochondria from mouse hearts in swelling buffer (containing 250 mmol/L sucrose, 10 mmol/L MOPS, 5 μmol/L EGTA, 2 mmol/L MgCl2, 5 mmol/L KH2PO4, 5 mmol/L pyruvate, and 5 mmol/L malate) were incubated with 150 μmol/L of calcium chloride in a final volume of 200 μL in a 96-well plate for 20 min. Mitochondrial swelling was assessed by reading absorbance every 30 seconds at 520 nm.

3.12 CHROMATIN IMMUNOPRECIPITATION ASSAY (STUDIES I- IV)

For chromatin immunoprecipitation (ChIP), mouse heart tissues were diced and fixed in formaldehyde solution (1%). The homogenate was cross-linked for 10 min at room temperature (RT), and excessive formaldehyde was quenched by using 125 mM of glycine. After quenching, the homogenate was resuspended in SDS-based lysis buffer and sonicated to obtain chromatin fragments of 200 to 500 bp using a water bath sonicator. Immunopurification of soluble chromatin was performed using antibodies against JunD, H3K4me1, H3K4me3, H3K9me2, H3K9me3 and H3K27me3. The non-specific mouse IgG

antibody was used as negative control. The antibody-bound chromatin fraction was pulled down using dynabeads coated with protein A. Washing steps, revere cross-linking and purification of DNA conjugates were performed according to previously described protocol.[101] ChIP-enriched DNA sequences were detected by using real-time PCR system.

3.13 METHYLATED DNA ENRICHMENT (STUDY III)

Isolated genomic DNA from mouse heart was fragmented to 150-300 bp using a Diagenode sonicator. Methyl-CpG-binding domin (MBD) protein was coupled with dynabeads. Fragmented genomic DNA was incubated with coupled MBD-beads on rotating mixer for 1 hour at RT. Methylated DNA was eluted by 2M of NaCl buffer and precipitated with ethanol. The obtained sequences were quantified by real-time PCR.

3.14 STATISTICAL METHODS

Results were confirmed to follow a normal distribution with the Kolmogorov-Smirnov test of normality. All normally distributed variables are presented as mean (standard deviation), unless otherwise stated. Data that failed the normality assumption 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 using the v^2 test. Data that passed the normality assumption were analyzed using unpaired 2-sample t, whereas multiple comparisons were performed by 1-way analysis of variance (ANOVA) followed by Bonferroni correction. A multiple t-test using the Benjamin–Hochberg false discovery rate procedure was employed for the analysis of gene expression data [real-time polymerase chain reaction (PCR) array]. Spearman ranked correlation test was used for correlation analysis. Probability values <0.05 were considered statistically significant. All statistical analyses were performed with GraphPad Prism Software (version 5, 6.03, 7.03).

4 RESULTS AND DISCUSSION

4.1. Interplay among H3K9-editing enzymes SUV39H1, JMJD2C and SRC-drives $p66^{Shc}$ transcription and vascular oxidative stress in obesity (STUDY I)

Current understanding of molecular pathways and biological processes unveiling vascular phenotype in patients with obesity is limited. Gene environment interaction as a putative

mechanism in vascular complications of obesity remains uncertain [111-113]. A strong body of evidence supports the notion that endothelial dysfunction contributes to the development of obesity-related CVD [114]. Accumulation of ROS generation is a key event preceding the development of endothelial dysfunction and vascular disease [114, 115]. In this regard, mitochondrial adaptor $p66^{Shc}$ protein, which is part of a mitochondrial complex, regulates endogenous production of free radicals and apoptosis [66].

Eukaryotic chromosome is composed of histone-DNA complexes forming the chromatin that are organized into subunits called nucleosomes. In nucleosomes, chromosomal DNA is packaged around histone proteins [15]. A key mechanism that regulates chromatin organization is the covalent modification such as acetylation and methylation of specific amino residues on histone tails [116]. These histone modifications may cluster in different orientations to regulate chromatin accessibility [117]. For instance, acetylation of histone 3 at lysine 9 (H3K9ac) is associated to open chromatin and active gene transcription whereas methylation of histone 3 at lysine 9 (H3K9me) is associated to heterochromatin, characterized by tightly packed form of DNA and inactive gene transcription [118]. Based on this background, we investigated whether chromatin modifications may regulate vascular ROS by modulating the transcription of the mammalian adaptor $p66^{Shc}$, a key redox gene implicated in mitochondrial generation of free radicals and translation of oxidative signals into apoptosis [50, 51].

First, we investigated the link between endothelial dysfunction, oxidative stress, and $p66^{Shc}$ gene expression in visceral fat arteries (VFAs) isolated from obese and control individuals. Obese subjects showed an impairment of acetylcholine-induced vasorelaxation as compare to control subjects (**Figure 1a**) Consistently, generation of mitochondrial superoxide anion (O_2^-) was higher in VFA from obese as compared to controls (**Figure 1b)**. However, pretreatment with antioxidant prevented impaired relaxation to acetylcholine, suggesting that oxidative stress contribute to the impairment of endothelial function in this setting (**Figure 1a**). $p66^{Shc}$ gene expression was significantly increased in obese VFA as compared to control and was significantly correlated with endothelial impairment and mitochondrial oxidative stress in obese VFA (**Figure 1d-e**).

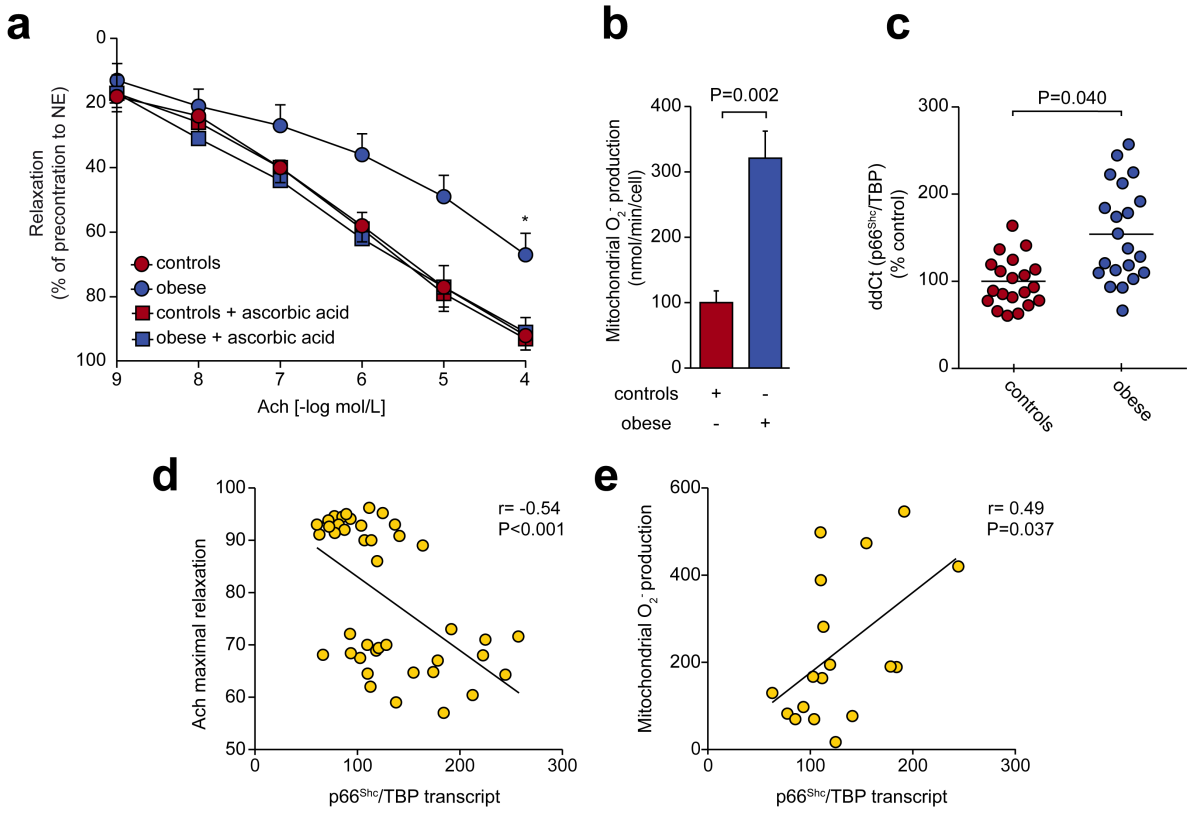

Figure 1. (**a**) Endothelium-dependent relaxation to Ach in small visceral fat arteries (VFA) isolated from obese subjects and age-matched healthy controls, in the presence or in the absence of antioxidant ascorbic acid; (**b**) ESR spectroscopy analysis of O_2^- generation in isolated mitochondria from VFA of obese patients and controls. (**c**) Gene expression of the mitochondrial adaptor $p66^{Shc}$ in isolated vessels from the two groups. (**d-e**) Spearman correlations of $p66^{Shc}$ gene expression with Ach maximal relaxations and mitochondrial O_2^-, respectively. (**a**) Repeated-measures analysis of variance (ANOVA) followed by Bonferroni's post-test; (**b-c**) Student's t-test; (**d-e**) Spearman correlations, r= correlation coefficient. Data are expressed as means ± SEM, n= 20-21 per group. Ach, acetylcholine; O_2^-, superoxide anion.

In order to investigate epigenetic regulation of $p66^{Shc}$ gene in obesity, we performed a real time PCR array for chromatin modifying enzymes in VFA isolated from obese and normal weight subjects. We observed 27 out 84 genes were deregulated in obese as compared to control VFA. Among these 84 genes, 21 were upregulated and 6 were downregulated in VFA from obese patients (**see Figure 2; Paper I**). Since previous studies have been shown that epigenetic regulations of $p66^{Shc}$ occur on histone 3 (H3), we performed chromatin immunopreciption (ChIP) to unveil H3 modifying enzymes interacting with $p66^{Shc}$ promoter. We found that only methyltransferase SUV39H1, demethylase JMJD2C and acetyltransferase SRC-1 were involved in epigenetic remodeling of H3K9 on $p66^{Shc}$ promoter (**Figure 2A**). Interestingly, acetylation of H3K9 on $p66^{Shc}$ promoter (H3K9ac) was increased, whereas di-methylation (H3K9me2) and tri-methylation (H3K9me3) of H3K9 on $p66^{Shc}$ promoter were reduced in obese as compared to control VFA (**Figure 2B**). These findings suggest that

SUV39H1, JMJD2C and SRC-1 may contribute to p66^{Shc} transcription, oxidative stress and vascular dysfunction in obese patients.

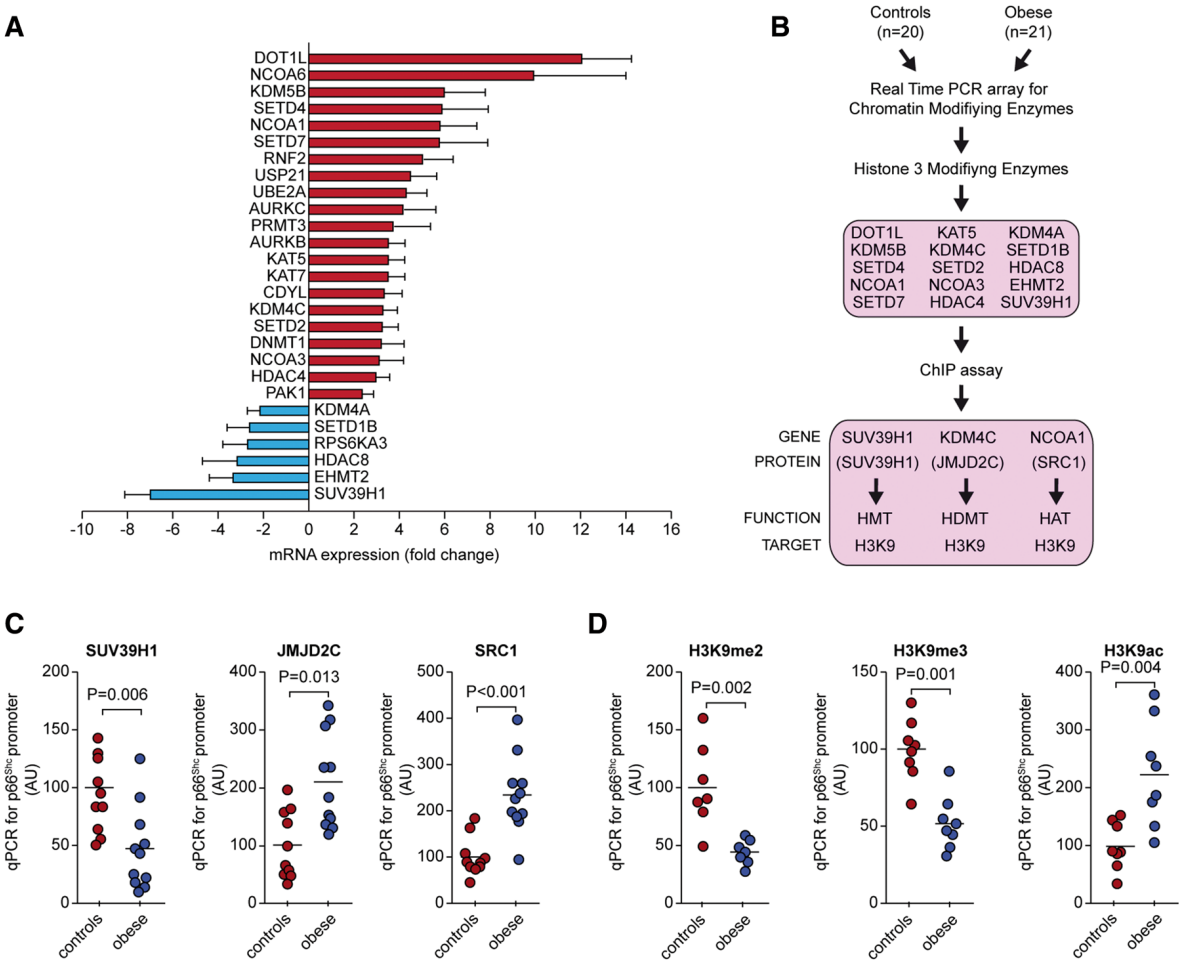

Figure 2. Adverse epigenetic remodelling of H3K9 on human p66^{Shc} promoter. (A) Real-time polymerase chain reaction array showing deregulated chromatin modifying enzymes in obese vs. control visceral fat artery. (A) change of at least two-fold (>2 or < −2) with $P < 0.05$ was considered significant; (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.

Similar to the human findings, p66^{Shc} gene expression was significantly increased in aorta of obese mice (*Lep*$^{Ob/Ob}$). Endothelial function was impaired in obese mice as compared to WT controls, whereas p66^{Shc} knockout obese mice were protective against endothelial dysfunction. Mitochondrial O_2^- levels were significantly increased in vasculature of *Lep*$^{Ob/Ob}$ but not in *Lep*$^{Ob/Ob}$*p66*$^{-/-}$ as assessed by ESR spectroscopy. We confirmed that H3K9 modifying enzymes SUV39H1, Jmjd2C and SRC-1 were deregulated in the aorta isolated

from $Lep^{Ob/Ob}$ as compared to WT controls. Furthermore, H3K9ac was increased on $p66^{Shc}$ promoter whereas H3K9me2 and H3K9me3 were reduced in $Lep^{Ob/Ob}$ as compared to WT controls (**see Figure 3; Paper I**).

To understand the contribution of H3K9 modifying enzymes SUV39H1, Jmjd2C and SRC-1 to vascular oxidative stress, we selectively reprogrammed their expressions in endothelial cells isolated from $Lep^{Ob/Ob}$ mice. Interestingly enough, we found that either overexpression of SUV39H1 or silencing of Jmjd2C and SRC-1 blunted $p66^{Shc}$ upregulation and vascular oxidative stress. These results were also confirmed in the *in vivo* setting. Our results demonstrated that SUV39H1, JMJD2C and SRC-1 were critically involved in $p66^{Shc}$ gene regulation. In order to understand how these enzymes functionally interact, we performed ChIP experiments to unveil functional molecular interactions among SUV39H1, JMJD2C and SRC-1. We found that binding of SRC-1 to $p66^{Shc}$ promoter was significantly reduced in the vasculature of $Lep^{Ob/Ob}$ mice treated either with SUV39H1 overexpressing vector or JMJD2C siRNA. In contrast, knockdown of SRC-1 did not affect the binding of SUV39H1 and JMJD2C to $p66^{Shc}$ promoter. Of interest, only SUV39H1 overexpression blunted $p66^{Shc}$ transcription by recruitment of both JMJD2C and SRC-1 to $p66^{Shc}$ promoter (**see Figure 4; Paper I**).

Previous studies have shown that H3K9 modifications are associated with impaired insulin signalling, deregulation of metabolic and inflammatory genes as well as changes in appetite [119-123]. More importantly, H3K9 and related histone marks have been found to contribute to intergenerational metabolic reprogramming with a profound impact on phenotype variation and evolution [124]. However, modifications of H3K9 in obesity-induced oxidative stress and vascular phenotypes remain unmasked. This study showed for the first time that both human and experimental obesity are strongly associated with specific remodelling of H3K9, characterized by increased AcH3K9 and reduced H3K9me2 as well as H3K9me3. Strikingly, these epigenetic changes resulted due to deregulation of three chromatin modifying enzymes such as the methyltransferase SUV39H1, demethylase JMJD2C and acetyltransferase SRC-1 interacting to the promoter of $p66^{Shc}$ gene. Among these three chromatin-modifying enzymes, we observed that SUV39H1 was downregulated whereas JMJD2C and SRC-1 were upregulated. This obesity-induced expression pattern led to increased H3K9 acetylation by SRC-1 and decreased methylation by SUV39H1 and JMJD2C, thus fostering the shift from heterochromatin to active euchromatin. Interestingly enough, either overexpression of SUV39H1 or gene silencing of JMJD2C and SCR-1 were able to edit the H3K9 landscape, thus blunting transcription of the adaptor $p66^{Sh}$ in endothelial cells both *in vitro* and *in vivo*

settings of obesity. Furthermore, we also found that SUV39H1 fosters JMJD2C/SRC-1 recruitment to p66^{Shc} promoter, however JMJD2C and SRC-1 do not affect SUV39H1 activity. This indicates that downregulation of SUV39H1 is the initial event in the setting of obesity responsible for increased JMJD2C and SRC-1 recruitment to p66^{Shc} promoter. Indeed, genetic disruption of SUV39H1 in lean control mice recapitulated obesity-induced epigenetic landscape on H3K9. Taken together, our results have unveiled a novel epigenetic mechanism underlying obesity-induced mitochondrial oxidative stress in vasculature (**Figure 3**).

The analyses of recent clinical trials of antioxidants for cardiovascular disorder targeting ROS (ROS scavengers) have shown that they are ineffective and sometimes harmful therapeutic strategy [125]. Accordingly, antioxidants partially scavenge cellular ROS without impacting mitochondrial redox signalling pathway [126]. On contrary, modulation of SUV39H1 expression may represent a novel therapeutic option to revert adverse H3K9 epigenetic modifications and oxidative stress in obese vasculature.

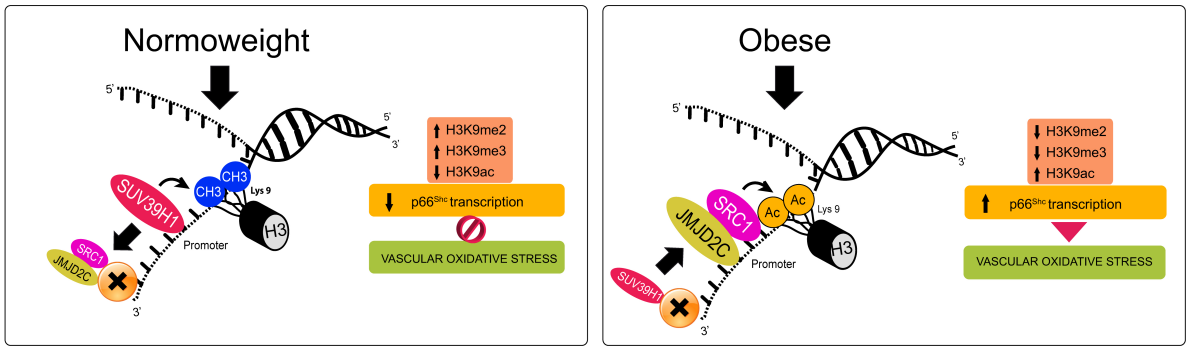

Figure 3. **Role of SUV39H1 in obesity-induced vascular oxidative stress**. In normal weight, SUV39H1 expression maintains H3K9 methylation levels preventing the binding of chromatin remodellers SRC-1 and JMJD2C to p66^{Shc} promoter. In the presence of obesity, down-regulation of SUV39H1 facilitates 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.

4.2. Hyperglycaemia-induced epigenetic changes drive persistant cardiac dysfunction via the adaptor $p66^{Shc}$ (STUDY II)

Recent randomized clinical trials have failed to demonstrate major CV benefits with intensive glycaemic control in patients with diabetes. Hyperglycaemia remains associated with an increased risk of impaired left ventricular function and HF, even after targeting HbA_{1c} levels <6.5% [127]. In contrast with these findings, very recent data from the clinical trials showed that gliflozins, selective inhibitors of the sodium glucose cotransporter 2 (SGLT2i), reduce HF-related outcomes in diabetic patients [128-130]. Although these drugs are effective in reducing hyperglycaemic burden, SGLT2i-related benefits on HF are likely driven by other mechanisms including osmotic diuresis, effects on plasma volume, sodium retention with modulation of the cardio-renal axis and neurohumoral activation. Collectively, the analysis of clinical trials conducted so far suggests that hyperglycaemia may have long-lasting effects which persist even after normalization of blood glucose levels. Out of different hyperglycaemia-related signalling pathways, redox pathway plays a major role in the development of diabetic cardiomyopathy and HF [63]. However, the molecular mechanisms regulating ROS generation in the diabetic heart are not yet fully understood. In the present study, we have investigated whether epigenetic changes may regulate derailed transcriptional programs in hyperglycaemia-induced mitochondrial oxidative stress and left ventricular dysfunction.

Diabetes was induced in C57/B6 male mice by streptozotocin and followed for six weeks. After three weeks of diabetes, an additional group of mice received insulin treatment to achieve optimal glycaemic control (**Figure 4A**). After six weeks, mice were sacrificed and left ventricular specimens were collected for molecular studies. We found that mRNA and protein expression of adaptor $p66^{Shc}$ was significantly increased in diabetic heart compared to controls. Interestingly, glycaemic control did not revert such upregulation of $p66^{Shc}$. $p66^{Shc}$ mitochondrial translocation and release of cytochrome *c* are important steps in ROS production and apoptosis [50]. Therefore, we assessed $p66^{Sh}$ mitochondrial translocation and its interaction with cytochrome *c* in the different experimental groups. Mitochondrial translocation of $p66^{Shc}$ and its interaction with cytochrome *c* was significantly increased in diabetic heart despite of glycaemic control. Accordingly, mitochondrial O_2^- production was also increased in diabetic heart despite of glucose normalization (**Figure 4B-E**). Moreover, mitochondria isolated from diabetic heart showed persistent mitochondrial swelling, even after glucose normalization (**see Figure 1; Paper II**).

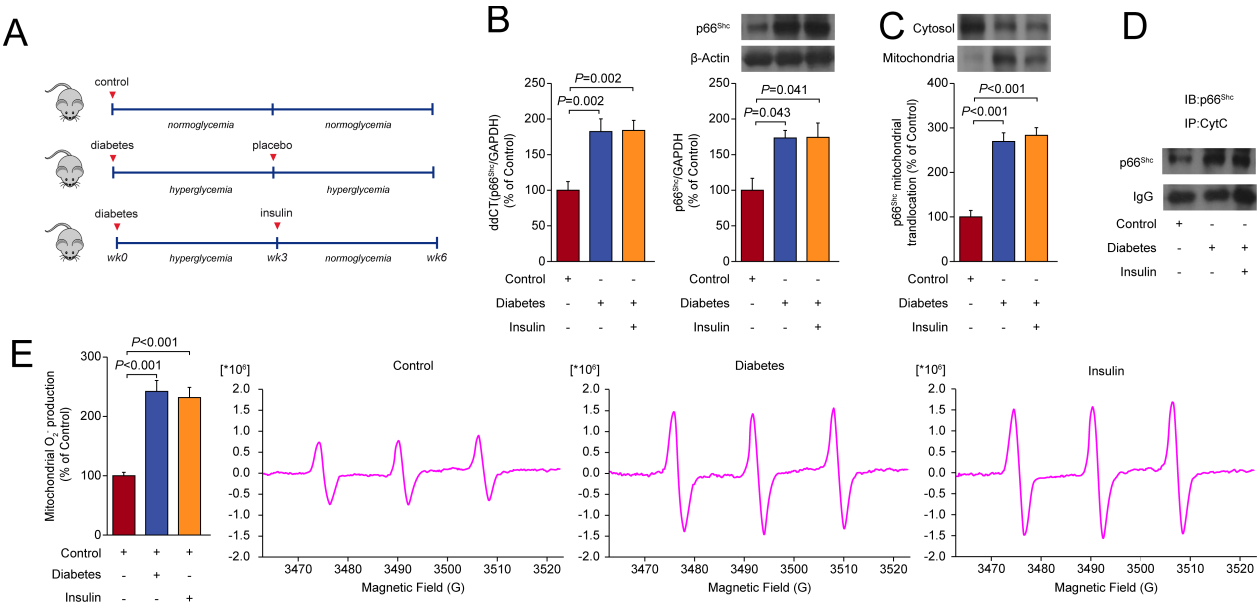

Figure 4. Diabetes-induced p66^{Shc} upregulation and mitochondrial oxidative stress are not reverted by glycaemic control. (A) Schematic showing study design. (**B**) Real time PCR and Western blot showing gene and protein expression of the adaptor p66^{Shc} in control and diabetic mice, with or without intensive glycaemic control with insulin (n=5/group). (**C**) Western blot and relative quantification showing p66^{Shc} mitochondrial translocation in left ventricular specimens from the different experimental groups (n=8/group). (**D**) Immunoprecipitation showing the interaction of p66^{Shc} with cytochrome *c*. (**E**) ESR spectroscopy analysis and representative spectra of mitochondrial superoxide (O_2^-) anion in the 3 experimental groups (n=5-8/group).

Given the ROS critical role in activation of inflammatory pathways [28, 29], we investigated myocardial inflammation in our setting. Interestingly, we found that NF-kB binding activity and its dependent genes VCAM-1, MCP-1 and IL-6 were significantly enhanced in diabetic heart despite of glucose restoration suggesting that ROS exerts myocardial inflammation (**see Figure 1; Paper II**).

To investigate cardiac function, we performed standard and speckle-tracking echocardiography. Compared to control, diabetic mice showed an impaired left ventricular function assessed by fractional shortening (FS) and ejection fraction (EF). In addition, advanced measures of global and systolic cardiac performance represented by myocardial strain in longitudinal axis showed a clear impairment of LV function in diabetic mice as compared to controls. Of interest, glucose restoration did not revert such abnormalities (**see Figure 2; Paper II**). In order to investigate whether p66^{Shc} drives myocardial dysfunction after glucose restoration, cardiac expression of p66^{Shc} was blunted by specific RNA interference. Interestingly enough, we found that cardiac mitochondrial ROS generation was significantly reduced in p66^{Shc} siRNA-treated mice as compared to mice treated with insulin alone . In line with these findings, mitochondrial swelling was persistent in diabetic mice that received insulin alone, while p66^{Shc} silencing reduced this phenomenon. Accordingly, we also

showed that glucose normalization did not revert $p66^{Shc}$-driven mitochondrial ROS generation, inflammatory pathways and left ventricular dysfunction. Indeed, silencing of $p66^{Shc}$ blunted mitochondrial oxidative stress, expression of VCAM-1, MCP-1 and IL-6 as compared to insulin alone. Of note, $p66^{Shc}$ knock-down during normoglycaemia was able to restore cardiac function. Thus, these findings indicate that persistent upregulation of $p66^{Shc}$ drive mitochondrial ROS despite of glucose restoration (**see Figure 3; Paper II**). To understand the epigenetic regulations of $p66^{Shc}$, we found that DNA methylation was significantly reduced at active regions of $p66^{Shc}$ promoter in diabetic heart (**see Figure 4; Paper II**). Interestingly, glucose restoration was not able to revert such detrimental signature. To dissect the mechanism, we assessed the expression of methyltransferase DNMT3b that is a methyl-writing enzyme critical involved in DNA methylation [15, 83]. We found that DNMT3b was downregulated in diabetic heart and such downregulation was not affected despite of glucose control. Moreover, interaction of DNMT3b to $p66^{Shc}$ promoter was reduced in diabetic heart despite of normoglycaemia restoration. DNA hypomethylation clustering with histone acetylation is a well-established posttranslational mechanism of active genes transcription [83]. Hence, we next investigated the acetylation of histone 3 (H3) on $p66^{Shc}$ promoter. We found that H3 acetylation was significantly increased in diabetic heart despite of glucose control. Of note, the chromatin-modifying enzyme SIRT1 that is H3 deacetylase was persistently downregulated in the diabetic heart, regardless of glucose control(**see Figure 4; Paper II**).. Accordingly, SIRT1-dependent deacetylation of $p66^{Shc}$ promoter was significantly reduced in diabetic heart and not improved by glucose normalization. To determine the impact of these chromatin modifiers on $p66^{Shc}$ cardiac transcription, we reprogrammed SIRT1 and DNMT3b in human cardiomyocytes. In line with our *in vivo* findings, we found that SIRT1 and DNMT3b were persistently downregulated in glucose-treated cardiomyocytes despite of glucose normalization. Interestingly, DNMT3b and SIRT1 blunted suppressed $p66^{Shc}$ upregulation and ROS production (**see Figure 4; Paper II**).

The pivotal role of miRNAs in posttranscriptional regulation has recently emerged as a key underlying mechanisms with direct impact on cardiovascular diseases [131]. For that reason, we investigated whether miRNAs modulate DNMT3b and SIRT1 expression, hence affecting chromatin architecture and cardiac $p66^{Shc}$ transcription. By using *in silico* prediction analysis, we found that DNMT3b and SIRT1 were targets of miR-218 and miR-34a, and expression of these miRNAs was markedly increased in diabetic heart despite of glucose normalization (**see Figure 4; Paper II**).. Interestingly enough, silencing of miR-218 and miR-34a blunted $p66^{Shc}$ transcription and oxidative stress as compare to restoration of normoglycaemia alone. Taken together, miR-218 and miR-34a target chromatin modifying enzymes DNMT3b and SIRT1

leading to persistent p66^{Shc} upregulation despite of glucose normalization (**see Figure 4; Paper II**).

Previous studies have shown that poor glycaemic control is associated with an increased risk of HF. In contrast to this observation, recent clinical trials have unexpectedly reported that intensive glycaemic treatment fails to reduce HF-related outcomes in patients with diabetes. Combined analysis of clinical trials suggests that hyperglycaemia may have long-lasting effects that may persist even after blood glucose normalization [132]. These findings support the notion that hyperglycaemic environment may be remembered in diabetic heart, and this might lead to persistent cardiac damage and dysfunction despite of glucose normalization. Mitochondrial redox signalling pathway may probably be involved in this effect. However, the underlying molecular mechanism remains to be unveiled. Mitochondrial overproduction of ROS alters cardiomyocytes function leading to cardiac damage. In the present study, we have postulated that dynamic epigenetic changes may contribute to affect transcription programs implicated in hyperglycaemia-induced oxidative stress and myocardial damage. Epigenetic changes are heritable changes in gene expression without alterations in the underlying DNA sequence [112]. These non-genetic changes are strictly attributed to three major epigenetic signatures: DNA methylation, histone modifications, and non-coding RNAs. In addition to other regulatory transcriptional mechanisms, epigenetic modifications modulate gene activity in development and differentiation, or in response to environmental stimuli [111]. Here in this study we show that epigenetic modifications, such as DNA hypomethylation and H3 acetylation, drive p66^{Shc} transcription, a key protein involved in mitochondrial ROS production and apoptosis [66]. Of interest, adverse epigenetic signatures on p66^{Shc} promoter were not erased by glucose normalization, suggestion that these epigenetic modifications are pivotal in maintenance of myocardial oxidative stress during subsequent normoglycaemia. Further mechanistic experiments revealed that persistent deregulation of chromatin modifiers DNMT3b and SIRT1 underlined p66^{Shc} promoter remodelling. Indeed, DNMT3b and SIRT1 overexpression in cardiomyocyte blunted p66^{Shc} persistent upregulation by regulating chromatin accessibility. Furthermore, we demonstrated that miR-218 and miR-34a were upstream regulators of DNMT3b and SIRT1. MiRNAs are a recently discovered class of noncoding RNAs that have emerged as important regulators of gene expression [133]. Albeit, previous studies have demonstrated that miRNAs are aberrantly expressed in cardiovascular pathologies [131], however their role in the modulation of chromatin modifying enzymes remains unknown. Our results unmask novel epigenetic mechanisms linking miRNAs with modifications of DNA-histone complexes and persistent upregulation of adaptor gene p66^{Shc}. We show that miR-218 upregulation in diabetic heart epigenetically

contributes to persistent p66^{Shc}-related mitochondrial oxidative stress via regulation of the methyltransferase DNMT3b. Although, the role of miR-218 have been investigated in heart development [134, 135] and vascular remodelling [136], its role in myocardial oxidative stress and cardiac dysfunction remains unclear. Our results indicate that diabetes-induced upregulation of miR-218 promotes adverse epigenetic remodelling of p66^{Shc} promoter in the heart. Indeed, upregulation of miR-218 causes downregulation of DNMT3b leading to hypomethylation of CpG dinucleotides, and increased transcription of p66^{Shc}. In line with these findings, we also found that miR-34a indirectly modulates H3 acetylation via epigenetic repression of the deacetylase SIRT1. MiR-34a inhibition blunted persistent p66^{Shc} upregulation and oxidative stress during subsequent normoglycaemia, suggesting that miR-34a is an important epigenetic regulator of mitochondrial ROS generation. Similarly, it has been shown recently that epigenetic silencing of miR-34a protects against maladaptive cardiac remodelling and myocardial damage following acute myocardial infarction [137]. In summary, our results suggest that targeting miR-218 and miR34a may represent a potential therapeutic strategy against mitochondrial oxidative stress in the diabetic heart, regardless of intensive glycaemic control (**Figure 5**).

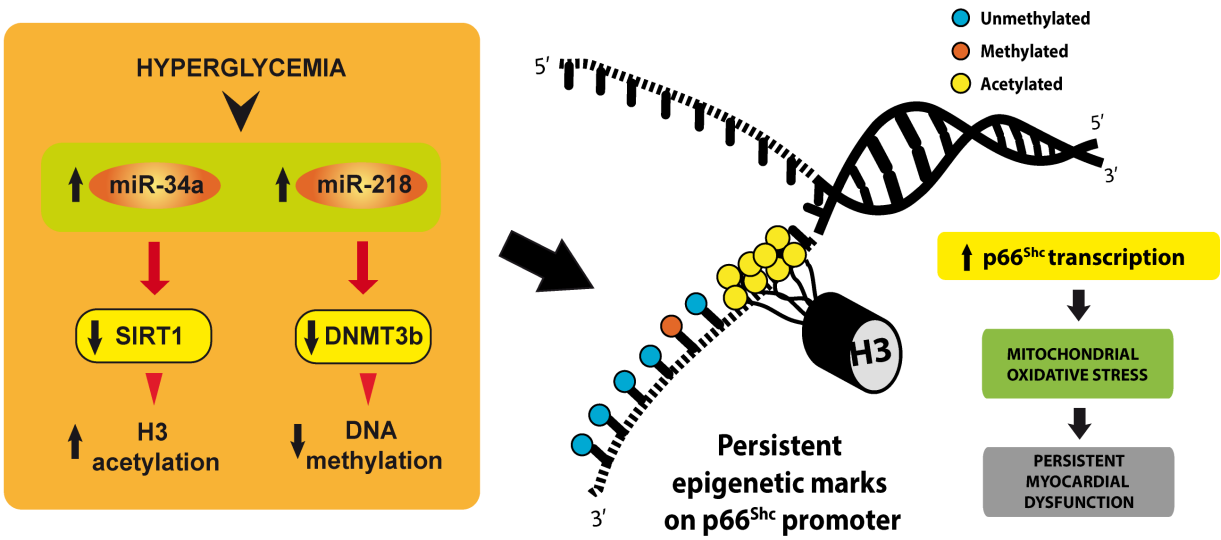

Figure 5. Schematic summarizing miR-218 and miR-34a orchestrate p66^{shc} transcription by targeting DNMT3b, DNA Methyltransferase 3 Beta and SIRT1, Sirutin 1.

4.3. Protective role of AP-1 transcription factor JunD in the diabetic heart p66^{Shc} (STUDY III)

Understanding the precise molecular mechanism that lead to oxidative stress in the diabetic heart is a major challenge to reduce cardiovascular disease burden over the next decades. Although ROS-dependent pathways have been intensively studied in hyperglycaemic conditions, the link between hyperglycaemia and free radical generation remain to be elucidated. The Activated Protein-1 (AP) transcription factor JunD is emerging as an important modulator of oxidative stress [78]. AP-1 is a family of dimeric complexes made by different members of three families of DNA-binding proteins: Jun, Fos, and ATF/CREB [78, 138-140]. These members assemble to form AP- 1 transcription factor and exert functional effects that are strongly influenced by their specific components as well as their cellular environment [78, 138]. In the present study we have investigated the role of JunD in diabetes-induced ROS-driven myocardial damage.

To investigate the role of JunD in the diabetic heart, diabetes was induced in WT and cardiac specific JunD transgenic mice (α-MHC-*JunD*^{tg}) of JunD by streptozotocin (**Figure 6 A**). After four weeks of diabetes, cardiac function was assessed by standard and speckle-tracking echocardiography in all four experimental groups. WT diabetic mice showed an impaired left ventricular function as compared to controls, as assessed by reduced FS and EF (**see Figure 3; Paper III**). Accordingly, myocardial strain in longitudinal axis revealed a clear impairment of LV function in WT diabetic mice as compared to controls. Of interest, such abnormalities were not observed in α-MHC-*JunD*^{tg} diabetic mice (**see Figure 3; Paper III**). Then, mice were euthanized and hearts were collected for molecular analyses. We found that gene and protein expression of JunD were significantly reduced in the heart of diabetic mice as compared to controls (**Figure 6 B-C**). This downregulation of JunD in WT mice was associated with increased myocardial O_2^- generation as assessed by ESR spectroscopy. In contrast, MHC-*JunD*^{tg} diabetic mice were protective against elevated O_2^- generation (**see Figure 4; Paper III**).

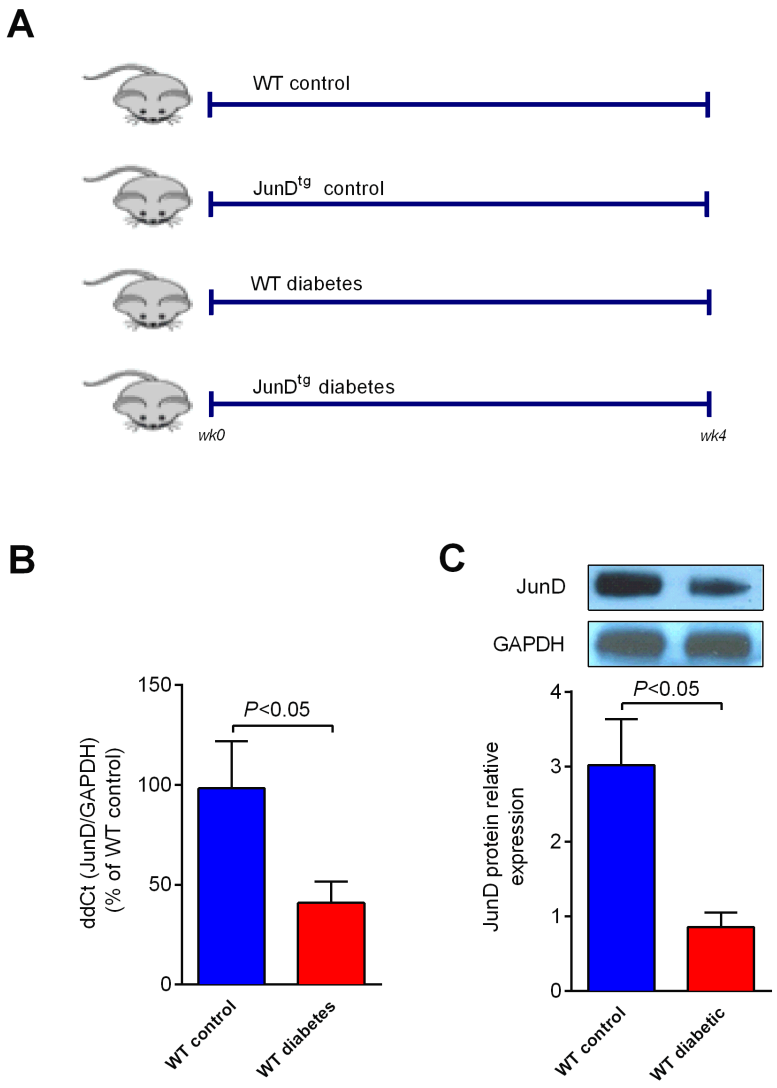

Figure 6. (**A**) Schematic representation of experimental groups. **(B)** Bar graphs showing downregulation of JunD mRNA and **(C)** protein expression in in wild-type (WT) diabetic mice as compared to controls. Results are presented as mean ± SEM; n=4-6 per group.

It is well established that diabetes alters the balance between pro- and anti-oxidants enzymes resulting in oxidative stress**.** Since JunD down-regulation was associated with an increased O_2^- generation in WT diabetic heart, we investigated the effect of JunD on ROS-generating and ROS-scavenging enzymes in diabetic heart in the presence or absence of cardiac overexpression of JunD. We found that gene and protein expression of superoxide dismutase 1 (SOD1) and aldehyde dehydrogenase 2 (ALDH2) were decreased in the heart of WT diabetic mice. While, NADPH oxidase subunits NOX2 and NOX4 were significantly upregulated as compared to WT controls. Interestingly enough, such detrimental changes were not found in the heart of MHC-*JunD*^{tg} diabetic mice suggesting that JunD is required for redox balance under diabetic conditions ((**see Figure 4; Paper III**)). In agreement with JunD downregulation, its binding on the promoter of anti-oxidant (*SOD1* and *ALDH2*) and pro-

oxidant (*NOX2* and *NOX4*) genes was significantly reduced in WT diabetic mice (**see Figure 5; Paper III**).

Given that ROS is a pivotal activator of inflammatory pathways, we investigated nuclear factor-kappa B (NF-κB)-dependent transcriptional programs in our setting. We found that NF-κB activity as well as NF-κB-dependent inflammatory genes *MCP-1*, *IL-6*, *TNFα* were significantly enhanced in the heart of WT diabetic mice as compared to controls (**Figure 4**). In line with these findings, gene expression of *IkB*, the inhibitory subunit of NF-κB was reduced. Of interest, MHC-*JunD*^{tg} diabetic mice were protected against the derangement of these inflammatory pathways (**see Figure 6; Paper III**).

Since it is well known that DNA methylation and histone modifications modulate gene expression, DNA methylation and posttranslational modifications of histone H3 at JunD promoter were investigated. We found that DNA methylation level of CpG islands in the promoter region of JunD was gene was significantly elevated in the heart of WT mice with diabetes as compared to controls. Accordingly, the active marks histone 3 lysine 4 trimethylation (H3K4me3) and histone 3 lysine 4 monomethylation (H3K4me1) were significantly reduced in WT diabetic heart as compared to controls. In contrast, we found that repressive histone 3 lysine 9 trimethylation (H3K9me3) was increased in the heart of WT diabetic mice as compared to controls (**see Figure 7; Paper III**). As a translational approach, left ventricular specimens were collected from patients with diabetes and heart failure and age-matched controls. Interestingly, JunD gene and protein levels were significantly reduced in patients compared to controls.

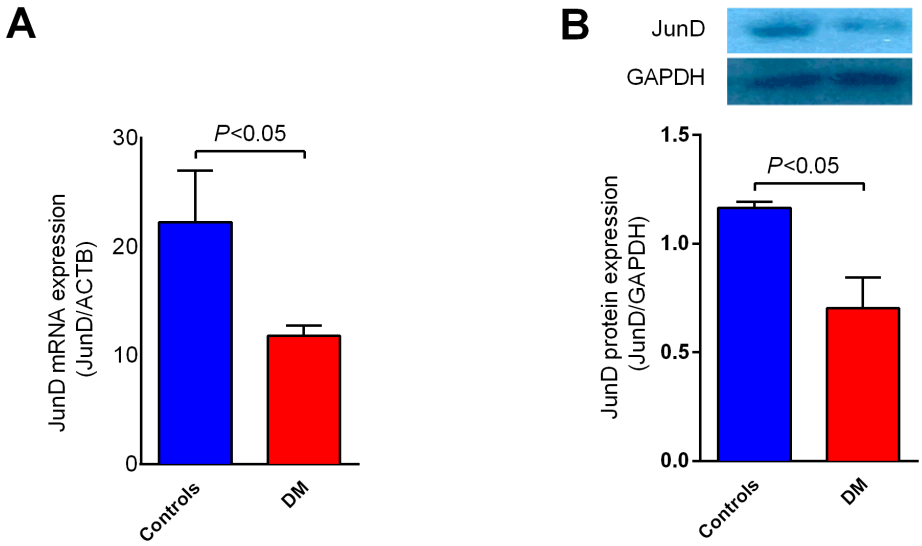

Figure 7. JunD expression in human heart. (A) mRNA and **(B)** protein expression of JunD in controls and in patients with diabetes and heart failure.

Ap-1 transcription factor JunD protects against oxidative stress and modulates different genes implicated in growth, proliferation and survival [140-142]. Previous studies have shown that ROS generation was increased in *JunD*$^{-/-}$ immortalized cells [75]. We have recently demonstrated that JunD deletion is associated with vascular oxidative stress and premature endothelial dysfunction in aging [78]. Of note, *JunD*$^{-/-}$ mice display reduced life span [76, 77, 143]. The observation that JunD gene and protein expression were reduced in the heart of diabetic WT mice prompted us to investigate the link between JunD downregulation and derailed oxidative/inflammatory myocardial pathways and its contribution to myocardial dysfunction. ROS accumulation alters myocytes function by triggering the cardiac inflammatory pathways and fibrosis [62, 63]. Here we have demonstrated that ROS accumulation in the myocardium was associated with impaired balance between ROS-scavenging and -producing enzymes. Indeed, SOD1 and ALDH2 were significantly reduced in WT diabetic mice suggesting that JunD is required for their transcription. It has been recently reported that ALDH2 protects against diabetes-induced myocardial dysfunction [144]. Accordingly, ALDH2 also protects against cardiac ischemia/reperfusion injury [145-147]. Here we showed that JunD downregulation in diabetic heart impairs ALDH2 expression, which may contribute to abnormal myocardial redox haemostasis. Our results are in line with the established role of JunD in transcriptional activation of genes involved in detoxification [148]. Of note, anti-oxidant response elements have been reported in the promoter regions of ROS-scavenging enzymes [149]. On the other side, we found that NOX2 and NOX4 were upregulated in the heart of WT diabetic mice. In agreement with ROS-induced NF-κB-dependent inflammatory pathway [150], inhibitory subunit of NF-κB was reduced, whereas *MCP-1*, *IL-6*, and *TNFα* genes were enhanced in the heart of WT diabetic mice. On contrary, α-MHC-*JunD*^{tg} mice were protected against myocardial oxidative stress, inflammation and left ventricular dysfunction suggesting that modulation of JunD expression plays a key role in ROS-driven left ventricular dysfunction. In line with our findings, it has been reported that adenoviral overexpression of wild-type JunD protects against phenylephrine-mediated cardiomyocyte hypertrophy [80]. Similarly, another study has shown that JunD also protect against myocardial apoptosis and hypertrophy-induced by pressure overload [81].

To dissect the molecular mechanism of JunD downregulation in diabetes, we investigated the modulation of JunD at the transcriptional level. Our analysis of JunD promoter showed a significant hypermethylation of CpG island in the heart WT diabetic mice compared to controls. Indeed, DNA methylation is a repressor of gene transcription in mammals [83]. In addition, we also found a deranged pattern of histone marks with reduced active marks such

as H3K4me3 and H3K4me1 and an increased level of the repressive mark H3K9me3. Histone posttranslational modifications, such as acetylation and methylation regulate gene expression by affecting their chromatin architecture [83]. Taken together, our results unveiled the epigenetic mechanisms responsible for JunD downregulation in diabetes, which could become novel therapeutic targets. Of note, JunD expression was significantly downregulated at both mRNA and protein level in heart tissues obtained from human diabetic patients. These findings indicate that downregulation of JunD may contribute to ROS-driven myocardial damage also in humans providing a translational perspective to our findings (**Figure 8**).

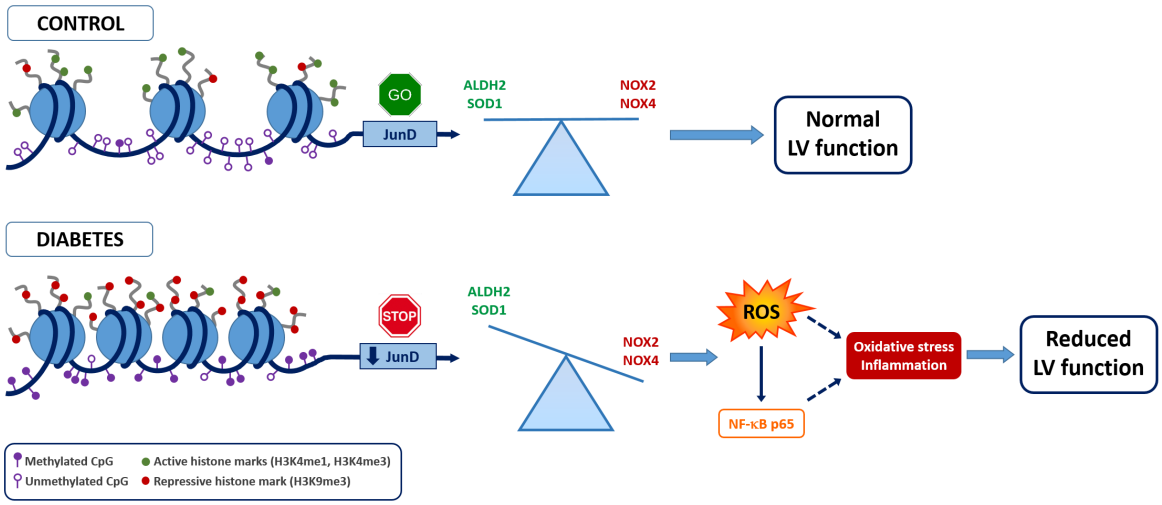

Figure 8 Schematic representation of JunD role in hyperglycemia-induced myocardial dysfunction. Under control conditions JunD protects against cardiac oxidative stress and inflammation by modulating the expression of genes involved in anti-oxidant defence system. However, in the setting of diabetes downregulation of JunD, mediated via DNA methylation and post-translational modifications of histones on JunD promoter, contributes to myocardial dysfunction by triggering overexpression of pro-oxidant and pro-inflammatory genes.

4.4. Inhibition of histone methyltransferase EZH2 blunts glucose-induce oxidative stress and inflammation in human aortic endothelial cells (STUDY IV)

Oxidative stress and inflammation are tightly interlinked processes involved in the pathogenesis of diabetes-associated endothelial dysfunction [148]. Previous studies have been reported that elevated ROS levels and inflammation are responsible for endothelial dysfunction in diabetes [26, 115]. However, little is known about the molecular mechanisms underpinning increased ROS production and inflammation in diabetes. Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that specifically catalyzes trimethylation of histone H3 at lysine residue 27 (H3K27me3) and, eventually, transcriptional repression [149]. Indeed, EZH2-depended chromatin remodeling fosters accumulation of free radicals and

inflammation [100, 108]. Pharmacological targeting of EZh2 in different cancer types has been found to be effective, however it remains unknown whether targeting EZH2 may reduce hyperglycemia-induced oxidative and inflammatory transcriptional programs. In the present study, we have investigated whether pharmacological inhibition of EZH2 by GSK126 attenuates hyperglycemia-induced oxidative stress and inflammation in human aortic endothelial cells (HAECs).

To investigate the effects of hyperglycemia on EZH2 activity, HAECs were exposed to high (HG) and normal glucose (NG) levels for 20 hours. We found a significant increase of histone H3K27me3 modification in HAECs exposed to a high glucose environment (**Figure 9A**). Indeed, EZH2 inhibition by GSK126 blunted EZH2 enzymatic activity as assessed by H3K27me3 protein analysis (**Figure 9A**). MTT is a calorimetric assay used for cell metabolic activity. MTT assay was performed to measure the cytotoxicity of the drug at different concentrations. As a result, no significant difference in HAEC viability was observed up to 5 μM concentration of GSK126. This 5 μM concentration of GSK126 was then used in subsequent experiments (**Figure 9B**).

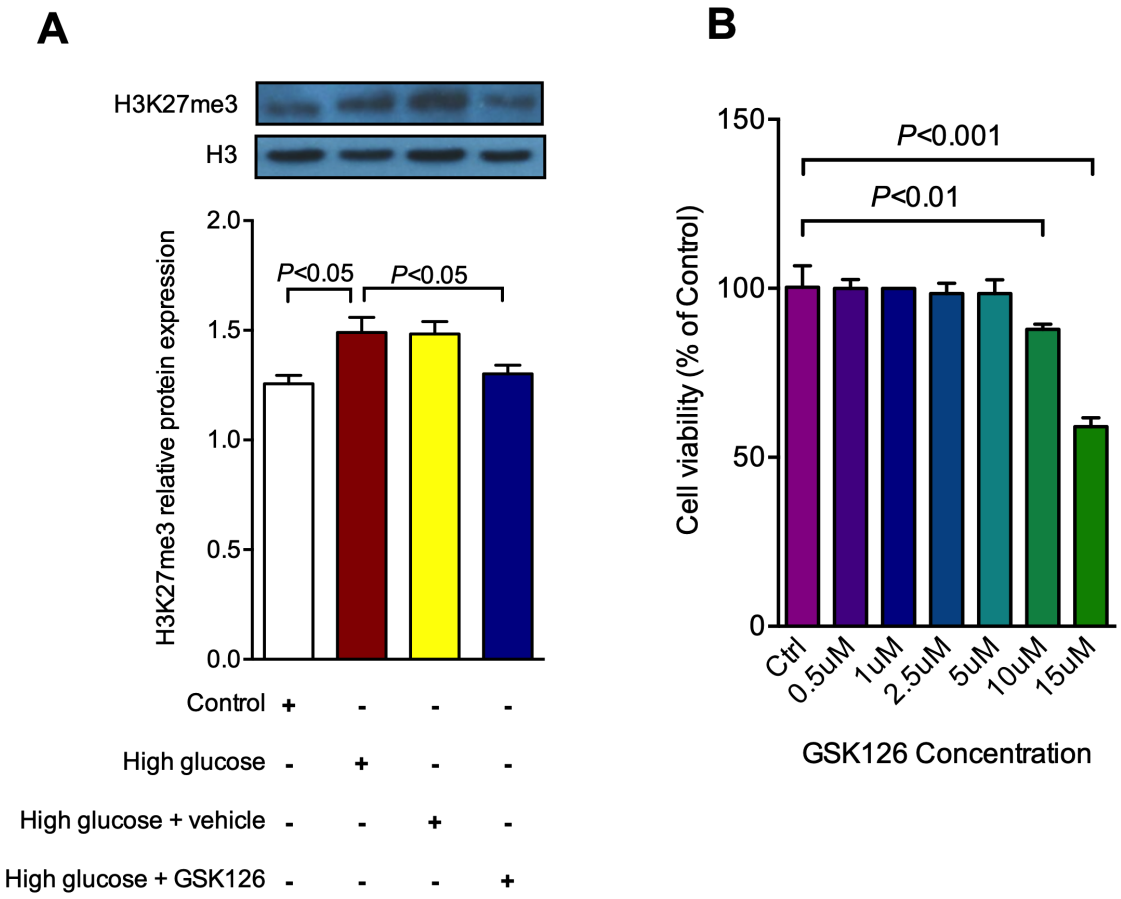

Figure 9. H3K27me3 expression and cell viability in the absence and the presence of GSK12 (A) EZH2 activity measured as H3K27me3 expression by western blot analysis. (**B**) Cell viability assessed by MTT assay. Results are presented as means ± SEM; n=3. Bonferroni-adjusted p values are shown for multiple comparisons.

HAECs exposed to HG showed a significant increase in endothelial O_2^- generation as compared to NG (**see Figure 2; Paper IV**). Interestingly enough, EZH2 inhibition by GSK126 abolished O_2^- generation in HAECs exposed to HG. Gene and protein expression of dismutase 1 (SOD1) and superoxide dismutase 2 (SOD2), major ROS-scavenging enzymes were significantly reduced in HAECs exposed to high glucose. By contrast, GSK126 treatment prevented glucose-induced down-regulation of ROS-scavengers. EZH2-mediated H3K27me3 was significantly increased on the promoter of SOD1 and SOD2 genes in HAECs exposed to high glucose (**see Figure 2; Paper IV**). Of interest, GSK126 blunted HG-induced trimethylation of H3K27 on promoter of SOD1 and SOD2 genes (**see Figure 2; Paper IV**). Accordingly, gene and protein expression of NOX4, a well-known source of ROS, were increased in HAECs exposed to HG. This increased expression of NOX4 was blunted by GSK126 treatment in HAECs exposed to HG (**see Figure 3; Paper IV**). Mechanistic studies have previously shown that AP-1 transcription factor JunD protects cells against oxidative stress by modulating NOX4 expression[75]. Therefore, we investigated binding of JunD on NOX4 promoter. Interestingly, we found that binding of JunD on NOX4 promoter was significantly reduced in HAECs exposed to HG. In contrast, GSK126 was able to restore the binding of JunD to NOX4 promoter. Furthermore, we also found that glucose-induced down-regulation of JunD was inhibited by GSK126. Indeed, high glucose-induced down-regulation of JunD was associated with increased H3K27me3 on JunD promoter. Interestingly, H3K27me3 was markedly reduced by GSK126 treatment (**see Figure 3; Paper IV**). Due to the effect of ROS on the activation of inflammatory pathways, NF-κB p65 binding activity and expression of MCP-1 and IL-6 genes were assessed and found to be enhanced in HAECs exposed to HG as compared to control cells [150]. Of interest, GSK126 abolished these detrimental inflammatory programs (**see Figure 4; Paper IV**). In line with these findings, we also found that the expression of SOD1, SOD2 and JunD genes was significantly reduced in human aortic endothelial cells isolated from patients with diabetes (D-HAECs) as compared to control HAECs**)**. In contrast, we found an up-regulation of NOX4, MCP-1 and IL-6 gene expression in D-HAECs, which could be reprogrammed by GSK126 treatment (**see Figure 5; Paper IV**).

Previous studies have demonstrated that oxidative stress and inflammation play a key role in the progression of endothelial dysfunction in diabetes [148, 151-153]. EZH2 is a methyltransferase belongs to SET1 family of methyltransferase, and is well known for its transcriptional repression via H3K27me3 histone modification [109]. The importance of EZH2 in maintenance of cellular haemostasis is outlined by the fact that its deregulation causes disease phonotypes,

such as cancer, neurodegenerative disease, cardiac and vascular dysfunction [100, 109, 154, 155]. A recent study showed an increased EZH2 expression and activity in endothelial cells exposed to high glucose [156]. Here we demonstrate that increased endothelial ROS generation was associated with derailed gene expression involved in redox signalling pathway. Our ChIP analysis revealed that the interaction of JunD and NOX4 promoter was significantly reduced in HAECs exposed to high glucose suggesting that HG deregulate NOX4 expression via JunD. NOX4 has been found a major source of ROS generation, and it contributes to endothelial dysfunction in diabetes [157]. Indeed, targeting NOX4 protects against ROS-driven endothelial dysfunction [158]. We found that down-regulation of JunD in HAECs exposed to high glucose is dependent on methyl-writing activity of EZH2. Targeting EZH2 by GSK126 in HAECs exposed to HG blunted EZH2 dependent signatures on JunD promoter and restored JunD expression. JunD was showed to protect against oxidative stress by modulating genes involved in redox signalling pathway [78]. Accordingly, *JunD*$^{-/-}$ immortalized cells showed increased ROS generation suggesting that JunD is crucial in maintaining the redox homeostasis [75]. ROS-scavengers such as SOD1 and SOD2 are important mediators of the redox balance. Our results have demonstrated that hyperglycaemia-induced endothelial ROS generation was associated with significantly down-regulation with SOD1 and SOD2. In line with our findings, it has been recently reported that SODs play a crucial role in oxidative inhibition of nitric oxide (NO), thus preventing peroxynitrite and preserving endothelial function [159]. Furthermore, we also demonstrate that active transcription of SODs depends on methyltransferase activity of EZH2. GSK126 preserved the endothelial redox balance by inhibiting glucose-induced down-regulation of SODs. We also provide strong evidence that EZH2 drives endothelial inflammation in HAECs in the setting of diabetes. Although, it has been shown recently that EZH2 promotes NF-kB signalling in liver failure [160], no previous studies have investigated whether EZH2 mediates inflammation in HAECs. In our study EZH2 induce NF-kB nuclear translocation and up-regulation of adhesion molecules in HAECs exposed to HG and also in D-HAECs. However, pharmacological inhibition of EZH2 by GSK126 suppressed NF-κB p65 activity and expression of IL-6 and MCP-1. These results deserve attention since NF-κB p65 activation has been recently reported in endothelium of patients with type 2 diabetes.[46] These results uncover adverse epigenetic signatures underlying endothelial dysfunction in diabetes. Targeting EZH2 may attenuate oxidative and inflammatory transcriptional programmes and thus prevent vascular disease in this setting (**Figure 10**)

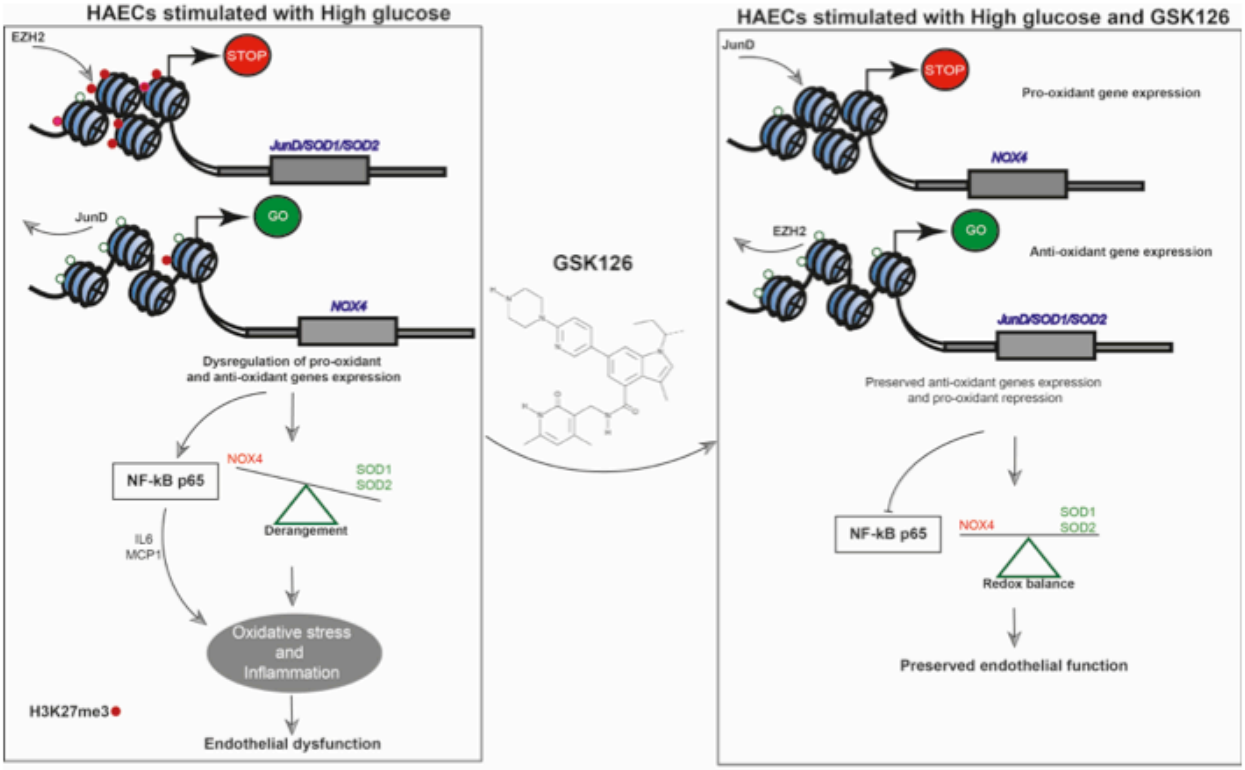

Figure 10. Schematic representation of the role of EZH2 in glucose-induced redox imbalance and inflammation. (A) High glucose–induced, EZH2-mediated H3K27me3 leads to oxidative stress and inflammation. **(B)** GSK126, an EZH2 specific inhibitor preserves redox balance and, hence, blunts the activation of inflammatory signalling. NADPH oxidase subunit NOX4 is regulated via recruitment of JunD on its promoter whereas SOD-1 and SOD-2 expression is maintained by inhibiting H3K27me3 writing activity of EZH2.

5 CONCLUDING REMARKS

The worldwide rising prevalence of diabetes and obesity is closely linked to cardiovascular morbidity and mortality. However, the current understanding of the signaling pathways triggering cardiovascular damage is still limited and, most of all, novel mechanism-based therapeutic approaches are in high demand. Oxidative stress and inflammation are well-established pathophysiological players. The epigenetic changes of DNA/histone complexes are emerging as critical regulators of transcriptional programs of oxidant and pro-inflammatory genes. Given this background the herewith thesis was designed to study the epigenetic mechanisms which may contribute to the onset of abnormal cardiovascular phenotypes. State-of-the-art techniques for the assessment of DNA methylation, histone modifications and non-coding RNAs (microRNAs) have been used both in obesity and diabetes experimental models as well as in human subjects to determine the role of such epigenetic signatures in triggering redox and inflammatory-mediated cardiovascular injury.

Specifically, we were able to identify a dynamic interplay among histone 3 lysine 9 (H3K9)-modifying enzymes SUV39H1, JMJD2C and SRC-1 orchestrating the transcriptional regulation of the adaptor $p66^{Shc}$, with subsequent mitochondrial ROS generation and vascular dysfunction. Moreover, we demonstrated that $p66^{Shc}$ transcription, ROS generation and left ventricular dysfunction in the heart of diabetic mice treated with high glucose is driven by a complex epigenetic mechanism linking miR-218/miR34a and chromatin editing enzymes DNMT3b/SIRT1. Interestingly, these promoter-related marks were not erased by intensive glycemic control, suggesting that epigenetic mechanisms are crucial for the maintenance of myocardial oxidative stress during subsequent normoglycaemia. These findings unveil novel epigenetic networks linking miRNAs with modifications of DNA/histone complexes. Targeting miR-218 and miR34a may thus represent a promising approach to blunt ROS generation in the diabetic heart, regardless of glycaemic control.

Recent evidence support the notion that the activator protein-1 (AP-1) transcription factor JunD is a key molecule implicated in ROS-driven cardiovascular aging. Here, we demonstrate that a reduced expression of JunD contributes to derangement of ROS homeostasis, inflammation and cardiac damage. Hyperglycemia-induced JunD downregulation is epigenetically regulated. Interestingly enough, diabetic mice with cardiac-specific overexpression of JunD were protected against ROS-driven damage. This work provides important molecular insights on the role of transcription factor JunD in diabetes-induced myocardial dysfunction and might open novel therapeutic perspectives in this setting.

It is indeed emerging that epigenetic changes are amenable to pharmacological intervention. To this aim we investigated the effect of selective inhibition of histone methyltransferase EZH2 on glucose-induced impairment of endothelial redox and inflammatory pathways. Interestingly enough, selective inhibitor GSK126 restored the transcription of anti-oxidant/anti-inflammatory genes, which was blunted by EZH2-mediated repressive H3K27me3 mark.

6 FUTURE PERSPECTIVES

All together our findings unravel a complex scenario linking enzymes implicated in chromatin remodelling, ROS generation and vascular inflammation. Undoubtedly they shed some light on epigenetic networks and open perspectives for their pharmacological targeting to correct deregulated gene expression as a strategy to alleviate the cardiovascular burden of

obesity and diabetes. However, it is important to consider that we are just starting to become aware of the role and complexity of epigenetic mechanisms in health and disease. Environmental factors potently influence epigenetic variations and altered gene expression over the life time. The causal nexus between epigenetic events and abnormal cardiovascular phenotype requires careful assessment. Larger studies combining epigenome, transcriptome and clinical data are needed to further elucidate the interaction between epigenetics and cardiovascular disease and eventually to develop personalized therapeutic strategies.

7 LIMITATIONS

Although in **study I** 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 but, $p66^{Shc}$ 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. As shown in **study II** modulation of miRNA expression represents a highly promising approach. However, it may leads to undesirable effects due to hybridization-associated off-target effects. This lack of target specificity highlights the need for caution in the development of therapeutics targeting a single miRNA. Although further work is warranted to address the potential of miRNA-targeting therapeutics in this setting, our study has the strength to unveil novel epigenetic connections and their potential role in diabetic cardiomyopathy. Another limitation of the present study is represented by the duration of glycemic control period (3 weeks), which may not be sufficient to exert a significant impact in restoring defects of cardiac performance initiated by hyperglycemia. However, an increasing body of evidence suggests that the bad legacy effect of hyperglycaemia is long-lasting and detrimental. We recently showed that oxidative stress and vascular dysfunction persist even after 6 months of glycemic control in patients with type 2 diabetes. Other recent reports *in vitro* and *in vivo* support this concept, by showing that short exposure to hyperglycemia induces long lasting effects on the endothelium and the myocardium. It is noteworthy that in the experimental setting of **study III** we have

used a STZ model of T1D to demonstrate the protective role of JunD in hyperglycemia-induced, ROS-mediated myocardial dysfunction. However, the translational perspective of our experimental findings was obtained only in a very limited number of patients with T2D and heart failure (n=5) as well as in age-and gender-matched controls (n=6) for which we had available specimens of the left ventricle. The **IV study** designed to investigate whether EZH2 may represent a novel therapeutic target in hyperglycemia-induced oxidative stress and inflammation was performed only *in vitro* in HAECs exposed to high glucose and in endothelial cells isolated from patients with diabetes (D-HAECs). In order to conclude that such reprogramming of adverse epigenetic signatures determines the observed attenuation of oxidative and inflammatory transcriptional programmes these results have to be confirmed *in vivo*.

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9 REFERENCES

1. International Diabetes Federation. *IDF Diabetes Atlas, 8th edn.* Brussels, Belgium:International Diabetes Federation, 2017. http://www.diabetesatlas.org
2. Hossain P, Kawar B, El Nahas M. Obesity and diabetes in the developing world--a growing challenge. *The New England journal of medicine*. 2007;356:213-215
3. Paneni F, Costantino S, Cosentino F. Insulin resistance, diabetes, and cardiovascular risk. *Current atherosclerosis reports*. 2014;16:419
4. Napoli C, Crudele V, Soricelli A, Al-Omran M, Vitale N, Infante T, et al. Primary prevention of atherosclerosis: A clinical challenge for the reversal of epigenetic mechanisms? *Circulation*. 2012;125:2363-2373
5. Barua S, Junaid MA. Lifestyle, pregnancy and epigenetic effects. *Epigenomics*. 2015;7:85-102
6. Caprio S. Development of type 2 diabetes mellitus in the obese adolescent: A growing challenge. *Endocrine practice : official journal of the American College of Endocrinology and the American Association of Clinical Endocrinologists*. 2012;18:791-795
7. Ogurtsova K, da Rocha Fernandes JD, Huang Y, Linnenkamp U, Guariguata L, Cho NH, et al. Idf diabetes atlas: Global estimates for the prevalence of diabetes for 2015 and 2040. *Diabetes research and clinical practice*. 2017;128:40-50
8. Colosia AD, Palencia R, Khan S. Prevalence of hypertension and obesity in patients with type 2 diabetes mellitus in observational studies: A systematic literature review. *Diabetes, metabolic syndrome and obesity : targets and therapy*. 2013;6:327-338
9. Stefan N, Fritsche A, Schick F, Haring HU. Phenotypes of prediabetes and stratification of cardiometabolic risk. *Lancet Diabetes Endocrinol*. 2016;4:789-798
10. WHO. Obesity and overweight. 2016;2018
11. Ortega FB, Lavie CJ, Blair SN. Obesity and cardiovascular disease. *Circulation research*. 2016;118:1752-1770
12. Dagenais GR, Yi Q, Mann JF, Bosch J, Pogue J, Yusuf S. Prognostic impact of body weight and abdominal obesity in women and men with cardiovascular disease. *Am Heart J*. 2005;149:54-60
13. Mellbin LG, Anselmino M, Ryden L. Diabetes, prediabetes and cardiovascular risk. *Eur J Cardiovasc Prev Rehabil*. 2010;17 Suppl 1:S9-14
14. Gregorio BM, De Souza DB, de Morais Nascimento FA, Pereira LM, Fernandes-Santos C. The potential role of antioxidants in metabolic syndrome. *Current pharmaceutical design*. 2016;22:859-869
15. Costantino S, Paneni F, Cosentino F. Targeting chromatin remodeling to prevent cardiovascular disease in diabetes. *Current pharmaceutical biotechnology*. 2015;16:531-543
16. American Diabetes A. Diagnosis and classification of diabetes mellitus. *Diabetes Care*. 2012;35 Suppl 1:S64-71
17. Ndisang JF, Vannacci A, Rastogi S. Insulin resistance, type 1 and type 2 diabetes, and related complications 2017. *Journal of diabetes research*. 2017;2017:1478294
18. Kawamura T, Umemura T, Hotta N. Cognitive impairment in diabetic patients: Can diabetic control prevent cognitive decline? *J Diabetes Investig*. 2012;3:413-423
19. Du YT, Rayner CK, Jones KL, Talley NJ, Horowitz M. Gastrointestinal symptoms in diabetes: Prevalence, assessment, pathogenesis, and management. *Diabetes Care*. 2018;41:627-637

20. American Diabetes A. Diagnosis and classification of diabetes mellitus. *Diabetes Care*. 2014;37 Suppl 1:S81-90
21. Relimpio F. "The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of type 2 diabetes", by kahn se. *Diabetologia*. 2003;46:1707
22. Neumann A, Norberg M, Schoffer O, Norstrom F, Johansson I, Klug SJ, et al. Risk equations for the development of worsened glucose status and type 2 diabetes mellitus in a swedish intervention program. *BMC public health*. 2013;13:1014
23. de Vegt F, Dekker JM, Jager A, Hienkens E, Kostense PJ, Stehouwer CD, et al. Relation of impaired fasting and postload glucose with incident type 2 diabetes in a dutch population: The hoorn study. *Jama*. 2001;285:2109-2113
24. Vaccaro O, Ruffa G, Imperatore G, Iovino V, Rivellese AA, Riccardi G. Risk of diabetes in the new diagnostic category of impaired fasting glucose: A prospective analysis. *Diabetes Care*. 1999;22:1490-1493
25. Qiao Q, Lindstrom J, Valle TT, Tuomilehto J. Progression to clinically diagnosed and treated diabetes from impaired glucose tolerance and impaired fasting glycaemia. *Diabetic medicine : a journal of the British Diabetic Association*. 2003;20:1027-1033
26. Paneni F, Costantino S, Cosentino F. Molecular mechanisms of vascular dysfunction and cardiovascular biomarkers in type 2 diabetes. *Cardiovasc Diagn Ther*. 2014;4:324-332
27. American Diabetes A. 8. Cardiovascular disease and risk management. *Diabetes Care*. 2016;39 Suppl 1:S60-71
28. Paneni F, Beckman JA, Creager MA, Cosentino F. Diabetes and vascular disease: Pathophysiology, clinical consequences, and medical therapy: Part i. *European heart journal*. 2013;34:2436-2443
29. Giacco F, Brownlee M. Oxidative stress and diabetic complications. *Circulation research*. 2010;107:1058-1070
30. Malmberg K, Yusuf S, Gerstein HC, Brown J, Zhao F, Hunt D, et al. Impact of diabetes on long-term prognosis in patients with unstable angina and non-q-wave myocardial infarction: Results of the oasis (organization to assess strategies for ischemic syndromes) registry. *Circulation*. 2000;102:1014-1019
31. Haffner SM, Lehto S, Ronnemaa T, Pyorala K, Laakso M. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *The New England journal of medicine*. 1998;339:229-234
32. Meschia JF, Bushnell C, Boden-Albala B, Braun LT, Bravata DM, Chaturvedi S, et al. Guidelines for the primary prevention of stroke: A statement for healthcare professionals from the american heart association/american stroke association. *Stroke*. 2014;45:3754-3832
33. Banerjee C, Moon YP, Paik MC, Rundek T, Mora-McLaughlin C, Vieira JR, et al. Duration of diabetes and risk of ischemic stroke: The northern manhattan study. *Stroke*. 2012;43:1212-1217
34. Tuomilehto J, Rastenyte D, Jousilahti P, Sarti C, Vartiainen E. Diabetes mellitus as a risk factor for death from stroke. Prospective study of the middle-aged finnish population. *Stroke*. 1996;27:210-215
35. Lenzen M, Ryden L, Ohrvik J, Bartnik M, Malmberg K, Scholte Op Reimer W, et al. Diabetes known or newly detected, but not impaired glucose regulation, has a negative influence on 1-year outcome in patients with coronary artery disease: A report from the euro heart survey on diabetes and the heart. *European heart journal*. 2006;27:2969-2974

36. Balkau B. The decode study. Diabetes epidemiology: Collaborative analysis of diagnostic criteria in europe. *Diabetes & metabolism*. 2000;26:282-286
37. Tominaga M, Eguchi H, Manaka H, Igarashi K, Kato T, Sekikawa A. Impaired glucose tolerance is a risk factor for cardiovascular disease, but not impaired fasting glucose. The funagata diabetes study. *Diabetes Care*. 1999;22:920-924
38. Beckman JA, Paneni F, Cosentino F, Creager MA. Diabetes and vascular disease: Pathophysiology, clinical consequences, and medical therapy: Part ii. *European heart journal*. 2013;34:2444-U2431
39. Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, et al. Mechanisms of increased vascular superoxide production in human diabetes mellitus: Role of nad(p)h oxidase and endothelial nitric oxide synthase. *Circulation*. 2002;105:1656-1662
40. Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, et al. Vascular superoxide production by nad(p)h oxidase: Association with endothelial dysfunction and clinical risk factors. *Circulation research*. 2000;86:E85-90
41. Szopa M, Osmenda G, Wilk G, Matejko B, Skupien J, Zapala B, et al. Intima-media thickness and endothelial dysfunction in gck and hnf1a-mody patients. *European journal of endocrinology / European Federation of Endocrine Societies*. 2015;172:277-283
42. Eckel RH, Kahn SE, Ferrannini E, Goldfine AB, Nathan DM, Schwartz MW, et al. Obesity and type 2 diabetes: What can be unified and what needs to be individualized? *J Clin Endocrinol Metab*. 2011;96:1654-1663
43. Shenouda SM, Widlansky ME, Chen K, Xu G, Holbrook M, Tabit CE, et al. Altered mitochondrial dynamics contributes to endothelial dysfunction in diabetes mellitus. *Circulation*. 2011;124:444-453
44. Wilk G, Osmenda G, Matusik P, Nowakowski D, Jasiewicz-Honkisz B, Ignacak A, et al. Endothelial function assessment in atherosclerosis: Comparison of brachial artery flowmediated vasodilation and peripheral arterial tonometry. *Polskie Archiwum Medycyny Wewnetrznej*. 2013;123:443-452
45. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature*. 2000;404:787-790
46. Tabit CE, Shenouda SM, Holbrook M, Fetterman JL, Kiani S, Frame AA, et al. Protein kinase c-beta contributes to impaired endothelial insulin signaling in humans with diabetes mellitus. *Circulation*. 2013;127:86-95
47. Kouroedov A, Eto M, Joch H, Volpe M, Luscher TF, Cosentino F. Selective inhibition of protein kinase cbeta2 prevents acute effects of high glucose on vascular cell adhesion molecule-1 expression in human endothelial cells. *Circulation*. 2004;110:91-96
48. Geraldes P, King GL. Activation of protein kinase c isoforms and its impact on diabetic complications. *Circulation research*. 2010;106:1319-1331
49. Sun L, Xiao L, Nie J, Liu FY, Ling GH, Zhu XJ, et al. P66shc mediates high-glucose and angiotensin ii-induced oxidative stress renal tubular injury via mitochondrial-dependent apoptotic pathway. *American journal of physiology. Renal physiology*. 2010;299:F1014-1025
50. Giorgio M, Migliaccio E, Orsini F, Paolucci D, Moroni M, Contursi C, et al. Electron transfer between cytochrome c and p66shc generates reactive oxygen species that trigger mitochondrial apoptosis. *Cell*. 2005;122:221-233
51. Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P, Pandolfi PP, et al. The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature*. 1999;402:309-313

52. Camici GG, Schiavoni M, Francia P, Bachschmid M, Martin-Padura I, Hersberger M, et al. Genetic deletion of p66(shc) adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104:5217-5222
53. Pagnin E, Fadini G, de Toni R, Tiengo A, Calo L, Avogaro A. Diabetes induces p66shc gene expression in human peripheral blood mononuclear cells: Relationship to oxidative stress. *J Clin Endocrinol Metab*. 2005;90:1130-1136
54. Paneni F, Volpe M, Luscher TF, Cosentino F. Sirt1, p66(shc), and set7/9 in vascular hyperglycemic memory: Bringing all the strands together. *Diabetes*. 2013;62:1800-1807
55. Ceriello A, Ihnat MA, Thorpe JE. Clinical review 2: The "metabolic memory": Is more than just tight glucose control necessary to prevent diabetic complications? *J Clin Endocrinol Metab*. 2009;94:410-415
56. Quagliaro L, Piconi L, Assaloni R, Martinelli L, Motz E, Ceriello A. Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells: The role of protein kinase c and nad(p)h-oxidase activation. *Diabetes*. 2003;52:2795-2804
57. Guzik B, Sagan A, Ludew D, Mrowiecki W, Chwala M, Bujak-Gizycka B, et al. Mechanisms of oxidative stress in human aortic aneurysms--association with clinical risk factors for atherosclerosis and disease severity. *International journal of cardiology*. 2013;168:2389-2396
58. Nichols GA, Gullion CM, Koro CE, Ephross SA, Brown JB. The incidence of congestive heart failure in type 2 diabetes: An update. *Diabetes Care*. 2004;27:1879-1884
59. Dei Cas A, Khan SS, Butler J, Mentz RJ, Bonow RO, Avogaro A, et al. Impact of diabetes on epidemiology, treatment, and outcomes of patients with heart failure. *JACC. Heart failure*. 2015;3:136-145
60. Seferovic PM, Paulus WJ. Clinical diabetic cardiomyopathy: A two-faced disease with restrictive and dilated phenotypes. *European heart journal*. 2015;36:1718-1727, 1727a-1727c
61. Iribarren C, Karter AJ, Go AS, Ferrara A, Liu JY, Sidney S, et al. Glycemic control and heart failure among adult patients with diabetes. *Circulation*. 2001;103:2668-2673
62. Boudina S, Abel ED. Diabetic cardiomyopathy revisited. *Circulation*. 2007;115:3213-3223
63. Munzel T, Gori T, Keaney JF, Jr., Maack C, Daiber A. Pathophysiological role of oxidative stress in systolic and diastolic heart failure and its therapeutic implications. *European heart journal*. 2015;36:2555-2564
64. Aon MA, Foster DB. Diabetic cardiomyopathy and the role of mitochondrial dysfunction: Novel insights, mechanisms, and therapeutic strategies. *Antioxid Redox Signal*. 2015;22:1499-1501
65. Camici GG, Cosentino F, Tanner FC, Luscher TF. The role of p66shc deletion in age-associated arterial dysfunction and disease states. *J Appl Physiol (1985)*. 2008;105:1628-1631
66. Cosentino F, Francia P, Camici GG, Pelicci PG, Luscher TF, Volpe M. Final common molecular pathways of aging and cardiovascular disease: Role of the p66shc protein. *Arteriosclerosis, thrombosis, and vascular biology*. 2008;28:622-628
67. Francia P, delli Gatti C, Bachschmid M, Martin-Padura I, Savoia C, Migliaccio E, et al. Deletion of p66shc gene protects against age-related endothelial dysfunction. *Circulation*. 2004;110:2889-2895

68. Berniakovich I, Trinei M, Stendardo M, Migliaccio E, Minucci S, Bernardi P, et al. P66shc-generated oxidative signal promotes fat accumulation. *The Journal of biological chemistry*. 2008;283:34283-34293
69. Paneni F, Costantino S, Cosentino F. P66(shc)-induced redox changes drive endothelial insulin resistance. *Atherosclerosis*. 2014;236:426-429
70. Rota M, LeCapitaine N, Hosoda T, Boni A, De Angelis A, Padin-Iruegas ME, et al. Diabetes promotes cardiac stem cell aging and heart failure, which are prevented by deletion of the p66shc gene. *Circulation research*. 2006;99:42-52
71. Franzeck FC, Hof D, Spescha RD, Hasun M, Akhmedov A, Steffel J, et al. Expression of the aging gene p66shc is increased in peripheral blood monocytes of patients with acute coronary syndrome but not with stable coronary artery disease. *Atherosclerosis*. 2012;220:282-286
72. Hasegawa Y, Saito T, Ogihara T, Ishigaki Y, Yamada T, Imai J, et al. Blockade of the nuclear factor-kappab pathway in the endothelium prevents insulin resistance and prolongs life spans. *Circulation*. 2012;125:1122-1133
73. Rask-Madsen C, King GL. Endothelium-dependent delivery of insulin to muscle interstitium. *Cell metabolism*. 2011;13:236-238
74. Hirai SI, Ryseck RP, Mechta F, Bravo R, Yaniv M. Characterization of jund: A new member of the jun proto-oncogene family. *The EMBO journal*. 1989;8:1433-1439
75. Gerald D, Berra E, Frapart YM, Chan DA, Giaccia AJ, Mansuy D, et al. Jund reduces tumor angiogenesis by protecting cells from oxidative stress. *Cell*. 2004;118:781-794
76. Laurent G, Solari F, Mateescu B, Karaca M, Castel J, Bourachot B, et al. Oxidative stress contributes to aging by enhancing pancreatic angiogenesis and insulin signaling. *Cell metabolism*. 2008;7:113-124
77. Toullec A, Gerald D, Despouy G, Bourachot B, Cardon M, Lefort S, et al. Oxidative stress promotes myofibroblast differentiation and tumour spreading. *EMBO molecular medicine*. 2010;2:211-230
78. Paneni F, Osto E, Costantino S, Mateescu B, Briand S, Coppolino G, et al. Deletion of the activated protein-1 transcription factor jund induces oxidative stress and accelerates age-related endothelial dysfunction. *Circulation*. 2013;127:1229-1240, e1221-1221
79. Ricci R, Eriksson U, Oudit GY, Eferl R, Akhmedov A, Sumara I, et al. Distinct functions of jund in cardiac hypertrophy and heart failure. *Genes & development*. 2005;19:208-213
80. Hilfiker-Kleiner D, Hilfiker A, Castellazzi M, Wollert KC, Trautwein C, Schunkert H, et al. Jund attenuates phenylephrine-mediated cardiomyocyte hypertrophy by negatively regulating ap-1 transcriptional activity. *Cardiovascular research*. 2006;71:108-117
81. Hilfiker-Kleiner D, Hilfiker A, Kaminski K, Schaefer A, Park JK, Michel K, et al. Lack of jund promotes pressure overload-induced apoptosis, hypertrophic growth, and angiogenesis in the heart. *Circulation*. 2005;112:1470-1477
82. Costantino S, Mohammed SA, Ambrosini S, Paneni F. Epigenetic processing in cardiometabolic disease. *Atherosclerosis*. 2018
83. Handy DE, Castro R, Loscalzo J. Epigenetic modifications: Basic mechanisms and role in cardiovascular disease. *Circulation*. 2011;123:2145-2156
84. Kohli RM, Zhang Y. Tet enzymes, tdg and the dynamics of DNA demethylation. *Nature*. 2013;502:472-479
85. Miranda TB, Jones PA. DNA methylation: The nuts and bolts of repression. *Journal of cellular physiology*. 2007;213:384-390
86. Jenuwein T, Allis CD. Translating the histone code. *Science*. 2001;293:1074-1080

87. Shahbazian MD, Grunstein M. Functions of site-specific histone acetylation and deacetylation. *Annual review of biochemistry*. 2007;76:75-100
88. Paneni F, Costantino S, Cosentino F. Molecular pathways of arterial aging. *Clinical science*. 2015;128:69-79
89. Gurha P, Marian AJ. Noncoding rnas in cardiovascular biology and disease. *Circulation research*. 2013;113:e115-120
90. Mathiyalagan P, Keating ST, Du XJ, El-Osta A. Interplay of chromatin modifications and non-coding rnas in the heart. *Epigenetics*. 2014;9:101-112
91. Magistri M, Faghihi MA, St Laurent G, 3rd, Wahlestedt C. Regulation of chromatin structure by long noncoding rnas: Focus on natural antisense transcripts. *Trends in genetics : TIG*. 2012;28:389-396
92. Cantone I, Fisher AG. Epigenetic programming and reprogramming during development. *Nature structural & molecular biology*. 2013;20:282-289
93. El-Osta A. Contemporary trends emerging in epigenomics and metabolism. *Antioxid Redox Signal*. 2018;29:1019-1022
94. Arguelles AO, Meruvu S, Bowman JD, Choudhury M. Are epigenetic drugs for diabetes and obesity at our door step? *Drug discovery today*. 2016;21:499-509
95. Makita S, Tobinai K. Targeting ezh2 with tazemetostat. *Lancet Oncol*. 2018;19:586-587
96. Shan L, Zhou X, Liu X, Wang Y, Su D, Hou Y, et al. Foxk2 elicits massive transcription repression and suppresses the hypoxic response and breast cancer carcinogenesis. *Cancer cell*. 2016;30:708-722
97. Peng D, Kryczek I, Nagarsheth N, Zhao L, Wei S, Wang W, et al. Epigenetic silencing of th1-type chemokines shapes tumour immunity and immunotherapy. *Nature*. 2015;527:249-253
98. Hasegawa N, Oshima M, Sashida G, Matsui H, Koide S, Saraya A, et al. Impact of combinatorial dysfunctions of tet2 and ezh2 on the epigenome in the pathogenesis of myelodysplastic syndrome. *Leukemia*. 2017;31:861-871
99. Comet I, Riising EM, Leblanc B, Helin K. Maintaining cell identity: Prc2-mediated regulation of transcription and cancer. *Nat Rev Cancer*. 2016;16:803-810
100. Shi ZL, Fang K, Li ZH, Ren DH, Zhang JY, Sun J. Ezh2 inhibition ameliorates transverse aortic constriction-induced pulmonary arterial hypertension in mice. *Can Respir J*. 2018;2018:9174926
101. Mathiyalagan P, Okabe J, Chang L, Su Y, Du XJ, El-Osta A. The primary microrna-208b interacts with polycomb-group protein, ezh2, to regulate gene expression in the heart. *Nucleic acids research*. 2014;42:790-803
102. Arifuzzaman S, Das A, Kim SH, Yoon T, Lee YS, Jung KH, et al. Selective inhibition of ezh2 by a small molecule inhibitor regulates microglial gene expression essential for inflammation. *Biochemical pharmacology*. 2017;137:61-80
103. Gao M, Liu Y, Chen Y, Yin C, Chen JJ, Liu S. Mir-214 protects erythroid cells against oxidative stress by targeting atf4 and ezh2. *Free radical biology & medicine*. 2016;92:39-49
104. Oh JY, Choi GE, Lee HJ, Jung YH, Ko SH, Chae CW, et al. High glucose-induced reactive oxygen species stimulates human mesenchymal stem cell migration through snail and ezh2-dependent e-cadherin repression. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. 2018;46:1749-1767
105. Ruiz MA, Feng B, Chakrabarti S. Polycomb repressive complex 2 regulates mir-200b in retinal endothelial cells: Potential relevance in diabetic retinopathy. *PloS one*. 2015;10:e0123987

106. Greissel A, Culmes M, Burgkart R, Zimmermann A, Eckstein HH, Zernecke A, et al. Histone acetylation and methylation significantly change with severity of atherosclerosis in human carotid plaques. *Cardiovasc Pathol*. 2016;25:79-86
107. Xu S, Xu Y, Yin M, Zhang S, Liu P, Koroleva M, et al. Flow-dependent epigenetic regulation of igfbp5 expression by h3k27me3 contributes to endothelial anti-inflammatory effects. *Theranostics*. 2018;8:3007-3021
108. Lee ST, Li Z, Wu Z, Aau M, Guan P, Karuturi RK, et al. Context-specific regulation of nf-kappab target gene expression by ezh2 in breast cancers. *Molecular cell*. 2011;43:798-810
109. McCabe MT, Ott HM, Ganji G, Korenchuk S, Thompson C, Van Aller GS, et al. Ezh2 inhibition as a therapeutic strategy for lymphoma with ezh2-activating mutations. *Nature*. 2012;492:108-112
110. Paneni F, Mocharla P, Akhmedov A, Costantino S, Osto E, Volpe M, et al. Gene silencing of the mitochondrial adaptor p66(shc) suppresses vascular hyperglycemic memory in diabetes. *Circulation research*. 2012;111:278-289
111. Paneni F, Costantino S, Volpe M, Luscher TF, Cosentino F. Epigenetic signatures and vascular risk in type 2 diabetes: A clinical perspective. *Atherosclerosis*. 2013;230:191-197
112. Baccarelli A, Rienstra M, Benjamin EJ. Cardiovascular epigenetics: Basic concepts and results from animal and human studies. *Circulation. Cardiovascular genetics*. 2010;3:567-573
113. Benayoun BA, Pollina EA, Brunet A. Epigenetic regulation of ageing: Linking environmental inputs to genomic stability. *Nature reviews. Molecular cell biology*. 2015;16:593-610
114. Kluge MA, Fetterman JL, Vita JA. Mitochondria and endothelial function. *Circulation research*. 2013;112:1171-1188
115. Paneni F, Costantino S, Cosentino F. Role of oxidative stress in endothelial insulin resistance. *World journal of diabetes*. 2015;6:326-332
116. Tessarz P, Kouzarides T. Histone core modifications regulating nucleosome structure and dynamics. *Nature reviews. Molecular cell biology*. 2014;15:703-708
117. Swygert SG, Peterson CL. Chromatin dynamics: Interplay between remodeling enzymes and histone modifications. *Biochimica et biophysica acta*. 2014;1839:728-736
118. Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, et al. Regulation of chromatin structure by site-specific histone h3 methyltransferases. *Nature*. 2000;406:593-599
119. Tateishi K, Okada Y, Kallin EM, Zhang Y. Role of jhdm2a in regulating metabolic gene expression and obesity resistance. *Nature*. 2009;458:757-761
120. Mahmood S, Smiraglia DJ, Srinivasan M, Patel MS. Epigenetic changes in hypothalamic appetite regulatory genes may underlie the developmental programming for obesity in rat neonates subjected to a high-carbohydrate dietary modification. *J Dev Orig Health Dis*. 2013;4:479-490
121. Zhong L, D'Urso A, Toiber D, Sebastian C, Henry RE, Vadysirisack DD, et al. The histone deacetylase sirt6 regulates glucose homeostasis via hif1alpha. *Cell*. 2010;140:280-293
122. Mikula M, Majewska A, Ledwon JK, Dzwonek A, Ostrowski J. Obesity increases histone h3 lysine 9 and 18 acetylation at tnfa and ccl2 genes in mouse liver. *International journal of molecular medicine*. 2014;34:1647-1654
123. Brasacchio D, Okabe J, Tikellis C, Balcerczyk A, George P, Baker EK, et al. Hyperglycemia induces a dynamic cooperativity of histone methylase and demethylase enzymes associated with gene-activating epigenetic marks that coexist on the lysine tail. *Diabetes*. 2009;58:1229-1236

124. Ost A, Lempradl A, Casas E, Weigert M, Tiko T, Deniz M, et al. Paternal diet defines offspring chromatin state and intergenerational obesity. *Cell*. 2014;159:1352-1364
125. Kornfeld OS, Hwang S, Disatnik MH, Chen CH, Qvit N, Mochly-Rosen D. Mitochondrial reactive oxygen species at the heart of the matter: New therapeutic approaches for cardiovascular diseases. *Circulation research*. 2015;116:1783-1799
126. Sugamura K, Keaney JF, Jr. Reactive oxygen species in cardiovascular disease. *Free radical biology & medicine*. 2011;51:978-992
127. Nasir S, Aguilar D. Congestive heart failure and diabetes mellitus: Balancing glycemic control with heart failure improvement. *The American journal of cardiology*. 2012;110:50B-57B
128. Fitchett D, Zinman B, Wanner C, Lachin JM, Hantel S, Salsali A, et al. Heart failure outcomes with empagliflozin in patients with type 2 diabetes at high cardiovascular risk: Results of the empa-reg outcome(r) trial. *European heart journal*. 2016;37:1526-1534
129. Wiviott SD, Raz I, Bonaca MP, Mosenzon O, Kato ET, Cahn A, et al. Dapagliflozin and cardiovascular outcomes in type 2 diabetes. *The New England journal of medicine*. 2019;380:347-357
130. Neal B, Perkovic V, Matthews DR. Canagliflozin and cardiovascular and renal events in type 2 diabetes. *The New England journal of medicine*. 2017;377:2099
131. Thum T, Condorelli G. Long noncoding rnas and microrna in cardiovascular pathophysiology. *Circulation research*. 2015;116:751-762
132. Ceriello A. Hypothesis: The "metabolic memory", the new challenge of diabetes. *Diabetes research and clinical practice*. 2009;86 Suppl 1:S2-6
133. van Rooij E, Kauppinen S. Development of microrna therapeutics is coming of age. *EMBO molecular medicine*. 2014;6:851-864
134. Chiavacci E, Dolfi L, Verduci L, Meghini F, Gestri G, Evangelista AM, et al. Microrna 218 mediates the effects of tbx5a over-expression on zebrafish heart development. *PloS one*. 2012;7:e50536
135. Fish JE, Wythe JD, Xiao T, Bruneau BG, Stainier DY, Srivastava D, et al. A slit/mir-218/robo regulatory loop is required during heart tube formation in zebrafish. *Development*. 2011;138:1409-1419
136. Small EM, Sutherland LB, Rajagopalan KN, Wang S, Olson EN. Microrna-218 regulates vascular patterning by modulation of slit-robo signaling. *Circulation research*. 2010;107:1336-1344
137. Boon RA, Iekushi K, Lechner S, Seeger T, Fischer A, Heydt S, et al. Microrna-34a regulates cardiac ageing and function. *Nature*. 2013;495:107-110
138. Persengiev SP, Green MR. The role of atf/creb family members in cell growth, survival and apoptosis. *Apoptosis : an international journal on programmed cell death*. 2003;8:225-228
139. Hai T, Curran T. Cross-family dimerization of transcription factors fos/jun and atf/creb alters DNA binding specificity. *Proceedings of the National Academy of Sciences of the United States of America*. 1991;88:3720-3724
140. Mechta-Grigoriou F, Gerald D, Yaniv M. The mammalian jun proteins: Redundancy and specificity. *Oncogene*. 2001;20:2378-2389
141. Hernandez JM, Floyd DH, Weilbaecher KN, Green PL, Boris-Lawrie K. Multiple facets of jund gene expression are atypical among ap-1 family members. *Oncogene*. 2008;27:4757-4767
142. Jochum W, Passegue E, Wagner EF. Ap-1 in mouse development and tumorigenesis. *Oncogene*. 2001;20:2401-2412

143. Thepot D, Weitzman JB, Barra J, Segretain D, Stinnakre MG, Babinet C, et al. Targeted disruption of the murine jund gene results in multiple defects in male reproductive function. *Development*. 2000;127:143-153
144. Zhang Y, Babcock SA, Hu N, Maris JR, Wang H, Ren J. Mitochondrial aldehyde dehydrogenase (aldh2) protects against streptozotocin-induced diabetic cardiomyopathy: Role of gsk3beta and mitochondrial function. *BMC medicine*. 2012;10:40
145. Wenzel P, Schuhmacher S, Kienhofer J, Muller J, Hortmann M, Oelze M, et al. Manganese superoxide dismutase and aldehyde dehydrogenase deficiency increase mitochondrial oxidative stress and aggravate age-dependent vascular dysfunction. *Cardiovascular research*. 2008;80:280-289
146. Chen CH, Budas GR, Churchill EN, Disatnik MH, Hurley TD, Mochly-Rosen D. Activation of aldehyde dehydrogenase-2 reduces ischemic damage to the heart. *Science*. 2008;321:1493-1495
147. Koda K, Salazar-Rodriguez M, Corti F, Chan NY, Estephan R, Silver RB, et al. Aldehyde dehydrogenase activation prevents reperfusion arrhythmias by inhibiting local renin release from cardiac mast cells. *Circulation*. 2010;122:771-781
148. Meixner A, Karreth F, Kenner L, Penninger JM, Wagner EF. Jun and jund-dependent functions in cell proliferation and stress response. *Cell death and differentiation*. 2010;17:1409-1419
149. Venugopal R, Jaiswal AK. Nrf2 and nrf1 in association with jun proteins regulate antioxidant response element-mediated expression and coordinated induction of genes encoding detoxifying enzymes. *Oncogene*. 1998;17:3145-3156
150. Cong W, Ruan D, Xuan Y, Niu C, Tao Y, Wang Y, et al. Cardiac-specific overexpression of catalase prevents diabetes-induced pathological changes by inhibiting nf-kappab signaling activation in the heart. *J Mol Cell Cardiol*. 2015;89:314-325