University of Milano-Bicocca School of Medicine and Faculty of Science

Ph.D PROGRAM IN TRANSLATIONAL AND MOLECULAR MEDICINE

DIMET

Epigenetics of Myocardial Physiology and Disease

Coordinator: Prof. Andrea Biondi
Tutor: Prof. Gianluigi Condorelli

Dr. Carolina GRECO
Matr. No. 760707

XXVII CYCLE
ACADEMIC YEAR
2013-2014

DIMET - Dr. Carolina GRECO - A.A. 2013-14

“The scientist is not a person who gives the right answers, he's one who asks the right questions.”

Claude Lévi-Strauss

Table of contents

Chapter 1 General Introduction 7
Heart Failure: the Clinical Problem 7
Cardiac Hypertrophy 8
Transcriptional control of cardiac Hypertrophy 10
Epigenetics 13
DNA Methylation 15
Histone Modifications 17
The Epigenetic Cofactor UHRF1 19
Epigenetics in Cardiac Hypertrophy 20
DNA Methylation and Cardiac Hypertrophy 21
Histone Modifications and Cardiac Hypertrophy 23
Cardiac Energetics and Metabolism 25
Cardiac metabolic pathways 26
Transcriptional Regulation of Cardiac Metabolism 26
Metabolic Remodeling in the Hypertrophic Heart 29
Scope of the thesis 31
References 33

Chapter 2 The DNA methylation cofactor UHRF1 controls the metabolic re-programming of hypertrophic cardiomyocytes 51
SUMMARY 52
INTRODUCTION 53
RESULTS 56
DISCUSSION 84
EXPERIMENTAL PROCEDURES 89
REFERENCES 100

Chapter 3 DNA hydroxymethylation controls cardiomyocyte gene expression in development and hypertrophy 109
Abstract 110
Introduction 111
Results 113
Discussion 136
Methods 139
References 147

Chapter 4 Summary, Conclusions and Future Perspectives 154
References 159
Publications 161

CHAPTER 1 GENERAL INTRODUCTION

HEART FAILURE: THE CLINICAL PROBLEM

Heart failure (HF) is a clinical syndrome resulting from several major cardiovascular disease entities and now constitutes the primary cause of hospitalization and death in Europe. A variety of cardiovascular diseases, including hypertension, coronary artery disease, myocardial infarction (MI) and genetic inherited forms of cardiomyopathies result in HF, and provoke pathological remodeling of the myocardium, pump failure and sudden death. While current pharmacological treatment strategies (e.g. β-blockers and ACE- inhibitors) have shown effectiveness in prolonging survival of heart failure patients(Packer et al., 2001), the prognosis of affected individuals remains poor and there is a dire need for new paradigms and therapeutic approaches for treatment of this devastating disease. One of the major obstacles in achieving a more effective HF clinical treatment is posed by the fact that therapy is currently mainly generic, a 'one-size-fits-all' approach for all patients with heart failure, ignoring the differences caused by underlying disease mechanisms, age, gender or different stages of HF pathophysiology. This is further emphasized by the fact that genome wide association studies (GWAS) in large scale patient studies focusing on protein coding genes have largely failed to discover common denominators for the HF syndrome, indicating that there is a dire need for stratification of

HF patients and novel therapeutic targets tailored to treat the underlying biological mechanisms of distinct subtypes of HF. Mechanisms that are similar to those that control gene expression in heart development are at the basis of hypertrophy and failure. Therefore, the regulatory networks that govern heart development and pathology are under intense investigation.

CARDIAC HYPERTROPHY

Cardiomyocytes are terminally differentiated cells and lose their ability to proliferate soon after birth. Thereafter, cardiomyocytes grow in cell size to adapt to a demand for an increased workload. In a number of pathological conditions that impose overwork on the heart, cardiomyocytes undergo
cardiac hypertrophy. Hypertrophy of cardiomyocytes represents the typical reaction of ventricular myocardium to the increased biomechanical stress induced by chronic pressure or volume overload. At the cellular level, cardiomyocyte hypertrophy is characterized by an increase in cell size, enhanced protein synthesis, and increased organization of the sarcomere. At the molecular level, these changes in cellular phenotype are preceded and accompanied by induction of the “fetal gene program”, a pattern of gene expression that resembles that observed during embryonic development. The functional significance of this global change in gene expression regarding cardiac remodeling remains unclear.

The “fetal gene program” includes the natriuretic peptides ANP and BNP, as well as alpha-skeletal actin (Schoenfeld et al., 1998). Their expression is closely correlated with the onset of heart disease and is currently used as diagnostic markers in patients suspected of having heart failure.

Similarly, alpha- myosin heavy chain gene expression is repressed while the fetal isoform, β-myosin heavy chain, is induced (“myosin switch”) (Lowes et al., 1997).Cardiac hypertrophy can be divided into morphologically distinct entities, including concentric and eccentric hypertrophy. Rarely seen clinically in pure form, volume overload (observed, for example, with large arteriovenous fistulae or thyrotoxicosis) typically leads to marked enlargement of left ventricular diastolic dimensions with relatively normal wall thickness (eccentric hypertrophy). In this setting, cardiomyocytes lengthen with longitudinal apposition of sarcomeres. In contrast, concentric hypertrophy due to pressure overload (e.g. hypertension, aortic stenosis) is initially characterized by normal diastolic dimensions but increased wall thickness; hypertrophied cardiomyocytes manifest an increase in width, indicative of parallel apposition of sarcomeres.

Pathological hypertrophy is characterized by a specific gene program and characteristic morphological features, such as cardiac fibrosis, which together promote progression to heart failure. The molecular changes at the gene expression level are an important part of remodeling in the diseased

myocardium. Therefore, improved knowledge of their regulatory mechanisms and the functional contribution of these genes to heart failure can be translated into new diagnostic tools and therapies.

TRANSCRIPTIONAL CONTROL OF CARDIAC HYPERTROPHY

Cardiac transcription factors play the crucial part in the transcriptional regulation of the hypertrophic response, as they directly regulate a number of cardiac genes that are up-regulated in hypertrophied myocardium. Cardiac transcription factors include the GATA family of transcription factors, myocyte enhancer factor 2 (MEF2) transcription factors, and the homeobox transcription factor Csx/Nkx2-5.

GATA transcription factors are characterized by the conserved double zinc fingers that are required for binding to the specific consensus DNA sequence (A/T)GA T A(A/G)(Patient and McGhee, 2002). The GA T A family of transcription factors comprises six members, GATA4, GATA5, and GATA6 are highly expressed in the heart(Molkentin, 2000).

Among these, GATA4 is the main member involved in the regulation of gene expression in hypertrophic cardiomyocytes. GA T A-binding elements are required for the up-regulation of β-MHC in response to aortic constriction(Hasegawa et al., 1997; Herzig et al., 1997) and of BNP in the hearts of bilaterally nephrectomized rats(Marttila et al., 2001). Even though an increase in the DNA-binding activity of GATA4 is observed in

response to pressure overload(Hasegawa et al., 1997; Hautala et al., 2001; Herzig et al., 1997) the expression levels of GATA4 are not affected by hypertrophic stimulation. In fact, the transcriptional activity of GATA4 is regulated through a phosphorylation dependent mechanism, which determines its shuttling from the nucleus to the cytoplasm(Morisco et al., 2001). In addition to phosphorylation, the transcriptional activity of GA T A4 is also regulated through interaction with cofactors such as p30048 and other transcription regulators such as serum response factor (SRF)(Belaguli et al., 2000), Csx/Nkx2-5(Durocher et al., 1997), MEF2 and nuclear factor of activated T cells (NFAT)(Molkentin et al., 1998).

MEF2 transcription factors contain a MADS domain and an adjacent MEF2- specific domain in the N-terminus, which together direct dimerization and binding to their target DNA sequence(McKinsey et al., 2002).MEF2-binding A/T-rich DNA sequences have been identified within the promoter regions of a number of cardiac genes, (eg, muscle creatine kinase gene, β-MHC, MLC1/3, MLC2v, skeletal α-actin, cardiac troponin T, cardiac troponin C, cardiac troponin I, desmin, and dystrophin (Bhavsar et al., 2000). MEF2 activity is enhanced in response to hypertrophic stimulation, and MEF2 functions as an essential effector of divergent intracellular signaling pathways mediating hypertrophic features.

Csx/Nkx2-5 is a homeodomain-containing transcriptional activator, originally identified as a potential homologue of

Drosophila tinman(Komuro and Izumo, 1993; Lints et al., 1993).In phenylephrine or isoproterenol-mediated hypertrophic hearts, expression of Csx/Nkx2-5 is increased as well as the expression of fetal genes, such as ANP and β-MHC(Saadane et al., 1999).

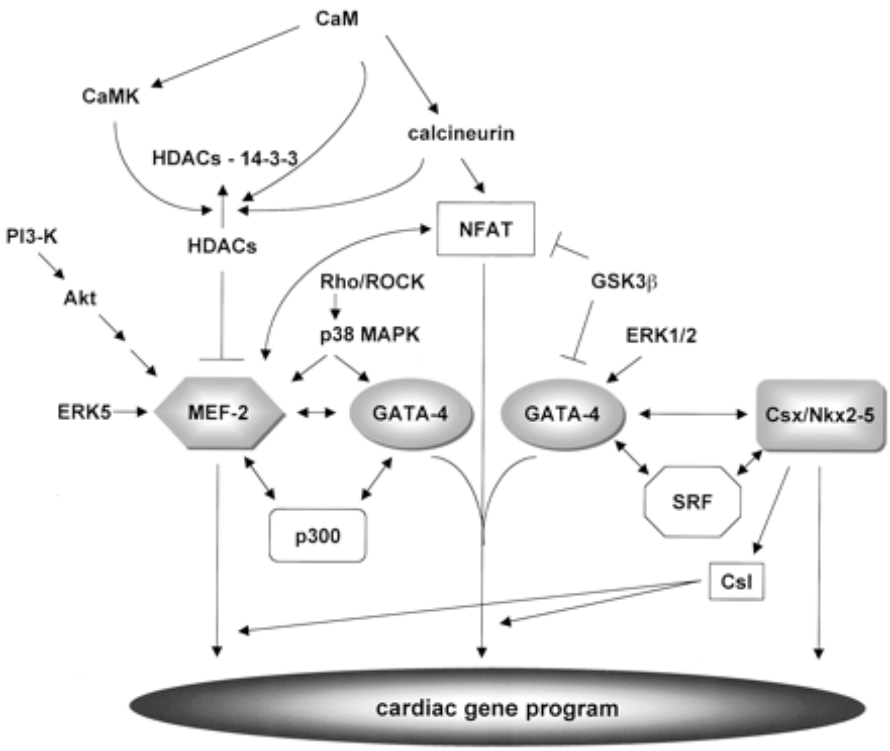

Figure 1: cardiac transcription factors during cardiac hypertrophy (Akazawa H, Circ Res 2003)

Several studies have demonstrated that these transcriptional regulators are involved in the reactivation of the fetal gene program in response to a variety of hypertrophic stimulation and that these factors function as important effectors during the

generation of cardiac hypertrophy. However, regulatory mechanisms of cardiac hypertrophy include the combination of additional factors, such as epigenetic modifications and non-coding RNA.

EPIGENETICS

Some of the latest advances in the field of gene expression regulation regard the study of the epigenome. The epigenome is the collection of alterations of the genome, which do not pertain to the DNA sequence itself but to secondary chemical modifications of either the DNA's cytosines or of histones, the proteins around which DNA is wound(Bernstein et al., 2007; Wu et al., 2009). These two types of modifications (i.e., methylation of DNA cytosines and post- translational modifications of histone proteins) induce changes to the state of the chromatin, and in doing so regulate transcription by rendering the genes' regulatory elements more or less permissive to the activity of transcription factors.

Chromatin is a macromolecular complex of DNA and histones. The basic unit of chromatin is the nucleosome, an octamer of four core histone proteins (two each of H2A, H2B, H3, and H4) around which 147 base-pairs of DNA are wrapped. This allows a 5-fold packaging of DNA to be achieved. The nucleosomes are then tightly packaged into higher-order structures to further condense DNA. The N-terminal tails of all eight core histone proteins are subjected to post-translational covalent

modifications, among which are acetylation, methylation, phosphorylation, ubiquitination and poly-ADP-ribosylation(Bannister and Kouzarides, 2011). Apart from modification of histones, chromatin structure can also be regulated by DNA methylation and ATP-dependent chromatin remodelling complexes. In this way, transcription, DNA repair, replication and recombination are controlled (Kouzarides, 2007). By modulating chromatin structure, epigenetic modifications regulate the accessibility of DNA to promoters and of distal regulatory regions, such as enhancers, and in this way control gene expression. Promoters and enhancers display, and are governed by, specific epigenetic signatures: active promoters are marked by high levels of mono-acetylated lysine 9 and lysine 14 (H3K9ac and H3K14ac) and by tri-methylated (me3) lysine 4 (H3K4me3); on the other hand, methylated DNA, tri-methylation of lysine 9 and lysine 27 on histone H3 (H3K9me3 and H3K27me3) and de-acetylation of histones are associated with inactive promoters (Barski et al., 2007). In contrast, active enhancers are mainly enriched in H3K27ac, whereas an absence of H3K27ac and a high level of H3K27me3 defines enhancers as being “poised”(Rada- Iglesias et al., 2011).

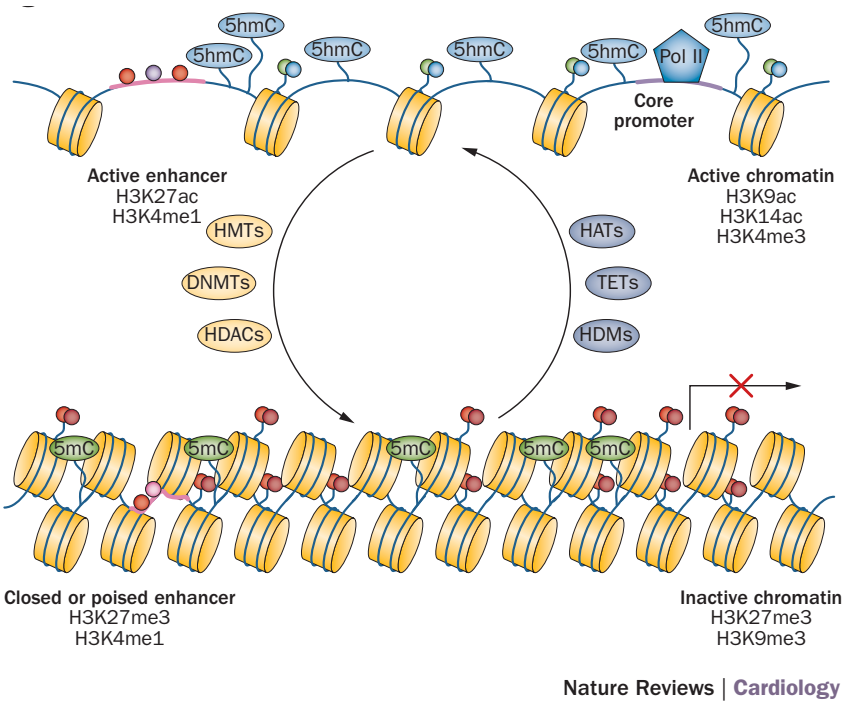

Figure 2: Epigenetic modifications in gene-expression regulation (Greco CM, Nat Rev Cardiol 2015)

DNA Methylation

Methylation of DNA at position 5 of cytosine (5-mC) is a highly conserved epigenetic mark that signals for transcriptional repression (Stein et al., 1982). It also plays a key role in transposon silencing, genomic imprinting, X-chromosome inactivation and development (Smith and Meissner, 2013). In mammals, methylated cytosines are found preferentially on CpG dinucleotides, which tend to cluster in regions called CpG islands (CpGIs) and on repetitive elements of the genome (e.g., satellite DNA, centromeric repeats, retrotransposon elements

and rDNA). In somatic cells, CpGIs are mainly distributed on the 5′ end of genes, and the majority of cytosine residues (60%–80%) are methylated (Wu and Zhang, 2014).

There are three mammalian DNA methyltransferases (DNMTs): DNA methyltransferase 1 (DNMT1), 3 alpha (DNMT3A) and 3 beta (DNMT3B). DNMT1 is involved in the maintenance of established DNA methylation and binds preferentially to hemi-methylated DNA, allowing symmetrical propagation of DNA methylation during mitosis (Reik et al., 2001; Smith and Meissner, 2013). In contrast, DNMT3A and DNMT3B establish de novo, or new, DNA methylation patterns (Okano et al., 1999), generally targeting unmethylated promoters and other epigenetic repressors(Smith and Meissner, 2013). Although DNA methylation has historically been considered a stable epigenetic mark, the discovery of 5-hydroxymethylcytosine (5-hmC) – an oxidation product of 5-mC generated by the ten-eleven translocation (TET) family of enzymes – has revealed the presence of an active DNA demethylation mechanism in mammalian cells (Tahiliani et al., 2009). Moreover, recent studies have shown that 5-hmC is not only an intermediate product of an active de-methylation process, but can also act as a relatively stable epigenetic mark whose role in transcription regulation is linked to its genomic location. Indeed, enrichment of 5-hmC on the gene body (GB) has been associated with transcriptional activation in several reports (Ficz et al., 2011; Gan et al., 2013; Ivanov et al., 2013; Pastor et al., 2011;

Szulwach et al., 2011b; Tsagaratou et al., 2014). However, its function on promoters and distal regulatory regions is less clear. In embryonic stem cells (ESCs), for example, 5-hmC can localize to the transcription start site (TSS) of repressed, but developmentally poised, genes(Pastor et al., 2011; Szulwach et al., 2011a; Wu et al., 2011). In addition, 5-hmC can be highly enriched at poised and active enhancers(Hon et al., 2014; Stroud et al., 2011; Yu et al., 2012), but only recently have two studies proven that TET-mediated 5-mC oxidation regulates the activity of these elements(Hon et al., 2014; Lu et al., 2014).

DNA methylation regulates gene expression either by directly blocking transcription factor binding or by recruiting methyl-binding proteins, namely methyl-CpG binding domain proteins (MBD) 1, 2 and 3, and methyl-CpG binding protein 2 (MECP2), which in turn interact with co-repressor complexes(Hendrich and Tweedie, 2003). Interestingly, several methyl- binding proteins, including MBD3, MBD4 and MECP2, bind also to hydroxymethylated DNA, enforcing the notion that 5-hmC is not only an intermediate of DNA demethylation but additionally a stable epigenetic markable to influence gene expression by attracting or precluding the binding of specific proteins(Wu and Zhang, 2014).

HISTONE MODIFICATIONS

Most eukaryotic cells contain five main histone proteins: histone H2A, H2B, H3 and H4 – known as the core histones – and H1 – called linker histone. Histones are small, basic proteins (11-20

KDa) and are formed by a globular domain and charged tails enriched in lysine and arginine residues that a subject to a large number of post-translational modification, including acetylation, methylation, ubiquitylation, phosphorylation, SUMOylation and ADP-ribosylation. Among the various histone modifications, acetylation and methylation are the best investigated.

Histone acetylation occurs on lysine residues. It enables relaxation of chromatin structure, thereby promoting transcriptional activation. Histone hypo-acetylation leads instead to chromatin condensation and repression of gene expression. Acetylation is a highly dynamic modification and is regulated by the opposing action of two families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs)(Wang and Hayes, 2008). HATs use acetyl coenzyme A as a cofactor to catalyse acetylation, and are divided into two major classes: type A are nuclear and include N-acetyltransferase 6 (NAT6), MYST – named after its founding members MOZ, Ybf2, Sas2 and Tip60 – and CBP/p300 proteins; in contrast, type B are predominantly cytoplasmic (Hodawadekar and Marmorstein, 2007). On the other hand, eleven HDAC members are known and are subdivided into 4 classes (I–IV) according to cellular localization, enzymatic activity and protein structure. Instead histone methylation occurs on lysine (K) and arginine residues on histones H3 and H4, and can be associated with either activation or repression of transcription, depending on the degree (mono-, di- and tri-

methylation) and location of the modification(Bannister and Kouzarides, 2005). In fact, methylation at H3K4, H4K36 or H3K79 is associated with transcriptional activation, with H3K4me and H3K36me implicated in mRNA elongation. On the other hand, methylation at H3K9, H3K27 or H3K20 is inked with transcriptional repression(Greer and Shi, 2012). Like histone acetylation, histone methylation is a dynamic process regulated by two classes of enzymes: histone methyltransferases (HMTs) and histone demethylases (HDMTs)(Cloos et al., 2008; Zhang and Reinberg, 2001).

THE EPIGENETIC COFACTOR UHRF1

UHRF1 – also known as Np95 or ICBP90 –encodes a cofactor involved in DNA methylation(Hashimoto et al., 2008; Sharif et al., 2007) (Hashimoto et al., 2008; Sharif et al., 2007).UHRF1 has five functional domains: an ubiquitin-like domain, followed by a tandem Tudor domain that binds H3K9me2/3 (Avvakumov et al., 2008; Rottach et al., 2010), an ADD-like domain that binds the histone H3 tail (Citterio et al., 2004; Karagianni et al., 2008), a SET- and RING-associated (SRA) domain that binds hemi-methylated DNA (Bostick et al., 2007; Hashimoto et al., 2008; Sharif et al., 2007) and a RING domain at the C-terminus(Citterio et al., 2004).

UHRF1 targets DNMT1 to hemi-methylated DNA, in order to ensure that DNA becomes re- methylated after replication. In UHRF1-deficient mice and ES cells, methylation of imprinted

genes and satellite repeats is decreased and UHRF1 knockout mice die during early embryogenesis, as Dnmt1 mutants(Sharif et al., 2007). Moreover, UHRF1 is also involved in the maintenance of histone marks: UHRF1 can complex and bind to the histone methyltransferase G9a and with the PHD finger bind to methylated H3K9(Karagianni et al., 2008; Sharif et al., 2007). Importantly, UHRF1 mediates epigenetic inheritance of DNA methylation by associating with DNMT1 and PCNA at replicating heterochromatic regions(Bostick et al., 2007).

UHRF1 is also involved in the regulation of the cell cycle and DNA damage control(Bonapace et al., 2002; Muto et al., 2002). It colocalizes with replication foci in mid-to late S phase and is required for proper heterochromatin formation in mammalian cells(Papait et al., 2007).

UHRF1 has been implicated in pathological processes related to cell proliferation: in cancer cells, over-expression of UHRF1 promotes cancer progression(Babbio et al., 2012; Mudbhary et al., 2014) and, more recently, UHRF1 has been linked to the regulation of local proliferation of regulatory T cells in response to bacterial colonization through the regulation of the growth inhibitory p21 gene(Furusawa et al., 2013).

EPIGENETICS IN CARDIAC HYPERTROPHY

The regulatory networks governing gene expression in the cardiomyocyte are under intense investigation, not least because dysregulation of the gene program has a fundamental role in the development of failing myocardium. Importantly

epigenetic mechanisms are emerging as crucial players in this process.

DNA METHYLATION AND CARDIAC HYPERTROPHY

To date, knowledge on the function of DNA methylation and the associated enzymes and cofactors in cardiac hypertrophy and failure is limited and restricted to the findings of preliminary studies. Immunoprecipitation of methylated DNA from cardiac biopsies, followed by massive DNA sequencing, has indicated that the profile of this epigenetic modification is altered in end-stage cardiomyopathic patients(Movassagh et al., 2011): in fact, DNA methylation differed significantly at the CpGs of promoters and gene bodies when compared with that of healthy heart; moreover, increased gene expression in failing hearts was associated with promoter demethylation, whereas hypermethylation did not clearly correlate with decreased expression. Further analysis revealed that CpGIs associated with double- homeobox 4 (DUX4) were heavily methylated in failing heart, and that hypermethylation of this region was associated with down-regulation of the DUX4 transcript. Because DNA methylation plays a key role in genome stability by repressing repetitive elements, another study investigated the impact of differential methylation of repetitive elements in end-stage cardiomyopathy(Haider et al., 2012): when compared with non-failing hearts, failing hearts were significantly hypomethylated at satellite regions, and this correlated with a

27-fold increase of the corresponding transcripts. Moreover, a low-resolution DNA methylation microarray analysis of the myocardium of idiopathic dilated cardiomyopathy patients revealed that altered DNA methylation patterns caused mis-expression of lymphocyte antigen 75 (LY75) and adenosine receptor A2A (ADORA2A)(Haas et al., 2013); in vivo studies of the zebrafish orthologues of these genes linked them with adaptive and maladaptive heart failure. However, cardiomyocytes make up only 20%–30% of the cells of the heart, so these initial studies conducted on human samples are limited because they analysed a mixed cell population. Investigation of the DNA methylome in specific cardiac cells, such as cardiomyocytes, fibroblasts, endothelial and immune cells, is thus needed.

A recent whole genome–bisulfite sequencing study has tackled this problem in an animal model by isolating cardiomyocytes from mouse heart subjected to pressure overload for 3 weeks(Gilsbach et al., 2014). Subtle but significant differences in the methylation profiles of normal and hypertrophic cells were found mostly in intergenic regions. The authors also determined that failing cardiomyocytes had a methylation profile that partially resembled the one observed in foetal cells.

Instead, whereas 5-hmC has been investigated extensively in several cell and tissue types, its relevance for myocardial pathophysiology is unknown.

HISTONE MODIFICATIONS AND CARDIAC HYPERTROPHY

Several studies have elucidated the importance of p300 in cardiac development(Shikama et al., 2003; Yao et al., 1998) and heart failure(Morimoto et al., 2008; Wei et al., 2008; Yanazume et al., 2003) through myocyte enhancer factor (MEF) 2- and GATA binding protein 4 (GATA4)- dependent transcription. Instead the role of HDACs in cardiac hypertrophy and failure is complex: on one hand, class II HDACs are anti-hypertrophic; on the other, certain HDACs promote hypertrophy. For example, loss of function of either HDAC5 or HDAC9 (two class II HDACs) has been associated with increased susceptibility to cardiac hypertrophy and failure because of the ability of theses enzymes to bind and silence MEF2C(Chang et al., 2004; Zhang et al., 2002). HDAC4 – another class II HDAC – was found under physiological conditions to repress the activities of MEF2 and serum response factor (SRF), but in cardiac hypertrophy it was oxidized, causing it to shuttle out of the nucleus and allow de-repression of pro-hypertrophy genes(Ago et al., 2008); moreover, nuclear export of HDAC4 has been linked to demethylation of H3K9, dissociation of chromobox protein homologue (CBX or HP1), and activation of the hypertrophy-related gene atrial natriuretic peptide (ANP)(Hohl et al., 2013). Thus, cardiac stress induces de-repression of class II HDAC target genes by triggering the nuclear export of these enzymes upon their oxidation.

Several reports have suggested an involvement of histone methylation in cardiac hypertrophy and failure. The histone trimethyllysine demethylase KDM4A (lysine-specific demethylase 4A) has been shown to promote cardiac hypertrophy under pathological conditions by binding to the promoter of Fhl1 (four and a half LIM domains 1) and up-regulating the gene's expression in a SRF- and myocardin-dependent manner(Zhang et al., 2011). Another study elucidated the importance of PAXIP1 (PAX-interacting protein 1) – a key component of the H3K4me complex – in maintenance of the gene expression profile of fully differentiated cardiomyocytes(Stein et al., 2011): one of the genes regulated by H3K4me3 was Kcnip2 (Kv channel interacting protein 2), which encodes a potassium channel down-regulated in heart failure and involved in arrhythmogenesis. Hohl and colleagues found that, despite nuclear export of HDAC4, up-regulation of the hypertrophy markers ANP and natriuretic peptide B (BNP) in patients and in a mouse model of heart failure was not caused by altered acetylation of their promoters but rather by decreased di- and tri-methylation of lysine 9 on histone H3(Hohl et al., 2013). Finally, a recent genome-wide study by our group has defined the profiles of seven histone modifications in cardiomyocytes isolated from mouse heart one week after pressure overload(Papait et al., 2013). Of the 1,109 genes found significantly modulated in hypertrophic cardiomyocytes, 50% were associated with an alteration of at least one histone

mark at their promoter region. Changes in histone acetylation and methylation during the hypertrophy response underscored the activation of a specific class of enhancer regulated by MEF2A and MEF2C.

CARDIAC ENERGETICS AND METABOLISM

The intermediary metabolism of energy providing substrates supports the rhythmic contraction of the heart. Metabolic regulation is inextricably linked with cardiac function. This metabolism-function relationship is relevant to diseases that lead to cardiac hypertrophy and heart failure. The progression to heart failure of any cause is associated with a gradual but progressive decline in the activity of mitochondrial respiratory pathways leading to diminished capacity for adenosine triphosphate (ATP) production.

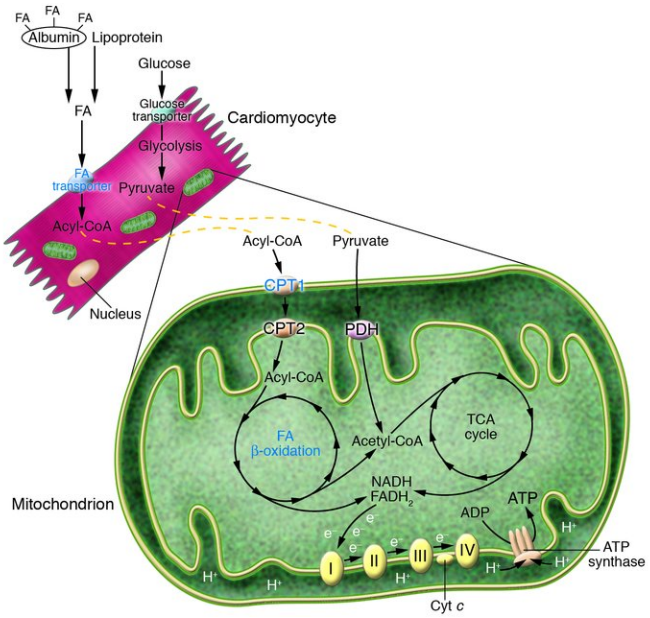

Figure 3: Cardiac energy metabolism (Huss, JCI 2005)

CARDIAC METABOLIC PATHWAYS

The healthy adult heart mainly depends on oxidation of fatty acids (FAs) and glucose in mitochondria for the vast majority of ATP generation (Huss and Kelly, 2005; Stanley and Chandler, 2002). The metabolic flexibility of the adult heart is high, with multiple substrates (e.g., fatty acids, glucose, lactate, and ketone bodies) potentially usable as an energy source in response to physiological changes in demand.

The fetal heart relies mainly on carbohydrate substrates, primarily lactate and glucose, as the major sources of ATP synthesis, while its ability to oxidize long chain fatty acids is limited (Bartelds et al., 2000).Conversely fatty acids (FAs) are the preferred substrate of the adult myocardium. FAs are oxidized in the mitochondrial matrix by the process of FA β-oxidation (FAO) and supply about 70% of total ATP (Lopaschuk et al., 2010). Acetyl-CoA, derived from both pathways, enters the tricarboxylic acid (TCA) cycle. Substrate flux, through the β-oxidation spiral and the TCA cycle respectively, generates flavin adenine dinucleotide (FADH2) and NADH. FADH2 and NADH then enter the electron transport/oxidative phosphorylation pathway and drive ATP synthesis.

TRANSCRIPTIONAL REGULATION OF CARDIAC METABOLISM

Mitochondrial oxidative metabolism and respiratory function are dynamically controlled by gene regulatory programs. One of the most important regulators of cardiac mitochondrial metabolism

and biogenesis is the transcriptional regulator PPARγ coactivator-1 alpha (PGC-1á). PGC-1á was first discovered in brown adipose tissue, where was shown to drive mitochondrial biogenesis and uncoupled respiration (Wu et al., 1999). One year later PGC-1á was shown to induce the production of mitochondria capable of high-level coupled respiration and ATP production also in cardiac myocytes (Lehman et al., 2000). In the heart, PGC-1á expression increases at birth coincident with an increase in cardiac oxidative capacity (Lehman et al., 2000). PGC-1á can be induced by several physiological stimuli that increase ATP demand, including exercise, cold exposure and fasting (Goto et al., 2000; Puigserver et al., 1998). PGC-1á activates a number of downstream regulatory networks by directly coactivating a specific array of the nuclear receptor (NR) superfamily and non-NR transcription factors involved in the control of cellular metabolism. PGC-1α regulates the expression of mitochondrial transcription factor A (Tfam), a nuclear-encoded transcription factor essential for replication, maintenance, and transcription of mitochondrial DNA(Fisher et al., 1992; Larsson et al., 1998) through the coactivation of nuclear respiratory factor-1 (NRF-1) and -2 (NRF-2)(Wu et al., 1999). Moreover, NRF-1 and NRF-2 also control the expression of nuclear genes encoding respiratory chain subunits and other proteins required for mitochondrial function(Scarpulla, 2002; Virbasius et al., 1993). In addition, PGC-1α regulates genes involved in the uptake and mithocondrial oxidation of FAs

through coactivation of PPARs and estrogen-related receptor (ERRs)(Huss et al., 2002; Schreiber et al., 2003; Vega et al., 2000). PPARs are FA-activated members of the NR family of transcription factors that serve as central regulators of cardiac FA metabolism(Desvergne and Wahli, 1999). There are three PPAR isoforms (á,â and ã). While the functional role of PPARá in the regulation of cardiac FA uptake and oxidation has been extensively investigated (Lee et al., 1995; Watanabe et al., 2000), the biological function of PPARâ in the heart is less clear.

ERRs are a subfamily of orphan nuclear receptors and include three members: ERRa, ERRb and ERRg(Giguere et al., 1988; Heard et al., 2000; Sladek et al., 1997). In the heart, ERRa regulates the expression of many genes involved in cardiac FAs oxidation and mitochondrial respiratory function(Sladek et al., 1997). Heart-specific disruption of these transcription factors results in cardiac dysfunction with reduced FAO and mitochondrial ATP production(Arany et al., 2006; Huss et al., 2007). In addition, impairment of the transcriptional programs dictated by these regulators is frequently observed in HF patients(Sihag et al., 2009).

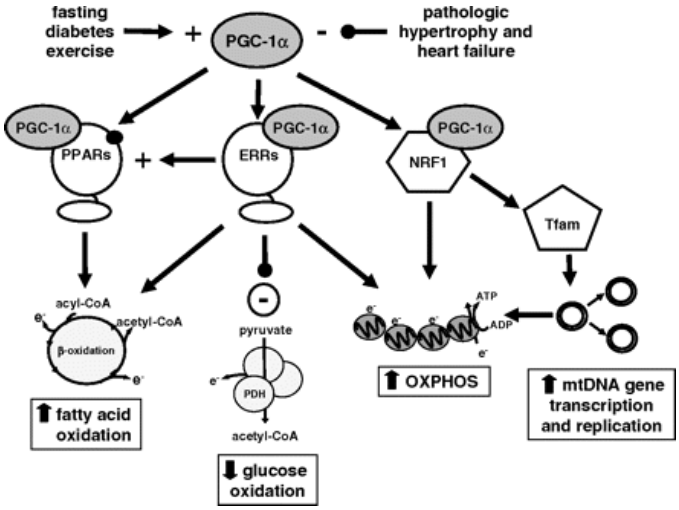

Figure 4: PGC-1a transcriptional network (Finck, Circulation 2007)

METABOLIC REMODELING IN THE HYPERTROPHIC HEART

The heart responds to hemodynamic stress by reverting to a fetal metabolic gene program. Features of the fetal heart reemerge, as for the preference of carbohydrates over fatty acids as substrates for energy. The shift from FAO toward glucose oxidation(Bishop and Altschuld, 1970; Finck and Kelly, 2007), is accompanied by changes in gene expression of mitochondrial FAO enzymes(Doenst et al., 2013; Razeghi et al., 2001), as well as repression of respiratory chain complexes and/or Krebs – or tricarboxylic acid (TCA) – cycle enzymes(Ventura-Clapier et al., 2004). Mice models of pressure-overload indicate that expression of PGC1a and PPARa is reduced in the hypertrophic and failing myocardium(Barger et al., 2000; Garnier et al., 2003). Altogether, this leads to an insufficient supply of ATP to the

hypertrophied heart, negatively affecting cardiomyocyte contractility.

Initially altered cardiac metabolism was considered a byproduct of the pathological states. However, emerging evidence, including observations of the phenotypic expression of genetic defects in humans and animal models, supports the notion that imbalances in mitochondrial energy metabolism actually contribute to cardiac dysfunction. In fact, mitochondrial DNA mutations which result in impairment in mitochondrial respiratory function cause hypertrophic or dilated cardiomyopathy and cardiac conduction defects(DiMauro and Schon, 2001; Triepels et al., 2001; Vallance et al., 2004). Moreover, mutations in nuclear genes encoding mitochondrial fatty acid oxydation enzyme also lead to a cardiomyopathic phenotype(Kelly and Strauss, 1994; Strauss et al., 1995).

Several genetically engineered mouse models have also evidenced a causal relationship between mitochondrial dysfunction and cardiomyopathy. Deletion of the adenine nucleotide translocator 1, which transports ATP generated in the mitochondrion to the cytosol, results in mitochondrial dysfunction and cardiomyopathy(Graham et al., 1997). Furthermore, cardiac-specific deletion of Tfam (transcription factor of activated mitochondria), exhibit marked impairments in mitochondrial metabolism, severe cardiomyopathy, and premature mortality(Wang et al., 1999).

SCOPE OF THE THESIS

Dysregulation of gene expression is implicated in many pathologies, including those of the cardiovascular system. In the last decade epigenetic modifications are emerging as fundamental regulatory mechanisms of cardiac pathophysiology. However, knowledge on if and how epigenetics affects the phenotype of cardiomyocytes is incomplete.

While preliminary findings support a role for DNA methylation in the gene expression deregulation associated with heart failure, whether this epigenetic mark contributes significantly to pathogenesis remains unclear, as are the key players in the process. During my PhD my work focused on determining the role of both 5-mC and 5-hmC in gene expression regulation during myocardial development and disease.

Chapter 2: This work was aimed to elucidate the role of DNA methylation in the development of cardiac hypertrophy and failure. We conducted initial studies on cardiomyocytes isolated from adult mice subjected to transverse aortic constriction (TAC), a pro-hypertrophy stimulus, aimed at defining their methylome in this state. Moreover, a candidate gene screening approach was used to identify the key player of this process.

Chapter 3: Using a genome-wide approach we sought to determine the role of 5-hmC in regulating cardiomyocyte gene expression in development and diseases.

REFERENCES

Ago, T., Liu, T., Zhai, P., Chen, W., Li, H., Molkentin, J.D., Vatner, S.F., and Sadoshima, J. (2008). A redox-dependent pathway for regulating class II HDACs and cardiac hypertrophy. Cell 133, 978-993.Arany, Z., Novikov, M., Chin, S., Ma, Y., Rosenzweig, A., and Spiegelman, B.M. (2006). Transverse aortic constriction leads to accelerated heart failure in mice lacking PPAR-gamma coactivator 1alpha. Proceedings of the National Academy of Sciences of the United States of America 103, 10086-10091.Avvakumov, G.V., Walker, J.R., Xue, S., Li, Y., Duan, S., Bronner, C., Arrowsmith, C.H., and Dhe-Paganon, S. (2008). Structural basis for recognition of hemi- methylated DNA by the SRA domain of human UHRF1. Nature 455, 822-825. Babbio, F., Pistore, C., Curti, L., Castiglioni, I., Kunderfranco, P., Brino, L., Oudet, P., Seiler, R., Thalman, G.N., Roggero, E., et al. (2012). The SRA protein UHRF1 promotes epigenetic crosstalks and is involved in prostate cancer progression. Oncogene 31, 4878-4887.Bannister, A.J., and Kouzarides, T. (2005). Reversing histone methylation. Nature 436, 1103-1106.Bannister, A.J., and Kouzarides, T. (2011). Regulation of chromatin by histone modifications. Cell research 21, 381-395.Barger, P.M., Brandt, J.M., Leone, T.C., Weinheimer, C.J., and Kelly, D.P. (2000). Deactivation of peroxisome proliferator-activated receptor-alpha during cardiac hypertrophic growth. The Journal of clinical investigation 105, 1723-1730.

Barski, A., Cuddapah, S., Cui, K., Roh, T.Y., Schones, D.E., Wang, Z., Wei, G., Chepelev, I., and Zhao, K. (2007). High-resolution profiling of histone methylations in the human genome. Cell 129, 823-837.Bartelds, B., Knoester, H., Smid, G.B., Takens, J., Visser, G.H., Penninga, L., van der Leij, F.R., Beaufort-Krol, G.C., Zijlstra, W.G., Heymans, H.S., et al. (2000). Perinatal changes in myocardial metabolism in lambs. Circulation 102, 926-931.

Belaguli, N.S., Sepulveda, J.L., Nigam, V., Charron, F., Nemer, M., and Schwartz, R.J. (2000). Cardiac tissue enriched factors serum response factor and GATA-4 are mutual coregulators. Molecular and cellular biology 20, 7550-7558.Bernstein, B.E., Meissner, A., and Lander, E.S. (2007). The mammalian epigenome. Cell 128, 669-681.

Bhavsar, P.K., Dellow, K.A., Yacoub, M.H., Brand, N.J., and Barton, P.J. (2000). Identification of cis-acting DNA elements required for expression of the human cardiac troponin I gene promoter. Journal of molecular and cellular cardiology 32, 95-108.

Bishop, S.P., and Altschuld, R.A. (1970). Increased glycolytic metabolism in cardiac hypertrophy and congestive failure. The American journal of physiology 218, 153-159.Bonapace, I.M., Latella, L., Papait, R., Nicassio, F., Sacco, A., Muto, M., Crescenzi, M., and Di Fiore, P.P. (2002). Np95 is regulated by E1A during mitotic reactivation of terminally differentiated cells and is essential for S phase entry. The Journal of cell biology

157, 909-914.Bostick, M., Kim, J.K., Esteve, P.O., Clark, A., Pradhan, S., and Jacobsen, S.E. (2007). UHRF1 plays a role in maintaining DNA methylation in mammalian cells. Science 317, 1760-1764.Chang, S., McKinsey, T.A., Zhang, C.L., Richardson, J.A., Hill, J.A., and Olson, E.N. (2004). Histone deacetylases 5 and 9 govern responsiveness of the heart to a subset of stress signals and play redundant roles in heart development. Molecular and cellular biology 24, 8467-8476.Citterio, E., Papait, R., Nicassio, F., Vecchi, M., Gomiero, P., Mantovani, R., Di Fiore, P.P., and Bonapace, I.M. (2004). Np95 is a histone-binding protein endowed with ubiquitin ligase activity. Molecular and cellular biology 24, 2526-2535.Cloos, P.A., Christensen, J., Agger, K., and Helin, K. (2008). Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. Genes & development 22, 1115-1140.Desvergne, B., and Wahli, W. (1999). Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocrine reviews 20, 649-688. DiMauro, S., and Schon, E.A. (2001). Mitochondrial DNA mutations in human disease. American journal of medical genetics 106, 18-26.Doenst, T., Nguyen, T.D., and Abel, E.D. (2013). Cardiac metabolism in heart failure: implications beyond ATP production. Circulation research 113, 709-724. Durocher, D., Charron, F., Warren, R., Schwartz, R.J., and Nemer, M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. The EMBO journal 16, 5687-5696.Ficz, G., Branco, M.R., Seisenberger, S.,

Santos, F., Krueger, F., Hore, T.A., Marques, C.J., Andrews, S., and Reik, W. (2011). Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473, 398-402.

Finck, B.N., and Kelly, D.P. (2007). Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) regulatory cascade in cardiac physiology and disease. Circulation 115, 2540-2548.Fisher, R.P., Lisowsky, T., Parisi, M.A., and Clayton, D.A. (1992). DNA wrapping and bending by a mitochondrial high mobility group-like transcriptional activator protein. The Journal of biological chemistry 267, 3358-3367. Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., et al. (2013). Commensal microbe- derived butyrate induces the differentiation of colonic regulatory T cells. Nature 504, 446-450.

Gan, H., Wen, L., Liao, S., Lin, X., Ma, T., Liu, J., Song, C.X., Wang, M., He, C., Han, C., et al. (2013). Dynamics of 5-hydroxymethylcytosine during mouse spermatogenesis. Nature communications 4, 1995.Garnier, A., Fortin, D., Delomenie, C., Momken, I., Veksler, V., and Ventura-Clapier, R. (2003). Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles. The Journal of physiology 551, 491- 501.

Giguere, V., Yang, N., Segui, P., and Evans, R.M. (1988). Identification of a new class of steroid hormone receptors.

Nature 331, 91-94.Gilsbach, R., Preissl, S., Gruning, B.A., Schnick, T., Burger, L., Benes, V., Wurch, A., Bonisch, U., Gunther, S., Backofen, R., et al. (2014). Dynamic DNA methylation orchestrates cardiomyocyte development, maturation and disease. Nature communications 5, 5288.

Goto, M., Terada, S., Kato, M., Katoh, M., Yokozeki, T., Tabata, I., and Shimokawa, T. (2000). cDNA Cloning and mRNA analysis of PGC-1 in epitrochlearis muscle in swimming-exercised rats. Biochemical and biophysical research communications 274, 350-354.

Graham, B.H., Waymire, K.G., Cottrell, B., Trounce, I.A., MacGregor, G.R., and Wallace, D.C. (1997). A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoform of the adenine nucleotide translocator. Nature genetics 16, 226-234.

Greer, E.L., and Shi, Y. (2012). Histone methylation: a dynamic mark in health, disease and inheritance. Nature reviews. Genetics 13, 343-357.Haas, J., Frese, K.S., Park, Y.J., Keller, A., Vogel, B., Lindroth, A.M., Weichenhan, D., Franke, J., Fischer, S., Bauer, A., et al. (2013). Alterations in cardiac DNA methylation in human dilated cardiomyopathy. EMBO molecular medicine 5, 413-429.

Haider, S., Cordeddu, L., Robinson, E., Movassagh, M., Siggens, L., Vujic, A., Choy, M.K., Goddard, M., Lio, P., and Foo, R. (2012). The landscape of DNA repeat elements in human heart failure. Genome biology 13, R90.Hasegawa, K.,

Lee, S.J., Jobe, S.M., Markham, B.E., and Kitsis, R.N. (1997). cis- Acting sequences that mediate induction of beta-myosin heavy chain gene expression during left ventricular hypertrophy due to aortic constriction. Circulation 96, 3943-3953.

Hashimoto, H., Horton, J.R., Zhang, X., Bostick, M., Jacobsen, S.E., and Cheng, X. (2008). The SRA domain of UHRF1 flips 5-methylcytosine out of the DNA helix. Nature 455, 826-829.

Hautala, N., Tokola, H., Luodonpaa, M., Puhakka, J., Romppanen, H., Vuolteenaho, O., and Ruskoaho, H. (2001). Pressure overload increases GATA4 binding activity via endothelin-1. Circulation 103, 730-735.Heard, D.J., Norby, P.L., Holloway, J., and Vissing, H. (2000). Human ERRgamma, a third member of the estrogen receptor-related receptor (ERR) subfamily of orphan nuclear receptors: tissue-specific isoforms are expressed during development and in the adult. Molecular endocrinology 14, 382-392.

Hendrich, B., and Tweedie, S. (2003). The methyl-CpG binding domain and the evolving role of DNA methylation in animals. Trends in genetics : TIG 19, 269- 277.Herzig, T.C., Jobe, S.M., Aoki, H., Molkentin, J.D., Cowley, A.W., Jr., Izumo, S., and Markham, B.E. (1997). Angiotensin II type1a receptor gene expression in the heart: AP-1 and GATA-4 participate in the response to pressure overload. Proceedings of the National Academy of Sciences of the United States of America 94, 7543-7548.

Hodawadekar, S.C., and Marmorstein, R. (2007). Chemistry of acetyl transfer by histone modifying enzymes: structure, mechanism and implications for effector design. Oncogene 26, 5528-5540.Hohl, M., Wagner, M., Reil, J.C., Muller, S.A., Tauchnitz, M., Zimmer, A.M., Lehmann, L.H., Thiel, G., Bohm, M., Backs, J., et al. (2013). HDAC4 controls histone methylation in response to elevated cardiac load. The Journal of clinical investigation 123, 1359-1370.

Hon, G.C., Song, C.X., Du, T., Jin, F., Selvaraj, S., Lee, A.Y., Yen, C.A., Ye, Z., Mao, S.Q., Wang, B.A., et al. (2014). 5mC oxidation by Tet2 modulates enhancer activity and timing of transcriptome reprogramming during differentiation. Molecular cell 56, 286-297.

Huss, J.M., Imahashi, K., Dufour, C.R., Weinheimer, C.J., Courtois, M., Kovacs, A., Giguere, V., Murphy, E., and Kelly, D.P. (2007). The nuclear receptor ERRalpha is required for the bioenergetic and functional adaptation to cardiac pressure overload. Cell metabolism 6, 25-37.

Huss, J.M., and Kelly, D.P. (2005). Mitochondrial energy metabolism in heart failure: a question of balance. The Journal of clinical investigation 115, 547-555. Huss, J.M., Kopp, R.P., and Kelly, D.P. (2002). Peroxisome proliferator-activated receptor coactivator-1alpha (PGC-1alpha) coactivates the cardiac-enriched nuclear receptors estrogen-related receptor-alpha and -gamma. Identification of novel leucine-rich interaction motif within PGC-1alpha. The Journal of biological

chemistry 277, 40265-40274.Ivanov, M., Kals, M., Kacevska, M., Barragan, I., Kasuga, K., Rane, A., Metspalu, A., Milani, L., and Ingelman-Sundberg, M. (2013). Ontogeny, distribution and potential roles of 5-hydroxymethylcytosine in human liver function. Genome biology 14, R83.Karagianni, P., Amazit, L., Qin, J., and Wong, J. (2008). ICBP90, a novel methyl K9 H3 binding protein linking protein ubiquitination with heterochromatin formation. Molecular and cellular biology 28, 705-717.Kelly, D.P., and Strauss, A.W. (1994). Inherited cardiomyopathies. The New England journal of medicine 330, 913-919.Komuro, I., and Izumo, S. (1993). Csx: a murine homeobox-containing gene specifically expressed in the developing heart. Proceedings of the National Academy of Sciences of the United States of America 90, 8145-8149.

Kouzarides, T. (2007). Chromatin modifications and their function. Cell 128, 693- 705.Larsson, N.G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G.S., and Clayton, D.A. (1998). Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. Nature genetics 18, 231-236.

Lee, S.S., Pineau, T., Drago, J., Lee, E.J., Owens, J.W., Kroetz, D.L., Fernandez- Salguero, P.M., Westphal, H., and Gonzalez, F.J. (1995). Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. Molecular and cellular biology 15, 3012-

3022.Lehman, J.J., Barger, P.M., Kovacs, A., Saffitz, J.E., Medeiros, D.M., and Kelly, D.P. (2000). Peroxisome proliferator-activated receptor gamma coactivator-1 promotes cardiac mitochondrial biogenesis. The Journal of clinical investigation 106, 847-856.Lints, T.J., Parsons, L.M., Hartley, L., Lyons, I., and Harvey, R.P. (1993). Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development 119, 969.Lopaschuk, G.D., Ussher, J.R., Folmes, C.D., Jaswal, J.S., and Stanley, W.C. (2010). Myocardial fatty acid metabolism in health and disease. Physiological reviews 90, 207-258.Lowes, B.D., Minobe, W., Abraham, W.T., Rizeq, M.N., Bohlmeyer, T.J., Quaife, R.A., Roden, R.L., Dutcher, D.L., Robertson, A.D., Voelkel, N.F., et al. (1997). Changes in gene expression in the intact human heart. Downregulation of alpha-myosin heavy chain in hypertrophied, failing ventricular myocardium. The Journal of clinical investigation 100, 2315-2324.Lu, F., Liu, Y., Jiang, L., Yamaguchi, S., and Zhang, Y. (2014). Role of Tet proteins in enhancer activity and telomere elongation. Genes & development 28, 2103-2119. Marttila, M., Hautala, N., Paradis, P., Toth, M., Vuolteenaho, O., Nemer, M., and Ruskoaho, H. (2001). GATA4 mediates activation of the B-type natriuretic peptide gene expression in response to hemodynamic stress. Endocrinology 142, 4693-4700.McKinsey, T.A., Zhang, C.L., and Olson, E.N. (2002). MEF2: a calcium-dependent regulator of cell division, differentiation and death. Trends in biochemical

sciences 27, 40-47.Molkentin, J.D. (2000). The zinc finger-containing transcription factors GATA-4, - 5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. The Journal of biological chemistry 275, 38949-38952.Molkentin, J.D., Lu, J.R., Antos, C.L., Markham, B., Richardson, J., Robbins, J., Grant, S.R., and Olson, E.N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 93, 215-228.Morimoto, T., Sunagawa, Y., Kawamura, T., Takaya, T., Wada, H., Nagasawa, A., Komeda, M., Fujita, M., Shimatsu, A., Kita, T., et al. (2008). The dietary compound curcumin inhibits p300 histone acetyltransferase activity and prevents heart failure in rats. The Journal of clinical investigation 118, 868-878.Morisco, C., Seta, K., Hardt, S.E., Lee, Y., Vatner, S.F., and Sadoshima, J. (2001). Glycogen synthase kinase 3beta regulates GATA4 in cardiac myocytes. The Journal of biological chemistry 276, 28586-28597.

Movassagh, M., Choy, M.K., Knowles, D.A., Cordeddu, L., Haider, S., Down, T., Siggens, L., Vujic, A., Simeoni, I., Penkett, C., et al. (2011). Distinct epigenomic features in end-stage failing human hearts. Circulation 124, 2411-2422. Mudbhary, R., Hoshida, Y., Chernyavskaya, Y., Jacob, V., Villanueva, A., Fiel, M.I., Chen, X., Kojima, K., Thung, S., Bronson, R.T., et al. (2014). UHRF1 overexpression drives DNA hypomethylation and hepatocellular carcinoma. Cancer cell 25, 196- 209.

Muto, M., Kanari, Y., Kubo, E., Takabe, T., Kurihara, T., Fujimori, A., and Tatsumi, K. (2002). Targeted disruption of Np95 gene renders murine embryonic stem cells hypersensitive to DNA damaging agents and DNA replication blocks. The Journal of biological chemistry 277, 34549-34555.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247-257.Packer, M., Coats, A.J., Fowler, M.B., Katus, H.A., Krum, H., Mohacsi, P., Rouleau, J.L., Tendera, M., Castaigne, A., Roecker, E.B., et al. (2001). Effect of carvedilol on survival in severe chronic heart failure. The New England journal of medicine 344, 1651-1658.

Papait, R., Cattaneo, P., Kunderfranco, P., Greco, C., Carullo, P., Guffanti, A., Vigano, V., Stirparo, G.G., Latronico, M.V., Hasenfuss, G., et al. (2013). Genome-wide analysis of histone marks identifying an epigenetic signature of promoters and enhancers underlying cardiac hypertrophy. Proceedings of the National Academy of Sciences of the United States of America 110, 20164-20169.

Papait, R., Pistore, C., Negri, D., Pecoraro, D., Cantarini, L., and Bonapace, I.M. (2007). Np95 is implicated in pericentromeric heterochromatin replication and in major satellite silencing. Molecular biology of the cell 18, 1098-1106.Pastor, W.A., Pape, U.J., Huang, Y., Henderson, H.R., Lister, R., Ko, M., McLoughlin, E.M., Brudno, Y., Mahapatra, S.,

Kapranov, P., et al. (2011). Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473, 394-397. Patient, R.K., and McGhee, J.D. (2002). The GATA family (vertebrates and invertebrates). Current opinion in genetics & development 12, 416-422. Puigserver, P., Wu, Z., Park, C.W., Graves, R., Wright, M., and Spiegelman, B.M. (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell 92, 829-839.Rada-Iglesias, A., Bajpai, R., Swigut, T., Brugmann, S.A., Flynn, R.A., and Wysocka, J. (2011). A unique chromatin signature uncovers early developmental enhancers in humans. Nature 470, 279-283.Razeghi, P., Young, M.E., Alcorn, J.L., Moravec, C.S., Frazier, O.H., and Taegtmeyer, H. (2001). Metabolic gene expression in fetal and failing human heart. Circulation 104, 2923-2931.Reik, W., Dean, W., and Walter, J. (2001). Epigenetic reprogramming in mammalian development. Science 293, 1089-1093.Rottach, A., Frauer, C., Pichler, G., Bonapace, I.M., Spada, F., and Leonhardt, H. (2010). The multi-domain protein Np95 connects DNA methylation and histone modification. Nucleic acids research 38, 1796-1804.Saadane, N., Alpert, L., and Chalifour, L.E. (1999). Expression of immediate early genes, GATA-4, and Nkx-2.5 in adrenergic-induced cardiac hypertrophy and during regression in adult mice. British journal of pharmacology 127, 1165-1176.

Scarpulla, R.C. (2002). Nuclear activators and coactivators in mammalian mitochondrial biogenesis. Biochimica et biophysica

acta 1576, 1-14.Schoenfeld, J.R., Vasser, M., Jhurani, P., Ng, P., Hunter, J.J., Ross, J., Jr., Chien, K.R., and Lowe, D.G. (1998). Distinct molecular phenotypes in murine cardiac muscle development, growth, and hypertrophy. Journal of molecular and cellular cardiology 30, 2269-2280.

Schreiber, S.N., Knutti, D., Brogli, K., Uhlmann, T., and Kralli, A. (2003). The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor estrogen-related receptor alpha (ERRalpha). The Journal of biological chemistry 278, 9013-9018.

Sharif, J., Muto, M., Takebayashi, S., Suetake, I., Iwamatsu, A., Endo, T.A., Shinga, J., Mizutani-Koseki, Y., Toyoda, T., Okamura, K., et al. (2007). The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. Nature 450, 908-912.

Shikama, N., Lutz, W., Kretzschmar, R., Sauter, N., Roth, J.F., Marino, S., Wittwer, J., Scheidweiler, A., and Eckner, R. (2003). Essential function of p300 acetyltransferase activity in heart, lung and small intestine formation. The EMBO journal 22, 5175-5185.

Sihag, S., Cresci, S., Li, A.Y., Sucharov, C.C., and Lehman, J.J. (2009). PGC-1alpha and ERRalpha target gene downregulation is a signature of the failing human heart. Journal of molecular and cellular cardiology 46, 201-212.Sladek, R., Bader, J.A., and Giguere, V. (1997). The orphan nuclear receptor estrogen-related receptor alpha is a transcriptional regulator of the

human medium-chain acyl coenzyme A dehydrogenase gene. Molecular and cellular biology 17, 5400-5409.

Smith, Z.D., and Meissner, A. (2013). DNA methylation: roles in mammalian development. Nature reviews. Genetics 14, 204-220.Stanley, W.C., and Chandler, M.P. (2002). Energy metabolism in the normal and failing heart: potential for therapeutic interventions. Heart failure reviews 7, 115-130.

Stein, A.B., Jones, T.A., Herron, T.J., Patel, S.R., Day, S.M., Noujaim, S.F., Milstein, M.L., Klos, M., Furspan, P.B., Jalife, J., et al. (2011). Loss of H3K4 methylation destabilizes gene expression patterns and physiological functions in adult murine cardiomyocytes. The Journal of clinical investigation 121, 2641-2650. Stein, R., Gruenbaum, Y., Pollack, Y., Razin, A., and Cedar, H. (1982). Clonal inheritance of the pattern of DNA methylation in mouse cells. Proceedings of the National Academy of Sciences of the United States of America 79, 61-65.

Strauss, A.W., Powell, C.K., Hale, D.E., Anderson, M.M., Ahuja, A., Brackett, J.C., and Sims, H.F. (1995). Molecular basis of human mitochondrial very-long-chain acyl- CoA dehydrogenase deficiency causing cardiomyopathy and sudden death in childhood. Proceedings of the National Academy of Sciences of the United States of America 92, 10496-10500.

Stroud, H., Feng, S., Morey Kinney, S., Pradhan, S., and Jacobsen, S.E. (2011). 5- Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem

cells. Genome biology 12, R54.Szulwach, K.E., Li, X., Li, Y., Song, C.X., Han, J.W., Kim, S., Namburi, S., Hermetz, K., Kim, J.J., Rudd, M.K., et al. (2011a). Integrating 5-hydroxymethylcytosine into the epigenomic landscape of human embryonic stem cells. PLoS genetics 7, e1002154.

Szulwach, K.E., Li, X., Li, Y., Song, C.X., Wu, H., Dai, Q., Irier, H., Upadhyay, A.K., Gearing, M., Levey, A.I., et al. (2011b). 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. Nature neuroscience 14, 1607-1616.

Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L.M., Liu, D.R., Aravind, L., et al. (2009). Conversion of 5- methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324, 930-935.

Triepels, R.H., Van Den Heuvel, L.P., Trijbels, J.M., and Smeitink, J.A. (2001). Respiratory chain complex I deficiency. American journal of medical genetics 106, 37-45.Tsagaratou, A., Aijo, T., Lio, C.W., Yue, X., Huang, Y., Jacobsen, S.E., Lahdesmaki, H., and Rao, A. (2014). Dissecting the dynamic changes of 5-hydroxymethylcytosine in T-cell development and differentiation. Proceedings of the National Academy of Sciences of the United States of America 111, E3306-3315.

Vallance, H.D., Jeven, G., Wallace, D.C., and Brown, M.D. (2004). A case of sporadic infantile histiocytoid cardiomyopathy caused by the A8344G (MERRF) mitochondrial DNA mutation.

Pediatric cardiology 25, 538-540.Vega, R.B., Huss, J.M., and Kelly, D.P. (2000). The coactivator PGC-1 cooperates with peroxisome proliferator-activated receptor alpha in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. Molecular and cellular biology 20, 1868-1876.

Ventura-Clapier, R., Garnier, A., and Veksler, V. (2004). Energy metabolism in heart failure. The Journal of physiology 555, 1-13.Virbasius, C.A., Virbasius, J.V., and Scarpulla, R.C. (1993). NRF-1, an activator involved in nuclear-mitochondrial interactions, utilizes a new DNA-binding domain conserved in a family of developmental regulators. Genes & development 7, 2431-2445.

Wang, J., Wilhelmsson, H., Graff, C., Li, H., Oldfors, A., Rustin, P., Bruning, J.C., Kahn, C.R., Clayton, D.A., Barsh, G.S., et al. (1999). Dilated cardiomyopathy and atrioventricular conduction blocks induced by heart-specific inactivation of mitochondrial DNA gene expression. Nature genetics 21, 133-137.

Wang, X., and Hayes, J.J. (2008). Acetylation mimics within individual core histone tail domains indicate distinct roles in regulating the stability of higher- order chromatin structure. Molecular and cellular biology 28, 227-236. Watanabe, K., Fujii, H., Takahashi, T., Kodama, M., Aizawa, Y., Ohta, Y., Ono, T., Hasegawa, G., Naito, M., Nakajima, T., et al. (2000). Constitutive regulation of cardiac fatty acid metabolism through peroxisome proliferator-activated receptor alpha associated

with age-dependent cardiac toxicity. The Journal of biological chemistry 275, 22293-22299.Wei, J.Q., Shehadeh, L.A., Mitrani, J.M., Pessanha, M., Slepak, T.I., Webster, K.A., and Bishopric, N.H. (2008). Quantitative control of adaptive cardiac hypertrophy by acetyltransferase p300. Circulation 118, 934-946.Wu, H., D'Alessio, A.C., Ito, S., Wang, Z., Cui, K., Zhao, K., Sun, Y.E., and Zhang, Y. (2011). Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. Genes & development 25, 679-684.Wu, H., and Zhang, Y. (2014). Reversing DNA methylation: mechanisms, genomics, and biological functions. Cell 156, 45-68.

Wu, J.I., Lessard, J., and Crabtree, G.R. (2009). Understanding the words of chromatin regulation. Cell 136, 200-206.Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R.C., et al. (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98, 115-124.Yanazume, T., Hasegawa, K., Morimoto, T., Kawamura, T., Wada, H., Matsumori, A., Kawase, Y., Hirai, M., and Kita, T. (2003). Cardiac p300 is involved in myocyte growth with decompensated heart failure. Molecular and cellular biology 23, 3593-3606.Yao, T.P., Oh, S.P., Fuchs, M., Zhou, N.D., Ch'ng, L.E., Newsome, D., Bronson, R.T., Li, E., Livingston, D.M., and Eckner, R. (1998). Gene dosage-dependent embryonic development and proliferation defects in

mice lacking the transcriptional integrator p300. Cell 93, 361-372.Yu, M., Hon, G.C., Szulwach, K.E., Song, C.X., Zhang, L., Kim, A., Li, X., Dai, Q., Shen, Y., Park, B., et al. (2012). Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. Cell 149, 1368-1380.Zhang, C.L., McKinsey, T.A., Chang, S., Antos, C.L., Hill, J.A., and Olson, E.N. (2002). Class II histone deacetylases act as signal-responsive repressors of cardiac hypertrophy. Cell 110, 479-488.Zhang, Q.J., Chen, H.Z., Wang, L., Liu, D.P., Hill, J.A., and Liu, Z.P. (2011). The histone trimethyllysine demethylase JMJD2A promotes cardiac hypertrophy in response to hypertrophic stimuli in mice. The Journal of clinical investigation 121, 2447-2456.Zhang, Y., and Reinberg, D. (2001). Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. Genes & development 15, 2343-2360.

CHAPTER 2 THE DNA METHYLATION COFACTOR UHRF1 CONTROLS THE METABOLIC RE-PROGRAMMING OF HYPERTROPHIC CARDIOMYOCYTES

Carolina M. Greco[1], Pierluigi Carullo[1,2], Paolo Kunderfranco[1,2], Giacomo Viggiani[1], Lorena Zentilin[3], Elisa di Pasquale[1,2], Paola Cattaneo[1,2], Diego Morone[1], Michael V. G. Latronico[1], Mauro Giacca[3], Roberto Papait[1,2],§ & Gianluigi Condorelli[1,2,4], §

1Cardiovascular Research, Humanitas Research Hospital, via Manzoni 56, 20089 Rozzano (MI), Italy.
2Institute of Genetic and Biomedical Research (IGBR), National Research Council of Italy, via Manzoni 56, 20089 Rozzano (MI), Italy.
3International Centre for Genetic Engineering and Biotechnology (ICGEB) Science Park, Padriciano 99, 34149 Trieste, Italy.
4University of Milan, via Manzoni 56, 20089 Rozzano (MI), Italy.

*Correspondence: gianluigi.condorelli@humanitasresearch.it or roberto.papait@humanitasresearch.it.

Short title: Epigenetic control of cardiac metabolic remodeling

Submitted

SUMMARY

Heart failure, a leading cause of mortality worldwide, is accompanied by cardiac hypertrophy, a process characterized by a specific gene expression program. Although DNA methylation is a key element of gene expression regulation, its role in this pathology remains unclear. Here, we report that DNA methylation governs the metabolic reprogramming of hypertrophic cardiomyocytes at multiple levels. We identify the epigenetic cofactor UHRF1 as a master regulator of this process by mediating the crosstalk between two major repressive marks – DNA methylation and H3K9 di-methylation – decreasing binding of the mitochondrial transcriptional regulator NRF1 to its target genes. Indeed, cardiac-specific silencing of Uhrf1 improved cardiac and mitochondrial function in pressure-overloaded mice. Taken together, our study identifies DNA methylation as a new layer of regulation of cardiac energy metabolism in the hemodynamically stressed heart, and indicates that inhibition of this epigenetic network could be a therapeutic strategy for heart failure.

INTRODUCTION

Heart failure (HF) is a major cause of mortality worldwide. Pathological cardiac hypertrophy almost invariably accompanies HF. Activation of a specific gene expression program is a hallmark of cardiac hypertrophy (Izumo et al., 1988; Lompre et al., 1979). Cardiac work depends strongly on ATP generation, which derives mainly from oxidative phosphorylation (OXPHOS) in mitochondria (Doenst et al., 2013). The metabolic flexibility of the adult heart is high, with multiple substrates (e.g., fatty acids, glucose, lactate, and ketone bodies) potentially usable as an energy source in response to physiological changes in demand. Despite this, HF is characterized by major alterations in the bioenergetics of cardiomyocytes, caused by a diminished metabolic plasticity (Razeghi et al., 2001; Taegtmeyer et al., 2005). In fact, in response to pressure overload, the heart reverts to a fetal-like metabolic pattern: mitochondrial fatty acid oxidation (FAO) genes are repressed, causing a switch from oxidation of fatty acids to an increased reliance on glucose metabolism (Doenst et al., 2013; Mohn et al., 2008; Rajabi et al., 2007). Moreover, decreased activity of respiratory chain complexes and/or Krebs – or tricarboxylic acid (TCA) – cycle enzymes have been described in humans and experimental models of HF (Ventura-Clapier et al., 2004). Altogether, this leads to an insufficient supply of ATP to the hypertrophied heart, negatively affecting cardiomyocyte contractility and resulting ultimately in HF.

Regulation of the metabolic profile by several members of the nuclear receptor superfamily and their co-activators, including estrogen-related receptors a and g (ERRa and g), proliferator-activated receptor a and g (PPARa and g), and PPAR coactivator-1, have been shown to play a key role in the metabolic switch from FAO to glycolysis (Huss et al., 2007; Madrazo and Kelly, 2008; Rowe et al., 2010). Heart-specific disruption of these transcription factors results in cardiac dysfunction with reduced FAO and mitochondrial ATP production (Arany et al., 2005; Huss et al., 2007). In addition, impairment of the transcriptional programs dictated by these regulators is frequently observed in HF patients (el Azzouzi et al., 2013; Sihag et al., 2009). These studies clearly illustrate the importance of these signaling pathways in the energy homeostasis and pathogenesis of HF. However, the molecular mechanisms that govern the metabolic remodeling of hypertrophic cardiomyocytes are not completely understood.

Methylation of DNA at position 5 of cytosine is a key marker of transcription repression (Mohn et al., 2008; Stein et al., 1982). In somatic cells, this occurs preferentially on CpG dinucleotides at CpG islands. In the vertebrate genome, approximately 70% of annotated gene promoters contain a CpG island, making them important genetic elements of gene expression regulation (Wu and Zhang, 2014). Alterations in the DNA methylation profile have been found in patients with dilated cardiomyopathy (Haas et al., 2013; Movassagh et al., 2011) and in a mice

model of pressure-overload HF (Gilsbach et al., 2014). Although these reports suggest a role for DNA methylation in the regulation of gene expression in the diseased heart, it remains unclear how this epigenetic mark is implicated in cardiac hypertrophy and which proteins govern this modification.

Here, we have investigated the role of DNA methylation in cardiac hypertrophy with a genome-wide approach. We demonstrate that this epigenetic mark governs metabolic remodeling of the hypertrophied and failing heart. Moreover, we identify UHRF1 (ubiquitin-like, containing PHD and RING finger domains, 1) as a critical mediator of the gene re-programming process of cardiac hypertrophy and suggest that inhibition of this epigenetic network could be a promising therapeutic strategy for heart failure.

RESULTS

DNA Methylation is Involved in the Re-Programming of the Metabolic Gene Profile in Pressure-Overloaded Cardiomyocytes

To address the role of DNA methylation in gene re-programming during cardiac hypertrophy, we assessed the genomic distribution of 5-methylcytosine (5-mC) in hypertrophic cardiomyocytes and then explored the relationships between this epigenetic modification and gene expression. To this end, we used a methylated DNA immunoprecipitation approach coupled with deep-sequencing (MeDIP-seq) on cardiomyocytes isolated from mice subjected or not to transverse aortic constriction (TAC) – a surgical procedure that causes cardiac hypertrophy through the induction of pressure overload – for seven days, a time-point corresponding to maximal activation of compensatory mechanisms (Kemi et al., 2008; Toischer et al., 2010) (Figure S1A). For the gene expression data, we employed our previously published RNA-seq dataset (Papait et al., 2013). As expected (Mohn et al., 2008), DNA methylation around the transcription start site (TSS) was negatively associated with gene expression, whilst the presence on the gene body was associated with transcriptional activation (Figure S1B).

We then asked whether loss or acquisition of DNA methylation at the promoter during hypertrophy significantly influenced the expression of subsets of genes. We found 109 up-regulated

genes that were associated with loss of methylation at their promoter (Figure 1A and Supplemental Dataset 1), and 185 repressed genes associated with acquisition of promoter methylation (Figure 1B). This finding was suggestive of a causal role for promoter DNA methylation in the differential expression of these genes in hypertrophic cardiomyocytes. The 109 hypo-methylated genes were mainly implicated in cell hemostasis, the TGFb signaling pathway, and cell cycle regulation (Figure 1C), whereas those that gained methylation were involved in multiple metabolic pathways, including the TCA cycle, OXPHOS, and the electron transport chain (ETC) (Figure 1D).

Furthermore, motif enrichment analysis – which identifies putative binding sites for transcription factors (TFs) – indicated that there was a specific and significant enrichment of the motifs for the E26 transformation-specific (ETS) TF family and for nuclear respiratory factor 1 (NRF1) at the promoter region of the hyper-methylated genes. In contrast, de-methylated promoters were specifically enriched in TATA-binding protein (TBP) and Kruppel-like factor 4 (KLF4) motifs (Figure 1E).

We used quantitative RT-PCR to confirm the down-regulation of several metabolism-related genes, including the pyruvate dehydrogenase Pdhb; Atp5g3, which is involved in mitochondrial ATP synthesis; Acss1, Etfa, Me3, and Acot7, which are involved in lipid metabolism; Ndufb2, Ndufs4, and Ndufs5, which encode subunits of the OXPHOS complex I; Cox8a, the terminal enzyme of the respiratory chain; Slc4a2,

which encodes GLUT4; and the mitochondrial- membrane carrier protein gene Slc25a20. Similarly, methylated-CpG island recovery assay (MIRA) was used to confirm the promoter DNA methylation pattern of the genes (Figure 1F).

These results indicate that pressure overload–induced cardiac hypertrophy is associated with a dynamic DNA methylation profile, and specifically that increased promoter DNA methylation is involved in metabolic remodeling of the heart.

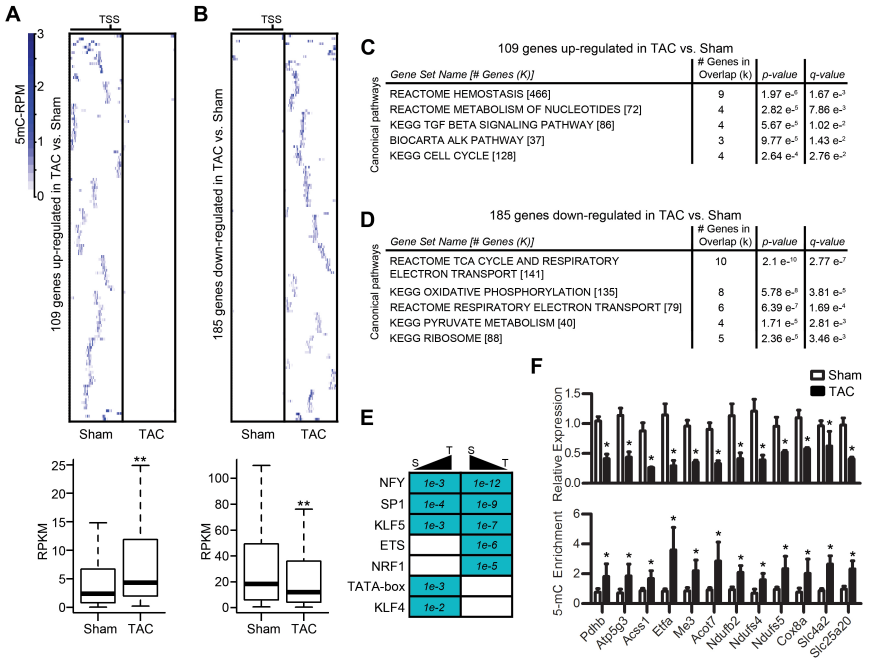

Figure 1. Analysis of genes regulated by promoter DNA methylation in adult cardiomyocytes isolated from pressure-overloaded and control mice

(A) Density heatmap of 5-mC reads around (–4 to +1 Kb) the hypomethylated transcription start site (TSS) of 109 genes found up-regulated (fold change >1.2) 7 days after tranverse aortic constriction (TAC) surgery (top), and boxplot of median expression in sham- and TAC-operated mice (bottom). (B) Heatmap of 5- mC levels of the 185 down-regulated genes associated with a hypermethylated TSS at 7 days post-TAC surgery (top), and boxplot of median expression (bottom). **, p <0.01, determined by Mann-Whitney test. (C) Gene set enrichment analyisis of the 109 up-regulated genes. (D) Gene set enrichment analyisis of the 185 down-regulated genes. (E) Transcription factor binding motifs identified for promoters regulated by DNA methylation in cardiac hypertrophy. Numbers indicate the enrichment p-value. Up-regulated (◄) and down-regulated (►) genes in TAC (T) vs sham (S); NFY, nuclear transcription factor Y; SP1, Sp1 transcription factor; ETS, KLF4/5, Kruppel-like factor 4/5; ETS, E26 transformation-specific transcription factor; NRF1, nuclear respiratory factor-1. (F) Quantitative RT-PCR of mRNA and 5-mC levels of several metabolic genes regulated by DNA methylation in cardiac hypertrophy (graphs of mean±SD, n=3- 6). *, p <0.05. See also Figure S1 and Supplemental Dataset 1.

A

Sample ID	# raw reads	# aligned & unique & mapq≥10	# of peaks
Input DNA	33214430	21080221	
5mC_Sham	55782184	12585289	135886
5mC_TAC	54532430	14658177	162107

B

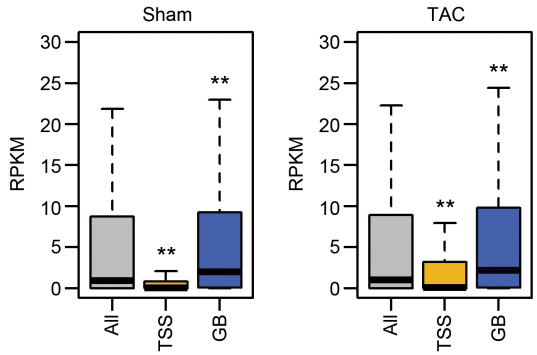

Figure S1, Related to Figure 1.

Methylation around transcription start sites and gene expression.(A) Sequencing statistics and number of peaks detected for each sample.(B) Boxplot of median expression of all genes, genes with a methylated region only on the transcription start site (TSS), and genes with a methylated region in the gene body (GB: exon-intron). P-value determined by Mann-Whitney test **, $p<0.01$

The Epigenetic Co-factor UHRF1 is Up-regulated in Hypertrophic Cardiomyocytes

To identify significantly modulated epigenetic players potentially involved in defining the DNA methylation pattern in cardiac hypertrophy, we analyzed the expression of 48 genes encoding enzymes and co-factors involved in DNA methylation/de-methylation. The analysis was carried out on cardiomyocytes isolated from mice subjected to TAC for 2, 4, and 7 days; the expression profile of the hypertrophy marker genes Nppb, Acta1, Myh7, and Des confirmed the presence of an on-going hypertrophic response from 4 days post-TAC surgery (Figure 2A). We found that while many of the 48 genes remained unmodified, a set of genes encoding several proteins involved in active de-methylation – consisting in the hydroxylation of 5-mC to 5-hydroxymethylcytosine – were significantly down-regulated (e.g., Idh1, Idh2, and Tet3). In addition, and more strikingly, one gene, namely Uhrf1 – also known as Np95 or ICBP90 – was strongly up-regulated already four days after TAC surgery (Figure 2A and Table S1). Uhrf1 encodes a cofactor involved in DNA methylation (Hashimoto et al., 2008; Sharif et al., 2007) and has been shown to play a critical role in regulating the expression of a large number of genes in T cells and cancer cell lines (Babbio et al., 2012; Obata et al., 2014; Sharif et al., 2007).

Cardiac-stress-mediated up-regulation of Uhrf1 was confirmed in vivo with qRT-PCR and immunostaining on whole left

ventricle tissue, and in vitro on neonatal cardiomyocytes exposed to a hypertrophy stimulus – either phenylephrine (PE) or fetal bovine serum (FBS) (Figure 2B). In addition, qRT- PCR on whole left ventricle revealed that Uhrf1 returned to its basal expression level by four weeks of TAC, indicating that UHRF1 may play a role during the initial phase of cardiac hypertrophy.

To assess whether modulation of UHRF1 was relevant also to the human setting, we studied functional cardiomyocytes differentiated from human induced pluripotent stem cells (Figure S2A). We found that UHRF1 expression was induced also in these cells after treatment with PE or FBS (Figure 2C and Figure S2B).

We then assessed whether TFs mediating cardiac hypertrophy regulated Uhrf1 expression. To this end, we analyzed published chromatin immunoprecipitation–DNA sequencing (ChIP–seq) data sets of cardiac TFs in HL1 cells (He et al., 2011), a transformed mouse cardiomyocyte line. We identified a binding site for serum response factor (SRF) – a key TF for the hypertrophy response – 2 Kb upstream of Uhrf1's TSS (Figure 2D). To confirm the functional relevance of this finding, we fused the promoter region of Uhrf1 to a luciferase reporter gene, generating a Uhrf1 expression reporter construct. Expression of SRF revealed that the response of Uhrf1 transcription to this TF was dose-dependent, an effect lost when the DNA-binding motif for SRF was deleted or mutated (Figure 2D and Figure S2C). To validate the SRF-dependent up-regulation of Uhrf1 also in

vivo, we performed chromatin IP using an anti-SRF antibody on mouse heart extracts. We found that SRF was not bound to the promoter region of Uhrf1 in sham operated mouse heart, but was strongly enriched in extracts from TAC mice (Figure 2E). Thus, data strongly indicate that SRF regulates the expression of Uhrf1 in cardiac hypertrophy, and identify Uhrf1 as a stress-induced gene controlled by cardiac-specific mechanisms.

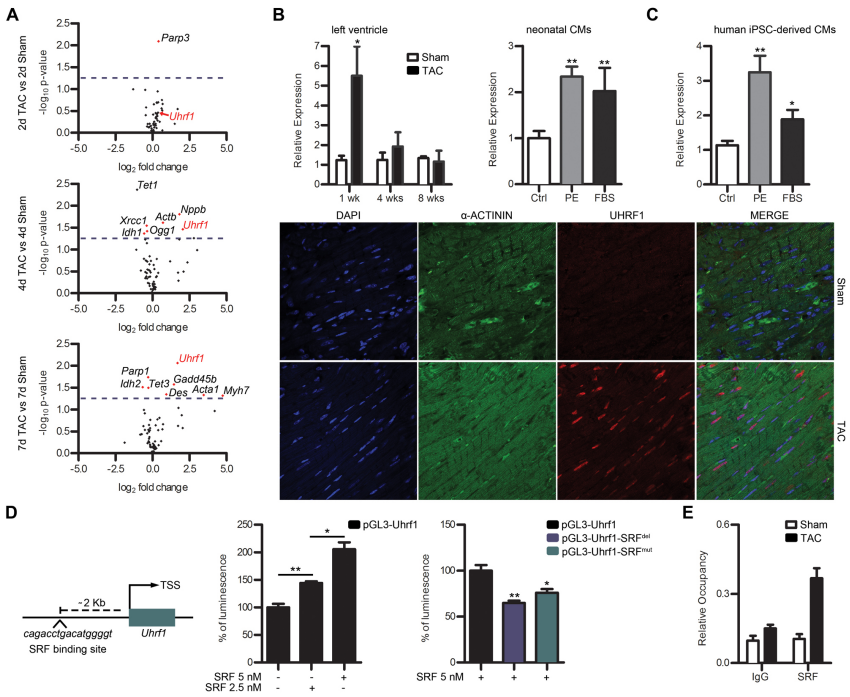

Figure 2. Uhrf1 is a stress-induced cardiac gene

(A) Gene expression profiling of hypertrophy markers (highlighted in green) and 48 epigenetic genes involved in DNA methylation/de-methylation was conducted on cardiomyocytes isolated from sham- and transverse aortic constriction (TAC)-operated mice. The plots give fold change at 2, 4 and 7 days after the surgical procedure. (B) qRT- PCR analyses of Uhrf1 expression in whole left ventricle tissue (left; mean±SD, n=5) and in in vitro neonatal cardiomyocytes (CMs) exposed to phenylephrine (PE) or fetal bovine serum (FBS). Ctrl, control. (right; mean±SD, n=3). Representative immunostaining for UHRF1 on whole left ventricle at one week of TAC (bottom); cardiomyocytes were identified with alpha-actinin, and nuclei were stained with diamidino-2-phenylindole (DAPI). *, $p<0.05$; **, $p<0.01$. (C) qRT- PCR analysis (mean±SD, n=3) of Uhrf1 in cardiomyocytes derived from human induced pluripotent stem cells (iPSC) after exposure to either PE or FBS. *, $p<0.05$; **, $p<0.01$. (D) Serum response factor (SRF) induces transcription of Uhrf1. A binding site for SRF is located upstream of the transcription start site (TSS) of the Uhrf1 gene (left). A luciferase construct with the promoter region of Uhrf1 (pGL3-Uhrf1) was cotransfected into NIH3T3 cells with different concentrations of a construct expressing SRF (middle). Cotransfection with deleted (del) or mutated (mut) versions of the pGL3-Uhrf1 construct significantly reduced luminescence activity (right). Graphs of mean±SD, n=3. *, $p<0.05$; **, $p<0.01$. (E) ChIP assay revealing the binding of SRF to the Uhrf1

promoter in 1- week TAC cardiomyocytes. Quantitative RT-PCR data plotted as mean±SD. The results shown are one of three indipendent immunoprecipitations prepared from a pool of 5 hearts. See also Figure S2, Table S1, and Supplemental Video.

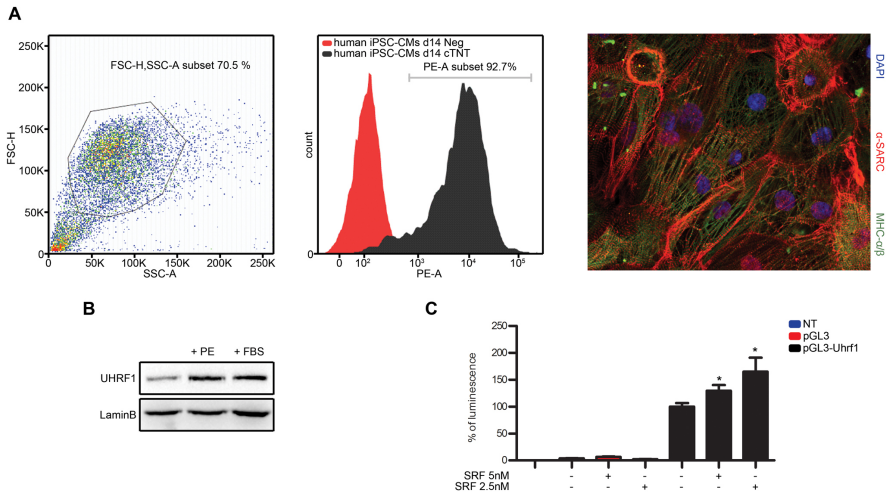

Figure S2, Related to Figure 2. Characterization of cardiomyocytes derived from human induced pluripotent stem cells (iPSC), and luciferase assay control.

(A) Human iPSC-derived cardiomyocytes (iPSC-CMs) assessed with FACS analysis for positivity to cardiac troponin T (cTNT) (left and middle) and with immunostaining for alpha- sarcomeric actin (α-SARC) and myosin heavy chain alpha/beta (MHCα/β) (right) after 14 days of diffentiation.

(B) Western blot showing that the level of UHRF1 was increased in human iPSC-derived cardiomyocytes by exposure to phenylephrine (+PE) or fetal bovine serum (+FBS).(C)

Cotransfection of serum response factor (SRF) with the pGL3-Uhrf1 construct but not with pGL3 alone induced dose-dependent activation of luciferase activity in NIH3T3 cells. NT, not treated. *, $p<0.05$.

Uhrf1 Silencing Attenuates Cardiac Remodeling and Dysfunction in Vivo

To assess any cause–effect relationship between UHRF1 and cardiac hypertrophy, we first pursued an antisense approach in vitro, transducing neonatal mouse cardiomyocytes with lentiviral particles expressing short hairpin (sh) RNAs targeting Uhrf1 (shUhrf1) or a scrambled shRNA (shscr) as a control, and then exposed cells to PE. After choosing the more efficient shRNA sequence (shUhrf1 #1), we analyzed the expression of the hypertrophy markers Acta1, Myh7, and Nppa and found that silencing of Uhrf1 inhibited the hypertrophy response in vitro (Figure S3A). We then silenced Uhrf1 in mice, utilizing a serotype 9 adeno-associated viral vector (AAV9), which efficiently transduces cardiomyocytes in vivo (Zacchigna et al., 2014). To this end, 10-day-old pups were administered AAV9 encoding either shscr (AAV9–scr) or shUhrf1 #1 (AAV9–shUhrf1) and subjected to TAC two months later (Figure 3A). The efficiency of Uhrf1 silencing was verified by qRT-PCR (Figure 3B). Cardiac enlargement due to pressure overload was significant in control mice, whereas it was prevented in AAV9–shUhrf1-treated mice; in fact, the latter had a reduced

left ventricular weight/body weight ratio (Figure 3C). Analysis of cardiac function by M-mode echocardiography revealed an increased left ventricular internal diameter (LVID) and decreased systolic contractility (percent fractional shortening and ejection fraction) in the two control groups TAC subjected to(mice treated with either PBS or AAV9−shscr), whereas exposure to AAV9−shUhrf1 improved cardiac function (Figure 3D and Table S2). Moreover, control mouse heart presented with interstitial fibrosis, enlarged cardiomyocytes, and increased expression of hypertrophy markers upon pressure overload, whereas that of TAC mice administered AAV9−shUhrf1 had reduced fibrosis, smaller cardiomyocytes, and significantly reduced re-expression of Acta1, Myh7, and Nppa (Figure 3E–3G and S3B). Notably, silencing of Uhrf1 did not produce any alteration of heart size or function in sham-operated mice, suggesting that UHRF1 is not required for cardiomyocyte homeostasis (Figure S3C), a finding supported by the absence of Uhrf1 expression in adult cardiomyocytes (Figure S3D) and in other post- mitotic cells, such as myotubes, and tissues such as bladder, lung, liver, and kidney (Hopfner et al., 2000). Thus, Uhrf1 expression is required for the induction of hypertrophy and cardiac dysfunction in vivo, but not for normal cardiac functioning.

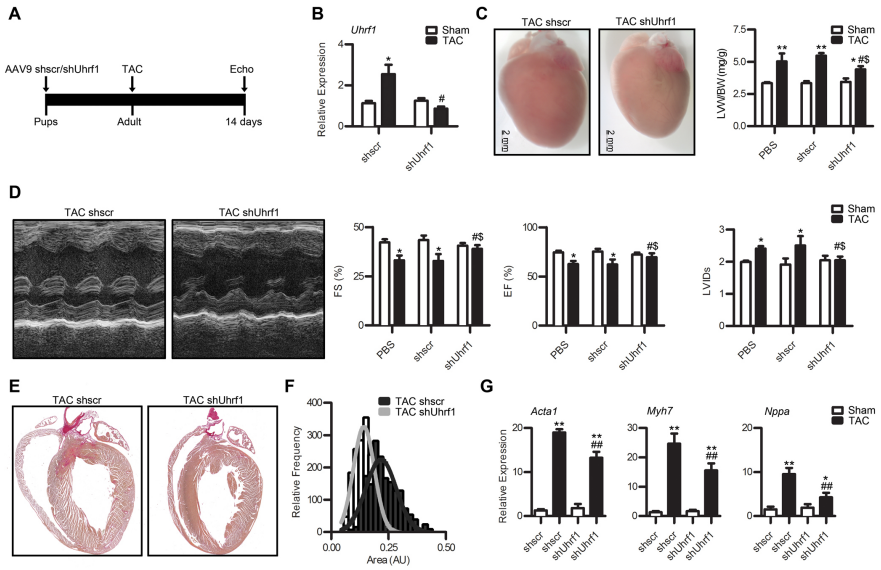

Figure 3. UHRF1 is required for the induction of cardiac hypertrophy by pressure overload

(A) Experimental strategy for silencing Uhrf1 in mice using cardiotropic adeno-associated virus subtype 9 (AAV9). TAC, transverse aortic constriction; ECHO, echographic analysis. (B) Quantitative RT-PCR analyses (mean±SD, n=5) of Uhrf1 expression after infection of mice with AAV9 encoding a short hairpin targeting Uhrf1 (shUhrf1); *, p <0.05, Sham vs TAC; #, p<0.05 AAV9–shUhrf1 TAC vs AAV9–shscr TAC. (C) Representative photographs of freshly excised whole hearts from 2-week TAC AAV9–shscr and AAV9–shUhrf1 mice (left); gravimetric analysis (corrected heart weights; mean±SD, n=5) of 2- week sham or TAC mice after administration of AAV9–shscr, AAV9–shUhrf1, or phosphate buffered saline (PBS) (right). (D) Echographic analyses of pressure-

overloaded heart with and without Uhrf1 silencing. From left to right: representative M-mode recordings at the level of the left ventricle; percent fractional shortening (FS) and ejection fraction (EF); LV internal diameter at systole (LVIDs) (mean±SD, n=8) in TAC mice with/without shRNA-mediated silencing of Uhrf1 or administered PBS. (E) Whole sections from hearts shown in C, stained with picro-sirius red for visualization of fibrosis. (F) Quantification of cardiomyocyte size two weeks after TAC in mice administered AAV9–shscr or AAV9–shUhrf1. (G) Quantitative RT-PCR analysis (mean±SD, n=5) of mRNA levels of the hypertrophy markers alpha-1 skeletal muscle actin (Acta1), beta- myosin heavy chain (Myh7) and atrial natriuretic peptide (Nppa). *, $p<0.05$, sham vs TAC; **, $p<0.01$, sham vs TAC; #, $p<0.05$, AAV9–sh-Uhrf1 TAC vs AAV9–shscr TAC; ##, $p <0.01$, AAV9–shUhrf1 TAC vs AAV9–shscr TAC. See also Figure S3 and Table S2.

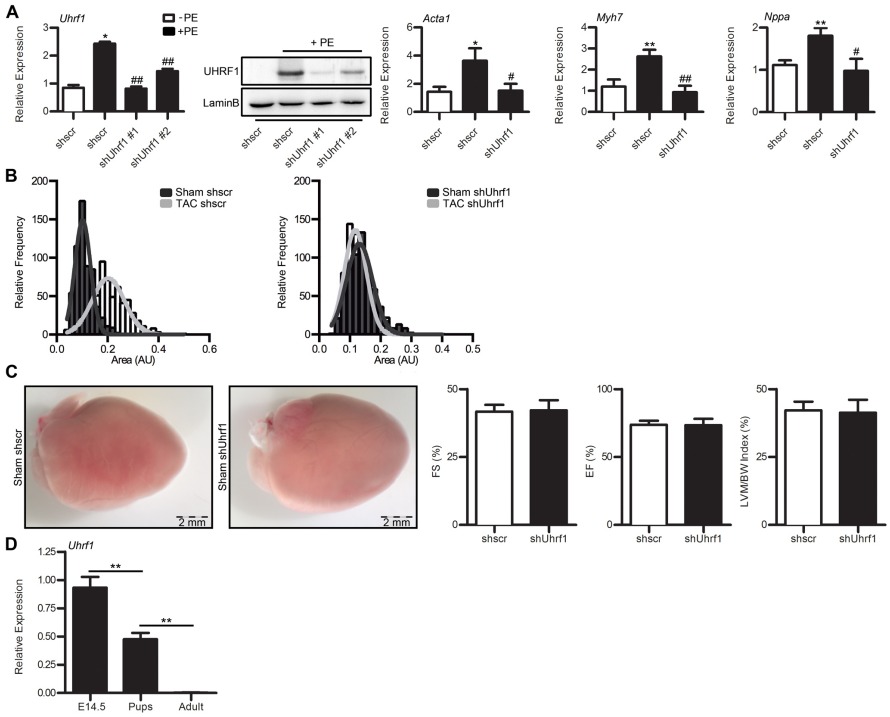

Figure S3, Related to Figure 3. Control experiments for Uhrf1 silencing.

(A) From left to right: neonatal cardiomyoctes were transduced with two independent short hairpin (sh) RNAs targeting Uhrf1 (shUhrf1 #1 and shUhrf2 #2) or a scrambled shRNA (shscr). Western blotting for UHRF1 confirmed down-regulation of the protein, especially with shUhrf1 #1. Analysis of the hypertrophy markers alpha-1 skeletal muscle actin (Acta1), beta-myosin heavy chain (Myh7), and atrial natriuretic peptide (Nppa) in neonatal cardiomyocytes treated with phenylephrine (PE) for 24 h (n=3). *, p<0.05 and **, p<0.01, – PE vs +PE; #, p <0.05 and ##, p <0.01, shUrf1 vs shscr.(B) Quantification of cardiomyocyte size in sham and TAC mice after two weeks, with/without

silencing of Uhrf1.(C) Representative photographs of freshly excised whole sham-operated hearts and echography analysis of per cent fractional shortening, ejection fraction, and left ventricle/body weight (n=8).(D) Graph of qRT-PCR data (mean±SD n=3) on Uhrf1 expression in embryonic (E14.5), neonatal (pups), and adult cardiomyocytes. **, $p<0.01$.

UHRF1 is Required for the Re-programming of Metabolism Gene Expression in Hypertrophic Cardiomyocytes

To gain insight on the mechanisms by which UHRF1 is involved in cardiac hypertrophy, we first identified UHRF1-regulated genes. To this end, we assessed gene expression with an Illumina MouseWG-6 v2.0 Expression BeadChip in one-week TAC mice after interference with Uhrf1. Silencing of Uhrf1 altered the expression of 1033 gene-specific probes in pressure-overloaded mice (Figure 4A and Supplemental Dataset 2). The expression profile was closer to that of sham than of TAC mice, as evidenced by clustering enrichment and principle component analyses (Figure S4A). Gene-set enrichment analysis indicated that the 479 significantly down-regulated probes in AAV9−shUhrf1 TAC compared with AAV9−shscr TAC mice were involved in cell hemostasis and collagen formation, a finding consistent with a blunted hypertrophic response; on the other hand, the 554 transcripts that were significantly more expressed in AAV9−shUhrf1 TAC cardiomyocytes were enriched for terms mainly concerning

metabolic pathways, such as the TCA cycle, OXPHOS/ETC, and ATP synthesis (Figure 4B). Quantitative RT-PCR confirmed the de-repression of genes involved in multiple metabolic pathways in pressure-overloaded AAV9–shUhrf1 mice, including the TCA cycle genes Idh3a, Idh3g, Suclg1, Sdhb, Sdhd, and Fh1; the OXPHOS/ETC genes Cox8a, Ndufb2, Ndufs4, Ndufs5, Atp5g3, and Atp5ah; and the lipid metabolism genes Acadl, Etfa, and Acss1 (Table 1). Of note, the promoter region of the up-regulated 554 genes had a significantly increased coverage of 5-mC in hypertrophic cardiomyocytes (Figure 4C), suggestive of a direct link between their repression and UHRF1-mediated methylation.

In accordance with our genome-wide data (Figure 1E), motif discovery analysis indicated enrichment for NRF1 binding sites on promoters of the up- regulated genes (p-value, 10-9). Moreover, most of the genes targeted by this TF were significantly more expressed in TAC mice upon silencing of Uhrf1 (Figure 4D).

To investigate the mechanism underlying the UHRF1-mediated gene expression program, we assessed the methylation status at the proximal promoter regions of Idh3a, Sdhd, Atp5g3, and Ndufs5. MIRA identified significant enrichment of 5-mC in control mice subjected to pressure overload, whereas Uhrf1 silencing impaired DNA methylation enrichment (Figure 4E).

Since it is known that UHRF1 links DNA methylation with deposition of the H3K9me2 and H3K9me3 repressive marks on

chromatin – particularly in cancer cell lines (Babbio et al., 2012a; Kim et al., 2009) – we analyzed the presence of these histone modifications at the promoter region of these genes. ChIP assay revealed that there was an enrichment of H3K9me2, but not of H3K9me3, for all the analyzed genes after the induction of hypertrophy (Figure S4B). Consistent with this, the increased occupancy of H3K9me2 was impaired in AAV9-shUhrf1 TAC mice (Figure 4E).

We then assessed whether UHRF1 bound directly to the promoters of these genes and if it mediated the binding of two of its direct interacting partners, namely DNMT3b and G9a, which are involved in de novo DNA methylation and di-methylation at lysine 9 of histone H3, respectively (Kim et al., 2009; Meilinger et al., 2009). ChIP assay revealed that UHRF1, DNMT3b, and G9a were bound to the promoters of these epigenetically repressed genes in hypertrophic cardiomyocytes (Figure S4C).

Overall, these findings indicate that UHRF1 plays a central role in metabolism-gene expression re-programming in response to hemodynamic stress, via chromatin remodeling mediated by the deposition not only of 5-mC on DNA but also of H3K9me2 on histones.

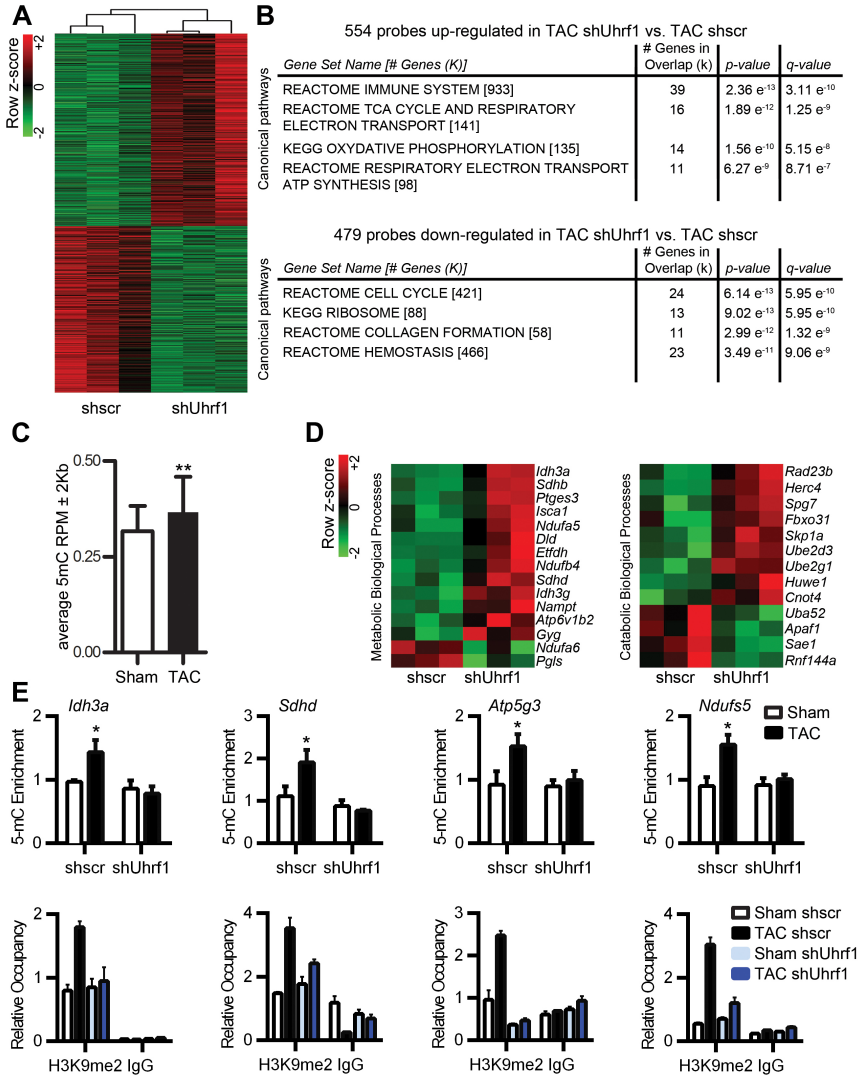

Figure 4. UHRF1 governs two repressive epigenetic marks (DNA methylation and H3K9me2), which are increased at the promoter regions of metabolism-related genes.

(A) Pups were administered either AAV9–shUhrf1 to silence Uhrf1 or AAV9–shscr as a control and when adult, were

subjected (TAC) or not (sham) to pressure overload for one week. Expression profile heatmap of significantly modulated cardiac genes (adjusted p-value ≤0.1). (B) Gene Set enrichment analysis of the significantly modulated genes. (C) Average 5-mC coverage on the promoter region of the 554 up-regulated genes. *, p<0.05. (D) Expression profile heatmap of genes with a promoter harboring an NRF1 binding site. (E) Quantitative RT-PCR (mean±SD, n=3) analysis of 5-mC enrichment at the promoter regions of metabolism-related genes. *, p <0.05 (upper panel). ChIP assay revealed enrichment of H3K9me2 at the promoter region of metabolism genes: quantitative RT-PCR data plotted as mean±SD. The results shown are one of three indipendent immunoprecipitations prepared from a pool of 5 hearts. See also Figure S4 and Supplemental Dataset 2.

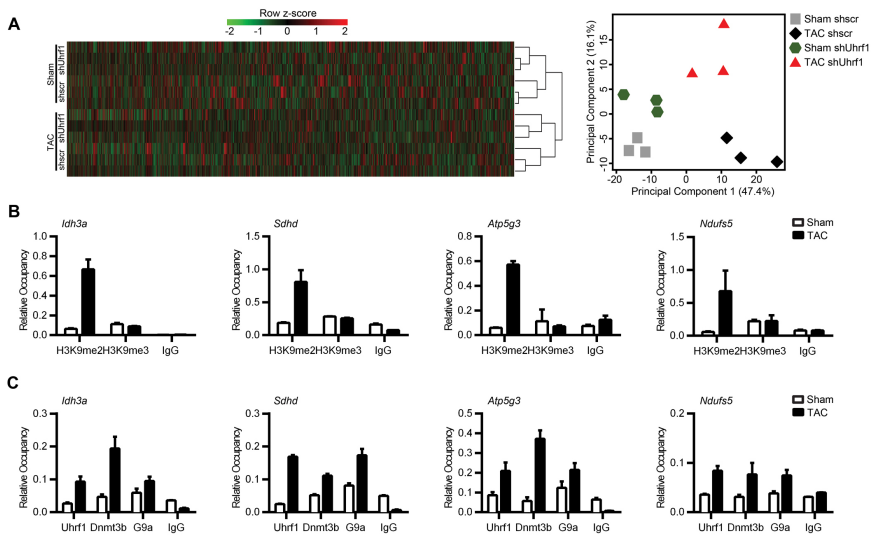

Figure S4, Related to Figure 4. Gene profile and histone modifications of pressure- overloaded cardiomyocytes upon silencing of Uhrf1.

(A) Heatmap of the expression profile of all genes in one-week sham and TAC mice with/without Uhrf1 silencing (left). PCA plot of all the samples used in the gene expression array (right).

(B) Analysis of H3K9me2 and H3K9me3 occupancy for each gene studied. Graph of mean±SD of qRT-PCRs, n=5.(C) qRT-PCR analysis of chromatin immunoprecipitation (ChIP) for UHRF1, DNMT3a, and G9a. Graphs of mean±SD, n=5.

Silencing of Uhrf1 Restores Binding of NRF1 and Mitochondrial Function in Pressure-Overloaded

HeartERRa and its co-activator PGC1a play a key role in the adaptive bioenergetics response to a hemodynamic stress such as pressure overload (Arany et al., 2006; Huss et al., 2007). Since we found that genes regulated by promoter DNA methylation were enriched in binding sites for NRF1 – a TF co-activated by PGC1a that regulates genes involved in mitochondrial OXPHOS and function (Finck and Kelly, 2007) – we investigated whether NRF1 activity was altered upon pressure overload and if this was DNA methylation dependent. We first assessed Nrf1 mRNA levels in cardiomyocytes isolated from mice after 7 days of TAC. We found that Nrf1 expression was not altered by pressure overload, but that Esrra – the gene encoding ERRa – was significantly down-regulated (Figure S5),

as previously described (Huss et al., 2007). Moreover, silencing of Uhrf1 did not affect Nrf1 expression (Figure 5A), and Western blotting confirmed that NRF1 was not modulated in response to pressure overload in either control mice or in those administered AAV9−shUhrf1 (Figure 5B).

Next, to assess whether NRF1 occupancy on target genes was modulated by pressure overload, we performed a ChIP assay. Indeed, binding of NRF1 to binding sites at the promoters of Idh3a, Sdhd, Atp5g3, and Ndufs5 was significantly reduced after TAC, an effect reversed by Uhrf1 silencing (Figure 5C). These results indicate that pressure overload leads to reduced occupancy of NRF1 at targeted promoters, a finding consistent with down-regulated expression of these genes; moreover, this phenomenon was due to methylation of the binding sites rather than to a decreased level of NRF1.

A major cause of cardiac dysfunction is inefficient energy production in cardiomyocytes (Neubauer, 2007) due largely to deficient FAO and OXPHOS in mitochondria. We thus investigated the functional outcome of restored metabolism gene expression in myocardial mitochondria of AAV9−shUhrf1 TAC mice. We found that the absolute activity of complex I (NADH:ubiquinone oxidoreductase) – the first enzyme of the mitochondrial respiratory chain – was reduced in TAC mouse heart, but was partially restored by Uhrf1 silencing (Figure 5D). Moreover, the significant ATP depletion observed in

mitochondria isolated from AAV9–shscr TAC mouse heart was also completely restored upon silencing of Uhrf1 (Figure 5E). Altogether, these results suggest a mechanism whereby cardiac stress increases the level of UHRF1, which in turn, through de novo DNA methylation and deposition of H3K9me2, prevents the binding of NRF1 to the promoters of several mitochondrial metabolism genes. This event is responsible for an alteration in cardiomyocyte bioenergetics that contributes to cardiac dysfunction and failure.

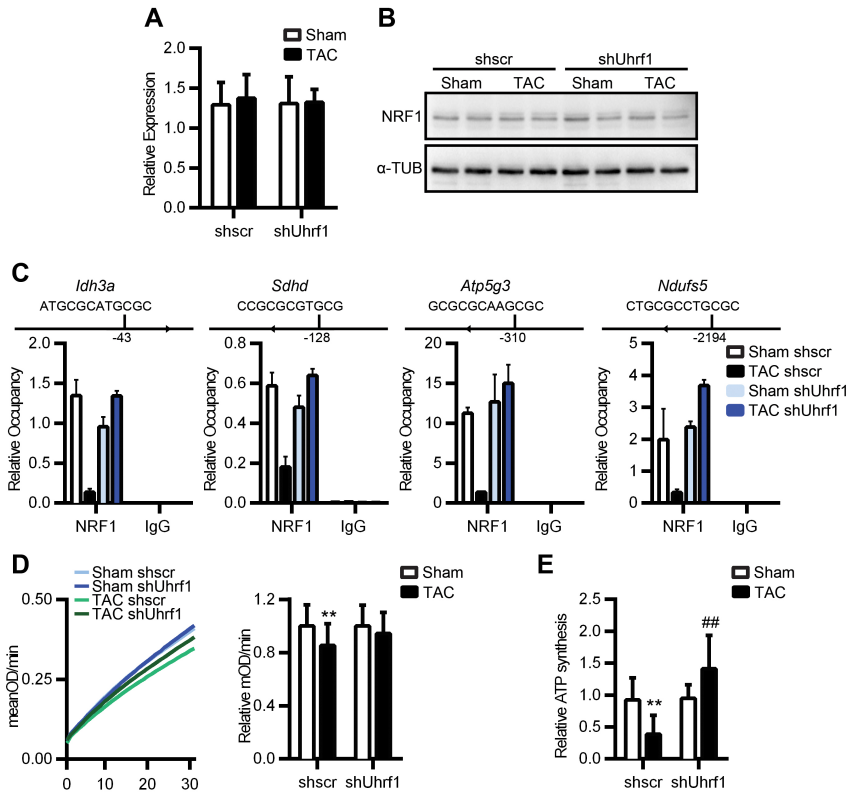

Figure 5. DNA Methylation Regulates the Binding of NRF1 to Promoters

(A) Quantitative RT-PCR analyses (mean±SD, n=3–5) of Nfr1 expression in cardiomyocytes isolated from one-week TAC mice with and without silencing of Uhrf1. The expression of Nfr1 was not altered by pressure overload. (B) Reppresentative Western blot for NRF1 in left ventricles of one-week sham- and TAC-operated mice with/without shRNA-mediated silencing of Uhrf1. (C) ChIP assay revealed decreased binding of NRF1 to its putative binding sites in one- week TAC cardiomyocytes, and that Uhrf1 silencing restored binding. Quantitative RT-PCR results (mean±SD) are of one from three indipendent immunoprecipitations prepared from a pool of 5 hearts. (D) Absolute complex I activity was decreased in control mice subjected to two weeks of pressure overload; it was partially restored with silencing of Uhrf1. Data plotted as mean±SD, n=6–7. (E) ATP synthesis in cardiac mitochondria isolated from mice subjected to two weeks of pressure overload with/without Uhrf1 silencing. Mean±SD, n=6–7. **, $p < 0.01$, sham vs TAC; ##, $p < 0.01$, AAV9−shUhrf1 TAC vs AAV9−shscr TAC. See also Figure S5.

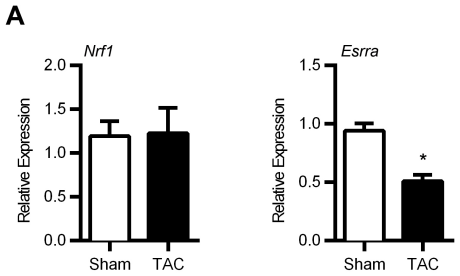

Figure S5, Related to Figure 5. Expression of nuclear respiratory factor-1 and estrogen-related receptors in cardiomyocytes.

qRT-PCR analysis of the expression of Nrf1 and Esrra in cardiomyocytes of mice subjected to TAC for 7 days. Graphs of mean±SD of qRT-PCR data (sham n=3 and TAC n=6). *, $p<0.05$ sham vs TAC.

Treatment with the DNA Methylation Inhibitor 5-Azacytidine Improves Cardiac Function In Vivo

Drugs targeting DNA methylation may have a therapeutic potential. To test this idea, we administered the DNA methyltransferase inhibitor 5-azacytidine (5-AZA) (Jones and Taylor, 1980) to mice 24 hours before inducing pressure overload and daily for two weeks thereafter (Figure 6A). Mice treated with 5-AZA had smaller hearts and a significantly reduced left ventricular weight/body weight ratio (Figure 6B), indicative of a blunted hypertrophy response. Moreover, treatment with the inhibitor improved cardiac function, as assessed by M-mode echocardiography (Figure 6C and Table S3), and significantly blunted the re- activation of the fetal genes Acta1, Myh7, and Nppa (Figure 6D).

Quantitative RT-PCR confirmed the de-repression of a set of metabolism genes, including Idh3a, Sdhd, Atp5g3, Ndufs5, Ndufb2, Ndufs4, Cox8a, Acadl, and Acss1, after pressure overload in the presence of 5-AZA (Figure 6E). These findings are in agreement with those observed in Uhrf1-silenced pressure- overloaded mice, suggesting that the effect is dependent upon impaired methylation and indicating the potential targeting of these epigenetic mechanisms for the therapy of heart failure.

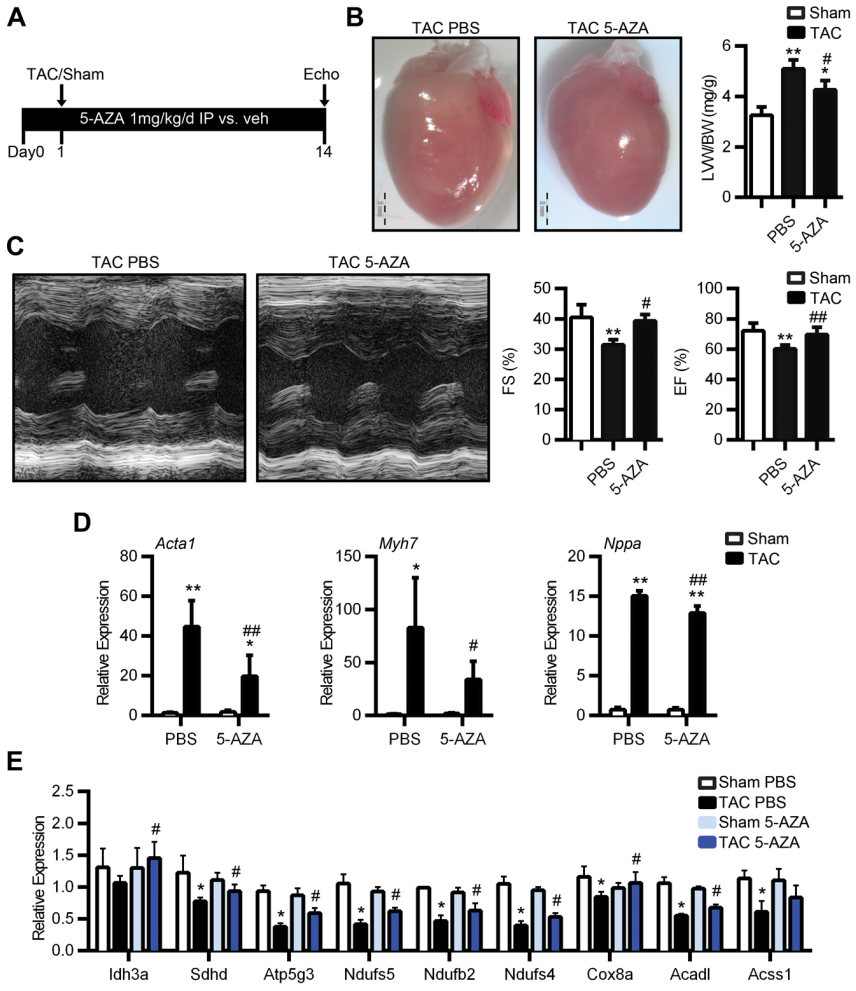

Figure 6. Treatment with 5-Azacytidine Improves Cardiac Function in the Presence of Pressure Overload

(A) Experimental strategy. TAC, transverse aortic constriction; Echo, echographic analysis; AZA, 5-Azacytidine. (B) Representative photographs of freshly excised whole

hearts from two-week TAC mice administered PBS or 5-AZA. Bar = 2 mm (left); gravimetric analysis (mean±SD, n=5) of corrected heart weights of mice after 2 weeks of sham or TAC surgery, treated with PBS or 5-AZA (right). (D) Echographic analysis of pressure-overloaded heart with and without Uhrf1 silencing. From left to right: representative M-mode recordings at the level of the left ventricle; per cent fractional shortening (FS) and ejection fraction (EF) (graphs of mean±SD, n=8) in TAC mice with/without 5-AZA administration. (D) Quantitative RT-PCR analysis of mRNA levels of the hypertrophy markers alpha-1 skeletal muscle actin (Acta1), beta-myosin heavy chain (Myh7), and atrial natriuretic peptide (Nppa) (graphs of mean±SD, n=3–6) (E) Quantitative RT-PCR analysis of mRNA levels of metabolism genes (graphs of mean±SD, n=3–6). *, $p < 0.05$, Sham vs TAC; **,$p < 0.01$, Sham vs TAC; #, $p < 0.05$, 5-AZA TAC vs PBS TAC; ##, $p < 0.015$, AZA TAC vs PBS TAC. See also Table S3.

DISCUSSION

Metabolism is regulated by a variety of factors, but the molecular mechanisms that govern metabolic reprogramming of the hypertrophic heart have not been fully delineated yet. Here we demonstrate that promoter DNA methylation is altered in the pathologically hypertrophied heart and contributes to the metabolic reprogramming of cardiomyocytes. We propose a mechanism whereby the epigenetic cofactor UHRF1 becomes re-expressed in cardiomyocytes upon cardiac stress and mediates crosstalk between two major repressive marks – DNA methylation and H3K9 di-methylation – leading to impaired binding of the transcription factor NRF1 to targeted metabolic genes and, hence, to their down regulation (Figure S6).

Although DNA methylation is considered to be a stable epigenetic mark (Reik et al., 2001), several reports have provided evidence that environmental factors – for instance acute exercise (Barres et al., 2012) and diet (Barres et al., 2009; Ghoshal et al., 2006) – can modify DNA methylation in skeletal muscle and liver, suggesting that this process is more dynamic than previously thought. The findings of the present study are in agreement with this notion, demonstrating that hemodynamic stress is sufficient to trigger genome-wide alterations in the DNA methylation pattern of cardiomyocytes. This profound modulation is due, at least partly, to significant re-expression of the DNA methylation co-factor UHRF1, which contributes to a de novo methylation process. This event is

controlled by SRF, a transcription factor the role of which is well established in cardiac hypertrophy (Heineke and Molkentin, 2006). In addition, cardiac stress induces
also the hypo-methylation of a large set of genes. Recent studies on mammalian cells have uncovered an active de-methylation mechanism involving the hydroxylation of methylated DNA to 5-hydroxymethylcytosine by the ten-eleven translocation (TET) family of hydroxylases (Tahiliani et al., 2009). Further investigation is needed to elucidate the role, and identify the key players, of active de-methylation in cardiac hypertrophy.

UHRF1 has been extensively studied in proliferating cells, in which it is required for cell cycle progression and for the maintenance of DNA methylation (Bonapace et al., 2002; Hashimoto et al., 2008). UHRF1 has also been implicated in pathological processes related to cell proliferation: in cancer cells, over-expression of UHRF1 promotes cancer progression (Babbio et al., 2012; Mudbhary et al., 2014) and, more recently, UHRF1 has been linked to the regulation of local proliferation of regulatory T cells in response to bacterial colonization through the regulation of the growth inhibitory p21 gene (Obata et al., 2014). Importantly, UHRF1 is not expressed in non-proliferating adult tissues. Here, we provide the first evidence of a role of UHRF1 in de novo methylation in non-proliferating adult cells in a pathological state. Specifically, we identify UHRF1 as a broad transcriptional regulator of genes involved in cardiac

metabolism and energetics, demonstrating that in hemodynamically stressed cardiomyocytes it targets genes involved in the TCA cycle, OXPHOS/ETC, and ATP synthesis pathways, contributing to dysfunction in the bioenergetics of these cells in a pathological condition. Of note, AAV-mediated gene silencing – a method that is being increasingly used for its reliability in transducing cardiomyocytes in vivo and its efficiency for long-term up- and down-regulation of gene expression (Gorski et al., 2015) – hindered the development of cardiac hypertrophy in TAC mice.

UHRF1 epigenetically silenced its target genes by mediating crosstalk between two repressive marks – DNA and histone H3K9 methylation – to form a reinforcing silencing loop, as previously suggested (Babbio et al., 2012; Cheng and Blumenthal, 2010). The notion that two repressive epigenetic marks cooperate in controlling the cardiac expression pattern of metabolism genes adds knowledge to the understanding of the regulatory networks. Indeed, distinct gene targets involved in cardiac energy metabolism have been demonstrated to be controlled at the gene expression level by transcriptional factors and co- activators, in particular PGC1a, ERRs, and PPARs (Kelly and Scarpulla, 2004). To date, most studies have indicated the importance of the level of the transcription regulator, rather than its ability to bind to the targeted DNA regions, as a major element of gene expression regulation of heart energetics (Arany et al., 2006; Huss et al., 2007).

Transcription can be inhibited by DNA methylation by preventing the binding of TFs to DNA, thus regulating the activity of constitutively active TFs (Bird and Wolffe, 1999). It is tempting to speculate that the epigenome may establish a previously unappreciated layer of regulation of cardiac metabolism. A hint of the proposed mechanism is given by the evidence we provide here that DNA methylation regulates the occupancy of NRF1 to its target genes to modulate their transcriptional activity.

NRF1 is co-activated by PGC1a (Wu et al., 1999) and is responsible for transcriptional activation of many mitochondrial proteins, including the majority of genes required for respiratory complexes, as well as those involved in mitochondrial DNA transcription and mitochondrial biogenesis (Kelly and Scarpulla, 2004). Evidence supports the concept that mitochondria are affected at an early phase during the development of cardiac hypertrophy and failure (Doenst et al., 2013), but the regulation of mitochondrial function in the hypertrophic cardiomyocyte is not completely understood. We found that many genes altered in the pressure-overloaded heart are targeted by NRF1. Thus, this TF has a role in the dysregulation of cardiac metabolism observed upon hypertrophy, opening the field to further investigations to elucidate its function in the heart.

Finally, we show that pharmacological inhibition of the epigenetic changes driven by UHRF1 improved the cardiac function of pressure-overloaded mice, a result demonstrating

the potential of epigenetic therapy in heart failure, as already suggested for histone deacetylases (Haberland et al., 2009). The use of currently available inhibitors is problematic for heart failure therapy, however, because of toxicity due to ubiquitous tissue-expression of their targets (Dawson and Kouzarides, 2012; Karberg, 2009). Nonetheless, the development of specific modulators of DNA methylation in cardiac cells might represent a new therapeutic avenue for myocardial disease.

Taken together, our study points to DNA methylation as a new layer of regulation of cardiac energy metabolism in the hemodynamically stressed heart, and identifies UHRF1 as a master regulator of this process. Our observations further the understanding of the regulatory networks that control cardiac metabolic activity, and, importantly, point to the potential of targeting epigenetic mechanisms for the therapy of heart failure.

EXPERIMENTAL PROCEDURES

In vitro experimentation

Isolation of adult cardiomyocytes and primary neonatal cardiomyocyte culture. The procedures used to obtain adult cardiomyocytes from sham- and TAC-operated mice, and for the culture of cardiomyocytes from neonatal mice, have been described elsewhere (Care et al., 2007). To induce hypertrophy, cultured cardiomyocytes were first serum-starved and then exposed either to 100 µM phenylephrine (PE) or to 10% fetal bovine serum (FBS) for 24 hours.

In vitro silencing of Uhrf1. Neonatal cardiomyocytes were isolated and cultured as previously described (Care et al., 2007). Cells were then transduced with lentiviral vectors encoding either of two distinct shRNAs targeting Uhrf1 (shUhrf1#1 or shUhrf1#2) or with a scrambled shRNA (sh-scr) as a control (Open Biosystems), and then treated with 100 µM PE for 24 hours. Results indicated that shUhrf1#1 was the more effective at silencing Uhrf1, and was thus utilized for subsequent in vivo experimentation.

Luciferase assay. The promoter region of Uhrf1 was cloned into the pGL3 enhancer vector (Promega) with the InFusion HD Cloning kit (Clontech). The 676 bp amplicon obtained from custom-designed primers and a High Fidelity Taq was inserted

upstream of the luc+ gene of the pGL3 enhancer vector, according to the manufacturer's protocol. After positive cloning asserted with enzymatic digestion and sequencing, 2.5x105 NIH3T3 cells were co-transfected with the pGL3 enhancer-Uhrf1, pCGN-SRF and pRL Renilla Luciferase Control plasmids. A carrier DNA, pcDNA plasmid, was used to balance transfected DNA quantity, whereas pCGN-renilla was used as the internal normalizer. Thirty-six hours post transfection, cells were lysed and luciferase activity measured, as instructed by the manufacturer (Dual-Luciferase Reporter Assay System, Promega). To confirm specific binding of SRF to the Uhrf1 sequence, site-specific mutagenesis at the predicted sites of binding was performed using the QuikChange Site-Directed Mutagenesis Kit as described by the manufacturer (Stratagene). Specifically, both a deletion and a simultaneous mutation of seven nucleotides in the sequence were used. Mutated constructs were then sequenced and tested in luciferase assays, and loss of chemiluminescent signal was assessed compared with the non-mutated condition.

Generation of human induced pluripotent stem cells (iPSC) and differentiation into cardiomyocytes. iPSC lines were generated and maintained as previously reported(Di Pasquale et al., 2013). For induction of cardiac differentiation, iPSC were first adapted to EDTA passaging and then grown on vitronectin-coated plates in E8 medium (Life Technologies). Differentiation

was induced as recently described, using a chemically defined, xeno-free protocol that relies on modulation of the Wnt pathway (Lian et al., 2013). Hypertrophy was induced with either FBS or PE for 24 hours.

Western blotting. Immunoblotting was carried out using standard protocols. The following primary antibodies were used overnight at 4°C: anti-human UHRF1 (Active Motif, 6134), anti-mouseUHRF1(Bonapace et al., 2002), anti lamin B (Santa Cruz, sc6216), anti-NRF1 (Abcam, ab34682).

Measurement of cell size. Surface areas, perimeter, length and width of isolated cardiomyocytes were measured using NIH Image J software (http://rsb.info.nih.gov/ij/). An average of 350–400 cardiomyocytes were chosen at random for measurements.

Fluorescence-activated cell sorting. FACS analysis was carried out using standard protocols. The following antibodies were used: 1:100 anti-troponin I (Millipore, MAB1691) and F(ab')2 goat anti-mouse IgG (H+L) conjugated with phycoerythrin as the secondary. Staining was performed on fixed cells after permeabilization with 0.5% saponin, and processed through a FACS Canto flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA). Data were analyzed with FlowJo software.

In vivo experimentation

Transverse aortic constriction. According to the New Directive 2010/63/EU, special attention was paid to animal welfare and to minimize animal suffering. Anesthetized mice were closely monitored during the procedure to assure that they were maintained in the proper anesthetic plane. In particular, the anesthetic efficacy was assessed by pinching the tail of the animal while monitoring body temperature and respiratory rate. Any reaction of the animal, hypothermia or increase in respiratory rate indicated that anesthesia was too light and that additional anesthetic should be given. All experiments were conducted in conformity with European Communities Council Directive and approved by our Institution. Adult cardiomyocytes were isolated and cultured as previously described (Care et al., 2007).

TAC surgery was adapted from Rockman HA et al (Rockman et al., 1997). Briefly, C57Bl6/J mice were anesthetized by intraperitoneal injection of a mixture ketamine (100mg/kg) and xylazine (10 mg/kg). The mice were then placed in a supine position, and the chest was shaved using a chemical hair remover. The aortic arch was exposed through the 1st intercostal space. A 8-0 Prolene suture was passed between the truncus anonymous and the left carotid artery, a blunted 27-gauge needle was placed against the aorta and the knot tightened along with the needle and secured with a second knot. The needle was removed to create a lumen with a fixed

stenotic diameter. The chest cavity was then closed with a 6-0 silk suture. A separate group of mice underwent the same surgical procedure but without any tightening of the knot (sham group). The pressure load caused by the knot was verified through the measurement of the pressure gradient across the aortic constriction with echocardiography. The mean pressure gradient in TAC mice made to be approximately 70 mmHg in order to induce a rapid deterioration of heart function.

AAV9 production. Recombinant adeno-associated viral vectors used in this study were prepared by the AAV Vector Unit at the International Centre for Genetic Engineering and Biotechnology Trieste (http://www.icgeb.org/avu-core-facility.html), as described previously(Arsic et al., 2004) with a few modifications. Briefly, infectious recombinant AAV vector particles were generated in HEK293T cells culture in roller bottles by a cross-packaging approach whereby the vector genome was packaged into AAV capsid serotype-9(Inagaki et al., 2006). Viral stocks were obtained by PEG precipitation and CsCl2 gradient centrifugation (Ayuso et al., 2010). The physical titer of recombinant AAVs was determined by quantifying vector genomes (vg) packaged into viral particles, by real-time PCR against a standard curve of a plasmid containing the vector genome(Zentilin et al., 2001); values obtained were in the range of 1x1012 to 1x1013 vg/ml.

AAV9 transduction. For cardiotropic silencing of Uhrf1, 10-day-old male mice received sh-scr-AAV9 or shUhrf1#1-AAV9 (1011 genome copies per mouse) by intraperitoneal injection. TAC was performed two months later. One or two weeks after the operation, mice were evaluated by echocardiography for heart function, and cardiomyocytes harvested for further analysis. Each group included 8–10 mice.

Drug treatments. 8-week-old male mice received intraperitoneal injections of 5-azacytidine (5-AZA; Sigma, A2385) at a dose of 1 mg/kg/day, or vehicle (PBS) for two weeks (8–10 mice/group). Sham or TAC procedures were performed 24 hours after the first injection. Mice were evaluated for heart function with echocardiography one and two weeks later. 5-AZA has been shown to have no significant effect on systolic blood pressure in mice (Kim et al., 2014).

Echocardiography. Echocardiography was performed on a Vevo 2100 (VisualSonics) system equipped with a with an MS550S probe "high frame" scanhead. Anesthesia was induced with 3% isoflurane, and maintained with a flow 1.0% isoflurane during constant monitoring of temperature, respiration rate and ECG while maintaining heart rate at 450–550 beats per minute. Two-dimensional cine loops with frame rates of 200 frames per second in long- and short-axis views of the left ventricles (LV) were recorded. End-diastolic (d) and end-

systolic (s) intraventricular septum (IVSTd, IVSTs), posterior wall thickness (PWTd, PWTs) and LV internal diameters (LVEDd, LVEDs) were measured. Percentage fractional shortening (%FS) and relative ejection fraction (EF) were calculated using standard formulas: %FS = [(LVEDd – LVEDs)/LVEDd] x 100; %EF = [(LVEDs – LVEDs)/LV LVEDd] x 100.

Histology and immunostaining. Histology and immunostaining were performed on 5µm-thick paraffin sections. Human iPSCs were fixed with 4% paraformaldehyde for 30 min. at 4°C, and then permeabilized with 1X PBS supplemented with 2% goat serum and 0.1% triton X-100. The following antibodies were used for immunostaining: anti-mouse-UHRF1(Bonapace et al., 2002), anti-sarcomeric alpha actinin (Abcam, ab 9465 and ab90776), anti-myosin heavy chain (abcam, ab15). Cardiac fibrosis was visualized with picro-sirius red staining.

ATP Synthesis and Complex I Activity

Cardiac mitochondria were isolated from freshly excised hearts using a mitochondrial isolation kit (SIGMA MITOISO1). ATP production was measured by luminometric assay using an ATP bioluminescent assay kit (Sigma FL-AA). Complex I activity was measured using a MitoSciences colorimetric assay kit (ab109721). Luminescence and absorbance were measured with Synergy H4 instrument (Biotek).

Molecular biology and statistical analysis

RNA isolation, qPCR and gene expression analysis. Total RNA was isolated using RNeasy Mini Kit (Qiagen), and treated with DNase according to the manufacture's protocol. cDNA was generated using the Super Script VILO cDNA Syntesis Kit (Invitrogen) and amplified by real-time quantitative PCR with SYBR Green PCR master mix (Applied Biosystem). Expression levels of target genes were analyzed using comparative CT methods and normalized to Gapdh.

Epigenetic enzyme TaqMan assay cards. RNA was extracted from cardiomyocytes isolated from sham- or TAC-operated mice. 1.2µg of cDNA of each sample was used to analyze the expression of 48 genes encoding proteins involved in DNA methylation/de-methylation. Custom microfluidic gene expression cards were drawn using TaqMan probes (Applied Biosystems). Each sample was analyzed in duplicate using Viaa7 (Applied Biosystems). Data were analyzed with DataAssist software.

MeDIP-seq Genome-wide localization of DNA methylation was determined via immunoprecipitation with an anti-5-mC antibody (Active Motif, AM39649). After immunoprecipitation, the DNA was amplified with barcoded Illumina primers to generate the final sequencing library. To generate the Input libraries, sonicated DNA with ligated adaptors was directly amplified

without the immunoprecipitation step. High-throughput sequencing with carried out as by the Active Motif Epigenetic services (http://www.activemotif.com/catalog/ 821/medip-sequencing-service) using the Illumina HiSeq platform. MeDIP-seq and Input reads were mapped to the mouse genome (version mm10) using bwa(Li and Durbin, 2010) (Li H 2010 Bioinformatics). Uniquely mapped reads and reads with Map quality >10 were used for peak detection. Enriched regions relative to input DNA were detected using “findPeaks” script in HOMER (Heinz et al., 2010) with the “histone” mode and default parameter values. Motif discovery was performed using “findMotifsGenome” script in HOMER with default parameter values.

Methylated-CpG island recovery assay. Genomic DNA, purified using the QIAamp DNA mini kit (Qiagen), was sonicated to obtain 200–500-bp fragments. The efficiency of sonication was assessed with agarose gel electrophoresis. Methylated CpG DNA fragments were enriched from 1 µg of sonicated DNA using the MethylCollector Ultra kit (Active Motif), according to the manufacturer’s protocol. Purified DNA was evaluated by qPCR with SYBR green PCR master mix (Applied Biosystem). Values obtained were normalized to the input.

Chromatin immunoprecipitation. Briefly, 5x106 cells were used for each ChIP. Cells were cross-linked for 10 minutes at

room temperature using 1% formaldehyde. Cross-linking was quenched by adding glycine to a final concentration of 0.125M. The cells were then collected, resuspended in lysis buffer (5mM PIPES pH 8, 85mM KCl, 0.5% NP40 and protease inhibitors), and incubated on ice for 15 min. before proceeding with sonication to generate 200–400bp fragments. The efficiency of sonication was assessed with agarose gel electrophoresis. Chromatin samples were pre-cleared for 1 hour with protein-G beads and then immunoprecipitated overnight at 4°C with the following specific antibodies: anti-H3 dimethyl lysine 9 (Abcam, ab1220), anti-H3 trimethyl lysine 9 (Millipore-Upstate, 07-442) anti-H3 (Abcam, ab1791), anti-UHRF1, anti-DMNT3b (Active Motif, 39207), anti-G9a (Sigma, G6919), anti-SRF (Cell Signaling, 5147), anti-NRF1 (Abcam, ab34682) and rabbit IgG (Millipore-Upstate, 12-370). After incubation, the immunocomplexes were bound to protein-G beads for 2 hours and subsequently washed with low-salt wash buffer (0.1% SDS, 2mM EDTA, 20mM Tris HCl pH8, 1% Triton X-100, 150mM NaCl and protease inhibitors), high-salt wash buffer (0.1% SDS, 2 mM EDTA, 20mM Tris HCl pH8, 1% Triton x-100, 500mM NaCl and protease inhibitors), and TE buffer. Immunocomplexes were then eluted in elution buffer (1% SDS, 100mM NaHCO3) and cross-linking reverted overnight at 65°C. Samples were then treated with proteinase K, extracted with phenol/chloroform and precipitated with ethanol. Purified DNA was evaluated by qPCR with SYBR green PCR master mix

(Applied Biosystem). Primers are listed in Supplementary Table 4. Values obtained were normalized to the input; histone modifications were also normalized to the H3 content.

Statistical Analysis

All data are expressed as mean±SD. Comparison between experimental groups was performed using unpaired Student's t-test or ANOVA tests, using Prism 6.0 software (GraphPad Software, Inc.). A value of $p < 0.05$ was considered statistically significant.

REFERENCES

Arany, Z., He, H., Lin, J., Hoyer, K., Handschin, C., Toka, O., Ahmad, F., Matsui, T., Chin, S., Wu, P.H., Rybkin, II, Shelton, J.M., Manieri, M., Cinti, S., Schoen, F.J., Bassel-Duby, R., Rosenzweig, A., Ingwall, J.S., and Spiegelman, B.M. (2005). Transcriptional coactivator PGC-1 alpha controls the energy state and contractile function of cardiac muscle. Cell Metab 1, 259-271.

Arany, Z., Novikov, M., Chin, S., Ma, Y., Rosenzweig, A., and Spiegelman, B.M. (2006). Transverse aortic constriction leads to accelerated heart failure in mice lacking PPAR-gamma coactivator 1alpha. Proc Natl Acad Sci U S A 103, 10086-10091.

Arsic, N., Zacchigna, S., Zentilin, L., Ramirez-Correa, G., Pattarini, L., Salvi, A., Sinagra, G., and Giacca, M. (2004). Vascular endothelial growth factor stimulates skeletal muscle regeneration in vivo. Mol Ther 10, 844-854.

Babbio, F., Pistore, C., Curti, L., Castiglioni, I., Kunderfranco, P., Brino, L., Oudet, P., Seiler, R., Thalman, G.N., Roggero, E., Sarti, M., Pinton, S., Mello- Grand, M., Chiorino, G., Catapano, C.V., Carbone, G.M., and Bonapace, I.M. (2012). The SRA protein UHRF1 promotes epigenetic crosstalks and is involved in prostate cancer progression. Oncogene 31, 4878-4887.

Barres, R., Osler, M.E., Yan, J., Rune, A., Fritz, T., Caidahl, K., Krook, A., and Zierath, J.R. (2009). Non-CpG methylation of the

PGC-1alpha promoter through DNMT3B controls mitochondrial density. Cell Metab 10, 189-198.

Barres, R., Yan, J., Egan, B., Treebak, J.T., Rasmussen, M., Fritz, T., Caidahl, K., Krook, A., O'Gorman, D.J., and Zierath, J.R. (2012). Acute exercise remodels promoter methylation in human skeletal muscle. Cell Metab 15, 405-411.

Bird, A.P., and Wolffe, A.P. (1999). Methylation-induced repression--belts, braces, and chromatin. Cell 99, 451-454.

Bonapace, I.M., Latella, L., Papait, R., Nicassio, F., Sacco, A., Muto, M., Crescenzi, M., and Di Fiore, P.P. (2002). Np95 is regulated by E1A during mitotic reactivation of terminally differentiated cells and is essential for S phase entry. J Cell Biol 157, 909-914.

Cheng, X., and Blumenthal, R.M. (2010). Coordinated chromatin control: structural and functional linkage of DNA and histone methylation. Biochemistry 49, 2999-3008.

Dawson, M.A., and Kouzarides, T. (2012). Cancer epigenetics: from mechanism to therapy. Cell 150, 12-27.

Doenst, T., Nguyen, T.D., and Abel, E.D. (2013). Cardiac metabolism in heart failure: implications beyond ATP production. Circ Res 113, 709-724.

Du, P., Kibbe, W.A., and Lin, S.M. (2008). lumi: a pipeline for processing Illumina microarray. Bioinformatics 24, 1547-1548.

el Azzouzi, H., Leptidis, S., Dirkx, E., Hoeks, J., van Bree, B., Brand, K., McClellan, E.A., Poels, E., Sluimer, J.C., van den Hoogenhof, M.M., Armand, A.S., Yin, X., Langley, S., Bourajjaj,

M., Olieslagers, S., Krishnan, J., Vooijs, M., Kurihara, H., Stubbs, A., Pinto, Y.M., Krek, W., Mayr, M., da Costa Martins, P.A., Schrauwen, P., and De Windt, L.J. (2013). The hypoxia-inducible microRNA cluster miR-199a approximately 214 targets myocardial PPARdelta and impairs mitochondrial fatty acid oxidation. Cell Metab 18, 341-354.

Finck, B.N., and Kelly, D.P. (2007). Peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) regulatory cascade in cardiac physiology and disease. Circulation 115, 2540-2548.

Ghoshal, K., Li, X., Datta, J., Bai, S., Pogribny, I., Pogribny, M., Huang, Y., Young, D., and Jacob, S.T. (2006). A folate- and methyl-deficient diet alters the expression of DNA methyltransferases and methyl CpG binding proteins involved in epigenetic gene silencing in livers of F344 rats. J Nutr 136, 1522-1527.

Gilsbach, R., Preissl, S., Gruning, B.A., Schnick, T., Burger, L., Benes, V., Wurch, A., Bonisch, U., Gunther, S., Backofen, R., Fleischmann, B.K., Schubeler, D., and Hein, L. (2014). Dynamic DNA methylation orchestrates cardiomyocyte development, maturation and disease. Nat Commun 5, 5288.

Gorski, Przemek A., Ceholski, Delaine K., and Hajjar, Roger J. (2015). Altered Myocardial Calcium Cycling and Energetics in Heart Failure—A Rational Approach for Disease Treatment. Cell Metab 21, 183-194.

Haas, J., Frese, K.S., Park, Y.J., Keller, A., Vogel, B., Lindroth, A.M., Weichenhan, D., Franke, J., Fischer, S., Bauer, A., Marquart, S., Sedaghat- Hamedani, F., Kayvanpour, E., Kohler, D., Wolf, N.M., Hassel, S., Nietsch, R., Wieland, T., Ehlermann, P., Schultz, J.H., Dosch, A., Mereles, D., Hardt, S., Backs, J., Hoheisel, J.D., Plass, C., Katus, H.A., and Meder, B. (2013). Alterations in cardiac DNA methylation in human dilated cardiomyopathy. EMBO Mol Med 5, 413-429.

Haberland, M., Montgomery, R.L., and Olson, E.N. (2009). The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat Rev Genet 10, 32-42.

Hashimoto, H., Horton, J.R., Zhang, X., Bostick, M., Jacobsen, S.E., and Cheng, X. (2008). The SRA domain of UHRF1 flips 5-methylcytosine out of the DNA helix. Nature 455, 826-829.

He, A., Kong, S.W., Ma, Q., and Pu, W.T. (2011). Co-occupancy by multiple cardiac transcription factors identifies transcriptional enhancers active in heart. Proceedings of the National Academy of Sciences 108, 5632-5637.

Heineke, J., and Molkentin, J.D. (2006). Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat Rev Mol Cell Biol 7, 589-600.

Hopfner, R., Mousli, M., Jeltsch, J.M., Voulgaris, A., Lutz, Y., Marin, C., Bellocq, J.P., Oudet, P., and Bronner, C. (2000). ICBP90, a novel human CCAAT binding protein, involved in the

regulation of topoisomerase IIalpha expression. Cancer Res 60, 121-128.

Huss, J.M., Imahashi, K., Dufour, C.R., Weinheimer, C.J., Courtois, M., Kovacs, A., Giguere, V., Murphy, E., and Kelly, D.P. (2007). The nuclear receptor ERRalpha is required for the bioenergetic and functional adaptation to cardiac pressure overload. Cell Metab 6, 25-37.

Izumo, S., Nadal-Ginard, B., and Mahdavi, V. (1988). Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. Proc Natl Acad Sci U S A 85, 339-343.

Jones, P.A., and Taylor, S.M. (1980). Cellular differentiation, cytidine analogs and DNA methylation. Cell 20, 85-93.

Karberg, S. (2009). Switching on epigenetic therapy. Cell 139, 1029-1031.

Kelly, D.P., and Scarpulla, R.C. (2004). Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev 18, 357-368.

Kemi, O.J., Ceci, M., Wisloff, U., Grimaldi, S., Gallo, P., Smith, G.L., Condorelli, G., and Ellingsen, O. (2008). Activation or inactivation of cardiac Akt/mTOR signaling diverges physiological from pathological hypertrophy. J Cell Physiol 214, 316-321.

Kim, J.K., Esteve, P.O., Jacobsen, S.E., and Pradhan, S. (2009). UHRF1 binds G9a and participates in p21

transcriptional regulation in mammalian cells. Nucleic Acids Res 37, 493-505.

Lompre, A.-M., Schwartz, K., d'Albis, A., Lacombe, G., Van Thiem, N., and Swynghedauw, B. (1979). Myosin isoenzyme redistribution in chronic heart overload. Nature 282, 105-107.

Madrazo, J.A., and Kelly, D.P. (2008). The PPAR trio: regulators of myocardial energy metabolism in health and disease. J Mol Cell Cardiol 44, 968-975.

Meilinger, D., Fellinger, K., Bultmann, S., Rothbauer, U., Bonapace, I.M., Klinkert, W.E., Spada, F., and Leonhardt, H. (2009). Np95 interacts with de novo DNA methyltransferases, Dnmt3a and Dnmt3b, and mediates epigenetic silencing of the viral CMV promoter in embryonic stem cells. EMBO Rep 10, 1259-1264.

Mohn, F., Weber, M., Rebhan, M., Roloff, T.C., Richter, J., Stadler, M.B., Bibel, M., and Schubeler, D. (2008). Lineage-specific polycomb targets and de novo DNA methylation define restriction and potential of neuronal progenitors. Mol Cell 30, 755-766.

Movassagh, M., Choy, M.K., Knowles, D.A., Cordeddu, L., Haider, S., Down, T., Siggens, L., Vujic, A., Simeoni, I., Penkett, C., Goddard, M., Lio, P., Bennett, M.R., and Foo, R.S. (2011). Distinct epigenomic features in end-stage failing human hearts. Circulation 124, 2411-2422.

Mudbhary, R., Hoshida, Y., Chernyavskaya, Y., Jacob, V., Villanueva, A., Fiel, M.I., Chen, X., Kojima, K., Thung, S.,

Bronson, R.T., Lachenmayer, A., Revill, K., Alsinet, C., Sachidanandam, R., Desai, A., SenBanerjee, S., Ukomadu, C., Llovet, J.M., and Sadler, K.C. (2014). UHRF1 overexpression drives DNA hypomethylation and hepatocellular carcinoma. Cancer Cell 25, 196-209.

Neubauer, S. (2007). The failing heart--an engine out of fuel. N Engl J Med 356, 1140-1151.

Obata, Y., Furusawa, Y., Endo, T.A., Sharif, J., Takahashi, D., Atarashi, K., Nakayama, M., Onawa, S., Fujimura, Y., Takahashi, M., Ikawa, T., Otsubo, T., Kawamura, Y.I., Dohi, T., Tajima, S., Masumoto, H., Ohara, O., Honda, K., Hori, S., Ohno, H., Koseki, H., and Hase, K. (2014). The epigenetic regulator Uhrf1 facilitates the proliferation and maturation of colonic regulatory T cells. Nat Immunol 15, 571-579.

Papait, R., Cattaneo, P., Kunderfranco, P., Greco, C., Carullo, P., Guffanti, A., Viganò, V., Stirparo, G.G., Latronico, M.V.G., Hasenfuss, G., Chen, J., and Condorelli, G. (2013). Genome-wide analysis of histone marks identifying an epigenetic signature of promoters and enhancers underlying cardiac hypertrophy. Proc Nat Acad Sci USA 110, 20164-20169.

Rajabi, M., Kassiotis, C., Razeghi, P., and Taegtmeyer, H. (2007). Return to the fetal gene program protects the stressed heart: a strong hypothesis. Heart Fail Rev 12, 331-343.

Razeghi, P., Young, M.E., Alcorn, J.L., Moravec, C.S., Frazier, O.H., and Taegtmeyer, H. (2001). Metabolic gene expression in fetal and failing human heart. Circulation 104, 2923-2931.

Reik, W., Dean, W., and Walter, J. (2001). Epigenetic reprogramming in mammalian development. Science 293, 1089-1093.

Rowe, G.C., Jiang, A., and Arany, Z. (2010). PGC-1 coactivators in cardiac development and disease. Circ Res 107, 825-838.

Sharif, J., Muto, M., Takebayashi, S., Suetake, I., Iwamatsu, A., Endo, T.A., Shinga, J., Mizutani-Koseki, Y., Toyoda, T., Okamura, K., Tajima, S., Mitsuya, K., Okano, M., and Koseki, H. (2007). The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. Nature 450, 908-912.

Sihag, S., Cresci, S., Li, A.Y., Sucharov, C.C., and Lehman, J.J. (2009). PGC- 1alpha and ERRalpha target gene downregulation is a signature of the failing human heart. J Mol Cell Cardiol 46, 201-212.

Stein, R., Gruenbaum, Y., Pollack, Y., Razin, A., and Cedar, H. (1982). Clonal inheritance of the pattern of DNA methylation in mouse cells. Proc Natl Acad Sci U S A 79, 61-65.

Taegtmeyer, H., Wilson, C.R., Razeghi, P., and Sharma, S. (2005). Metabolic energetics and genetics in the heart. Ann N Y Acad Sci 1047, 208-218.

Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L.M., Liu, D.R., Aravind, L., and Rao, A. (2009). Conversion of 5-methylcytosine to 5-

hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324, 930-935.

Toischer, K., Rokita, A.G., Unsold, B., Zhu, W., Kararigas, G., Sossalla, S., Reuter, S.P., Becker, A., Teucher, N., Seidler, T., Grebe, C., Preuss, L., Gupta, S.N., Schmidt, K., Lehnart, S.E., Kruger, M., Linke, W.A., Backs, J., Regitz- Zagrosek, V., Schafer, K., Field, L.J., Maier, L.S., and Hasenfuss, G. (2010). Differential cardiac remodeling in preload versus afterload. Circulation 122, 993- 1003.

Ventura-Clapier, R., Garnier, A., and Veksler, V. (2004). Energy metabolism in heart failure. J Physiol 555, 1-13.

Wu, H., and Zhang, Y. (2014). Reversing DNA methylation: mechanisms, genomics, and biological functions. Cell 156, 45-68.

Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R.C., and Spiegelman, B.M. (1999).

Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98, 115-124.

Zacchigna, S., Zentilin, L., and Giacca, M. (2014). Adeno-associated virus vectors as therapeutic and investigational tools in the cardiovascular system. Circ Res 114, 1827-1846.

CHAPTER 3 DNA HYDROXYMETHYLATION CONTROLS CARDIOMYOCYTE GENE EXPRESSION IN DEVELOPMENT AND HYPERTROPHY

Carolina M. Greco[1], Paolo Kunderfranco[1], Pierluigi Carullo[1], Andrea Angius[2], Michael V.G Latronico[1], Roberto Papait[1,2] & Gianluigi Condorelli[1–4]

1Humanitas Clinical and Research Center, Rozzano 20089 (MI), Italy; 2Institute of Genetics and Biomedical Research, National Research Council of Italy, Milan, Italy; 3University of Milan, Milan, Italy; 4University of Leicester, Leicester, UK. Correspondence and requests for materials should be addressed to G.C. (email: gianluigi.condorelli@humanitasresearch.it) or to R.P. (email: roberto.papait@humanitasresearch.it).

Submitted

ABSTRACT

Methylation at 5-cytosine (5-mC) is a fundamental epigenetic DNA modification recently associated with cardiac disease. In contrast, the role of 5- hydroxymethylcytosine (5-hmC) – 5-mC's oxidation product – is unknown in the context of the heart. Here, we assess the hydroxymethylome in embryonic, neonatal, adult and hypertrophic mouse cardiomyocytes, demonstrating that dynamic modulation of hydroxymethylated DNA is important for cardiomyocyte gene expression programming in heart development and failure. In fact, 5-hmC deposited on gene bodies potentiated the effect of activating histone marks on gene transcription. In pathological hypertrophy 5-hmC was found redistributed to intergenic regions, resembling the profile of embryonic cells, and defined a subset of enhancers with increased activity proximal to several key cardiac transcription regulators. We thus provide the first genome-wide analysis of 5-hmC in the cardiomyocyte, and establish the role of this epigenetic modification in heart development and disease.

INTRODUCTION

The heart can adapt to stimuli via the activation of specific transcriptional programmes. The epigenome – the sum of the chemical modifications occurring on DNA and the histone proteins it is wrapped around – has emerged as a key regulatory element of this process during heart development[1] and disease[2]. Among the epigenetic modifications that make up the epigenome, methylation of DNA at position 5 of cytosine (5-mC) has been found involved in X-chromosome inactivation, transposon silencing and genomic imprinting[3]. Regarding the heart, whole genome-bisulfite sequencing of DNA methylation has recently demonstrated the plasticity of the 5-mC profile in the cardiac genome[4].

DNA methylation was initially considered a stable epigenetic mark and, until recently, the only known chemical modification of DNA. However, the discovery of 5-hydroxymethylcytosine (5-hmC) – an oxidation product of 5-mC catalysed by the ten-eleven translocation (TET) family proteins – has revised this notion[5]. In fact, studies have shown that 5-hmC is not only an intermediate product of an active de-methylation process, but can also act as a relatively stable epigenetic mark whose role in transcription regulation is linked to its genomic location. Indeed, enrichment of 5-hmC on the gene body (GB) has been associated with transcriptional activation in several reports[6-11]. However, its function on promoters and distal regulatory regions is less clear. In embryonic stem cells (ESCs), for example, 5-

hmC can localize to the transcription start site (TSS) of repressed, but developmentally poised, genes[7, 12, 13]; moreover, it can be highly enriched at poised and active enhancers[14-16], but only recently have two studies proven that TET-mediated 5-mC oxidation regulates the activity of these elements[16, 17].

5-hmC has been investigated extensively in several cell and tissue types, but its relevance for myocardial pathophysiology is unknown. Thus, we decided to profile the distribution of 5-hmC in the cardiomyocyte genome. We found that DNA hydroxymethylation is highly dynamic during cardiac development and disease. Deposition of 5-hmC on the GB was strongly positively correlated with gene expression, and marked cardiac-specific genes. Moreover, cardiomyocyte hypertrophy was associated with a profound re-distribution of 5-hmC to distal regulatory regions; 5-hmC-enriched enhancers were found in the proximity of many genes encoding transcription factors known to play pivotal roles in the cardiomyocyte (i.e., Mef2c, Hif1a, Nkx2.5 and Hand2), strongly inducing their expression.

RESULTS

Genomic distribution of 5-hmC in cardiomyocytes during development and pathological hypertrophic growth.

To profile 5-hmC distribution in the cardiomyocyte (CMC), we performed hydroxymethylated-DNA immunoprecipitation coupled with high- throughput sequencing (hMeDIP-seq) on CMCs isolated from mice at four experimentalpoints: E14.5 embryos, 1- or 2-day-old pups, 2-month-old adult mice, and adult micesubjected for 1 week to transverse aortic constriction (TAC), a surgical procedure that induces pathological cardiac hypertrophy due to pressure overload[18]. We found 5-hmC-enriched sequences to result in >30 million reads in each condition. When mapped to the mouse genome, these 5-hmC fragments generated >22 million unique reads (mapQ ≥10) (Supplementary Fig. 1a). 5-hmC density was similar across autosomes and the X chromosome, whereas the Y chromosome was largely depleted of 5-hmC, as previously observed in mouse ESCs[19] (Fig. 1a); 5-hmC content correlated positively with the number of protein-coding genes on each chromosome at all experimental points (Fig. 1b and Supplementary Fig. 1b).

HOMER software was then used to identify the peaks of 5-hmC-enriched regions (Supplementary Fig. 1a). These mapped to a total of 16,354 protein-coding genes (Supplementary Fig. 1c and Supplemental Dataset 1). Around half of these genes underwent a re-distribution in 5-hmC deposition during

development and hypertrophy (Supplementary Fig.1d). Most of the 5-hmC peaks were found on introns and intergenic regions: notably, 5-hmC was distributed mainly (61%) on the GB (introns plus exons) in adult CMCs, whereas hypertrophy was characterized by a shift of 5-hmC to intergenic regions, resembling the distribution found in embryonic and neonatal CMCs (Fig. 1c). Intragenic 5-hmC density was highest in adult CMCs and lowest in embryonic CMCs, and was greatly reduced at TSSs and transcription termination sites (TTSs) at all the experimental points studied (Fig 1d,e). Integrative analyses of histone modifications supported these findings: dimethylation of lysine 79 on histone H3 (H3K79me2) – a modification distributed preferentially on the GB and recently implicated in cardiomyocyte differentiation[20] – strongly co-localized with 5-hmC, whereas trimethylation of lysine 4 (H3K4me3) – a mark mainly enriched around the TSS – displayed an opposite pattern (Fig.1f).

These results indicate that 5-hmC is strongly present in the CMC genome, that it tends to accumulate gradually on the GB during cardiac development, and that hypertrophy is characterized by re-establishment of an embryonic-like distribution pattern.

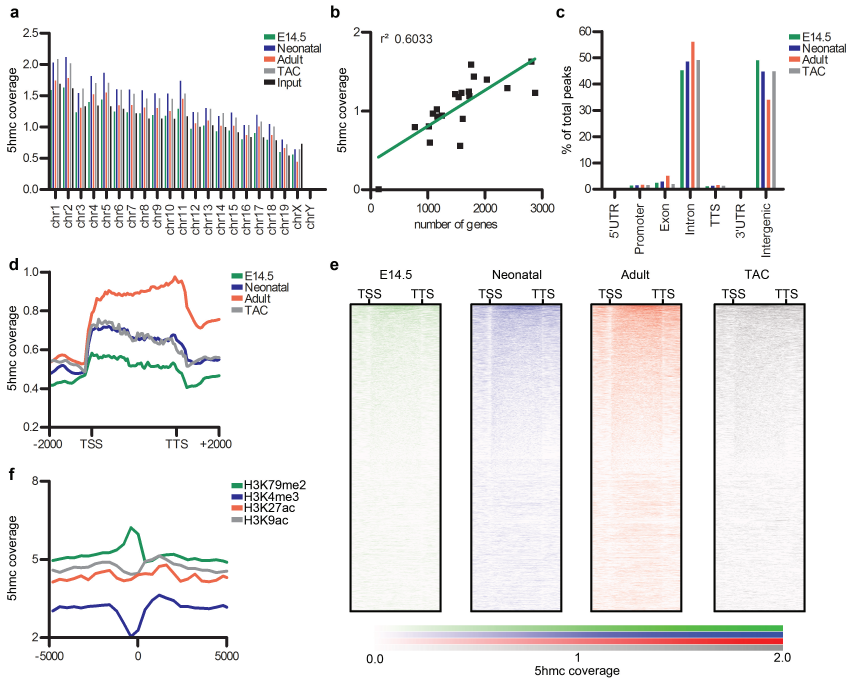

Figure 1 | Genomic distribution of 5-hydroxymethylcytosine in mouse cardiomyocytes.

(a) 5-hmC and input coverage on the 19 autosomal chromosomes and the X and Y chromosomes. Cardiomyocytes were isolated from embryonic (E14.5), neonatal (day 1), adult and transverse aortic constricted (TAC) mice. (b) Scatter plot showing positive correlation between 5-hmC coverage and the number of genes on each chromosome at the embryonic time point. (c) HOMER software analysis of the distribution 5-hmC peaks at different genomic elements at the four points studied. Promoters were defined as –1Kb to +1Kb relative to the TSS. (d) 5-hmC coverage across the gene body of all reference genes (build mm10 of the UCSC mouse genome browser) at

each studied point. (e) Heatmap of 5-hmC densities on gene bodies and flanking regions (±2Kb). (f) Profiles of 5-hmC distribution around the H3K79me2, H3K4me3, H3K27ac and H3K9ac peaks identified with MACS software in adult CMCs.

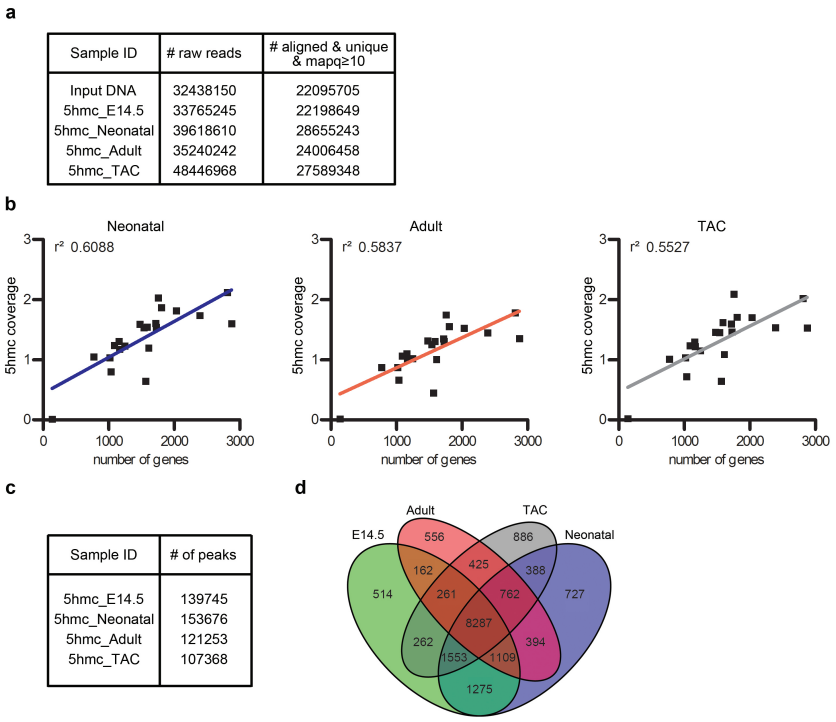

Figure S1 | Characterization of hMeDIP sequencing results.

(a) Summary of raw and mapped hMeDIP sequencing reads and number of peaks identified with HOMER software (fold enrichment ≥4; FDR <10-4; p-value <10-5) at each of the four points studied, i.e., cardiomyocytes from embryonic (E14.5), neonatal (day 1/2), adult and transverse aortic constricted (TAC) mice. Reads were mapped to the mouse genome

(UCSC, build mm10). (b) Scatter plots showing the positive correlation between 5-hmC coverage and the number of genes on each chromosome in neonatal, adult and TAC cardiomyocytes. (c) Venn diagram of the number of genes associated with the identified peaks. Peaks overlapping with 5′UTR, promoter, exon, intron, TTS and 3′UTR genomic regions were considered for the analysis.

Correlation of 5-hmC and transcription.

To assess the relationship between 5-hmC and gene expression, we first performed deep RNA sequencing (RNA-Seq) (Supplementary Fig. 2a). Overall, we found more than 11,500 expressed protein transcripts (RPKM ≥1) at the four studied points. Pairwise differential analysis identified 10,303 differentially expressed transcripts among the four biological conditions (adjusted p-value ≤0.05) (Fig. 2a and Supplementary Fig. 2b and Supplementary Dataset 2). The differentially modulated genes were grouped by SOTA software into 11 clusters with distinct patterns of expression (Fig. 2b and Supplementary Fig. 2c). Each cluster encoded for unique biological features, as assessed by gene ontology analysis (Fig. 2c and Supplementary Fig. 2d). For example, the set of neonatally expressed transcripts that became down-regulated in the adult but that were re-expressed upon hypertrophy (cluster 6), were found enriched for terms related to cell adhesion, extracellular matrix organization and response to

wounding; gene ontology analysis of adult-specific transcripts that were up-regulated upon hypertrophy (cluster 8) indicated strong activation of the immune response in stressed CMCs, consistent with a systemic inflammatory response[21]; and transcripts highly expressed in normal adult CMCs but down-regulated at the embryonic stage and in hypertrophic cells (clusters 10 and 11), were involved in metabolic pathways such as the tricarboxylic acid (TCA) cycle, oxidative phosphorylation and fatty acid metabolism, in agreement with the known shift to a foetal metabolic phenotype of the hypertrophic heart[22].

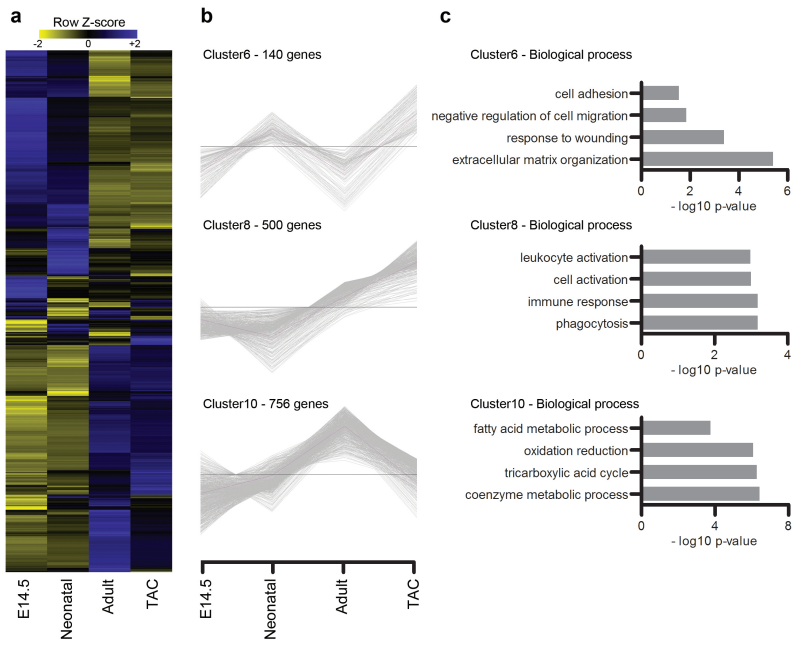

Figure 2 | Transcriptional analysis during cardiac development and hypertrophy.

(a) Hierarchical clustering of the 10,303 differentially expressed transcripts (adjusted p-value ≤0.05) among the studied points. (b) Examples of SOTA clustering analysis of the 10,303 differentially expressed genes. (c) Enriched gene ontology terms of the relative SOTA cluster.

We next assessed the correlation between gene expression and 5-hmC. Genes that contained 5-hmC on the GB were significantly more expressed than the median of all genes (Fig. 3a). The effect of 5-hmC on gene expression was a cumulative: the deeper the intragenic coverage, the higher the gene expression (Fig. 3b). Moreover, in post-mitotic CMCs – i.e., neonatal, adult and TAC cells – the median of 5-hmC coverage on the GB increased progressively along the expression quartiles; this relationship was not evidently present in embryonic CMCs (Supplementary Fig. 3a). In contrast, we found that genes enriched for this modification exclusively at the promoter were significantly repressed (Fig. 3a). These findings were suggestive of different transcriptional roles of 5-hmC in embryonic and post-mitotic (non-proliferating) CMCs, and indicated that the effect of 5- hmC on transcription was position dependent.

The strong enrichment of 5-hmC on GBs of highly expressed cardiac-specific genes is illustrated in Fig. 3c, which gives the profiles of Tnni1, the foetal isoform of troponin I; Myh7, the foetal isoform of myosin heavy chain; Idh3a, a component of

the TCA cycle; and desmin (Des), a muscle-specific gene up-regulated in hypertrophy.

Given the strong association of intragenic 5-hmC with gene expression and its broad distribution in the cardiac genome, we hypothesized that it may also play a role in regulating constitutively active genes. Indeed, genes found hydroxymethylated at all experimental points (Supplementary Fig. 3b) were consistently more expressed during cardiac development and hypertrophy (Supplementary Fig. 3c). Importantly, these genes encoded proteins for fundamental cellular processes, such as transcription, chromatin modification, intracellular signalling cascades protein transport and actin cytoskeleton organization, as well as for processes specific to the cardiac cell such as heart development (Supplementary Fig. 3d). Thus, as reported for other cell types[6, 8, 9, 11], 5-hmC marks highly expressed CMC genes involved in ubiquitary and cardiac-specific functions.

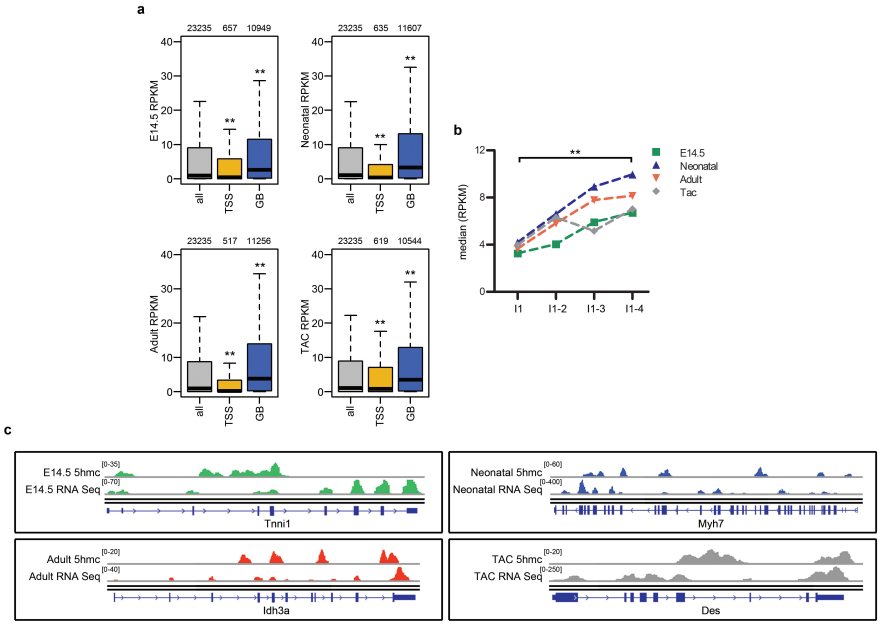

Figure 3 | Correlation of 5-hydroxymethylcytosine and gene expression.

(a) Boxplots of RNA expression of all genes (grey bars), genes with 5-hmC exclusively present on the promoter (P, yellow bars) and genes with 5-hmC exclusively on the gene body (GB, blue bars). Thick lines indicate the median, with whiskers extending to ±1.5 of the interquartile range. The numbers of genes in each group are shown above the plots. **, p-value ≤0.01(Mann-Whitney test). RPKM: reads per kilobase per million mapped reads. Promoters were defined as –1Kb to +1Kb relative to the TSS. (b) Plot showing the median RPKM value of genes harbouring 5-hmC only at one intron (1), at two introns (2), at three introns (3) and ≥4 introns (3+n). **, p-value ≤0.01 (Mann-

Whitney test). (c) Examples of IGV (Integrative Genome Viewer) snapshots of 5-hmC-enriched regions and RNA-Seq profiles. Scale for each track is shown on the left.

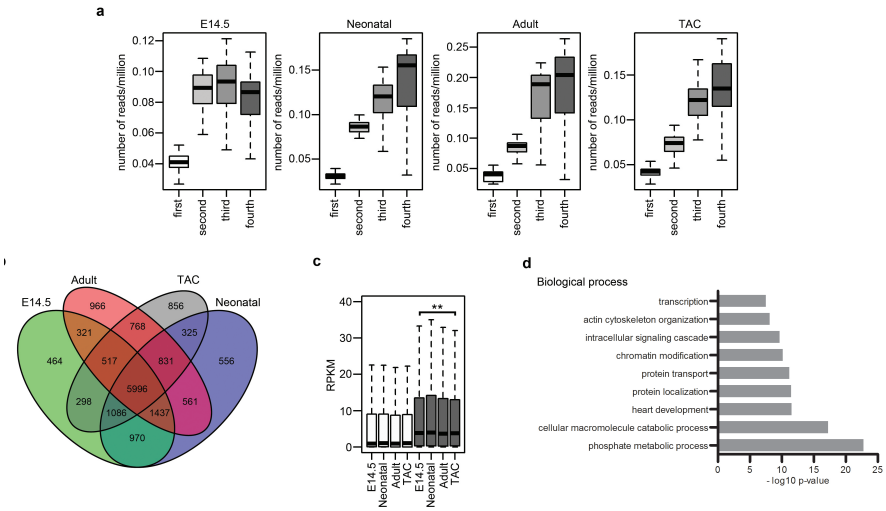

Figure S3 | Correlation of 5-hydroxymethylcytosine with gene expression during cardiomyocyte development and hypertrophy.

(a) 5-hmC coverage (from the TSS to the TTS) on genes clustered into quartiles of expression. Boxplots of median, with whiskers extending to ±1.5 of the interquartile range. (b) Venn diagram of the number of genes associated with the identified peaks at the four studied points; peaks overlapping with exons and introns were considered for the analysis. (c) RNA sequencing expression of all genes (light grey bars) and genes enriched for 5-hmC in intragenic regions (dark grey bars) at the

four studied points. Boxplots of median, with whiskers extending to ±1.5 of the interquartile range. **, p-value ≤0.01 (Mann-Whitney test). (d) Enriched gene ontology terms of genes enriched in 5-hmC.

5-hmC is dynamically modulated during cardiac development and hypertrophy.

To identify differentially hydroxymethylated (DhMRs) regions, we performed differential enrichment analysis. We found a large number of DhMRs and noticed greater dynamicity upon hypertrophy than during cardiac development (Supplementary Fig. 4a and Supplementary Dataset 3). The transition from the embryonic to the neonatal stage (Figure4, upper panel) was characterized by a predominant gain of 5-hmC within intragenic elements (Figure 4a). Given the positive correlation of GB 5-hmC with gene expression, we focused our attention on intragenic DhMRs that were accompanied with significant variations in the expression of the corresponding gene. 5-hmC was acquired by 830 up- regulated genes mainly related to energy production and metabolism (Figure 4b), such as Acaa2 and Acadl – which encode proteins involved in fatty acid metabolism (Figure 4c and Supplementary Fig. 4b). Loss of 5-hmC was associated with down-regulation of 107 genes enriched for biological processes related to tissue morphogenesis, embryonic development and Wnt signalling, as

exemplified by Dkk2 and Sfrp1, two members of the Wnt signalling pathway.

Further gain of 5-hmC on intragenic elements occurred in the neonatal to adult transition (Figure 4a, middle panel). These DhMRs were found on 1067 up-regulated genes enriched for energetic and metabolic pathways (Figure 4b), such as Acadsb and Ndufa10 (Figure 4c and Supplementary Fig. 4b). Loss of 5-hmC was instead associated with the down-regulation of 1064 genes involved in heart development – such as the transcription factor Tbx20 and the Notch family member Notch1 – angiogenesis and cell adhesion.

In contrast to the above, hypertrophy was associated with an opposite trend, i.e., a pronounced loss of 5-hmC on intragenic elements and a marked gain on intergenic regions (Figure 4a, lower panel). Despite the large overall loss of intragenic hydroxymethylated DNA in hypertrophic CMCs, the modification was gained by a set of up-regulated genes: these were mainly involved in extracellular matrix organization – such as Col3a1, which encodes type III procollagen – and actin cytoskeleton organization (Figure 4b,c and Supplementary Fig. 4b), two important processes for the development of hypertrophy[23]. Moreover, loss of 5-hmC was associated with repression of 652 genes belonging to processes related to the TCA cycle – such as Idh3g – fatty acid metabolism – [7] such as Acadsb – and the generation of energy, a finding consistent with the impaired energy production of hypertrophic CMCs[24]. Thus, 5-hmC follows

a specific distribution pattern during CMC maturation and disease, contributing to the specification of transcriptional programmes tightly linked to the biology of these cardiac cells.

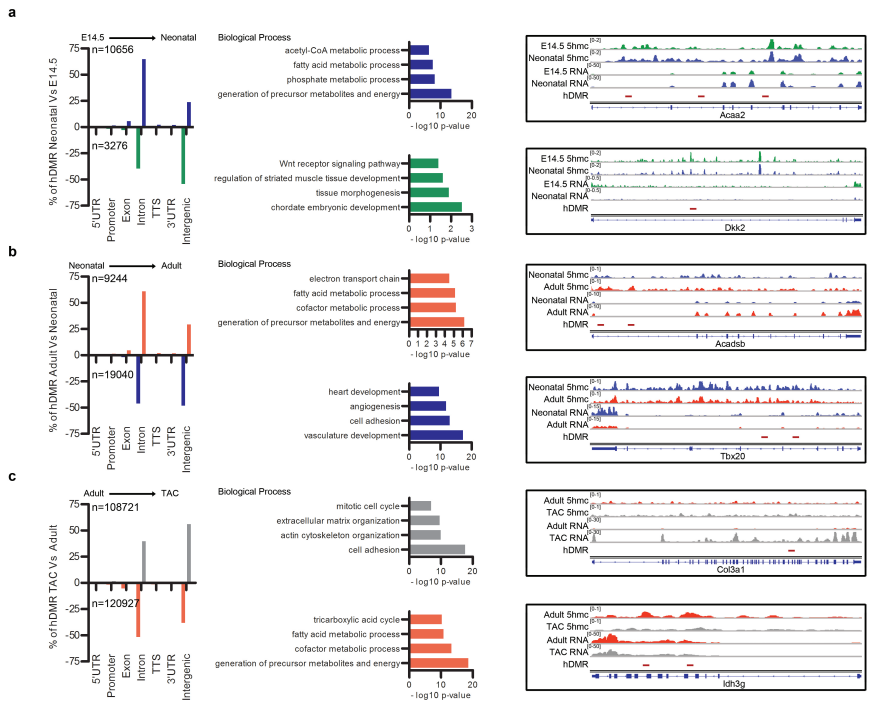

Figure 4 | Identification and characterization of differentially hydroxymethylated regions (DhMRs).

(a) Association of all identified DhMRs within different genomic elements: upper panel, in the embryonic to neonatal transition; middle panel, in the neonatal to adult transition; lower panel, between normal adult and hypertrophic (TAC) cardiomyocytes. (b) Upper panel, enriched gene ontology terms related to significantly up- regulated (blue) and down-regulated (green)

genes with DhMRs during the embryonic to neonatal transition. Middle panel, enriched gene ontology terms related to significantly up- regulated (red) and down-regulated (blue) genes with DhMRs during the neonatal to adult transition. Lower panel, enriched gene ontology terms related to significantly up-regulated (grey) and down-regulated (red) genes with DhMRs between normal adult and hypertrophic cardiomyocytes. Number of genes considered for the analyses are shown, and genes are listed in Supplementary Table 3. (c) IGV snapshot of 5-hmC distribution and RNA-Seq profiles of representative genes for each transition. DhMRs are indicated by the dark red dashes. Scale for each track is shown to the left.

a

# hDMR	E14.5	Neonatal	Adult	TAC
E14.5		3276	23438	176993
Neonatal	10656		19040	192101
Adult	26058	9244		120927
TAC	134190	128108	108721	

b

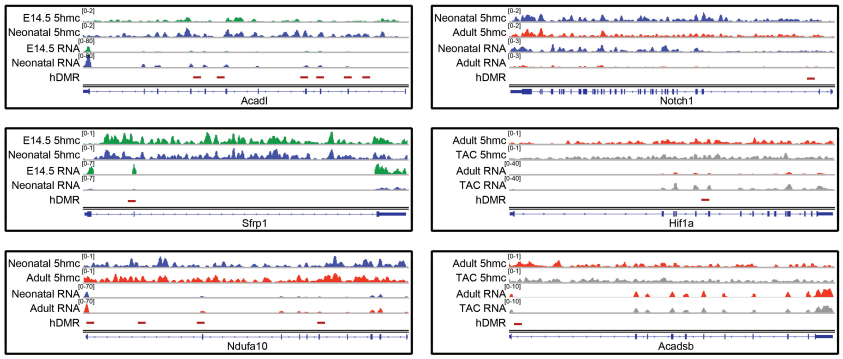

Figure S4 | Analysis of differentially hydroxymethylated (DhMR) regions.

(a) Summary of the number of identified DhMRs between embryonic (E), neonatal (N), normal adult (A) and hypertrophic (T) cardiomyocytes (fold enrichment ≥4; FDR<10-4; p-value <10-5). Green, regions losing hydroxymethylation; red, regions gaining hydroxymethylation. (b) Examples of IGV snapshots of 5-hmC distribution and RNA-Seq profiles of representative genes for each transition. DhMRs are indicated by dark red dashes. Scale for each track is shown on the left.

Correlation between 5-hmC and chromatin marks of active transcription.

We have previously defined the profile of the active histone marks H3K79me2, H3K9ac, H3K27ac and H3K4me3 in adult and hypertrophic CMCs[25]. For the present study, we investigated the relationship of GB 5-hmC with these modifications. Density plots of pairwise correlation coefficients pointed to a clear positive association between 5-hmC and all the analysed histone marks in adult CMCs (Figure 5a). This positive correlation was reduced in TAC CMCs (Supplementary Figure 5a).

We then assessed whether the co-presence of 5-hmC with the analysed active histone modifications led to transcriptional changes. Of note, in the absence of an active histone mark, GB 5-hmC was not associated with increased gene expression in adult CMCs (Figure 5b). On the other hand, the presence of 5-hmC along with H3K79me2, H3K9ac, H3K27ac or H3K4me3

was associated with enhanced transcription (Figure 5b). These results suggest that in adult cardiomyocytes, 5-hmC potentiates the effect of activating histone marks on gene transcription.

Of note, this cooperative effect on transcription was not seen with hypertrophy: in fact, in contrast to what was observed in normal adult CMCs, the presence of 5-hmC did not further modulate the expression of genes marked with an activating histone modification in TAC cells (Supplementary Figure 5b). This finding could be explained by the strong redistribution of 5-hmC to intergenic regions detected in TAC CMCs. Dynamic changes of 5-hmC on repeated sequences. DNA repetitive elements have been shown to be differentially methylated in hearts of cardiomyopathic patients[26].

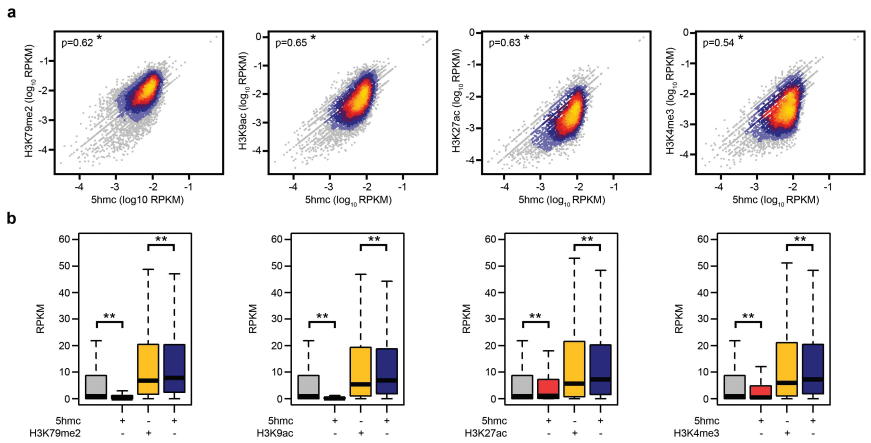

Figure 5 | Correlation of 5-hydroxymethylcytosine with activating histone marks in adult cardiomyocytes.

(a) Density plots illustrating the correlation of gene body 5-hmC level in adult CMCs with gene body levels of H3K79me2, H3K9ac, H3K27ac and H3K4me3 histone marks. For each plot, Spearman rank coefficient ρ is shown (exact
permutation test for testing the null hypothesis that there is no correlation). *, p-value <2×10−16 (two-tailed). (b) RNA sequencing expression values in adult cardiomyocytes for all genes (grey bars), genes positive for 5-hmC on the gene body (red bars), genes positive the indicated histone mark (yellow bars), and genes positive for both 5-hmC and the indicated histone mark (blue bars). Boxplots give the median (bold line), with whiskers extending to ±1.5 of the interquartile range. **, p-value ≤0.01 (Mann-Whitney test).

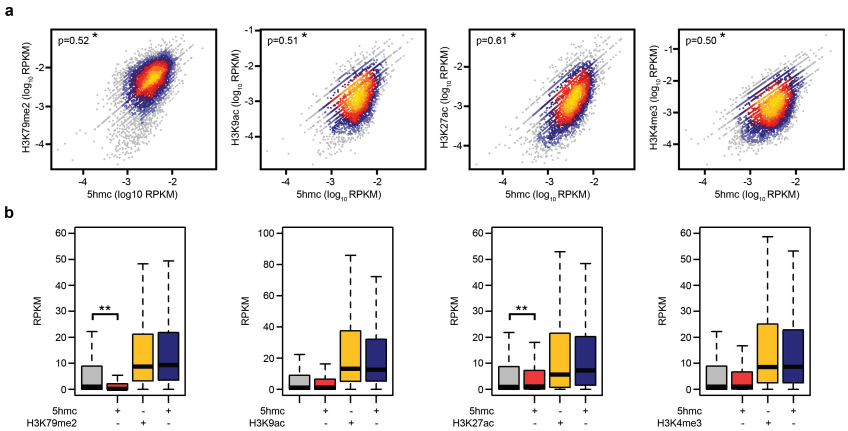

Figure S5 | Correlation of 5-hydroxymethylcytosine with activating histone marks in TAC cardiomyocytes.

(a) Correlation of 5-hmC with H3K79me2, H3K9ac, H3K27ac and H3K4me3 at the gene body in hypertrophic cardiomyocytes. The Spearman rank coefficient, ρ, is given for each density plot (exact permutation test for testing the null hypothesis that there is no correlation. *, two-tailed p-value $<2\times10-16$). (b) Expression of RNA (values given as RPKM) in hypertrophic cardiomyocytes for all genes (grey bars), genes positive for 5-hmC on the gene body (red bars), genes positive for the indicated histone mark (yellow bars), and genes with both 5-hmC and the indicated histone mark (blue bars). Boxplots giving median (thick line), with whiskers extending to ±1.5 of the interquartile range. **, p-value ≤0.01 (Mann-Whitney test).

Therefore, we examined 5-hmC distribution on repeated sequences. We found that almost all classes of repeats in CMCs were generally enriched in 5-hmC (Supplementary Figure 6a). However, the overall coverage of 5-hmC on these elements was markedly reduced during cardiac development (Supplementary Figure 6b). Repetitive elements are also known to undergo transcription in different tissues[27, 28]. Indeed, we found a strong positive correlation between the coverage of 5-hmC on these elements and their expression level (Supplementary Figure 6c).

With the induction of hypertrophy, the overall coverage of 5-hmC on repetitive elements became partially restored

(Supplementary Figure 6b), with long interspersed nuclear elements (LINEs) undergoing the greatest increase among the various classes (Supplementary Figure 6d,e). Coverage of H3K9me3 – a key indicator of LINE repression[29] – was significantly reduced only on hydroxymethylated LINEs (Supplementary Figure 6f), indicating a possible role of 5-hmC at LINES in rendering chromatin structure more permissive in the hypertrophic heart.

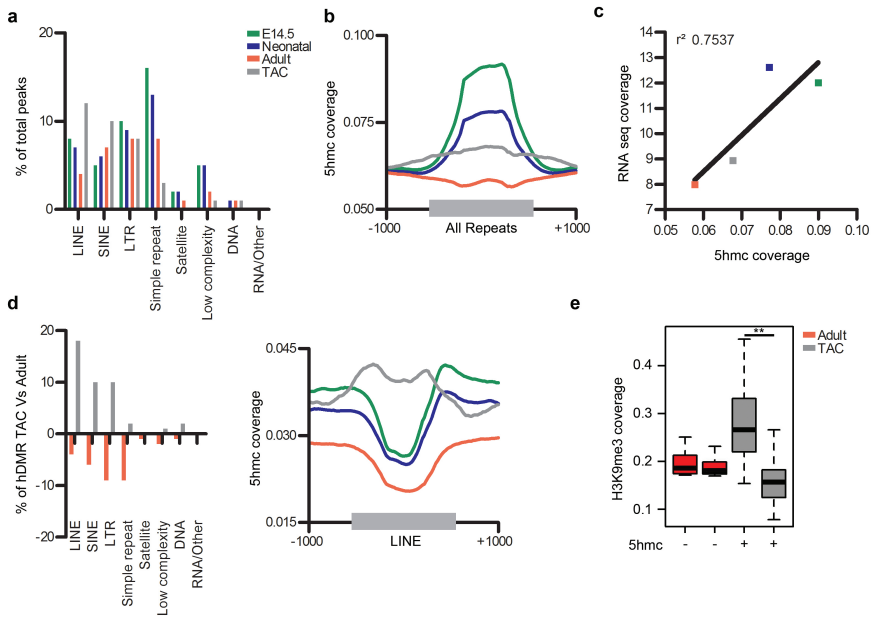

Figure S6 | Association of 5-hmC with repetitive elements of the genome.

(a) Analysis of repeat masker elements (mm10) associated with 5-hmC peaks (assessed by HOMER) at the four studied points. (b) 5-hmC coverage across the pooled repeat masker elements. (c) Positive correlation between 5-hmC coverage and

expression of repeat masker elements (RPKM). (d) Differentially hydroxymethylated regions (DhMRs) found in the adult-to-TAC transition, for each repeat masker class. (e) 5-hmC coverage across LINEs at the four studied points. (f) Coverage of H3K9me3 on LINEs (± 2Kb) in adult and hypertrophic (TAC) cardiomyocytes. The boxplot gives the median (thick line), with whiskers extending to ±1.5 of the interquartile range. LINEs enriched (+) or not (-) in 5-hmC. **, p-value ≤0.01 (Mann-Whitney test).

5-hmC activates cardiac-specific enhancers in hypertrophy.

Enhancers are key regulators of tissue-specific gene expression programmes. Sets of distal enhancer regions were recently reported for cardiac differentiation30 and hypertrophy[25]. Moreover, studies on ESCs have reported high levels of 5-hmC at enhancers[15, 31]. Thus, we decided to assess the presence of 5-hmC on these genomic regulatory regions in cardiac cells. We found strong associations between 5-hmC and regions classified as active (H3K27ac+, H3K4me1+/-) or poised (H3K4me1+ only) enhancers by Bruneau's group in CMCs differentiated from ESCs in vitro [30] (Supplementary Figure 7a). To determine whether the presence of 5-hmC on enhancers influenced gene expression in adult CMCs, we measured the level of expression of the nearest gene. We found that genes associated with 5-hmC+ enhancers were

expressed at higher levels than those near 5-hmC– ones (Supplementary Figure 7b). Gene ontology analysis revealed enrichment of distinct

biological processes in these two sets of genes (Supplementary Figure7c).

We then dissected the functional role of enhancer hydroxymethylation in TAC CMCs. In the 9027 enhancer regions we previously characterized in hypertrophy25, we identified a subset (n=914) enriched in 5-hmC (Figure 7a). Motif discovery analysis identified a significant over-representation of binding sites for myocyte enhancer factor family members (MEF2A and MEF2C) within these regions (Figure 7b). Importantly, 5- hmC+ enhancers in TAC CMCs were located in the proximity of many transcription factor genes known to play a pivotal role in cardiac cells (i.e. Mef2c, Hif1a, Nkx2.5, Hand2). Consistent with this, the activity of these cardiac-specific enhancers was boosted by the presence of 5-hmC, resulting in an increased expression of the neighbouring genes (Figure 7c).

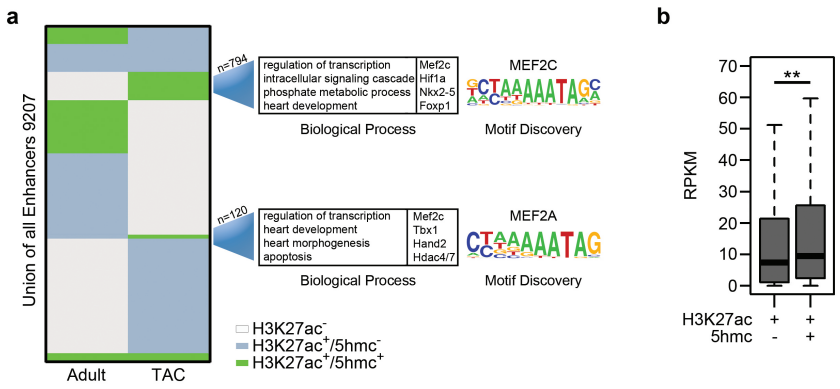

Figure 6 | 5-hmC-enriched enhancers in cardiac hypertrophy.

(a) Heatmap illustrating the distribution of 5-hmC in the 9207 hypertrophy-related enhancers identified by Papait et al.25 in normal (adult) and hypertrophic (TAC) cardiomyocytes. Enriched gene ontology terms related to genes nearby hypertrophy-specific H3K27ac+/5-hmC+ enhancers are shown together with representative genes. (b) Top-ranked transcription factor motifs found at H3K27ac+/5-hmC+ enhancers. (c) RNA sequencing expression of genes nearby hypertrophy-specific H3K27ac+/5-hmC+/- enhancers. The boxplot gives the median (bold line), with whiskers extending to ±1.5 of the interquartile range. **, p-value ≤0.01 (Mann- Whitney test).

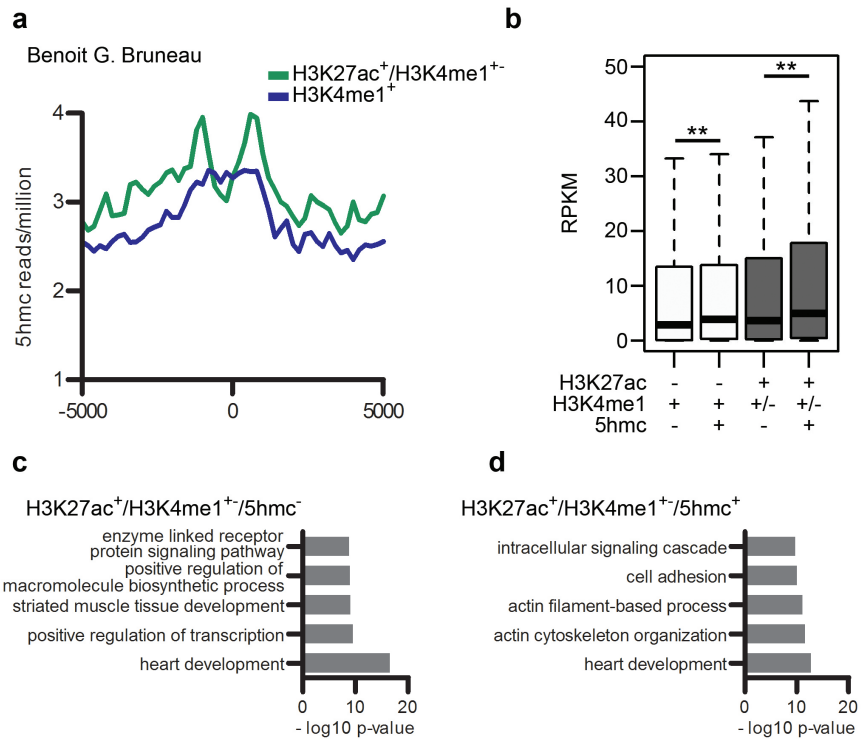

Figure S7 | Association of 5-hmC with enhancers.

(a) 5-hmC coverage across poised (H3K4me+) and active (H3K27ac+/H3K4me1+/-) enhancers identified in cardiomyocytes differentiated in vitro from embryonic stem cells, as defined in Wamstad et al. (b) Boxplots (whiskers extending to ±1.5 of the interquartile range) of adult RNA sequencing expression values (RPKM) for genes nearby poised/active enhancers enriched or not for 5-hmC. **, p-value ≤0.01 (Mann-Whitney test). (c) Enriched gene ontology terms of genes nearby active 5-hmC-depleted enhancers. (d) Enriched

gene ontology terms of genes nearby active 5-hmC-enriched enhancers.

DISCUSSION

The present study defines the genome-wide map of hydroxymethylated DNA during cardiac development and disease, providing new insights into how the epigenetic signature modulates cardiac gene expression. In fact, we report for the first time that 5- hmC acts on gene bodies as a co-activating mark in post-mitotic cardiomyocytes, and that it defines a set of enhancers with increased activity in cardiac hypertrophy.

An investigation of the methylome of embryonic, postnatal and failing cardiomyocytes carried out with whole-genome bisulfite sequencing identified a large number of differentially methylated regions mainly on gene bodies and cis-regulatory sequences, and highlighted a previously unappreciated plasticity of cardiac DNA methylation[4]. However, even though bisulfite sequencing allows resolution at the base level, it does not discriminate between 5-mC and 5-hmC[32]. We therefore asked whether 5-hmC could be an additional determinant in the cardiomyocyte methylome. In fact, the high abundance of 5-hmC in the central nervous system (CNS)[33, 34] has encouraged several investigations of its function during neurogenesis[35], postnatal neuronal development and aging [9, 36, 37]: in those contexts, 5-hmC was found to be a stable, but dynamic, epigenetic

mark. Moreover, quantification of 5-hmC in mammalian tissues suggested that heart and skeletal muscle contain intermediate levels of hydroxymethylated DNA [38, 39]. Our results reveal that 5-hmC marks active genes in cardiomyocytes, regulating distinct transcriptional networks during cardiac maturation – i.e., genes involved in embryonic and heart development during the E14.5 to neonatal transition, and genes involved in energetic and metabolic pathways during the neonatal to adult transition – as well as in the development of cardiac hypertrophy. This suggests that the dynamic modulation of hydroxymethylated DNA at gene bodies is an important feature of the control of the gene expression programme of cardiomyocytes.

However, the sole presence of 5-hmC on gene bodies is not sufficient to induce transcriptional activation: it must be co-expressed with an activating histone mark, such as H3K79me2, H3K9ac, H3K27ac or H3K4me3, potentiating the effect of the latter. Thus, 5- hmC may be a pre-activating mark, attracting or precluding the binding of chromatin remodelling proteins.

The exact mechanism through which TETs and 5-hmC regulate transcription remains elusive. TET2 and TET3 have been shown to directly interact with O-linked β-N- acetylglucosamine (O-GlcNAc) transferase (OGT) mediating histone O-GlcNAcylation[40], and to influence the deposition of H3K4me3, which in turn promotes transcription of target genes[41]. Moreover, mass spectrometry-based analysis has identified a large set of tissue- specific proteins selectively bound to or

repelled by hydroxymethylated DNA[42]. Further studies aimed at elucidating the specific role of TET proteins and their mechanism of action in cardiac cells are therefore warranted.

In embryonic stem cells, TET2-mediated hydroxymethylation has also been associated with the modulation of enhancer activity [16, 17]. We show here that enhancer- associated 5-hmC plays a role in transcriptional regulation in the development of hypertrophy: in fact, 5-hmC defined a subset of enhancers with increased activity in cardiomyocytes. Of note, the top biological function of genes in the proximity of hydroxymethylated enhancers was “regulation of transcription”, and included several key cardiac regulators. Moreover, transcription network analysis revealed that the activity of cardiac-specific enhancers was regulated by MEF2C and MEF2A in hypertrophic cardiomyocytes. Another transcription factor, GATA-4, has already been reported to shape the chromatin environment during cardiac development and disease and, in turn, regulate enhancer activity.[43] Similarly, MEF2C and MEF2A may be involved in establishing the epigenetic signature, in particular the deposition of 5-hmC on enhancers by binding with TETs, as recently observed for the transcription factor WT1 in HEK293T cells[44].

The findings of the present study set the basis for the understanding of the functional role of 5-hmC in cardiac physiology and disease, opening the field to the investigation of novel mechanisms governing cardiac transcription regulation.

METHODS

Animal procedures. All experiments were performed according to the 2010/63/EU Directive and approved by the local ethical committee. The study was performed using genomic material obtained from cardiomyocytes purified from embryonic (E14.5), neonatal (1–2 days old) and adult (2-month-old) mouse heart, and from adult mouse heart one week after transverse aortic constriction (TAC). Mice were housed in a controlled environment on a 12 h light/dark illumination schedule and fed with a chow diet.

TAC surgery was adapted from Rockman HA et al45. Briefly, C57Bl6/J mice were anaesthetized by intraperitoneal injection of a mixture ketamine (100mg/kg) and xylazine (10 mg/kg). The aortic arch was exposed through the 1st intercostal space. A 8-0 suturetightened around a blunted 27-gauge needle (TAC group). The chest cavity was then

closed with 6-0 silk sutures. A separate group of mice underwent the same surgical procedure but without any tightening of the knot (sham group). The pressure load caused by the knot was verified through measurement of the pressure gradient across the aortic constriction with echocardiography (Vevo 2100, VisualSonics).

Mice were sacrificed according to the protocol of the internal ethics committee, the hearts excised, and primary embryonic, neonatal, adult and TAC cardiomyocytes isolated as described elsewhere46, 47.

ChIP-sequencing data processing and analysis. Previously published25 ChIP-Seq datasets from adult and TAC cardiomyocytes were used. Mapped reads were converted from the UCSC mouse genome mm9 build to mm10 using the Batch Coordinate Conversion tool (LiftOver: https://genome.ucsc.edu/cgi-bin/hgLiftOver).

Enriched regions relative to input DNA were detected using the MACS (Model- based Analysis of ChIP-Seq) software48 with the following parameters: --pvalue=1e-3 -- mfold=5.30.

Intragenic levels of H3K79me2, H3K4me3, H3K9ac and H3K27ac were quantified by counting the number of reads falling into RefSeq genes defined by the corresponding annotated transcription start and termination sites. The RPKM (reads per kilobase of transcript per million reads mapped) were calculated based on the read counts overlapping the H3K79me2-, H3K4me3-, H3K9ac- and H3K27ac-enriched regions relative to input DNA. Density plots were generated in R, and Spearman rank coefficient ρ was calculated in R for each plot.

To profile 5-hmC distribution around the H3K79me2, H3K4me3, H3K9ac and H3K27ac MACS peaks identified in adult CMCs, we used the “annoatePeaks” routine in HOMER v4.5, a software suite for ChIP-Seq analysis, with -size 5000 -hist 200 parameters49.

Boxplots of RPKM for genes enriched in 5-hmC and/or the indicated histone markwere generated in R, and the difference in RPKM between groups tested for significance
using the Mann-Whitney test in GraphPad PrismTM.

Preparation of 5-hydroxymethylcytosine DNA immunoprecipitations (hMeDIP) and sequencing.

DNA was isolated from a pool of biological replicates at each experimental point using the QIAamp DNA mini kit (Qiagen). DNA was sonicated to ~150–400 bp (peak of distribution ~250 bp), Illumina adaptors ligated to the ends, and immunoprecipitated with a 5-hmC antibody (Active Motif, AM39791). The IP reaction was performed with Active Motif kit reagents and protocol.

After immunoprecipitation, DNA fragments were amplified with barcoded Illumina primers to generate the final sequencing library. To generate the input libraries, sonicated DNA with ligated adaptors was directly amplified without the immunoprecipitation step. High-throughput sequencing with carried out by Active Motif's epigenetic service (http://www.activemotif.com/catalog/821/medip-sequencing-service) using the Illumina HiSeq 2000 platform.5-hmC data processing and analysis. Raw sequencing reads were mapped to the UCSC mouse genome mm10 build using BWA 0.7.4-r385 (ref 50) with the default parameter setting. Multiple mapped reads were filtered out using the MarkDuplicates module inside

the Picard command-line tools (http://broadinstitute.github.io/picard/). Reads with “mapQ” <10 were filtered out using bamtools API 2.2.3 tool. Detection of 5-hmC- enriched regions relative to input DNA was carried out with the “findPeaks” routine of HOMER v4.5 with the “histone” mode and default parameter values. Analysis of differential 5-hmC regions (DhMRs) was carried out with the “findPeaks” routine in HOMER v4.5, with the “histone” mode and default parameter values. Intragenic levels of 5-hmC were quantified by counting the number of reads falling into RefSeq genes defined by the corresponding annotated transcription start and termination sites. The RPKMs were calculated based on the read counts overlapping the 5-hmC-enriched regions relative to input DNA. 5-hmC coverage profile heatmaps across different genomic regions were obtained with the ngsplot tool v 2.02, considering only enriched regions51. To compute 5- hmC and input DNA coverage across mouse chromosomes for each sample, the number of mapped reads in 100 Kb bins was calculated and normalized for the total number of mapped reads. Boxplots of RPKM for genes enriched in 5-hmC on the TSS or in the GB were generated in R, and the difference in RPKM between groups tested for significance using the Mann-Whitney test in GraphPad PrismTM.

RNA preparation and sequencing.

RNA was isolated using the RNeasy Mini Kit (Qiagen) and treated with DNase according to the manufacturer’s protocol.

Total RNA quality was evaluated with a 2100 Agilent Bioanalyzer and the RNA 6000 Nano kit: only samples with RIN score >7 were processed further. RNA-Seq libraries were created using the Epicentre Script-Seq Complete Gold Kit-Low Input (Epicentre), composed of two modules: the Ribo-Zero Magnetic Kit depletion protocol for rRNA removal and the Script- Seq v2 Kit to prepare the libraries. 150 ng of total RNA was depleted and concentrated with RNeasy MinElute Cleanup Kit (Qiagen). Purified RNA was fragmented and first-strand cDNA synthesized using random hexamer with 5′ tagging sequences. After RNase digestion, a 3′ tag was introduced with random terminal tagging oligos. The obtained double-tagged strand-specific cDNA was purified using AMPure XP beads (Beckman Coulter).

Libraries were enriched and indexed by 15 cycles of PCR amplification (ABI GeneAmp PCR system 9700) using FailSafe PCR enzyme and ScriptSeq Index PCR Primers for Illumina (Epicentre). After purification with AMPure XP beads, library quality and size distribution were checked with an Agilent Bioanalyzer and DNA 1000 chip. All libraries were quantified with a Qubit 2.0 Fluorometer (Life Technologies) using dsDNA BR Assay Kit (Life Technologies). RNA-Seq libraries were sequenced using TruSeq PE Cluster Kit v3 and TruSeq SBS Kit v3 (Illumina). According to index compatibility, the libraries were grouped into 2 pools and clustered on a TruSeq v3 flow cell at a concentration of 9pM to obtain >50 million clusters for each

sample. Paired end sequence reads (50 bp in length) were generated on an Illumina HiSeq 2000.RNA sequencing analysis. Two biological replicates were profiled at each experimental point. The correlation coefficient of the transcriptome between duplicates at the same experimental point ranged from 0.97 to 0.99, indicating a strong reproducibility of the technique. Number of raw and mapped reads are reported in Supplementary Figure 2.

The sequencing reads were processed to remove Illumina barcodes and aligned to the UCSC Mus musculus reference genome (build mm10), using TopHat v2.0.10 (ref 52) with the following parameters: -r 200; --mate-std-dev 150; --library-type fr-firststrand. The reference genome was downloaded from the UCSC site, and the mapped read files processed with the bamtools API 2.2.3 tool with the following parameters: -isMapped true; -isPaired true; -isProperPair. Only reads with "mapQ" ≥20 retained for further analysis. Reads were then processed by Cufflinks v2.1.1 (ref53) with the following parameters: -b; -u; --library-type fr-firststrand; --max-bundle-frags 10000000; --compatible-hits-norm. The RNA expression level of a gene was calculated in RPKM using the Cufflinks package. Differentially expressed genes were identified using the Cuffdiff package with the following parameters: --compatible-hits-norm; -b; u; --library-type fr-firststrand; --max-bundle-frags 10000000. Results of differential analysis are included in Supplementary Table 2. Hierarchical clustering of

the 10,303 differentially expressed genes between the experimental points was carried out and visualized using MeV v4.8.1 (MultiExperiment Viewer software) with complete linkage method. SOTA (Self Organizing Tree Algorithm) analysis was performed with MeV v4.8.1.Annotation of enriched regions. To annotate 5-hmC-enriched regions and ChIP sequencing, we used the “annotatePeaks” routine of HOMER v4.5.

Gene ontology analysis. The Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis software54, 55 was used to find the gene ontology (GO) terms that were significantly enriched (FDR-adjusted, p-value ≤0.05).Repetitive elements analysis. To study genome-wide transcriptional regulation of repetitive elements, we used the RepEnrich tool56. Briefly, the mouse genomic repetitive annotation required by RepEnrich was built and prepared using Repeat Masker elements dataset downloaded from UCSC table browsers. Raw RNA sequencing reads of each sample were mapped to the mouse mm10 genome using bowtie 4.1.2 (ref57) and the custom repetitive annotation prepared above with the following parameters: -m 1; --max. RepEnrich was then run with the number of total mapping reads calculated as number of reads processed minus the number of reads that failed to align.

For differential enrichment analysis of repetitive elements, we used EdgeR (ref58) in the R Bioconductor package.

To determine the correlation of 5-hmC coverage with repetitive element expression, we computed the average number of reads

overlapping all repetitive elements for each replicate normalized for the library size (total number of reads).

To compute the coverage of H3K9me3 over LINEs with or without 5-hmC in TAC cardiomyocytes, we used the ngsplot tool (ref51), considering only H3K9me3 MACS- enriched regions. Boxplots of the median H3K9me3 coverage over LINEs was generated in R, and the difference in values between groups tested for significance with the Mann- Whitney test in GraphPad PrismTM.

Enhancer analysis. To profile 5-hmC distribution in distal regulatory regions identified in the CM stage30 and in adult and TAC cardiomyocytes25, enhancer regions were downloaded and converted from the mouse genome mm9 build to mm10 using the UCSC Batch Coordinate Conversion tool (LiftOver: https://genome.ucsc.edu/cgi-bin/hgLiftOver).

Boxplots of RPKM for genes proximal to enhancers enriched or not in 5-hmC were generated in R, and the difference in RPKM between groups tested for significance with the Mann-Whitney Test in GraphPad PrismTM.Motif Discovery analysis. Motif enrichment analysis within 5-hmC-associated enhancers was carried out with the “findMotifsGenome” routine in HOMER v4.5.

REFERENCES

1. Han, P., Hang, C.T., Yang, J. & Chang, C.P. Chromatin remodeling in cardiovascular development and physiology. Circ Res 108, 378-96 (2011).

2. Papait, R., Greco, C., Kunderfranco, P., Latronico, M.V. & Condorelli, G. Epigenetics: a new mechanism of regulation of heart failure? Basic Res Cardiol 108, 361 (2013).

3. Smith, Z.D. & Meissner, A. DNA methylation: roles in mammalian development. Nat Rev Genet 14, 204-20 (2013).

4. Gilsbach, R. et al. Dynamic DNA methylation orchestrates cardiomyocyte development, maturation and disease. Nat Commun 5, 5288 (2014).

5. Tahiliani, M. et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324, 930-5 (2009).

6. Ficz, G. et al. Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature 473, 398-402 (2011).

7. Pastor, W.A. et al. Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature 473, 394-7 (2011).

8. Gan, H. et al. Dynamics of 5-hydroxymethylcytosine during mouse spermatogenesis. Nat Commun 4, 1995 (2013).

9. Szulwach, K.E. et al. 5-hmC-mediated epigenetic dynamics during postnatal neurodevelopment and aging. Nat Neurosci 14, 1607-16 (2011).

10. Ivanov, M. et al. Ontogeny, distribution and potential roles of 5-hydroxymethylcytosine in human liver function. Genome Biol 14, R83 (2013).
11. Tsagaratou, A. et al. Dissecting the dynamic changes of 5-hydroxymethylcytosine in T- cell development and differentiation. Proc Natl Acad Sci U S A 111, E3306-15 (2014).
12. Wu, H. et al. Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells. Genes Dev 25, 679-84 (2011).
13. Szulwach, K.E. et al. Integrating 5-hydroxymethylcytosine into the epigenomic landscape of human embryonic stem cells. PLoS Genet 7, e1002154 (2011).
14. Yu, M. et al. Base-resolution analysis of 5-hydroxymethylcytosine in the mammalian genome. Cell 149, 1368-80 (2012).
15. Stroud, H., Feng, S., Morey Kinney, S., Pradhan, S. & Jacobsen, S.E. 5- Hydroxymethylcytosine is associated with enhancers and gene bodies in human embryonic stem cells. Genome Biol 12, R54 (2011).
16. Hon, G.C. et al. 5mC oxidation by Tet2 modulates enhancer activity and timing of transcriptome reprogramming during differentiation. Mol Cell 56, 286-97 (2014).
17. Lu, F., Liu, Y., Jiang, L., Yamaguchi, S. & Zhang, Y. Role of Tet proteins in enhancer activity and telomere elongation. Genes Dev 28, 2103-19 (2014).

18. Verma, S.K., Krishnamurthy, P. & Kishore, R. in Manual of Research Techniques in Cardiovascular Medicine 164-169 (John Wiley & Sons, Ltd, 2014).
19. Sun, Z. et al. High-resolution enzymatic mapping of genomic 5-hydroxymethylcytosine in mouse embryonic stem cells. Cell Rep 3, 567-76 (2013).
20. Cattaneo, P. et al. DOT1L-mediated H3K79me2 modification critically regulates gene expression during cardiomyocyte differentiation. Cell Death Differ (2014).
21. Yndestad, A. et al. Systemic inflammation in heart failure--the whys and wherefores. Heart Fail Rev 11, 83-92 (2006).
22. Doenst, T., Nguyen, T.D. & Abel, E.D. Cardiac metabolism in heart failure: implications beyond ATP production. Circ Res 113, 709-24 (2013).
23. Jane-Lise, S., Corda, S., Chassagne, C. & Rappaport, L. The extracellular matrix and the cytoskeleton in heart hypertrophy and failure. Heart Fail Rev 5, 239-50 (2000).
24. Chatham, J.C. & Young, M.E. Metabolic remodeling in the hypertrophic heart: fuel for thought. Circ Res 111, 666-8 (2012).
25. Papait, R. et al. Genome-wide analysis of histone marks identifying an epigenetic signature of promoters and enhancers underlying cardiac hypertrophy. Proc Natl Acad Sci U S A 110, 20164-9 (2013).
26. Haider, S. et al. The landscape of DNA repeat elements in human heart failure. Genome Biol 13, R90 (2012).

27. Carninci, P. et al. The transcriptional landscape of the mammalian genome. Science 309, 1559-63 (2005).
28. Faulkner, G.J. et al. The regulated retrotransposon transcriptome of mammalian cells. Nat Genet 41, 563-71 (2009).
29. Bulut-Karslioglu, A. et al. Suv39h-dependent H3K9me3 marks intact retrotransposons and silences LINE elements in mouse embryonic stem cells. Mol Cell 55, 277-90 (2014).
30. Wamstad, J.A. et al. Dynamic and coordinated epigenetic regulation of developmental transitions in the cardiac lineage. Cell 151, 206-20 (2012).
31. Stadler, M.B. et al. DNA-binding factors shape the mouse methylome at distal regulatory regions. Nature 480, 490-5 (2011).
32. Jin, S.G., Kadam, S. & Pfeifer, G.P. Examination of the specificity of DNA methylation profiling techniques towards 5-methylcytosine and 5-hydroxymethylcytosine. Nucleic Acids Res 38, e125 (2010).
33. Jin, S.G., Wu, X., Li, A.X. & Pfeifer, G.P. Genomic mapping of 5- hydroxymethylcytosine in the human brain. Nucleic Acids Res 39, 5015-24 (2011).
34. Kriaucionis, S. & Heintz, N. The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 324, 929-30 (2009).

35. Hahn, M.A. et al. Dynamics of 5-hydroxymethylcytosine and chromatin marks in Mammalian neurogenesis. Cell Rep 3, 291-300 (2013).
36. Wen, L. et al. Whole-genome analysis of 5-hydroxymethylcytosine and 5- methylcytosine at base resolution in the human brain. Genome Biol 15, R49 (2014).
37. Lister, R. et al. Global epigenomic reconfiguration during mammalian brain development. Science 341, 1237905 (2013).
38. Globisch, D. et al. Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PLoS One 5, e15367 (2010).
39. Khare, T. et al. 5-hmC in the brain is abundant in synaptic genes and shows differences at the exon-intron boundary. Nat Struct Mol Biol 19, 1037-43 (2012).
40. Chen, Q., Chen, Y., Bian, C., Fujiki, R. & Yu, X. TET2 promotes histone O- GlcNAcylation during gene transcription. Nature 493, 561-4 (2013).
41. Deplus, R. et al. TET2 and TET3 regulate GlcNAcylation and H3K4 methylation through OGT and SET1/COMPASS. EMBO J 32, 645-55 (2013).
42. Spruijt, C.G. et al. Dynamic readers for 5-(hydroxy)methylcytosine and its oxidized derivatives. Cell 152, 1146-59 (2013).
43. He, A. et al. Dynamic GATA4 enhancers shape the chromatin landscape central to heart development and disease. Nat Commun 5, 4907 (2014).

44. Wang, Y. et al. WT1 Recruits TET2 to Regulate Its Target Gene Expression and Suppress Leukemia Cell Proliferation. Mol Cell 57, 662-73 (2015).
45. Rockman, H.A., Koch, W.J. & Lefkowitz, R.J. Cardiac function in genetically engineered mice with altered adrenergic receptor signaling. Am J Physiol 272, H1553- 9 (1997).
46. Care, A. et al. MicroRNA-133 controls cardiac hypertrophy. Nat Med 13, 613-8 (2007).
47. Rodgers, L.S., Schnurr, D.C., Broka, D. & Camenisch, T.D. An improved protocol for the isolation and cultivation of embryonic mouse myocytes. Cytotechnology 59, 93-102 (2009).
48. Zhang, Y. et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol 9, R137 (2008).
49. Heinz, S. et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell 38, 576-89 (2010).
50. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25, 1754-60 (2009).
51. Shen, L., Shao, N., Liu, X. & Nestler, E. ngs.plot: Quick mining and visualization of next-generation sequencing data by integrating genomic databases. BMC Genomics 15, 284 (2014).
52. Trapnell, C., Pachter, L. & Salzberg, S.L. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25, 1105-11 (2009).

53. Trapnell, C. et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 28, 511-5 (2010).
54. Huang da, W., Sherman, B.T. & Lempicki, R.A. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37, 1-13 (2009).
55. Huang da, W., Sherman, B.T. & Lempicki, R.A. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4, 44-57 (2009).
56. Criscione, S.W., Zhang, Y., Thompson, W., Sedivy, J.M. & Neretti, N. Transcriptional landscape of repetitive elements in normal and cancer human cells. BMC Genomics 15, 583 (2014).
57. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S.L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10, R25 (2009).
58. Robinson, M.D., McCarthy, D.J. & Smyth, G.K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139- 40 (2010).

CHAPTER 4 SUMMARY, CONCLUSIONS AND FUTURE PERSPECTIVES

Over the last few years, our understanding of the role played by epigenetic modifications in the regulation of myocardial function in health and disease has improved greatly.

DNA methylation is a key element of transcriptional repression; genome-wide studies have indicated that this epigenetic modification could be involved in the pathogenesis of heart failure. However it remains unclear how this epigenetic mark is implicated in cardiac hypertrophy and which proteins govern this modification.

Durind my thesis, by performing massive DNA sequencing on cardiomyocytes isolated from adult mice subjected to transverse aortic constriction (TAC) for one week, we found genome-wide alterations in the DNA methylation profile, and, more specifically, increased DNA methylation at promoters of genes involved in the metabolic remodeling of the hypertrophic heart. Moreover, expression screening of genes involved in DNA modifications revealed that the epigenetic co-factor Uhrf1 was highly re-expressed in hypertrophic cardiomyocytes. UHRF1 epigenetically silenced genes involved in the TCA cycle, OXPHOS/ETC, and ATP synthesis pathways. In addition, hyper-methylated promoters were specifically enriched in binding sites for nuclear respiratory factor-1 (NRF1), a transcription factor co-activated by PGC1a and responsible for transcriptional activation of many genes encoding mitochondrial

proteins. Importantly, we found that methylation prevented the binding of NRF1 to the promoters of several metabolic genes.

Finally, in vivo knock down of Uhrf1 with serotype 9 adeno-associated vectors (AAV9) expressing anti-Uhrf1 shRNAs, prevented the development of cardiac and mitochondrial dysfunction in TAC mice. This is the first study showing a causal role of DNA methylation in the metabolic remodelling of the hypertrophic heart and identifying a key player and its underlying mechanism of action during this process.

Pharmacological modulation of epigenetic mechanisms has a great therapeutic potential: epigenetic therapy in fact is based on the reversibility of the epigenetic profile of cells. Drugs that inhibit the activity of epigenetic enzymes can thus restore a correct gene expression in the affected cells(Karberg, 2009).

The biggest clinical impact of epigenetic drugs has been in the treatment of cancer. The DNA methylation inhibitors 5-azacytidine and decitabine are approved for use in the myelodysplastic syndromes and have improved the survival of patients with this disease(Fenaux et al., 2009). Moreover, several HDAC inhibitors are currently being tested in phase II-III trials, while two of them, vorinostat and romidepsin have been approved for the treatment of progressive or recurrent cutaneous T cell lymphoma (CTCL)(Prince et al., 2009).

Studies conducted on several in vivo models of cardiac hypertrophy and myocardial infarction have suggested HDAC inhibition as a possible therapeutic strategy (Kee et al., 2006;

Kong et al., 2006; Xie et al., 2014) and more recently inhibition of BET with the small molecule JQ1 prevented pathological hypertrophy and improved cardiac dysfunction induced by pressure overload(Anand et al., 2013).

Unfortunately, the ubiquitous nature of epigenetic modifiers and the lack of knowledge of the epigenetic mechanisms involved in cardiovascular disease, currently poses a major challenge to the development of cardiac-specific therapeutic applications. A better comprehension of the intimate, tissue- specific epigenetic mechanisms implicated in cardiovascular pathologies, could thus help overcome these problems and allow the development of more specific therapeutic strategies for heart disease based on the identification of organ-specific targets. On this point, because UHRF1 is absent in post-mitotic cells and in adult normal tissues(Hopfner et al., 2000), it represents an optimal target for the development of a specific inhibitor, which might lead to a new therapeutic avenue for myocardial disease. Epigenetic modifications could also play an important role in the diagnosis of cardiovascular pathologies. Contrarily from the abrupt nature of genetic alterations, epigenetic changes occur gradually. Therefore, epigenetic alterations are a good target for the identification of preventive strategies. However, due to lack in knowledge on the mechanisms involved in the alteration of the epigenetic landscape of cardiomyocytes in heart disease, we are far from a possible clinical application.

Further studies are needed in order to shed light on the events underlying transcriptional alterations in cardiac pathologies.

Historically, DNA methylation was considered a stable epigenetic mark. However, high-resolution genome wide mapping in pluripotent and differentiated cells has uncovered the dynamic nature of DNA methylation. In fact 5-methylcytosine can undergo oxidation to 5-hydroxymethylcytosine (5-hmC), catalysed by ten–eleven translocation (TET) enzymes. 5-hmC is not only an intermediate of DNA demethylation but additionally a stable epigenetic mark able to influence gene expression by attracting or precluding the binding of specific proteins. While in embryonic stem cells and cancer the role of 5-hydroxymethylation in modulating gene expression programs has been extensively investigated, whether this epigenetic modification is of relevance in myocardial physiology and disease is still unknown.

We performed hydroxymethylated DNA immunoprecipitation coupled with high-throughput sequencing (hMeDIP-seq) in cardiomyocytes to elucidate the function of this modification in this cell type in normal state and under stress, in particular cardiac hypertrophy induced by pressure overload. Our data indicate that the presence of 5-hmC within the gene-body strongly correlates with gene expression and cooperates with activating histone marks marking cardiac specific genes.

Moreover, during hypertrophy a profound genomic re-distribution of 5-hmC on distal regulatory regions such as enhancers and repetitive elements takes place. We then dissected the functional role of enhancer-associated hydroxymethylation in hypertrophy, defining a subset strongly enriched for 5-hmC. Enhancers harboring 5-hmC strongly influenced the expression of the associated genes and were found in the proximity of many transcription factors known to play a pivotal role in cardiac cells (i.e. Mef2c, Hif1a, Nkx2.5, Hand2).

These results provide the first genome-wide analysis of 5-hmC in cardiac cells and strongly support a regulatory function of this epigenetic modification in the specification of the transcriptional profile of cardiomyocytes.

In summary, this thesis identifies DNA methylation and the recently discovered DNA hydroxymethylation, as a new level of regulation of cardiac gene expression, opening the field to the investigation of novel mechanisms governing cardiac transcription regulation.

REFERENCES

Anand, P., Brown, J.D., Lin, C.Y., Qi, J., Zhang, R., Artero, P.C., Alaiti, M.A., Bullard, J., Alazem, K., Margulies, K.B., et al. (2013). BET bromodomains mediate transcriptional pause release in heart failure. Cell 154, 569-582.Fenaux, P., Mufti, G.J., Hellstrom-Lindberg, E., Santini, V., Finelli, C., Giagounidis, A., Schoch, R., Gattermann, N., Sanz, G., List, A., et al. (2009). Efficacy of azacitidine compared with that of conventional care regimens in the treatment of higher- risk myelodysplastic syndromes: a randomised, open-label, phase III study. The Lancet. Oncology 10, 223-232.

Hopfner, R., Mousli, M., Jeltsch, J.M., Voulgaris, A., Lutz, Y., Marin, C., Bellocq, J.P., Oudet, P., and Bronner, C. (2000). ICBP90, a novel human CCAAT binding protein, involved in the regulation of topoisomerase IIalpha expression. Cancer research 60, 121-128.

Karberg, S. (2009). Switching on epigenetic therapy. Cell 139, 1029-1031.Kee, H.J., Sohn, I.S., Nam, K.I., Park, J.E., Qian, Y.R., Yin, Z., Ahn, Y., Jeong, M.H., Bang, Y.J., Kim, N., et al. (2006). Inhibition of histone deacetylation blocks cardiac hypertrophy induced by angiotensin II infusion and aortic banding. Circulation 113, 51-59.Kong, Y., Tannous, P., Lu, G., Berenji, K., Rothermel, B.A., Olson, E.N., and Hill, J.A. (2006). Suppression of class I and II histone deacetylases blunts pressure- overload cardiac hypertrophy. Circulation 113, 2579-2588.

Prince, H.M., Bishton, M.J., and Harrison, S.J. (2009). Clinical studies of histone deacetylase inhibitors. Clinical cancer research : an official journal of the American Association for Cancer Research 15, 3958-3969.Xie, M., Kong, Y., Tan, W., May, H., Battiprolu, P.K., Pedrozo, Z., Wang, Z.V., Morales, C., Luo, X., Cho, G., et al. (2014). Histone deacetylase inhibition blunts ischemia/reperfusion injury by inducing cardiomyocyte autophagy. Circulation 129, 1139-1151.

PUBLICATIONS

"Aliskiren reduces prorenin receptor expression and activity in cultured human aortic smooth muscle cells." Ferri N, **Greco CM**, Maiocchi G, Corsini A. **J Renin Angiotensin Aldosterone Syst. 2011**

"Chemotactic effect of prorenin on human aortic smooth muscle cells: a novel function of the (pro)renin receptor." **Greco CM**, Camera M, Facchinetti L, Brambilla M, Pellegrino S, Gelmi ML, Tremoli E, Corsini A, Ferri N. **Cardiovasc Res**. **2012**

"Epigenetics: a new mechanism of regulation of heart failure?" Papait R, **Greco C**, Kunderfranco P, Latronico MV, Condorelli G. **Basic Res Cardiol**. **2013**

“Genome-wide analysis of histone marks identifying an epigenetic signature of promoters and enhancers underlying cardiac hypertrophy.” Papait R, Cattaneo P, Kunderfranco P, **Greco C**, Carullo P, Guffanti A, Viganò V, Stirparo GG, Latronico MV, Hasenfuss G, Chen J, Condorelli G. **Proc Natl Acad Sci U S A. 2013**

“DOT1L-mediated H3K79me2 modification critically regulates gene expression during cardiomyocyte differentiation.” Cattaneo P, Kunderfranco P, **Greco C**, Guffanti A, Stirparo GG, Rusconi F, Rizzi R, Di Pasquale E, Locatelli SL, Latronico MV, Bearzi C, Papait R, Condorelli G. **Cell Death Differ. 2014**

“PTX3 is an extrinsic oncosuppressor regulating complement-dependent inflammation in cancer.” Bonavita E, Gentile S, Rubino M, Maina V, Papait R, Kunderfranco P, **Greco C**, Feruglio F, Molgora M, Laface I, Tartari S, Doni A, Pasqualini F, Barbati E, Basso G, Galdiero MR, Nebuloni M, Roncalli M, Colombo P, Laghi L, Lambris JD, Jaillon S, Garlanda C, Mantovani A. **Cell**. **2015**

“Epigenetic modifications and noncoding RNAs in cardiac hypertrophy and failure.” **Greco CM**, Condorelli G. **Nat Rev Cardiol**. **2015**