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Epigenetics in Cardiac Disease

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Epigenetics in Cardiac Disease

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Preface

Cardiovascular disease is the leading cause of death worldwide. This book focuses on heart muscle disease, with an emphasis on heart failure, which is a syndrome defined by the heart's inability to adequately pump blood to supply the body's needs. Risk factors for the development of heart failure include coronary artery disease (CAD), high blood pressure, diabetes, and aging. Based on current guidelines, most patients with heart failure are treated with three classes of drugs: β-blockers, drugs targeting the angiotensin II type-I receptor (ACE inhibitors or ARBs), and a mineralocorticoid antagonist. Collectively referred to as neurohormonal blockade, this treatment regimen has clearly been shown to improve outcomes in heart failure patients. Furthermore, LCZ696, which is a dual angiotensin receptor blocker/neprilysin inhibitor, was recently found to be efficacious in a clinical trial with heart failure patients. Indeed, the primary outcome of cardiovascular death or a first hospitalization for heart failure occurred in 21.8 % patients treated with LCZ696, compared with 26.5 % of patients in the control treatment arm. While these findings are encouraging, they also serve to illustrate that disease progression persists in many patients treated with the full armamentarium of heart failure drugs, including LCZ696. Thus, heart failure remains a major unmet medical need, and the elucidation of novel mechanisms involved in heart failure pathogenesis holds promise for identifying new therapies for this prevalent and deadly syndrome. This book focuses on the role of epigenetics and chromatin remodeling for heart muscle disease.

The basic unit of chromatin is the nucleosome, which is a histone octamer wrapped in a 147-bp stretch of DNA. Modifications of nucleosomal DNA or protein, without changes to the underlying nucleotide sequence, can have a profound effect on gene expression. This mode of gene regulation is referred to as epigenetics. Based on data that are extensively reviewed in this book, we strongly believe that epigenetics represents a crucial, untapped reservoir for heart failure drug discovery and development. Our position is founded on the fact that epigenetic regulators function as "nodal" points that integrate upstream signals to convey a common pathway for heart failure pathogenesis. Furthermore, many epigenetic regulators have proven to be ideally suited for small molecule-mediated manipulation, and a multitude of epigenetic therapies are approved or are being tested for the treatment of noncardiac indications in humans, establishing the feasibility of this approach.

The book covers findings that validate roles for fundamental epigenetic processes (e.g., DNA methylation, histone acetylation, and histone methylation) in the control of heart failure. Furthermore, several chapters delve into chromatin signaling and nongenomic roles for classical epigenetic effectors. For example, it is now clear that histone deacetylases (HDACs) have many nonhistone substrates, and deacetylation of these substrates has profound effects on fundamental cellular processes other than transcription. Finally, the book concludes with a discussion of a newly emerging class of epigenetic regulators referred to as long nonprotein-coding RNAs (lncRNAs), which influence gene expression by altering chromatin organization and function.

We are grateful to the authors of the outstanding chapters in this book. The book illustrates that work done by the field over the last 15 years has significantly advanced our understanding of the role of epigenetics in the pathogenesis of heart failure. However, we expect the next 10 years to be a truly golden era for researchers in this field, where fundamental mechanistic studies are bridged with state-of-the-art drug discovery approaches to yield innovative 'epigenetic therapies' to combat the worldwide epidemic of heart failure.

Timothy A. McKinsey and Johannes Backs

The original version of the book was revised. The correction to the book is available at https://doi.org/10.1007/978-3-319-41457-7_14

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Epigenetics: Chromatin Organization and Function

1

Genevieve P. Delcuve, Dilshad H. Khan, Vichithra R.B. Liyanage, Sanzida Jahan, Mojgan Rastegar, Lorrie A. Kirshenbaum, and James R. Davie

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Abstract

Epigenetics refer to processes such as histone post-translational modifications (PTMs), DNA methylation and RNA that regulate gene activity and expression but are not dependent on alterations in DNA sequence. Herein, we review histone PTMs, histone variants and DNA modifications in the functioning of the nucleosome as an epigenetic signalling module. The majority of the human genome is transcribed, with most of the genome producing non-coding RNA, some of which is a component of the nuclear matrix, a dynamic RNA protein nuclear sub-structure. Non-coding RNA and coding RNA are associated with epigenetic modifiers, architectural chromatin proteins, coactivators and corepressors. The impact of changes in DNA sequence (single nucleotide polymorphisms) on the epigenome is discussed.

1.1 Basic and Higher-Order Levels of Chromatin Structure

In a mammalian nucleus, the bulk of genomic DNA is condensed into higher-order chromatin structures. The basic level of chromatin organization consists of a nucleosome array, the 11-nm ‘beads-on-a-string’ fibre. The nucleosome consists of a histone octamer, arranged as a $(H3–H4)_2$ tetramer and two H2A–H2B dimers, around which a 147-bp stretch of DNA is wrapped in almost two left-handed superhelical turns. Nucleosomes are joined by a 20–50-bp stretch of DNA called linker DNA. Core histones are primarily globular, containing a histone-fold structure, except for their highly positively charged N-terminal tails, which emanate from the nucleosome in all directions to interact with linker DNA, nearby nucleosomes or other proteins (Luger et al. 1997; Luger and Richmond 1998). Linker histones, H1/H5, bind to the DNA entry/exit points of nucleosomes and to the linker DNA region between nucleosomes and are crucial for the formation of higher-order chromatin structure (Felsenfeld and Groudine 2003; Li and Reinberg 2011).

Except for H4, all histones have variants, some of which are expressed at the time of DNA synthesis (H3.1, H2A.1, replication-dependent) and others expressed throughout the cell cycle (H2A.Z, H3.3, replication-independent) (Maze et al. 2014; Law and Cheung 2013). The insertion of histone variants affects chromatin structure and function (Talbert and Henikoff 2010). H3.3 is mostly deposited during transcription and is enriched at active genes, promoters and regulatory elements. Accordingly, H3.3 is enriched in active marks. However, H3.3 is also present at telomeres where it is required for the transcriptional repression of telomeric repeats. Different histone chaperones are involved in the deposition of H3.3 at different genomic loci (Elsaesser et al. 2010; Goldberg et al. 2010; Szenker et al. 2011). Similarly, the outcome of H2A.Z incorporation is context-dependent (Altaf et al. 2009; Draker et al. 2012). H2A.Z stabilizes nucleosome structure and facilitates chromatin compaction (Li et al. 1993; Chen et al. 2013a, b). The abundance of the replacement histones (e.g. H3.3) increases in differentiated non-cycling cells (e.g. cardiomyocytes) and in cells as they age (Wunsch and Lough 1987; Rogakou and Sekeri-Pataryas 1999; Feser and Tyler 2011; Pina and Suau 1987). Of note, while

specific antibodies can be raised against H2A.Z, this is not the case with H3.3. Therefore, studies on H3.3 have used exogenously expressed tagged H3.3, which raises the concern of results being skewed as a consequence of H3.3 overexpression. However, this caveat has been avoided in a recent study of genome-wide profiles of H3 variants by the use of genome editing with zinc-finger nucleases to tag endogenous H3.3 (Goldberg et al. 2010). Only some of the variants are mentioned here. A histone sequence database is available (Marino-Ramirez et al. 2011).

Histone PTMs and DNA methylation are two epigenetic mechanisms involved in the regulation of gene expression (Capell and Berger 2013; Delcuve et al. 2009). Epigenetics is defined as modifications in gene function without a change in DNA sequence. Histones undergo at least eight different classes of reversible PTMs, including acetylation, methylation, ubiquitination and phosphorylation, at many sites mostly on their N-terminal tails, but also in their globular domains (Mersfelder and Parthun 2006; Kouzarides 2007; Taverna et al. 2007; Cosgrove 2007; Huang et al. 2014). These PTMs alter chromatin structure and/or provide a 'code' for the recruitment or occlusion of proteins to chromatin (Gardner et al. 2011). Thus, much more than a unit with a structural role, the nucleosome is a signalling module, which regulates transcription and other genomic functions (Turner 2014).

An under-researched area concerns the composition of individual nucleosomes with regard to histone variants and PTMs. Mononucleosomes contain two copies of each of the four core histones. Are both H3s modified exactly the same? Do specific variants or PTMs occur together in a nucleosome? The early ChIP studies isolated di- and tri-nucleosomes with highly acetylated H4 or highly acetylated H3 (Myers et al. 2001). In these short chromatin fragments, both H3 and H4 were highly acetylated. Cross-linking studies have shown that uH2A and uH2B are together in nucleosomes associated with transcription-active chromatin (Li et al. 1993). Analyses of H3K27me2/3-modified mononucleosomes demonstrated that the two H3s were modified symmetrically and asymmetrically with the other H3 being monomethylated at H3K27 or unmodified at this site (Voigt et al. 2012). Although the H3 kinase, mitogen- and stress-activated kinase, phosphorylates H3 at S10 and S28, stretches of di- and tri-nucleosomes have either H3 modified at S10 or S28 (Dyson et al. 2005). It is not known, however, if both H3s in a nucleosome are symmetrically phosphorylated at S10.

From a simplistic point of view, chromatin is described by its state of condensation and is divided into heterochromatin (repressive state) and euchromatin (active state) (Jenuwein and Allis 2001; Trojer and Reinberg 2007). Euchromatin is characterized by low compaction, the presence of specific PTMs (active marks) and non-canonical histone variants, as well as accessibility to transcriptional regulators. In contrast, heterochromatin is more condensed, rich in repetitive sequences and is associated with a set of PTMs and histone variants regarded as repressive (Peterson and Laniel 2004; Jin et al. 2005; Sims and Reinberg 2006; Jin and Felsenfeld 2006; Loyola and Almouzni 2007).

Further, the vertebrate genome is organized into chromosomal domains, and specialized elements called barrier (insulator) elements are located at the boundaries of the domains. Barrier elements block the spread of heterochromatin into active

chromatin domains (Dixon et al. 2012; Phillips-Cremins and Corces 2013). A general feature of transcriptionally active/competent chromatin is its sensitivity to digestion by the nuclease DNase I. Both histone acetylation and torsional stress are contributing factors to the DNase I sensitivity of transcriptionally active chromatin (Villeponteau et al. 1984; Villeponteau and Martinson 1987). Studies initiated in chicken erythrocytes demonstrated that DNase I sensitivity was not necessarily confined to the transcribed gene but ranged from 15 to 100 kb in length and contained one or several genes. The boundaries of where the DNase I sensitivity was absent defined the length of the chromosomal domain (for review, see (Jahan and Davie 2014)). Using a chromosome conformation capture protocol called Hi-C, Bing Ren and colleagues demonstrated that the mammalian genome is organized into topologically associated domains (Dixon et al. 2012). The boundaries of these domains contained insulator-binding protein CCCTC-binding factor (CTCF), cohesin complex, housekeeping genes, transfer RNAs, short interspersed element (SINE) retrotransposons and double-strand break repair protein rad21 homologue (RAD21) (Dixon et al. 2012; Zuin et al. 2014). CTCF, which is referred to as an architectural protein, is associated with the cohesion complex and the nuclear matrix (Ong and Corces 2014; Dunn et al. 2003). CTCF plays a key role in enhancer-promoter interactions. Boundary formation requires several proteins in addition to CTCF, possibly TFIIIC (transcription factor for RNA polymerase III C), cohesion and condensin (Phillips-Cremins and Corces 2013). Disruption of boundary element or insulator may result in disease (Ibn-Salem et al. 2014; Phillips-Cremins and Corces 2013).

It has been widely assumed that higher levels of compaction are achieved through the formation of a 30-nm fibre (Li and Reinberg 2011). However, recent evidence suggests that the 30-nm chromatin fibre does not exist *in vivo* (Eltsov et al. 2008; Dekker 2008; Ahmed et al. 2009, 2010; Lieberman-Aiden et al. 2009; Maeshima et al. 2010). Instead, the various models proposed for higher-order chromatin arrangements imply the dense packing of only 11-nm chromatin fibres (Fussner et al. 2011, 2012; Nishino et al. 2012; Hubner et al. 2013). While chromatin readily folds into 30-nm fibres under physiological conditions *in vitro*, we hypothesize that *in vivo* chromatin must be in a high-energy state, which prevents the formation of the preferred 30-nm fibre, possibly through anchorage to the nuclear matrix including the nuclear lamina.

In recent years, an increasing diversity of non-coding RNAs (ncRNA) has been shown to play a role in the regulation of chromatin states. In particular, specific long ncRNAs (lncRNAs) serve as scaffolds, assembling chromatin-modifying complexes, and direct them to target sites by RNA–DNA interactions. For example, XIST (X-inactive-specific transcript) RNA initiates and maintains heterochromatin formation in the inactivation of the X chromosome (Turner and Morris 2010; Mattick 2011; Geisler and Coller 2013). What is more, a recent study showed that non-coding, repetitive RNAs, some derived from long interspersed nuclear elements L1 (LINE1), are stably associated with euchromatin and copurify with the nuclear matrix (Hall et al. 2014). It was suggested that these RNAs impact the interphase chromosome architecture, helping to maintain an open chromatin configuration (Hall et al. 2014; Nozawa and Gilbert 2014).

1.2 Nucleosome Dynamics and Gene Regulation

Nucleosomes limit access to DNA and thus serve as negative regulators, and their positioning and occupancy play an important role in the regulation of gene expression (Segal and Widom 2009). A recent study examining nucleosome positioning and gene expression in single cells revealed a significant level of heterogeneity within a cell population, and indicated a correlation between nucleosome occupancy and gene expression (Small et al. 2014). This study highlights the underlying complexity and dynamic nature of nucleosome positioning.

What determines nucleosome positioning, however, is poorly understood. The current view is that nucleosome positioning and occupancy are governed by the dynamic interplay of several factors, namely DNA sequence properties (including DNA methylation), cell-specific trans-acting regulatory proteins, histone-modifying enzymes and nucleosome remodellers (Bell et al. 2011; Jiang and Pugh 2009; Radman-Livaja and Rando 2010; Valouev et al. 2011; Zhang et al. 2011; Zhang and Pugh 2011). The next paragraph presents a brief summary of chromatin remodelling, whereas DNA methylation and histones PTMs are discussed in more detail in the following chapters.

Chromatin remodellers, found in multiprotein complexes, use the energy of adenosine triphosphate (ATP) hydrolysis to reshape the chromatin environment. Depending on the domain(s) located next to the ATPase domain of their catalytic subunit, remodellers are grouped into four families: SWI/SNF (bromodomain and helicase-SANT domain), ISWI (SANT-SLIDE domain), CHD (tandem chromodomains) and INO80 (helicase-SANT domain) (Clapier and Cairns 2009; Hargreaves and Crabtree 2011). Remodellers move or evict nucleosomes to establish regular spacing or to provide or deny accessibility of transcription factors to DNA. Some remodellers also restructure nucleosomes by promoting the exchange of nucleosomal histones with their variants, imparting different properties to nucleosomes (Clapier and Cairns 2009). Furthermore, mammalian BAF complexes (of the SWI/SNF family) also repress transcription through long-range interactions from distal regulatory sites (Ho et al. 2009; Hargreaves and Crabtree 2011).

The different remodellers share a wide range of biological functions, but some also specialize in specific processes, for example, SWR1 is involved in variant H2A.Z incorporation (Krogan et al. 2003; Kobor et al. 2004; Mizuguchi et al. 2004), yeast ISW2 prevents transcription initiation from cryptic sites (Whitehouse et al. 2007). Depending on the developmental context, BAF complexes can either activate or repress the transcription of the same gene (Chi et al. 2003). Contrary to yeast SWI/SNF remodellers, mammalian BAF complexes are polymorphic and thus attain more functional diversity through combinatorial assembly. BAF-selective assemblies contribute to cell fate decisions as they correlate with lineage-specific gene expression programmes (Ho and Crabtree 2010).

Remodellers act in concert with histone-modifying complexes and are recruited to specific nucleosomes by transcription factors or histone PTMs. There is evidence that DNA features (sequence, unusual structures like quadruplexes or methylation) as well as RNA are also involved in the targeting of remodellers (Erdel et al. 2011).

Histone PTMs are recognized by specific protein domains sometimes called reader modules (Taverna et al. 2007; Yun et al. 2011). Moreover, multivalent interactions are thought to occur between combinations of histone PTMs and remodelling complexes presenting different reading domains (Ruthenburg et al. 2007).

Also cooperating with chromatin remodellers and histone modifiers are histone chaperones, which facilitate nucleosome assembly/disassembly and histone exchange (De Koning et al. 2007; Das et al. 2010; Burgess and Zhang 2013). The role of histone chaperones has recently been reviewed, with an emphasis on their crosstalk with histone PTMs (Avvakumov et al. 2011). Furthermore, Biochim Biophys Acta published a special issue entitled 'Histone chaperones and Chromatin assembly' in March–April 2013.

Until recently, the bulk of research on the role of chromatin structure in the regulation of gene expression in response to extracellular and intracellular stimuli has focused on the recruitment of RNA polymerase II (RNAPII) to promoters and consequent transcription initiation. However, there is increasing evidence pointing to a precise regulation of elongation rates. First, RNAPII pausing immediately downstream of promoters is a prevalent regulation mechanism in higher eukaryotes, with differences across different genes in the rate of release of paused RNAPII. Then, there is variability in RNAPII elongation rates along a single gene as well as across the genome. Elongation rates are regulated through the dynamic interplay between histone PTMs, chromatin remodellers, histone chaperones, incorporation of histone variants into nucleosomes and DNA-binding proteins to generate discrete chromatin landscapes (Petesch and Lis 2012; Adelman and Lis 2012; Danko et al. 2013; Kwak and Lis 2013; Kwak et al. 2013; Weber and Henikoff 2014; Jonkers et al. 2014).

Alternative RNA splicing, which is widespread in multicellular eukaryotes, is another level where the chromatin state regulates gene expression. Recent studies indicate that co-transcriptional-splicing decisions correlate with the rates of RNAPII elongation through splice junctions. Indeed, RNAPII tends to slow down through splicing sites at spliced exons, but not at skipped exons (Khan et al. 2012; Kornblihtt et al. 2013; Kwak et al. 2013; Acuna and Kornblihtt 2014; Jonkers et al. 2014). However, exceptions have been reported (Jonkers et al. 2014; Dujardin et al. 2014). Thus, splicing decisions are probably the consequences of splicing complex assembly, RNA secondary and tertiary structure formation, and regulatory factor binding as well as chromatin state.

1.3 Histone PTMs

Histones undergo a wide variety of reversible covalent PTMs that regulate the structure and function of chromatin in a context-dependent manner and thereby regulate various biological processes (Kouzarides 2007; Zlatanova et al. 2009). These modifications occur primarily within the N-terminal tails of histones protruding from the surface of the nucleosomes as well as on the globular domains (Kouzarides 2007; Cosgrove and Wolberger 2005; Jack and Hake 2014). So far with the usage of mass

spectrometry, antibody-based and metabolic-labelling techniques, more than 70 different sites for PTMs and several types of histone PTMs have been described (Young et al. 2009; Kouzarides 2007). These include lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination and sumoylation, ADP ribosylation, glycosylation, carbonylation and many others (Huang et al. 2014). These modifications have a major influence on chromatin structure by affecting the local environment, facilitating the binding of transcription factors that regulate gene expression or allowing the interaction with various chromatin remodelling enzymes (Kouzarides 2007; Berger 2007).

Given the number of sites, the wide array of possible histone PTMs and their involvement in different cellular processes, the 'histone-code hypothesis' has been proposed (Rothbart and Strahl 2014; Strahl and Allis 2000). The code postulated that these PTMs on the same or different histone tails may be interdependent and can generate various combinations of PTMs on a single nucleosome. Further, distinct histone PTMs may culminate in specific landscape that can render the interaction sites for different chromatin-binding proteins (Rothbart and Strahl 2014). For example, many chromatin-associated proteins contain the bromodomains or chromodomains, which recognize acetylated lysines or methylated lysine residues, respectively. However, the role of a specific or a combination of histone PTMs may be different pending on the cellular context or gene of interest.

It has been long known that histone PTMs exert profound control over a variety of nuclear processes, including gene transcription, DNA repair, DNA replication and chromosome segregation (Dawson and Kouzarides 2012). However, the functional significance of each PTM, particularly histone acetylation and methylation in other regulatory processes, for example, in facilitating pre-mRNA splicing, alternative splicing, RNA stability or editing, is just beginning to come to light (Hnilicova et al. 2011; Hnilicova and Stanek 2011; Zhou et al. 2014; Delcuve et al. 2012; Gomez Acuna et al. 2013). A number of methods are applied to determine the function of a histone PTM including chromatin reconstitution with a specific histone PTM (e.g. H4K16ac) (Shogren-Knaak et al. 2006); affinity methods to identify a reader of the histone PTM (14-3-3 binding to H3S10ph) (Winter et al. 2008) and knockout, knockdown or application of CRISPR technology to reduce or eliminate the expression (or use of specific enzyme inhibitors) of an enzyme that catalyses a specific histone PTM (e.g. mitogen- and stress-activated protein kinases and H3S10ph) (Soloaga et al. 2003). ChIP assays with antibodies to specific histone PTMs will determine the genomic location of a histone PTM and provide hints at what the function of that histone PTM might be but other assays are required to determine the biological role of the histone PTM.

Aberrations in histone PTMs frequently occur in cancer and other diseases, the nature of which could be therapeutically exploited (Dawson and Kouzarides 2012; Zane et al. 2014; Hattori and Ushijima 2014). Therefore, expanding the understanding of histone PTMs in various regulatory processes will have a powerful impact on identification and development of new therapeutic targets in diseases and cancers, in addition to deciphering their mechanistic role in chromatin architecture and function.

1.3.1 Histone Acetylation

One of the most extensively studied histone PTMs is acetylation, which occurs at the ε-amino groups of lysine residues within the core histones. The 50 years of histone acetylation and the seminal contributions of Vincent Allfrey were recently celebrated at a Conference on Epigenetics and Cancer in October (http://www.bdebate.org/en/forum/50-years-histone-acetylation-barcelona-conference-epigenetics-and-cancer). Acetylation is a reversible, dynamic process, which is regulated by two classes of antagonizing histone-modifying enzymes, lysine acetyltransferases (KATs) and histone deacetylases (HDACs), which add or remove acetyl groups to/from lysine residues within the N-terminal tails of target histones, respectively. The global acetylation level of histones influences chromatin conformation and affects the accessibility of transcription factors and effector proteins to the DNA, thereby modulating gene expression. KATs and HDACs are associated with the nuclear matrix, likely in complexes containing the transcription apparatus (Hendzel et al. 1991, 1994). Interestingly, the treatment of the nuclear matrix with RNase releases the HDACs from the residual nuclear matrix (largely the nuclear pore complex and lamin). This observation suggests that the nuclear matrix-bound HDAC may be associated with the nuclear matrix-associated RNA (Khan et al. 2014; Hall et al. 2014).

Histone acetylation promotes increased transcriptional activity by two well-defined mechanisms. Firstly, lysine acetylation by KATs neutralizes the positive charge of the histone tails and reduces the affinity of histones for the negatively charged DNA, thus weakening histone–DNA or nucleosome–nucleosome interactions as well as inducing a conformation change. This results in destabilizing and loosening of nucleosome and chromatin structure, enables the transcriptional machinery to access the DNA and enhances gene transcription. Conversely, deacetylation by HDACs removes the acetyl group from the histone tails, presumably restores the positive charge to the specific lysine residue, thereby increasing the interaction of histones with negatively charged DNA, and thus reverses the effects of KATs and alters transcription. The role of these enzymes in transcription, their recruitment mechanisms to the regulatory elements of genes as well as their compositions in multiprotein complexes are well documented (Thompson et al. 2013; Delcuve et al. 2013). Overall, the acetylation levels of histones result from the interplay between histone acetylation and deacetylation reactions catalysed by KATs and HDACs. Secondly, histone acetylation can act as recognition-docking sites for bromodomain containing effector proteins that can interact with the modified residues (Marmorstein and Zhou 2014; Sanchez et al. 2014). Subsequently, the recruited effector proteins modulate DNA transcription.

One of the hallmarks of transcriptionally active genes is the presence of acetylated histones predominantly at upstream-promoter regions (UPR), indicating that the level of acetylation corresponds to the rate of transcription. However, histone acetylation also occurs throughout transcribed gene body regions, suggesting that histone acetylation is involved in transcription elongation. In human CD4+ T-cells, with chromatin immunoprecipitation sequencing (ChIP-seq), it has been reported

that acetylation of K9, K18, K27 and K36 of H3 is predominantly found at UPR, while acetylation of K14 and K23 of H3, which also localized at promoters, extended significantly downstream of the promoters to the transcribed units (Wang et al. 2009). Similar patterns were observed with acetylated H4, acetylated H4K5 and H4K8 being localized at promoters, while acetylated H4K12 was detected throughout the gene body. Furthermore, several KATs and HDACs are also identified in UPR and in the coding regions of transcribed genes (Wang et al. 2009).

KATs and HDACs are typically recruited to regulatory regions of genes in large multi-protein complexes. For example, HDAC1 and HDAC2 are present in the Sin3A, NuRD and CoREST complexes (Delcuve et al. 2013). HDAC1 and HDAC2 need to be phosphorylated at sites located in C-terminal part of the protein to form these complexes. Preventing the protein kinase CK2-mediated phosphorylation of HDAC1 or HDAC2, also precipitously reduces the activity of the enzyme (Delcuve et al. 2013). Presumably, the organization/structure of phosphorylated HDAC1/2 in these complexes stimulates HDAC activity. When the KAT and HDAC complexes are recruited together to the regulatory regions of genes, then dynamic acetylation of the histones and non-histone proteins in those regions will occur. However, a change in the balance of KATs to HDACs or change in activity of the KAT or HDAC will result in a shift in the acetylation state of the histones as well as other acetylated proteins such as transcription factors. For example, in cardiac myocytes, HDAC1 interacts with the transcription factor nuclear factor kappa B (NF-kB), which has an important role in regulating genes supporting cardiac myocyte survival (Shaw et al. 2006). HDAC1 deacetylates NF-kB, reducing its transcriptional activity. Acetylation and increased transcriptional activity are required in the tumour necrosis factor alpha (TNFα) pathway for cardiac myocyte survival. Interestingly, TNFα reduced HDAC activity but not the enzyme's nuclear location. Possibly, the TNFα-signalling pathway reduces HDAC1/2 phosphorylation (Gang et al. 2013, 2011). For substrate specificity of KATs and HDACs, see Anamika et al. (2010), Berndsen and Denu (2008), Li et al. (2014), Moser et al. (2014), Feldman et al. (2012), Riester et al. (2007), Yao and Yang (2011).

Although the functional significances of the KATs and HDACs in the gene body are not very well defined, there are several reports indicating their role in transcription regulation. Moreover, the loss of HDAC activity caused the transcription initiation from cryptic intragenic promoters in yeast (Carrozza et al. 2005). Interactions between KATs and components of the transcription elongation machinery have also been reported (Wery et al. 2004). Moreover, the role(s) of histone acetylation in other cellular processes, such as in co-transcriptional-splicing mechanisms, RNA processing and editing, are recently emerging, indicating their role(s) in diverse biological processes, yet to be discovered.

Studies with chicken erythrocytes have shown that transcribed regions of the genome are undergoing high rates of acetylation and deacetylation that are not present at other regions of the erythrocyte genome (Zhang and Nelson 1988a, b). In the first direct demonstration of acetylated histones being bound to competent/transcribed DNA, Colyn Crane-Robinson and colleagues reported that highly acetylated H4 was not only bound to the transcribed β-globin gene in 15-day chicken

embryo erythrocytes but was located along the entire 33-kb DNase I-sensitive β-globin domain (Hebbes et al. 1994). In contrast, highly acetylated H3 and H4 were located primarily at the 5′ regulatory region, which contained a CpG island, of housekeeping genes (e.g. *GAPDH*) (Myers et al. 2001). The understudied highly acetylated H2B was associated with the β-globin domain but not with the regulatory regions of the housekeeping genes (Myers et al. 2003). Interestingly, H2B undergoes the highest rate of acetylation/deacetylation of the core histones in chicken immature erythrocytes (Zhang and Nelson 1988a, b). The highly acetylated state of the histones associated with transcribed genes alters the capacity of histone H1/H5 to render the chromatin containing these genes insoluble at physiological ionic strength (Ridsdale et al. 1990). This feature of the chicken erythroid-transcribed chromatin made it possible to isolate transcriptionally active chromatin from these G0-phase non-replicating cells (Delcuve and Davie 1989). The salt-soluble, active gene-enriched polynucleosomes were associated with H3K4me2/3, uH2B, newly synthesized H2A/H2B (via histone exchange) and atypical nucleosomes (Locklear et al. 1990). There will be more mentioned about the U-shaped nucleosome in the section on nucleosomes and transcription.

1.3.2 Histone Phosphorylation

Phosphorylation on serine or threonine residues of the N-terminal of all core histones has been reported with implications in several biological processes such as mitosis/meiosis, DNA-damage repair, transcriptional induction, apoptosis and heterochromatin formation (Perez-Cadahia et al. 2010; Sawicka and Seiser 2012). Among the core histones, phosphorylation of H3 is very well studied and the conserved phospho-residues of H3 are Thr3, Ser10, Thr11 and Ser28 (Cerutti and Casas-Mollano 2009). All of these phospho-marks are constituents of condensed mitotic chromatin. However, phosphorylated H3Ser10 and Ser28 (referred to as inducible PTMs) are also involved in transcriptional activation of specific genes (immediate early genes) during interphase in response to various stimuli, such as mitogens, growth factors, stress, ultraviolet (UV) radiation and cytokines (Clayton et al. 2000; Cerutti and Casas-Mollano 2009; Khan et al. 2013; Healy et al. 2012). Overall, the effect of H3 phosphorylation on chromatin is context-dependent and is associated with two different chromatin states: chromatin condensation (mitosis) and accessible chromatin structure (transcription).

1.3.3 Histone Methylation

Methylation of histones can occur in the lysine and arginine residues on the N-terminal tails as well as on the histone-fold domains of histones H3 and H4. The ε-amino group of lysine residues is methylated in the states of mono-, di- or trimethylation by lysine methyltransferases (KMTs), whereas arginine residues are mono- or di-methylated in a symmetrical or asymmetrical manner by protein

arginine methyltransferases (PRMTs) (Agger et al. 2008; Zhang and Reinberg 2001; Jahan and Davie 2014). PRMT1, through asymmetric di-methylation of H4 Arg 3, recruits the Set1 complex via the WDR5 subunit. Set1 will di- and tri-methylate H3K4, which is 'read' by KATs, resulting in the acetylation of histones associated with that nucleosome. Several studies have shown that H3 di- and tri-methylated at K4 are preferentially acetylated (Wang et al. 2009; Edmunds et al. 2008; Hsu et al. 2012). The transcription factor USF1 recruits PRMT1 to the HS4 insulator/barrier element of the β-globin gene in chicken erythrocytes, an activity that is important in conferring barrier properties to this element (Huang et al. 2005, 2007; Li et al. 2010). In addition to H4R3me2a, PRMT (five or nine)-symmetric methylation of H3 at R2 may serve a similar function as H4R3me2a, in that the H3R2me2s is 'read' by Set enzymes, resulting in the strong linkage between this H3 PTM and H3K4me3 (Yuan et al. 2012; Migliori et al. 2012).

There are several mechanisms by which Set1-family members (H3K4 methyltransferases; SET1A/B, MLL1/2/3/4) are recruited to transcriptionally active genes. In addition to the mechanism described above, CpG islands, a feature associated with upstream promoter and 5′ end of the gene body of transcribed genes, associate with Set1. A zinc-finger protein, CXXC1, binds to the CpG island and recruits Set1-family members (Hashimoto et al. 2010; Blackledge et al. 2013). Previous studies have documented the association between the CpG island and H3K4me3 (Maunakea et al. 2010). It is curious, however, that the H3K4me3 often appears as two peaks, with one located with the CpG island and the other 3′ to the island. For example, the human *MCL1* has one peak of H3K4me3 at the exon 1/intron 1 boundary that overlaps with a CpG island, while a much greater peak of H3K4me3 is located over exon 2, which is downstream of the CpG island (Fig. 1.1). The two peaks of H3K4me cover two to three nucleosomes.

Once established, H3K4me3 is a relatively stable mark, which will increase and decrease with transcription induction and cessation, respectively, of inducible genes (Edmunds et al. 2008). The transcription-dependent increase in H3K4me3 at the 5′ end of the inducible gene body is likely linked to RNF20-mediated ubiquitination of H2B and stimulation of Set 1 activity to tri-methylate H3K4 (Kim et al. 2009). Ubiquitination of H2B is dependent upon transcription (Davie and Murphy 1990). The H3K4me3 mark remains associated with mitotic chromosomes acting presumably as a mitotic bookmark for the expression of specific genes in the daughter cell (Terrenoire et al. 2010; Delcuve et al. 2009; Zaidi et al. 2011, 2014).

Histone methylation is a dynamic modification with enzymatic conversion of methylated arginine residues to citrulline and with lysine demethylases that can remove the mono-, di- and tri-methylated groups from lysine (Bicker and Thompson 2013; Shmakova et al. 2014; Hojfeldt et al. 2013; Van Rechem and Whetstine 2014). Furthermore, the Jumonji family of histone demethylases is able to demethylate specific H3 lysines (tri-methylated residues) as well as methylated arginine residues (Shmakova et al. 2014). The functional consequences of the different degree of methylation result in various biological outcomes including transcriptional activation, elongation or repression, imprinting, DNA replication and DNA-damage repair.

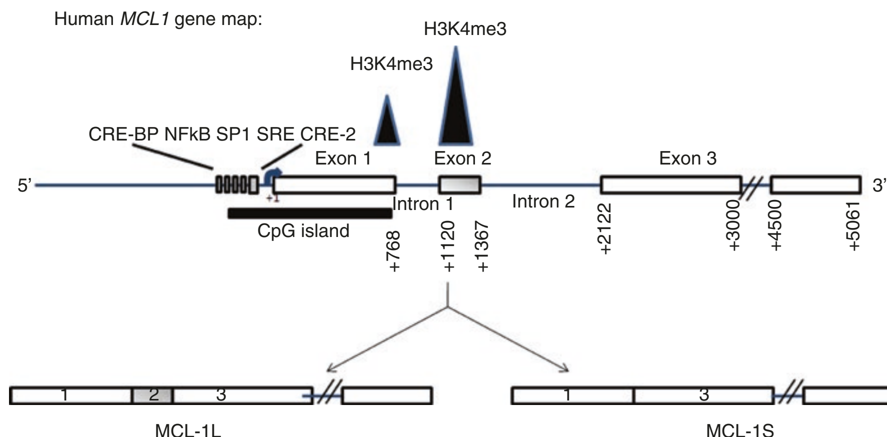

Fig. 1.1 Histone H3K4 tri-methylated nucleosomes and the *MCL1* gene. Exon 2 of the *MCL1* transcript is alternatively spliced. When exon 2 is retained, the *MCL-1L* transcript is produced, coding for a protein with anti-apoptotic function. When exon 2 is excluded, the short transcript *MCL-1S* is synthesized, yielding the MCL-1S protein with pro-apoptotic activity. A CpG island is located over the UPR and 5′ end of the gene body. One of the two peaks of H3K4me3 covers the CpG islands. The nucleosome positioned at exon 2 has high levels of H3K4me3 and is dynamically acetylated

Deregulation of lysine methyltransferases and lysine demethylases is common in disease states such as cancer. For example, EZH2, which tri-methylates H3K27 (a repressive mark), is mutated in some cancer, resulting in an enzyme that is more active in forming H3K27me3 (Yap et al. 2011). Lysine demethylase KDM5B, which demethylates H3K4me3, is expressed at a greater level in oestrogen receptor-positive breast cancer cells resulting in a lowering of genomic H3K4me3 levels (Klein et al. 2014; Yamane et al. 2007; Lu et al. 1999).

1.3.4 Other PTMs of Histones

Histones ubiquitinated on lysine residues have diverse biological functions. Ubiquitination involves the covalent linkage of ubiquitin monomers to histones as either a single addition (monoubiquitination) or as a polyubiquitin chain. Monoubiquitination of the C-terminal tail of H2B (H2BK120), catalysed by the mammalian RAD6/RNF20 complex, is a mark linked to transcriptional stimulation, elongation and nucleosome remodelling (Cole et al. 2014; Davie and Murphy 1990). RNF20 and H2B ubiquitination was shown to play a role in establishing domain boundary (HS4) at the chicken erythrocyte β-globin domain as well as establishing H3K4 methylation and highly acetylated H3, H4 and H2A.Z (Ma et al. 2011). While the dynamic regulation of H2B ubiquitination is essential for gene transcription, ubiquitination of H2A is considered as a repressive mark (Wright et al. 2012).

The small ubiquitin-like modifier (SUMO) is a large modification (10Ka in mass), which is covalently linked, and is found on all core histones (Shiio and Eisenman 2003). Recent studies suggest that sumoylation of histone H4 promotes chromatin decondensation (Dhall et al. 2014).

Histones can also be reversibly modified by ADP ribosylation on the arginine and glutamate residues in either mono- or poly-ribosylated forms. Distinct enzymes are responsible for this modification including mono-ADP ribosyltransferases and poly(ADP-ribose) polymerases (Allis et al. 2007; Messner and Hottiger 2011). ADP ribosylation of nucleosomes is involved in the epigenetic regulation of higher-order structural organization of chromatin (Rouleau et al. 2010).

1.4 DNA Methylation

DNA methylation is a dynamic and reversible epigenetic mechanism, which is involved in diverse biological processes such as gene expression regulation, alternative splicing, genomic imprinting, chromatin organization and X-chromosome inactivation (Wu and Zhang 2014; Liyanage et al. 2014). DNA methylation can be of different forms with respect to the nucleotide, which undergoes methylation. The two forms of DNA methylation are (1) CpG methylation where cytosine (C) residues in the context of CpG dinucleotides are methylated and (2) non-CpG methylation or CpH methylation (H=A, T or C) (Fig. 1.2). The distribution and functions of DNA methylation are highly dependent on their genomic location (context) and cell type, which will be discussed briefly in this section.

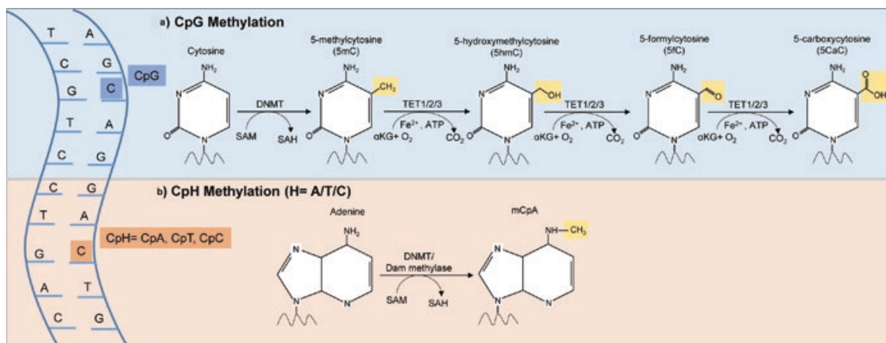

Fig. 1.2 Different types of DNA methyl modifications. (**a**) CpG methylation. DNMT transfer methyl group from SAM to cytosines to generate 5-methylcytosine (*5mC*). Ten eleven translocation (*TET*) proteins carry out oxidation of 5mC into 5-hydroxymethylcytosine (*5hmC*; 'the fifth base'), 5-formylcytosine (*5fC*) and 5-carboxycytosine (*5CaC*) through a series of oxidation steps which involve oxygen (*O2*), *alpha*-ketoglutarate (*αKG*), adenosine triphosphate (*ATP*) and Fe^{2+}. (**b**) Non-CpG methylation or CpH methylation where H=A/T/C. As an example of CpH methylation, transfer of methyl group from SAM to adenine by either DNMTs or Dam methylase is shown

1.4.1 CpG Methylation

In mammals, 70–80 % of the DNA methylation occurring in the context of CpGs is due to the transfer of a methyl group to the carbon-5 position of cytosine from the methyl donor S-adenosyl methionine (SAM) by DNA methyltransferases (DNMTs) (Fig. 1.2a). This basic DNA modification is referred to as 5-methylcytosine (5mC) and is considered as the 'fifth base' of the genome. There are three major types of DNMTs, DNMT1 which maintains the DNA methylation patterns during replication and, DNMT3A and DNMT3B, the de novo DNMTs. The 5mC modification can be oxidized by Ten eleven translocation proteins (TET1-3) to generate 5-hydroxymethylcytosine (5hmC), which is known as the 'sixth base' of the genome. A series of subsequent oxidation reactions mediated by TET proteins generate 5-formylcytosine (5fC) and 5-carboxycytosine (5CaC) (Fig. 1.2a). TET-based oxidation of 5mC to other DNA modifications is also considered as DNA demethylation, which involves oxygen (O_2), alpha-ketoglutarate (αKG), Fe^{2+} and ATP (Liyanage et al. 2014).

The 5mC methylation is predominantly associated with transcriptional repression when it is localized to promoter elements of genes. This repressive DNA modification is known to recruit transcription-repressor complexes, such as SIN3 (HDAC), which includes methyl CpG-binding proteins (MBPs). One of the major MBPs, which binds to 5mC, is MeCP2. This DNA modification can also mediate transcription repression through crosstalks with repressive histone PTMs such as H3K9me2/3, H3K27me3 and H4K20me3 (Rottach et al. 2009). The crosstalks between DNA methylation and histone PTMs may occur in two ways: (1) DNA methylation, followed by binding of MBPs, recruits repressor complex, which in turn loads writers of histone PTMs such as lysine methyltransferases, or (2) histone PTMs established by writers (lysine methyltransferases) or erasers (HDACs) of histone PTMs lead to the recruitment of DNMTs onto DNA, which subsequently establish DNA methylation (Kondo 2009; Vaissiere et al. 2008). The steric hindrance caused by DNA methylation could prevent the loading of transcription factors to the gene promoters, preventing transcriptional activation. Increased 5mC methylation, subsequent chromatin compaction and recruitment of MBPs and heterochromatin-associated proteins such as HP1 form a more compact chromatin structure leading to the formation of heterochromatin or transcriptionally inactive chromatin domains (Liyanage et al. 2014). Moreover, DNA methylation contributes to the regulation of splicing through the definition of exons/introns junctions and regulation of RNA polymerase elongation rate during co-transcriptional splicing (Gelfman and Ast 2013; Kornblihtt et al. 2013).

Most studies on 5hmC distribution within the genome report a preferential distribution of 5hmC in transcriptionally active chromatin regions and enhancers (Mellen et al. 2012). Although regions with high CpG density (CpG islands) are known to cause transcriptional repression when hypermethylated, a recent study showed the enrichment of 5hmC methylation in CpG islands of transcriptionally active genes in embryonic stem cells (Booth et al. 2012). However, there is evidence to suggest that 5hmC has an opposite role as well. For instance, MeCP2-bound 5hmC was shown

to be enriched at the promoters of the transcriptionally repressed *GAD1* and *RELN* genes (Zhubi et al. 2014). Moreover, TET1, which drives the 5hmC generation, interacts with polycomb-repressive complex 2 within the repressed promoters of several genes in mouse embryonic stem cells (Wu and Zhang 2011). Similar to 5mC, 5hmC seems to have genomic context-dependent and cell-type-specific roles in regulating gene expression.

The 5fC and 5CaC modifications have been investigated in many recent studies. The two modifications recruit different proteins to mediate their functions. For example, 5fC is recognized by MBD3 in mouse embryonic stem cells, while 5CaC is targeted by a wide range of regulatory proteins such as CTCF, EHMT1, NCOR2, PRP31 and DNMT1, which are involved in transcription repression and regulation of alternative splicing (Spruijt et al. 2013; Iurlaro et al. 2013). However, 5CaC was also enriched in transcriptionally active euchromatin regions within ovarian follicular cells, suggesting a role of 5CaC in transcriptional activation (Liyanage et al. 2014).

Altogether, the diverse roles of different DNA modifications are dependent on the genomic context (promoter, exons/introns, and enhancer), type of DNA modification (5mC, 5hmC, 5fC and 5CaC), interacting protein partners or recruited proteins (MeCP2, CTCF, DNMTs, splice factors) and cell type.

1.4.2 CpH Methylation

The CpH methylation is prevalent in embryonic stem cells, induced pluripotent stem cells, adult brain and mature neurons, while it is less abundant in somatic cell types (Guo et al. 2014b). The CpH methylation is carried out by DNMT3A and DNMT3B (Guo et al. 2014b) or Dam methylase (Amine Aloui et al. 2013) (Fig. 1.2b). The CpH methylation can either occur as mCHH or mCHG. Four per cent (4 %) of the metazoan genome contains CpGs, and thus the presence of CpH methylation was postulated to expand the nucleotides, which are methylated allowing a more in-depth regulation of gene expression (Guo et al. 2014b). Similar to 5mC methylation, CpH methylation is associated with gene silencing and genomic imprinting (Mellen et al. 2012). Regulatory proteins recruited onto DNA by CpH methylation mediate transcriptional regulation. Examples of major regulatory proteins, which recognize and bind to CpH, include MeCP2 and DNMT3A (Guo et al. 2014b).

1.4.3 DNA Methylation-Related Regulatory Proteins

The MBP protein family consists of five members, namely MBD1, MBD2, MBD3, MBD4 and MeCP2 (Liyanage and Rastegar 2014; Liyanage et al. 2012), which bind to methylated DNA through their methyl-binding domains. Unlike other MBPs, MBD3 is unable to bind to methylated DNA, yet plays a role in transcriptional regulation through protein interactions with repressor complexes

(Liyanage et al. 2014). Proteins, which lack MBD but contain Zn-finger domains, are also capable of binding to methylated DNA and Kaiso is one such protein. The initial concept was that MBPs once bound to methylated DNA (5mC) facilitate transcriptional repression. However, recent discovery of MBPs recruitment on 5hmC and interactions with TET proteins have led to a paradigm shift in the role of MBPs in transcriptional regulation (Mellen et al. 2012; Cartron et al. 2013). The vast variety of transcriptional regulatory roles of MBPs are determined by the preference of underlying DNA sequence (e.g. CpGG or CpGC for MBD2), type of DNA methyl modification (5mC/5hmC/5fC and 5CaC) and interacting protein partners (e.g. TET1, polycomb complexes) (Liyanage et al. 2012, 2014). Several members of the MBP family are also found to be recruited onto non-CpG-methylated regions and unmethylated DNA regions (Hansen et al. 2010).

Other than their well-known functions in transcriptional regulation, MBP proteins are also known as chromatin-architectural proteins because of their role in modulating chromatin structure (Liyanage et al. 2012). For example, once bound to methylated DNA, MeCP2 is able to mediate the formation of chromatin bridges, loops and higher-order chromatin structure. Disruption of the methyl-binding domains by mutations leads to alterations in binding to DNA and changes in chromatin structure (reviewed in (Liyanage and Rastegar 2014)).

1.5 Nucleosomes, Transcription and RNA Splicing

Throughout the transcription elongation process, RNAPII has to contend with nucleosomes. A model for transcription proposed by Ken van Holde has been supported by experimental data from Vasily Studitsky and colleagues (van Holde et al. 1992; Kulaeva et al. 2013). In this model, a dimer of H2A/H2B is displaced from the nucleosome during encounter with the RNAPII, explaining why the exchange of H2AH2B dimer is so much greater than the H3H4 tetramer (Thiriet and Hayes 2005; Jackson 1990; Das and Tyler 2013). In avian erythrocytes, which are G0 phase non-replicating cells, newly synthesized H2A and H2B rapidly exchange with transcriptionally active/competent regions; however, this exchange is not dependent upon transcription (Hendzel and Davie 1990). Interestingly, the newly synthesized histones incorporated into active/competent chromatin regions are also ubiquitinated. It is not clear whether the ubiquitination occurred before or after the newly synthesized histones were incorporated into chromatin. Replication-independent-synthesized histone variants, such as H3.3 and H2A.Z, exchange with the nucleosomal histones of transcriptionally active chromatin (Teves and Henikoff 2011). In G0 phase chicken erythrocytes, the newly synthesized histones are of the replication-independent type.

Clearly, the process of transcription is disruptive to nucleosome structure. Vincent Allfrey and colleagues demonstrated that transcription through a nucleosome resulted in the exposure of the sulphydryl groups of the H3 cysteine, which

is typically buried in the nucleosome (Walker et al. 1990). The exposure of the cysteine sulphydryl made it possible to isolate the atypical nucleosomes on mercury-affinity columns, which used to be available from BioRad. Characterization of these atypical nucleosomes showed that the nucleosomes had a U-shaped structure and had acetylated histones. Passage of RNAPII is required to form the U-shaped nucleosome. Further, the 'open' conformation of the U-shaped nucleosome will be maintained as long as the nucleosomal histones remain in a highly acetylated state (Walia et al. 1998). The salt-soluble polynucleosomes, containing active/competent DNA, isolated from chicken erythrocytes are rich in U-shaped nucleosomes (Locklear et al. 1990).

In concert with transcription, splicing of the pre-mRNA occurs. There has been a recent burst of research activity demonstrating the relationship between histone PTMs on the body of the transcribed gene and proteins involved in splicing. In human cells, most genes are multi-exonic and undergo alternative splicing, which can give rise to proteins (or RNA) with quite different functions and cellular locations (Khan et al. 2012; Kornblihtt et al. 2013). Also of interest is that most internal exons are short, about the length of nucleosomal DNA. The regulation of alternative splicing is quite complex, and is influenced by transcription rates, histone PTMs and a variety of splicing factors. The types of histone PTMs located along a gene body are polarized. At the 5' end of the gene body is found H3K4me3, which is followed by H3K4me2 and H3K36me3 further down the gene body. Tom Mistelli and colleagues reported that MRG15, which binds to H3K36me3, associates with the RNA-binding protein PTB (Luco et al. 2010). H3K4me3 recruits the chromatin remodeller CHD1, which influences splicing (Sims et al. 2007). The rate of elongation through the gene body is not uniform. John Lis and colleagues developed a method called precision nuclear run-on and sequencing to determine the positions of transcriptionally engaged RNAPII in the genome. In addition to promoter-proximal pausing, RNAPII pausing occurred at intron–exon boundaries and over 3′ polyadenylation sites (Kwak et al. 2013). Interestingly, RNAPII pausing was less acute at skipped exons. The elongation rate through a nucleosome may be influenced by histone PTMs.

We recently demonstrated that the nucleosome associated with the alternative exon 2 of the human *MCL1* gene is preferentially tri-methylated at H3K4 and is dynamically acetylated (Fig. 1.1). Inhibition of HDAC activity rapidly results in the histones of the exon 2 nucleosome becoming highly acetylated. Although not yet shown, it is quite possible that the highly acetylated exon 2 nucleosome has an 'open' U-shaped structure. The rate of elongation is increased through the exon 2 nucleosome, which prevents certain RNA-splicing factors required for exon 2 inclusion from binding to the *MCL1* transcript. The strategic placement of H3K4me3 on the exon 2 nucleosome confers properties onto this nucleosome to respond to environmental changes that differ from the neighbouring nucleosomes. Thus, in the context as a signalling module, the exon 2 nucleosome is quite different from its neighbouring nucleosomes.

1.6 Enhancers

Enhancers play a critical role in the regulation of transcription. For any given promoter, there may be several enhancers (e.g. *FOS*) (Kim et al. 2010). Enhancers interact with specific promoters by looping, resulting in the juxtapositioning of the enhancer with the promoter. Enhancers with nucleosomes containing H3.3 and H2A.Z undergo rapid histone turnover (Kraushaar et al. 2013). Other chromatin features of an enhancer include H3K4me1/2, p300 (a KAT), H3K27ac, p300, mediator, cohesin and enhancer RNA (eRNA) (Zentner and Scacheri 2012). Which of these features signifies an active enhancer is disputed; however, elegant approaches provide compelling evidence that chromatin looping, eRNA and mediator are required for transcriptional activation of the targeted promoter (Fanucchi et al. 2013; Lam et al. 2014; Zentner and Scacheri 2012). Enhancer RNAs are typically short-lived and low in abundance. Global run-on sequencing (GRO-Seq) rather than RNA sequencing is the preferred method to detect eRNA (Core et al. 2008; Lai and Shiekhattar 2014).

To be discussed in more detail in the next section, there is a growing body of literature reporting the important role of ncRNA in chromatin organization and function. Although there is still much to learn about the role of eRNA in mediating enhancer-promoter chromatin loop formation, it has been suggested that eRNA recruits mediator, leading to chromatin looping.

In oestrogen-responsive breast cancer cells, enhancers with oestrogen receptor element bind to oestrogen receptor alpha as well as many other transcription factors and coactivators, forming a large 1–2 megadalton complex. Several of the transcription factors in this complex were not associated with DNA. Interestingly, DNA-PK is a component of this complex (Liu et al. 2014). This megadalton complex may be instrumental in bringing together oestrogen-responsive genes in a cluster (Liu et al. 2014). The bromodomain-containing protein BRD4 is bound to a subset of enhancers, known as super enhancers, which represent less than 5 % of a cell's enhancers (Hnisz et al. 2013; Loven et al. 2013). A super enhancer is an extended genomic region (up to tens of kilobases in length) that has a high density of enhancer-associated marks and proteins such as H3K27ac, H3K4me1, mediator and BRD4 (Heinz et al. 2015). During pro-inflammatory stimulation by TNFα, NF-kB and BRD4 establish the super enhancers, which drive pro-inflammatory gene transcription (Brown et al. 2014). The activity of some enhancers is dependent upon recruitment to nuclear matrix-associated proteins. For example, the Pit1-bound enhancers are recruited to the nuclear matrin-3-rich network, an event that is critical for enhancer activity and expression of Pit1-regulated genes (Skowronska-Krawczyk et al. 2014).

Genome-wide association studies have identified single nucleotide polymorphisms (SNPs) that are associated with various disease states. Interestingly, these changes in nucleotide sequences are found in enhancers and super enhancers, which may impact the binding of key transcription factors and enhancer activity, and have a role in disease states (Corradin et al. 2014; Hnisz et al. 2013).

1.7 Epigenetics and Metabolism

There is increasing awareness of the relationship between DNA/histone PTMs and metabolism (Liyanage et al. 2014; Kaochar and Tu 2012). In mammalian cells, histone acetylation is dependent on ATP-citrate lyase. This enzyme produces acetyl-CoA from glucose-derived citrate (Wellen et al. 2009). Metabolites of intermediary metabolism, such as acetyl-CoA, SAM, FAD, α-ketoglutarate and ATP, are required to drive epigenetic processes (Kaochar and Tu 2012). Further, the metabolites themselves may regulate epigenetic modifiers. For example, Eric Verdin and colleagues demonstrated that the keto acid β-hydroxybutyrate inhibits HDAC activity (Newman and Verdin 2014). Vitamin C enhances TET activity and the conversion of 5meC to 5hmeC (Blaschke et al. 2013). Further, the TET enzymes and several of the KDMs require two cofactors Fe(II) and 2-oxoglutarate (Shmakova et al. 2014; Hojfeldt et al. 2013). Both diet and environmental factors can impact the epigenome (for review (Kaur et al. 2013; Kanherkar et al. 2014)).

1.8 ncRNA and Epigenetic Modifiers

RNA-sequencing studies have demonstrated that over 85 % of the human genome is transcribed (Hangauer et al. 2013; Mercer et al. 2014). A low level of transcription takes place between genes, producing long intergenic ncRNA (lincRNA). ncRNA also arises from enhancers, promoters and antisense transcripts (transcripts produced on the opposite strand as the protein coding or sense strand) (Guil and Esteller 2012; Carninci et al. 2005; Katayama et al. 2005). In some cases, the sense and antisense transcripts are coordinately regulated (Luther et al. 2005). The ncRNA has secondary structure and is bound to a variety of RNA-binding proteins (Quinn et al. 2014). Interestingly, the ncRNA may also be circular (Guo et al. 2014a).

Nuclear RNA, whether coding or ncRNA, regulates the structure and function of chromatin (Guil and Esteller 2012; Gardini and Shiekhattar 2014). RNA may have a role as genome-organizing-architectural factors of transcribed chromosomal domains (Caudron-Herger et al. 2011; Caudron-Herger and Rippe 2012; Guil and Esteller 2012) and/or have a functional role such as ncRNA originating from enhancers (eRNAs) (Natoli and Andrau 2012). RNA called chromatin-interlinking RNA (ciRNA) has been isolated from chromatin. The ciRNA was proposed to act as genome-organizing-architectural factors of transcribed chromatin regions (Rodriguez-Campos and Azorin 2007; Caudron-Herger et al. 2011). Microinjection of RNase A into the nucleus of mammalian cells resulted in the rearrangement of chromatin distribution with the aggregation of chromatin at the nuclear periphery. Current evidence suggests that lncRNA is involved in regulating gene expression and the nuclear/chromatin structure of active genes. Genes producing the lncRNA have H3K4me3 at their 5′ end and H3K36me3 throughout the gene body (Orom and Shiekhattar 2011; Gardini and Shiekhattar 2014).

There is mounting evidence that chromatin modifiers and remodellers associated with nuclear RNA play a key role in determining histone PTM positions along

chromatin. RNA-bound chromatin modifiers, architectural proteins and chromatin-associated proteins include KAT2B, HDAC1/2, SWI/SNF, mediator, CTCF and MeCP2 (Khan et al. 2012, 2014; Sjolinder et al. 2005; Tyagi et al. 2009; Guil et al. 2012; Maxwell et al. 2013; Tsai et al. 2010). The ncRNA may associate with chromatin modifiers that are activators (e.g. MLL, an H3K4 methyltransferase) or repressors (EZH2) (Guil and Esteller 2012; Guil et al. 2012). HOTAIR is an example of a lncRNA with a repressive function that silences nearby genes (Gardini and Shiekhattar 2014). The polycomb-repressive complex (EZH2, SUZ12, EED) binds to the 5′ domain of HOTAIR, while the LSD1/CoREST/REST complex binds to the 3′ domain of this lncRNA (Tsai et al. 2010). LSD1 is an H3K4me2 demethylase, and CoREST is associated with HDAC1/2. A class of lncRNAs, which do not overlap with protein-coding genes and activate transcription, is referred to as ncRNA activating (Orom and Shiekhattar 2011). Interestingly, the knockdown of a ncRNA-activating specifically attenuated the expression of a 'nearby' protein-coding gene; the median distance of a ncRNA-activating to the impacted protein-coding gene was over 100 kb in mammalian cells. The ncRNA-activating binds to mediator, a large multiprotein transcriptional coactivator (Ansari and Morse 2013; Lai et al. 2013). The association of these chromatin modifiers with RNA is often indirect, with the factor being recruited to RNA via a RNA-binding protein. For example, the KAT, PCAF or KAT2B is recruited to RNA via hnRNP U (Obrdlik et al. 2008). The H3K4 methyltransferase MLL is associated with RNA, with specific RNAs bringing MLL to a targeted promoter.

We recently reported that HDAC1 and HDAC2 are recruited to the gene body of a transcribed gene, with the recruitment of HDAC being transcription-dependent (Khan et al. 2014). However, the HDAC1/2 were not associated with the chromatin but with the transcript. Using a dual-cross-linking ChIP assay, HDAC1 and 2 appeared to be bound to the gene body of the transcribed gene. However, when the fragmented chromatin was digested with RNase A before adding the anti-HDAC1 or 2 antibodies, the association of the HDACs with the transcribed gene body was lost. These studies highlighted a caution in interpreting ChIP assay data. RNA-binding proteins involved in pre-mRNA splicing will be cross-linked to DNA with formaldehyde and will appear to be associated with the gene body of transcribed genes (Sapra et al. 2009). However, the addition of RNase digestion step before doing the immunoprecipitation will demonstrate that the RNA-binding protein was bound to RNA and not chromatin.

Importantly, lincRNAs are enriched (fivefold compared to non-expressed intergenic regions) in SNPs associated with specific traits. It has been proposed that the lincRNAs may play a significant role in human diseases (Hangauer et al. 2013).

1.9 Concluding Remarks

Looking back at the history of chromatin, it was not all that long ago that histones were considered as the most boring proteins to study. These 'boring' proteins are now the subject of intense investigation. To quote David Allis, 'every amino acid matters' (Maze et al. 2014). The devastating impact of a change in the amino acid

sequence of the H3 variant H3.3 is clearly demonstrated in paediatric gliomas and chondroblastomas (Behjati et al. 2013; Schwartzentruber et al. 2012). Every histone PTM and DNA modification also matters. Continuing studies investigating the role of the histone PTMs, histone variants and DNA modifications in chromatin structure and function, recruitment or removal of readers, and cross-talk with other histone/DNA PTMs and readers will lead to a better understanding of the full potential of the nucleosome as a signalling module in normal and disease states.

The role of the nuclear matrix, as a dynamic RNA-protein nuclear substructure, in the organization and function of the genome continues to be debated (Nozawa and Gilbert 2014). The nuclear matrix was dismissed by some research groups as a high salt artefact. However, these researchers are reminded of the studies by Dean Jackson and Peter Cook, who used a gentle method involving encapsulating cells in agarose beads, restriction endonuclease digestion of chromatin followed by chromatin removal by electroelution, to prepare the nuclear matrix (Jackson and Cook 1988). The recent studies by Lisa Hall and colleagues identified the stable ncRNA associated with the nuclear matrix (Hall et al. 2014). Initial studies by this group provide evidence that the scaffold/matrix attachment region-binding protein scaffold attachment factor 1 (SAF-A; also known as hnRNP U), which is a major protein of the nuclear matrix, plays a role in anchoring LINE1 to chromatin (Martens et al. 2002). SAF-A/hnRNP U also binds to KAT2B and perhaps other KATs (Obrdlik et al. 2008; Martens et al. 2002). It is possible that in addition to KATs, HDACs are associated with the nuclear matrix RNA.

There is building excitement as to the role of ncRNA in regulating genome organization and function. Current evidence shows that specific ncRNAs are targeting epigenetic modifiers, architectural proteins and coactivators/corepressors to genome targets to activate or silence genes. Changes in DNA sequence (that is SNPs) are likely to influence the production of these ncRNA, altering gene activity and may be important contributors to an individual's susceptibility to disease. Further, one's genetic make-up, diet, environment and lifestyle will all influence an individual's epigenome and his/her disease risk and longevity (Heyn et al. 2013, 2014; Corella and Ordovas 2014). It will be exciting times ahead as the field of epigenetics unravels the mysteries of these processes that impact human health.

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Epigenome Dynamics and Reader Proteins in Cardiomyocyte Development and Heart Failure

2

Lutz Hein

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Abstract

Epigenetic mechanisms are essential for cardiomyocyte function and adaptation in development, postnatal growth, and during cardiac remodeling in heart failure. Recent advances in the identification of cell-type-specific markers have facilitated the development of techniques to purify cardiomyocytes and their nuclei from intact heart tissue and to study DNA methylation and posttranslational histone modifications on a genome-wide basis. Cardiomyocyte development and postnatal maturation were accompanied by DNA demethylation of gene bodies of sarcomeric genes until adulthood. Genes that are expressed during the fetal period were also demethylated but were postnatally repressed by trimethylation of histone 3 at

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lysine 27 (developmental genes) or by de novo DNA methylation mediated by DNA methyltransferases 3A/3B (fetal sarcomeric genes). Dynamic changes in DNA methylation and histone modifications can be recognized by specialized "reader" proteins which modulate cardiomyocyte function. Future studies will be important to unravel the complex interplay between epigenetic modifications of DNA and histones and to identify the involved signaling pathways.

2.1 Epigenetic Adaptations During the Life Cycle of Cardiomyocytes

The heart is the first organ which develops during embryonic development. While it continuously beats to supply the body with blood, oxygen, and nutrients, it has to grow and adapt to multiple physiological and pathological situations. Despite these challenges, cardiomyocytes lose their proliferative capacity soon after birth (Bergmann et al. 2009; Senyo et al. 2013). In order to adapt to diverse demands, cardiomyocytes adjust their gene expression program. Long-term changes in gene expression are mediated by several epigenetic processes, including DNA methylation (Hon et al. 2013), chromatin, and histone modifications (Anand et al. 2013; Wamstad et al. 2012; Zhang et al. 2002) as well as microRNAs (Grueter et al. 2012).

2.1.1 DNA Methylation

During embryonic development, cells differentiate from progenitors into a broad spectrum of >200 specialized cell types in the body. DNA methylation is considered to be a stable mark to maintain cell-type identity after differentiation (Bird 2002; Jones 2012). DNA methylation is established at cytosines, mostly in CpG sequences, by de novo DNA methyltransferases 3A and 3B (DNMT3A, DNMT3B) (Reik et al. 2001). The maintenance methyltransferase DNMT1 is responsible for symmetrical methylation of the newly synthesized DNA strand during cell division, ensuring that DNA methylation will be stably transferred to the daughter cells (Reik et al. 2001). Several enzymes have been identified, which may actively remove DNA methylation marks, including the ten-eleven-translocation enzymes TET1, TET2, and TET3 (Branco et al. 2012). Methylated cytosines serve as binding partners for a number of methyl-CpG-binding domain (MBD) proteins, which associate with other proteins to modulate gene expression.

2.1.2 Histone Modifications

In every nucleated cell, the genomic DNA is complexed with histone proteins in nucleosomes (Allis et al. 2015). In each nucleosome, double-stranded DNA is wrapped 1.4-times around an octamer complex consisting of histone proteins. The octamer consists of two of the histone isoforms H2A, H2B, H3, and H4 (Allis et al.

2015). Modulation of the packing density of nucleosomes may allow to maintain an open chromatin structure (euchromatin) for active genomic regions as well as a condensed chromatin compaction (heterochromatin) for regions of the genome which are currently not in use. Nucleosome packing and gene activity may be modulated by posttranslational modifications of histones (Allis et al. 2015). So far, a large variety of histone modifications have been described, including methylation, acetylation, phosphorylation, ubiquitination, and many others. Specific modifications have been linked with gene activity, for example, H3K4me3 was found at active promoters and transcription start sites, H3K27ac at active enhancers and promoters and H3K36me3 at actively transcribed gene bodies (Rivera and Ren 2013). In contrast, H3K9me3 and H3K27me3 mark repressed or heterochromatic gene regions (Rivera and Ren 2013).

This chapter will focus on DNA methylation and histone modifications and their recognition by reader proteins in cardiomyocytes during development and in disease.

2.1.3 Methods to Study Epigenetic Processes in the Heart

The heart is a complex tissue which is composed of multiple cell types, including cardiomyocytes, endothelial cells, fibroblasts, vascular smooth muscle cells, and many other cells. Importantly, cardiomyocytes contribute only 20–30 % of the total cardiac cell population (Rubart and Field 2006). Depending on the type of DNA or histone mark, epigenomic profiles may greatly differ between individual cell types (Allis et al. 2015). Thus, in order to interrogate specific epigenetic features in one particular cell type, it is essential to isolate the cell type of interest from the tissue for further analysis (Jaffe and Irizarry 2014; Liang and Cookson 2014; Lister et al. 2013; Michels et al. 2013). For purification of cardiomyocytes from mouse heart tissue, we used an antibody against pericentriolar material 1 (PCM1), which specifically labels cardiomyocyte nuclei in the heart (Bergmann et al. 2011). For this method, cardiac nuclei were prepared from frozen mouse heart tissue, labeled with an anti-PCM1 antibody and cardiomyocyte nuclei were purified by flow cytometry (fluorescence-activated cell sorting (FACS)) or by magnetic-assisted sorting (MACS) (Gilsbach et al. 2014). FACS and MACS resulted in highly pure cardiomyocyte nuclei preparations (>97 %), which were further processed for bisulfite sequencing to generate DNA methylomes and histone maps at base-pair resolution (Gilsbach et al. 2014).

2.2 DNA Methylation in Cardiomyocytes

2.2.1 Dynamics of DNA Methylation During Cardiomyocyte Development and in Disease

Using PCM1-based purification of cardiomyocyte nuclei, we generated high-coverage maps of DNA methylation in mouse cardiomyocytes at postnatal day 1 (P1) and in normal and failing adult cardiomyocytes (Gilsbach et al. 2014). DNA

methylomes from mouse embryonic stem (ES) cells (Stadler et al. 2011) and from total cardiac tissue (Hon et al. 2013) were used for comparison. Compared with whole heart tissue (Hon et al. 2013), purified cardiomyocyte nuclei revealed distinct patterns of DNA methylation of cardiac enhancers and gene bodies (Gilsbach et al. 2014). Many highly expressed cardiomyocyte genes were strongly demethylated during development (Fig. 2.1). Most interestingly, gene body demethylation proceeded during postnatal maturation until adulthood (Gilsbach et al. 2014). Furthermore, demethylation of gene bodies correlated inversely with gene expression and active histone marks, suggesting a direct link between DNA methylation and RNA expression. Interestingly, the perinatal isoform switch of genes involved in sarcomere function, energy metabolism, or Ca^{2+} homeostasis coincided with opposite changes in DNA methylation of these genes (Fig. 2.1). Sarcomeric proteins are well known to shift their isoform expression around birth to adapt to the postnatal needs (Krüger et al. 2006; Siedner et al. 2003). In mouse cardiomyocytes, 313 gene bodies were demethylated and 127 were de novo methylated after birth (Gilsbach et al. 2014). As examples, the fetally expressed isoform of troponin I1 gene (*Tnni1*) was remethylated during the postnatal period while the adult troponin I3 gene (*Tnni3*) was demethylated starting after birth (Fig. 2.1) (Gilsbach et al. 2014). Importantly, de novo methylation of the *Tnni1* gene was mediated by DNMT3A/B enzymes. Cardiomyocyte-specific ablation of DNMT3A/B completely prevented remethylation of the *Tnni1* gene body and partially relieved gene repression (Gilsbach et al. 2014). These results indicate that DNA methylation of gene bodies of highly expressed cardiomyocte genes affects RNA expression of these genes. However, the underlying molecular mechanisms are unclear at present.

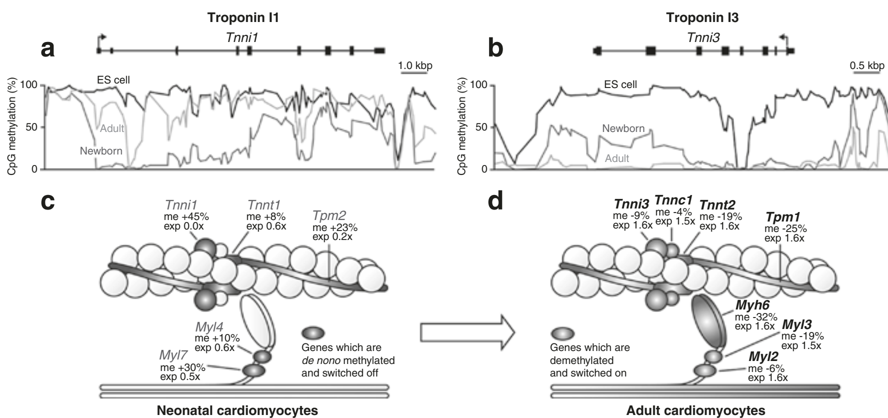

Fig. 2.1 DNA methylation of the troponin I1 and I3 genes in cardiomyocytes. (**a**, **b**) DNA methylation of the troponin I1 gene (*Tnni1*, **a**) and the troponin I3 gene (*Tnni3*, **b**) in embryonic stem cells, neonatal, and adult mouse cardiomyocytes. (**c**, **d**) Cardiac sarcomere at birth (**c**) and in the adult heart (**d**). Sarcomere genes which are differentially methylated and expressed between birth and adulthood are marked. *Numbers* indicate the differences in gene expression (exp) and CpG methylation (me) (Modified from Gilsbach et al. (2014))

Furthermore, not all genes, which were demethylated in their gene bodies, were expressed in adult cardiomyocytes. Many genes that are involved in cardiac differentiation, for example, *Isl1* (Zhuang et al. 2013), remained demethylated until adulthood despite the absence of significant levels of RNA expression (Fig. 2.2) (Gilsbach et al. 2014). These genes were decorated and repressed by the inactive histone mark H3K27me3, which is generated by the polycomb-repressive complex 2 (PRC2) (Fig. 2.2) (He et al. 2012; Delgado-Olguin et al. 2012). Ablation of the enzymatic component of the PRC2 complex, EZH2, led to reactivation of some of these genes (Gilsbach et al. 2014).

In addition to these alterations in gene body DNA methylation, a large number of cardiomyocyte enhancers were found to be demethylated during cardiomyocyte development. These short stretches of DNA demethylation contained motifs for cardiac transcription factors, for example, MEF2C, GATA1-4, as well as histone modifications (H3K4me1, H3K27ac), which are hallmarks of cis-regulatory elements (Creyghton et al. 2010; Stadler et al. 2011). Conversely, enhancers that carried motifs for pluripotency factors, which are active in ES cells, were de novo methylated in cardiomyocytes versus ES cells (Gilsbach et al. 2014). Furthermore, in failing cardiomyocytes several features of altered DNA methylation could be identified (Gilsbach et al. 2014). These alterations did not affect the gene body but were mostly localized outside of genes (Gilsbach et al. 2014). Interestingly, the DNA

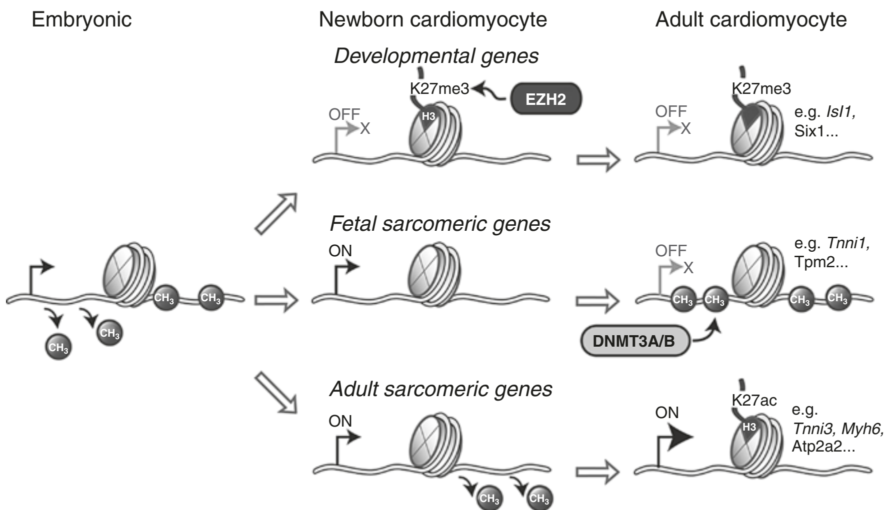

Fig. 2.2 Schematic representation of DNA methylation and histone modifications in gene bodies of cardiomyocytes. The scheme indicates gene body (de)methylation in embryonic stem cells (*left*), neonatal cardiomyocytes (*middle*), and adult mouse cardiomyocytes (*right*). Developmental genes (*upper row*) are demethylated but repressed by H3K27me3. Fetal sarcomeric genes (*middle row*) are demethylated and de novo methylated by DNMT3A/B. Adult sarcomeric genes (*lower row*) are completely demethylated during cardiac development and postnatal maturation (Modified from Gilsbach et al. (2014))

methylation pattern of failing cardiomyocytes partially resembled the fetal methylation pattern (Gilsbach et al. 2014). These observations are in line with several other reports, which suggest that DNA methylation may be altered during development and in heart failure (Haas et al. 2013; Movassagh et al. 2011a, b; Koczor et al. 2013; Sim et al. 2015). However, further studies will be required to determine which cell types contribute to changes in DNA methylation in cardiac tissue and which are the underlying molecular mechanisms.

2.2.2 Readers of DNA Methylation

DNA methylation can be recognized by specialized proteins. Three families of methyl-CpG-binding proteins have been identified in mammals, the MBD (methyl-CpG-binding domain) family, Kaiso and Kaiso-like proteins, and SRA (SET and RING-associated domain) domain proteins (Fournier et al. 2012). Within the MBD family of proteins, MeCP2, MBD1, MBD2, and MBD4 recognize methylated DNA, while MBD3, MBD5, and MBD6 do not bind to methylated DNA sequences (Fournier et al. 2012).

Methyl-CpG-binding protein 2 (MeCP2) binds specifically to methylated DNA (Fig. 2.3) and regulates transcription, chromatin organization, and RNA splicing (Song et al. 2014; Maunakea et al. 2013). Mutations in the MeCP2 gene have been

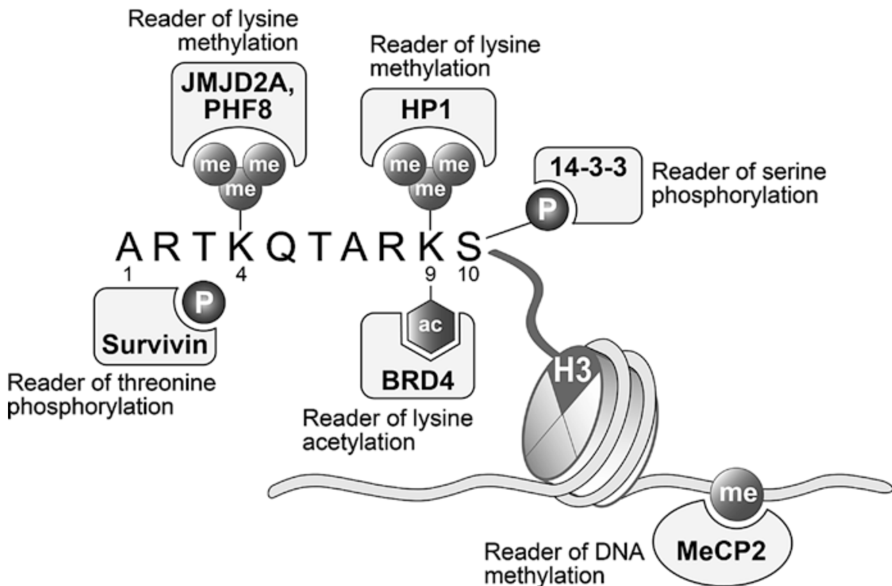

Fig. 2.3 Reader proteins involved in the recognition of DNA methylation and histone modifications. For clarity, only examples for epigenetic readers which have been associated with cardiac function are indicated. *Single letters* represent amino acid sequence of the aminoterminus of histone H3 with posttranslational modifications

identified as genetic causes of Rett syndrome, a neurodevelopmental disorder (Song et al. 2014; Bird 2002). MeCP2 is strongly expressed in neurons, but high levels of expression have also been identified in cardiovascular tissue including cardiomyocytes (Song et al. 2014). MeCP2 is a member of the MBD (methylated DNA-binding domain) family of proteins, which specifically recognize and bind to methylated DNA sequences (Guy et al. 2011; Baubec et al. 2013). The MeCP2 protein is built up of several domains, including a methyl-DNA-binding domain (MBD). This domain binds methylated CpG sequences with 100-fold selectivity over 5-hydroxymethylated or unmethylated CpG (Khrapunov et al. 2014). MeCP2 may repress transcription, but it can also activate gene expression by recruiting coactivators (Chahrour et al. 2008; Ebert et al. 2013).

In a mouse model with left ventricular pressure overload due to transverse aortic constriction (TAC), histone marks and MeCP2 binding to SERCA (*Atp2a2*) and β-MHC (*Myh7*) gene promoters were investigated (Angrisano et al. 2014). Downregulation of SERCA expression was accompanied by increased promoter binding of DNA methyltransferases DNMT1 and DNMT3B as well as elevated MeCP2 levels at the SERCA promoter (Angrisano et al. 2014). Opposite changes were observed at the *Myh7* promoter, suggesting that DNA methylation and/or altered MeCP2 binding was involved in gene regulation after TAC (Angrisano et al. 2014). Similarly, the loss of MeCP2 binding was also observed in H9c2 cardiomyocytes at the IGF-II/mannose 6-phosphate receptor gene after stimulation with tumor necrosis factor (TNF)-α, lipopolysaccharide, or angiotensin II (Chu et al. 2012). However, cardiomyocyte-specific analysis of DNA methylation did not reveal alterations in DNA methylation at the gene body or promoter of these two genes, indicating that more likely MeCP2 levels are important for the modulation of hypertrophic gene expression (Gilsbach et al. 2014).

Transgenic overexpression of MeCP2 in the heart (and other organs) led to lethal embryonic cardiomyopathy after E14.5 (Angrisano et al. 2014). After cardiac ischemia, the transfer of miR-22 contained in exosomes from mesenchymal stem cells to cardiomyocytes was observed (Feng et al. 2014). In cardiomyocytes, miR-22 repressed MeCP2, leading to reduced apoptosis and decreased interstitial fibrosis (Feng et al. 2014). MeCP2 may also be expressed in non-cardiomyocyte cells, including fibroblasts (Song et al. 2014). MeCP2 stimulates myofibroblast transdifferentiation and inhibition of its expression by miR-132 was found to be protective in a mouse model of myocardial infarction (Katare et al. 2011). These data suggest that the repression of MeCP2 is cardioprotective. Altered levels of global DNA methylation and methyl-CpG-binding proteins 1, 2, and 3 were identified during differentiation of neonatal rat cardiomyocytes (Kou et al. 2010).

Rett syndrome has been associated with an increased risk of sudden cardiac death, but the underlying pathogenesis has not been fully identified. In patients with Rett syndrome, subclinical cardiac dysfunction has been reported by echocardiographic measurements (De Felice et al. 2012). Studies in MeCP2-deficient mice have identified prolongation of the QT interval similar to clinical observations in Rett patients (McCauley et al. 2011). However, measures of autonomic cardiovascular regulation did not differ between wild-type and MeCP2-deficient mice

(Bissonnette et al. 2007). Thus, MeCP2 defects may predispose Rett patients to develop lethal cardiac arrhythmias.

In addition to MeCP2, several other methyl-DNA-binding proteins have been identified (Fournier et al. 2012). However, none of these readers of DNA methylation have been investigated in the context of heart development or failure, yet.

2.3 Histone Modifications in Cardiomyocytes

2.3.1 Histone Modifications in Cardiomyocyte Development and in Disease

As mentioned above, multiple posttranslational modifications have been identified in histone proteins. For several – but not all – of these modifications, specific enzymes have been identified which generate the mark ("writer") or remove it ("eraser"). Detailed descriptions of the function and cardiovascular significance of these histone-modifying enzymes are discussed in detail in other chapters of this volume. The present chapter focuses on "reader" proteins, which have been identified to bind to specific histone modifications and which affect cardiomyocyte function (Fig. 2.3). In contrast to writer and eraser enzymes, the group of reader proteins is very large and less well defined. This is due to the fact that a large variety of nuclear proteins contain domains which specifically recognize and bind to one or more histone modifications. Excellent reviews about the structure and functions of these "reader" domains have been published (Patel and Wang 2013; Du and Patel 2014; Gillette and Hill 2015; Yun et al. 2011; Gayatri and Bedford 2014; Taverna et al. 2007).

Recently, an unbiased approach has identified additional proteins which recognize specific features of modified histones and/or DNA (Bartke et al. 2010). Thus, the family or "reader" proteins – including those which are relevant for cardiomyocyte function – is likely to expand significantly.

Posttranslational modifications of histones have been mapped on a genome-wide basis in cardiomyocytes, which were obtained by Langendorff dissociation or by FACS-/MACS-based nuclei purification, respectively (Gilsbach et al. 2014; Papait et al. 2013). These studies identified specific features of histone marks which are associated with gene activation or silencing in cardiomyocytes.

2.3.2 Active Histone Marks: Acetyl-Lysine Readers

Acetylation of lysines in histones is usually associated with gene activation. Bromodomain proteins contain specific domains for recognition of acetylated lysines (Fig. 2.3) (Patel and Wang 2013). In cardiomyocytes, phenylephrine induced the expression of the bromodomain protein BRD4, but not of BRD2 and BRD3, as well as phosphorylation of RNA polymerase II (RNA Pol II) (Spiltoir et al. 2013). The small molecule compound JQ1 inhibited phenylephrine-induced

hypertrophy and induction of ANP protein synthesis in cardiomyocytes in vitro (Anand et al. 2013; Spiltoir et al. 2013). In vivo, JQ1 prevented cardiac hypertrophy, contractile dysfunction, and hypertrophic gene expression in response to cardiac pressure overload (TAC) (Anand et al. 2013; Spiltoir et al. 2013). By chromatin immunoprecipitation, BRD4 was found to be enriched at enhancers and promoters of actively transcribed genes (Anand et al. 2013). Previous studies had shown that BRD4 binds to CDK9 (cyclin-dependent kinase 9), which phosphorylates RNA Pol II to initiate RNA Pol II pause release and thus stimulate elongation of nascent RNA transcripts (Kwak and Lis 2013). JQ1 effectively blocked BRD4-mediated pause release and thus inhibited pathologic gene transcription in cardiomyocytes (Anand et al. 2013). Liu et al. have developed a model in HEK293 cells involving a link between anti-pause enhancers with promoters by BRD4 in a complex with CDK9, HEXIM, JMJD6, CCNT1/2, and 7SK snRNA (Liu et al. 2013). BRD4 recruits JMJD6, a demethylase with dual activities toward methylated histones and the 7SK snRNA cap. JMJD6 (jumonji C-domain-containing protein 6) thus removes the inhibitory complex of HEXIM and 7SK snRNA and initiates RNA Pol II pause release (Liu et al. 2013). However, whether the same molecular components are also responsible for transcriptional pause release in cardiomyocytes remains to be determined.

2.3.3 Active and Inactive Histone Marks: Methyl-Lysine Readers

2.3.3.1 Recognition of H3K4me3

Trimethylation of histone H3 at lysine 4 (H3K4me3) is a chromatin mark known to label transcription start sites of active genes (Fig. 2.3) (Benayoun et al. 2014). Multiple protein domains have been identified, which can bind to H3K4me3, including chromodomains, PHD fingers, Tudor, MBT, and Zf-CW domains (Yun et al. 2011). Many of the proteins that contain these recognition domains have diverse additional biological activities, for example, ATPase, DNA recombination, HDAC, demethylase, or acetyltransferase functions (Yun et al. 2011). Only few of the reader proteins binding to H3K4me3 have been studied in the context of cardiac function. The histone lysine demethylase JMJD2A has been associated with cardiac remodeling in response to cardiac stress (Fig. 2.3). Heart-specific ablation of JMJD2A attenuated hypertrophic remodeling after TAC in mice (Zhang et al. 2011). JMJD2A contains a double Tudor domain, which mediates binding to H3K4me3 while the enzymatic domain of JMJD2A demethylates H3K9me3 and H3K36me3 (Huang et al. 2006). Thus, JMJD2A is one example of a protein linking two different histone activities – it binds to H3K4me3 and demethylates H3K9me3 (Huang et al. 2006). Similar properties were observed for the histone demethylase PHF8 which contains a PHD finger domain to bind to H3K4me3. PHF8 overexpression in the heart of transgenic mice attenuated cardiac remodeling after pressure overload (Fig. 2.3) (Liu et al. 2015).

Also, several chromatin-remodeling proteins of the SWI/SNF complex, including Brg1, Baf60, Baf180, and Baf250, contain reader domains recognizing modified

lysines in histones. These proteins are important for ATP-dependent chromatin remodeling and nucleosome positioning and several of these factors are essential for cardiac development (for recent reviews, see Gillette and Hill 2015; Nuhrenberg et al. 2014).

2.3.3.2 Recognition of H3K9me3

H3K9me3 is associated with heterochromatin and thus gene repression (Fig. 2.3). This modification may be specifically recognized by a number of different proteins and protein domains, including heterochromatin protein 1 (HP1; chromobox homolog 1, CBX1, Fig. 2.3) (Eissenberg and Elgin 2014; Nishibuchi and Nakayama 2014). HP1 proteins consist of an amino-terminal chromodomain and a carboxyl-terminal chromo shadow domain, which are linked by a hinge region (Eissenberg and Elgin 2014; Nishibuchi and Nakayama 2014). The chromodomain recognizes and binds to trimethylated lysine-9 in histone H3 (H3K9me3), while the carboxyl terminus of HP1 acts as a dimerization and recognition domain for other interacting proteins (Eissenberg and Elgin 2014; Nishibuchi and Nakayama 2014). HP1 is an essential reader protein to mediate gene silencing in heterochromatin. Demethylation of H3K9me3 and loss of HP1 binding have been identified as key mechanisms of induction of the ANP gene (*Nppa*) in failing hearts in vivo and in response to increased cardiac preload in vitro (Hohl et al. 2013). As an initial event, phosphorylation of HDAC4 by CaMKIIδB was identified, which led to nuclear export of HDAC4 into the cytosol (Hohl et al. 2013). This led to demethylation of H3K9me3 by JMJC domain-containing demethylases, to dissociation of HP1 protein from chromatin and to the activation of the fetal gene program, including induction of expression of ANP and BNP (Hohl et al. 2013).

2.3.3.3 Phosphoserine Readers

Increased levels of histone phosphorylation are a hallmark of mitosis and immunofluorescence assays using antibodies directed against phospho-histones are frequently used to detect mitotic cells, including cardiomyocytes (Porrello et al. 2011). Recently, the role of CaMKIIδB in phosphorylation of histone H3 and its recognition by 14-3-3 adapter proteins (Fig. 2.3) in response to cardiac stress has been described (Awad et al. 2015). Histone H3 phosphorylation at serine-10 can be detected in fetal and adult cardiomyocytes (Awad et al. 2013). Phosphorylation of H3S10 increases after cardiac injury (Awad et al. 2013). 14-3-3 binds to H3S10-P at the promoter regions of fetal genes to activate their transcription, thus facilitating the expression of the hypertrophic gene program.

During mitosis (and in response to other stimuli), threonine-3 of histone H3 is phosphorylated and this event is being recognized by survivin (Fig. 2.3) followed by the formation of the chromosomal passenger complex (CPC) and recruitment of Aurora B kinase (Kelly et al. 2010). This complex leads to progression of mitosis with spindle formation. Interestingly, survivin is also expressed in cardiomyocytes. Ablation of survivin in mouse cardiomyocytes led to postnatal lethal cardiomyopathy (Schrickel et al. 2012; Levkau et al. 2008). Survivin-deficient cardiomyocytes displayed lower mitotic rates of cardiomyocytes, thus resulting in hearts with

smaller total cardiomyocyte numbers (Levkau et al. 2008). In addition to its function as a reader of phosphorylated histones, survivin has been characterized as an antiapoptotic protein. In survivin-deficient hearts, cardiomyocyte apoptosis occurred at similar levels as in wild-type cardiomyocytes (Levkau et al. 2008). However, further experiments are required to distinguish between the antiapoptotic role and the histone reader function of survivin in cardiomyocytes.

Conclusions

DNA methylation and histone modifications in cardiomyocytes are highly dynamic during development and in disease. Development of novel techniques for the isolation of pure cardiomyocyte nuclei together with advanced DNA-sequencing technologies has led to the first epigenome maps of mouse cardiomyocytes during development and in disease. Modifications of genomic DNA and histones can be recognized by specialized "reader" proteins, which modulate cardiomyocyte function. The first published studies demonstrate that several reader proteins are important for cardiomyocyte development and heart failure. Due to the large variety of epigenetic readers, considerable efforts will be required to uncover the complex interplay between epigenetic modifications of DNA and histones and to identify the involved signaling pathways. In addition, these techniques should now be applied to study the dynamics of the epigenome in human cardiomyocytes.

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Epigenetic Regulations in Cardiac Development

3

Mei Xin and Kunhua Song

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Abstract

Abnormalities in heart development lead to congenital heart disease affecting ~1 % of newborns. Severe congenital heart disease can cause heart dysfunction at very early development stages, whereas mild heart defects may trigger heart dysfunction until adulthood. The adult mammalian heart has very limited regenerative capacity. Designing therapeutic strategies for congenital heart disease is likely dependent on understanding the mechanisms of heart development. Heart development is orchestrated by complicated biological processes, including signal transduction, transcriptional regulation, and epigenetic regulation. In this chapter, we review the current processes of epigenetic modifications during heart development.

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3.1 Heart Development

During embryo development, heart formation precedes that of all other organs, providing an essential pump for future circulation and distribution of nutrients and oxygen throughout the embryo. Initiation of heart development occurs following embryonic gastrulation, the formation of three germ layers: ectoderm, mesoderm, and endoderm.

Cardiac development is an orchestrated process that begins with specification of splanchnic mesoderm into cardiogenic mesoderm in the primitive streak stage embryo (Fig. 3.1). At embryonic day 6.5 (E6.5), mouse cardiac progenitor cells form at the anterior region of the primitive streak during gastrulation (Garcia-Martinez and Schoenwolf 1993; Tam et al. 1997). These cells express the transcription factors mesoderm posterior 1 and 2 (Mesp1 and Mesp2), the early cardiac progenitor markers. At E7.5, cardiac progenitor cells migrate in an anterior-lateral direction under the head folds to form two groups of cells on either side of the midline: the cardiac crescent (also called the first heart field or FHF) and the second heart field (SHF) which lies medial and anterior to the FHF. Cardiac precursors at the FHF differentiate and incorporate into the heart tube with low proliferation capability. Transcription factors NKx2.5 and Gata4 are the early genes that are expressed throughout the cardiac crescent. The SHF is composed of the undifferentiated subpopulation of the cardiac progenitors and marked by Isl1, a LIM homeodomain transcription factor. By E8.0, cardiac precursor cells from the FHF migrate

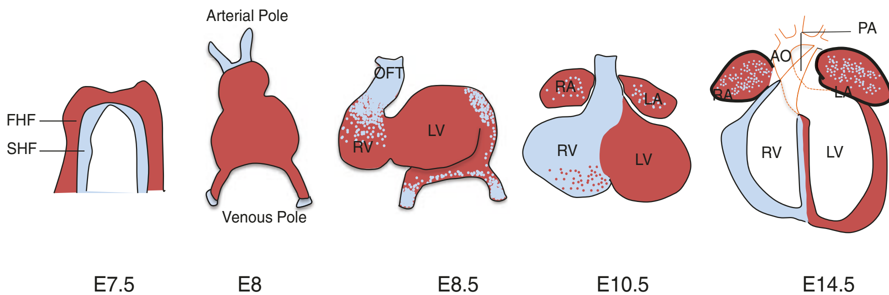

Fig. 3.1 Mouse heart development. During mouse embryogenesis, heart formation begins with two cardiac progenitor cell populations derived from common progenitors in the mesoderm. At E7.5, the cardiac crescent, which is also referred to as the first heart field (*FHF*), resides anterior to the embryo, with the secondary heart field (*SHF*) located medial and anterior to the cardiac crescent. The FHF contributes to the future left ventricle (*LV*), right atrium (*RA*), and left atrium (*LA*). The SHF contributes to the future right ventricle (*RV*), part of the atria and the outflow tract (*OFT*). By E8.0, cardiac progenitor cells from the FHF migrate to the midline to form a linear heart tube, which is further expanded posteriorly and anteriorly with cells migrating from the SHF, giving rise to arterial pole (*AP*) and the venous pole (*VP*). At E8.5, the linear heart tube undergoes a rightward looping to give rise to primitive ventricles and atria. Subsequently, heart maturation occurs with septum formation in the ventricles and atria. Neural crest cells migrating from the dorsal neural tube are involved in the septation of the OFT into the aorta (*AO*) and the pulmonary artery (*PA*)

to the midline to form a linear beating heart tube containing cardiomyoctyes and the underlying endothelial cells serve as a scaffold for subsequent heart growth. Blood enters the heart through the inflow tract (venous pole) located at the posterior of the heart tube and exits the heart through the outflow tract (arterial pole) located at the anterior of the heart tube. The elongation of the heart tube is achieved by an active recruitment of the cells from the SHF at both the arterial and venous poles of the heart. At E8.5, the heart tube undergoes uneven growth resulting in a rightward looping, followed by an anterior movement of the venous pole. This leads to the alignment of the primitive ventricles and atria with the inflow and outflow segments at the anterior pole of the heart, which is necessary for the proper development of future cardiac chambers (Garry and Olson 2006; Srivastava 2006). The FHF contributes to the future left ventricle as well as the future right and left atria. The SHF contributes to the future right ventricle, as well as the outflow tract and future right and left atria. Recent genetic study has suggested a third lineage population, the sinus venous progenitors that are positive for Tbx18 and negative for Nkx2.5 (Christoffels et al. 2006; Lepore et al. 2006; Mommersteeg et al. 2010), which contribute to the future conduction system. The subsequent heart maturation process involves valve formation as well as the septum development. At E14.5, the heart is covered by an epithelial layer named epicardium, and chambers are separated by interventricular septum (IVS). At later fetal developmental stages (from E16.5 to the birth), the four chambers are separated by IVS, interatria septum (IAS), and the atrioventricular valves.

Abnormalities in cardiac development lead to congenital heart defect, a defect in heart and vessel structure at birth. Congenital heart defect affects ~1 % of newborns. It has been shown that a fraction of congenital heart disease is associated with mutations in genes involved in cardiac development and structures. For examples, some mutations in *Myh6* encoding a cardiomyocyte structure protein α-myosin heavy chain are associated with atrial septal defects (ASD) (Granados-Riveron et al. 2010). Mutations in cardiac transcription factors including Nkx2-5, GATA4, and Tbx5 are also associated with ASD, ventricular septal defects (VSD), and abnormalities of conduction system (Garry and Olson 2006; Mohan et al. 2014; Reamon-Buettner and Borlak 2004; Stallmeyer et al. 2010; Xiang et al. 2013). Severe congenital heart defects can cause death in childhood or heart failure in adulthood. Therefore, understanding the transcriptional regulatory network during cardiogenesis will shed light on developing therapeutic tools for preventing and treating human congenital heart disease.

3.1.1 Transcriptional Regulation of Cardiac Development

The process of cardiac development is precisely regulated through activating and inhibitory pathways spanning multiple tissues. Recent studies have implicated many signaling pathways and transcriptional regulations involved, though more complicated molecular mechanisms need to be uncovered to better understand the process. It has been suggested that inductive signals such as bone morphorgenetic

protein (BMP), Notch, and fibroblast growth factor (FGF), are important to promote cardiomyocyte fate commitment, and balance cardiac differentiation and progenitor cell expansion (Klaus et al. 2007; Prall et al. 2007). For example, Notch1 activity is required for cardiac progenitor cell differentiation and for impeding the expansion of cardiac progenitor cells (Kwon et al. 2009). Wnt signaling promotes early cardiogenesis and plays an inhibitory role later as cardiomyocyte differentiation occurs. The Wnt/β-catenin dependent expansion of cardiac progenitors requires the downregulation of genes promoting cardiomyocyte differentiation (Kwon et al. 2007, 2009; Naito et al. 2006).

These complex signaling networks control cardiogenesis via downstream transcription factors, which regulate cardiac gene expression to influence differentiation, proliferation, and cell migration. It has been shown that evolutionarily conserved regulatory machinery consisting of a network of core transcription factors activated by upstream signaling regulates the expression of genes required for heart growth and morphogenesis during heart development (Olson 2006). Transcription factors Nkx2-5, MEF2, GATA, Tbx, and Hand are the key players in this core regulatory network. The T-box transcription factor eomesodermin (Eomes), expressed in the earliest cardiac precursor, induces cardiac mesoderm by activating the helix-loop-helix transcription factors Mesp1 and Mesp2, which regulate cardiac specification and migration (Bondue and Blanpain 2010; Bondue et al. 2008; Kitajima et al. 2000; Saga et al. 1999). Cardiogenic signals and Mesp transcription factors are able to activate the transcription factor Nkx2-5, which is necessary for specification of the cardiac lineage and activation of other transcription factors such as the myocyte differentiation gene (*Mef2),* and a zinc finger-containing transcription factor, GATA4 (Bodmer 1993; Gajewski et al. 1998; Harvey 1996). Cardiac transcription factors are able to physically interact with each other and bind to the same regulatory region in the genome, synergistically activating cardiac gene expression (Bruneau et al. 2001; Garg et al. 2003; Ghosh et al. 2001; He et al. 2011; Hiroi et al. 2001).

3.1.1.1 Nkx2-5

The homeobox gene *Nkx2-5*, one of the earliest genes expressed in the cardiac lineage, is activated by the inductive signals for cardiogenesis initiation, and is expressed in the cardiogenic mesoderm upon specification of the lineage (Komuro and Izumo 1993; Lints et al. 1993; Prall et al. 2007; Stanley et al. 2002). Nkx2.5 is expressed early in the cardiac crescent, later in both the FHF and SHF, and remains expressed in the adult heart. *Nkx2-5*, whose *Drosophila* orthologue is the *Tinman* gene, is evolutionarily conserved and has been shown to be necessary for specification of the cardiac lineage in flies (Bodmer 1993; Gajewski et al. 1998). Unlike the *Tinman* gene in Drsophila, *Nkx2-5* in mice is not required for the initial events of cardiogenesis. Mice lacking *Nkx2-5* are embryonically lethal between E9 and E10. Cardiac specification is not affected, and the heart tube was formed in the mutant embryos. However, the morphogenesis of the heart tube and left ventricular development failed to occur (Lyons et al. 1995). This suggests that other *Nkx* genes may play redundant roles in controlling early cardiac development. In humans,

mutations of the *NKX2-5* gene have been associated with congenital heart disease (Schott et al. 1998). Ventriclular deletion of *Nkx2.5* leads to defects in the conduction system (Pashmforoush et al. 2004). Nkx2.5 negatively regulates cardiac inductive genes such as *BMP2* to inhibit cardiac progenitor cell expansion, is essential in balancing SHF progenitor cell proliferation and differentiation, and is required for maintaining morphogenesis of OFT (Prall et al. 2007). Overexpression of *Nkx2-5* in zebrafish or frog embryos results in upregulation of cardiac gene expression and expansion of the heart field (Chen and Fishman 1996; Cleaver et al. 1996).

3.1.1.2 GATA

GATA transcription factors contain two class IV zinc finger domains, and bind to HGATAR DNA motifs. They can be divided into two subgroups. *GATA1*, *GATA2*, and *GATA3* are expressed predominantly in hematopoietic cells, and regulate differentiation-specific gene expression in T-lymphocytes, erythroid cells, and megakaryocytes (Orkin et al. 1998). Subgroup 2 consists of *GATA4*, *GATA5*, and *GATA6*, which are expressed in several endodermal and mesodermal lineages, such as the developing heart, liver, lung, gonad, and gut. They play critical roles in regulating tissue-specific gene expression (Molkentin 2000).

Null mutations in the Drosophila *GATA4* orthologue, *pannier* (Reiter et al. 1999), or mouse *GATA4* (Kuo et al. 1997; Molkentin et al. 1997) result in early defects in cardiogenesis. Mouse embryos lacking *GATA4* die at E8.5 due to cardiac bifida and failure of ventral foregut closure. Early cardiac-specific deletion of *GATA4* also results in myocardial thinning, abnormal endocardial cushion development, and right ventricular hypoplasia (Zeisberg et al. 2005), while cardiac-specific deletion at later time points results in reduced cardiac function and an inability to undergo hypertrophy following pressure overload or exercise (Oka et al. 2006).

Dose-sensitivity of *GATA4* in regulating cardiac function has been revealed. Reduction in *GATA4* dosage from a hypomorphic *GATA4* allele causes cardiac septal and other congenital heart defects (Pu et al. 2004). Heterozygous mutations in *GATA4* are also associated with congenital heart defects in humans (Garg et al. 2003). These mutations not only reduced GATA4 DNA binding affinity and transcriptional activity of downstream target genes, but also disrupt the interaction between GATA4 and Tbx5, suggesting interactions between GATA and other transcription factors play important roles in heart development. GATA4 is an important cardiac lineage marker. Specifically, GATA4 not only regulates the expression of genes critical for cardiac contractility, such as *α-MHC* and *β-MHC* (Charron et al. 1999), but also the expression of important cardiac transcription factors, such as Nkx2.5, MEF2, and Hand2 (Dodou et al. 2004; Jiang et al. 1999; Lien et al. 1999; McFadden et al. 2000; Molkentin et al. 1997; Nemer and Nemer 2001, 2003; Pikkarainen et al. 2004; Reecy et al. 1999).

GATA6 null mice die post implantation due to defects in visceral endoderm function and extra embryonic development (Morrisey et al. 1998). Tetraploid rescue experiments implicate a role for GATA6 in liver differentiation and growth, and suggest that GATA4 provides functional redundancy in liver specification (Zhao et al. 2005). Tissue-specific deletion of *GATA6* in smooth muscle or the neural crest

suggests a role for this factor in patterning the cardiac OFT and the aortic arch (Lepore et al. 2006). To date, defects in myocardial development have not been observed in *GATA6* mutant mice.

Moreover, in vitro studies suggest GATA4 and GATA6 interact and function synergistically in activating atrial natriuretic factor (*ANF*) and *BNP* gene expression in cardiomyocytes (Charron et al. 1999). Furthermore, GATA4 and 6 have been shown to be sufficient in driving extraembryonic endoderm differentiation in embryonic stem cells, suggesting a critical role for these factors in early development. GATA4 and GATA6 play redundant roles in cardiogenesis. Compound heterozygous mutations in *GATA4* and *GATA6* genes results in embryonic lethality at E13.5, accompanied by a spectrum of defects in liver development, hematopoiesis, and the cardiovascular system (Xin et al. 2006). Loss of both alleles of GATA4 and GATA6 totally blocks cardiomyocyte differentiation and heart development (Zhao et al. 2008).

3.1.1.3 MEF2

The myocyte enhance factor 2 (MEF2) belongs to the family of MADS box transcription factors, which play critical roles in muscle development (Gossett et al. 1989), chondrocyte maturation (Arnold et al. 2007), and brain development (Flavell et al. 2006; Mao et al. 1999). The four members of the MEF2 protein family (MEF2A-D) share similar protein structures consisting of the MADS box followed by the MEF2 domain, which mediates dimerization, and the transactivation domain, which mediates the protein-protein interaction with activators or repressors.

MEF2C is the first detectable *Mef2* gene during cardiogenesis. It is detected in the early precardiac mesoderm of the FHF and SHF at E7.5 (Dodou et al. 2004). *MEF2* enhancer studies have identified a cardiac-specific enhancer for the SHF containing GATA and Isl1 consensus binding sites. Mutations of these sites disrupt *MEF2C* expression in the SHF. MEF2C could also be regulated by FoxH1 and Nkx2.5 through another regulatory element (von Both et al. 2004).

MEF2C knockout mice die at E9.5 with severe cardiovascular defects (Lin et al. 1997, 1998), including failure of cardiac looping, pericardial effusion, and disorganized vessel formation. This phenotype suggests a role for *MEF2C* in the SHF, which leads to an undeveloped right ventricle, and a short OFT. The absence of sinus venousus in the MEF2C mutant suggests that MEF2C plays an important role in the SHF contribution to the venous and arterial poles during heart development. Some cardiac markers for heart development such as cardiac α-actin, Mlc1A, and Hand2 are significantly down-regulated in *MEF2C* null embryos.

MEF2A null mice die within the first two weeks of birth due to cardiac dilation and right ventricle failure, associated with a decrease in the number and dysfunction of mitochondria in cardiomyocytes (Naya et al. 2002).

3.1.1.4 Tbx

T-box transcription factors (Tbx) direct transcription of their target genes in different tissues via binding to the consensus sequence GGTGT by their highly conserved DNA binding domain T-box. Out of the 18 T-box proteins in mice, Tbx1, Tbx2,

Tbx3, Tbx5, Tbx18, and Tbx20 are expressed in the developing heart. T-box proteins are essential for early cardiac lineage determination, chamber formation, and differentiation of the specialized conduction system. Tbx transcription factors are able to regulate cardiac gene expression and development by physically interacting with other core cardiac factors including Nkx2-5 and GATA4 (Stennard et al. 2003). *TBX1*, *TBX3*, *TBX5*, and *TBX20* are associated with human congenital heart diseases and cardiac dialated cardiomyopathy (Alby et al. 2013; Kirk et al. 2007; Merscher et al. 2001; Mori and Bruneau 2004; Postma et al. 2008; Yagi et al. 2003).

Tbx1 is expressed in the endoderm and core mesoderm of the pharyngeal arches, contributing to the myocardial wall OFT and part of the right ventricle. Contribution of the SHF to the OFT is diminished in Tbx1 null mice suggesting that Tbx1 is required for the promoting SHF cell proliferation and contribution to the OFT (Xu et al. 2004).

Tbx5 is expressed in the precardiac venous mesoderm, and is essential for development of inflow tract, atrioventricular canal, and left ventricular myocardium. Mice lacking the *Tbx5* gene die at E9.5 with a deformed linear heart tube acompanied by a hypoplastic posterior heart segment (Bruneau et al. 2001). *ANF* and *Connexin 40* (*Cx40*) are down-regulated in *Tbx5* mutant mice. Moreover, Tbx5 interacts with other important cardiac transcription factors, such as Nkx2-5 and GATA, to synergize transcriptional efficacy (Bruneau et al. 2001; Garg et al. 2003).

Tbx2 and Tbx3 are expressed in primary myocardial cells (Yamada et al. 2000). Mice lacking Tbx2 display a defect in chamber formation and OFT morphogenesis. In the atrioventricular canal, Tbx2 antagonizes Tbx5 function by competing with Tbx5 for DNA binding and gene regulation (Yamada et al. 2000).

Tbx20 is expressed in the cardaic cresent and looping heart tube and is required for development from the heart tube to the four chamber organ (Chakraborty and Yutzey 2012; Singh et al. 2005). Deletion of *Tbx20* in mice leads to hypoplastic heart tissue and death at E10.5 (Singh et al. 2005). Ablation of *Tbx20* in adult cardiomyocytes results in cardiac dysfunction, progressing to heart failure and death through dys-regulating expression of genes important for excitation-contraction function in cardiomyocytes (Shen et al. 2011). During cardiomyogenesis, Tbx20 is able to regulate gene expression by physically interacting with other transcription factors including GATA4, GATA5, Nkx2-5, and Tbx5 (Brown et al. 2005; Stennard et al. 2003).

3.1.1.5 Hand

The basic helix-loop-helix (bHLH) family of transcription factors have been shown to play important roles in specification, differentiation, and morphogenesis during embryogenesis (Massari and Murre 2000). Hand1 (heart and neural crest derivatives expressed transcript 1, or (eHand) and Hand2 (also referred to dHand) are among the earliest cardiac chamber-specific transcription factors (Cserjesi et al. 1995; Srivastava et al. 1995, 1997). Hand1 is first detected at the cardiac cresent stage, and later exclusively in the outer curvatures of the left ventricle and OFT (Biben and Harvey 1997; Srivastava et al. 1997). In contrast, Hand2 expression in the heart is detected in the right ventricle. *Hand1* null mouse embryos die at E8.0 due to severe extraembryonic defects. In contrast, targeted deletion of *Hand2* results in

embryonic lethality at E10.5 due to cardiac and vascular defects (Srivastava et al. 1997; Yamagishi et al. 2000). Furthermore, these mice display right ventricular hypoplasia. This suggests that the *Hand2* gene is essential for morphogenesis of the right ventricle, and formation of the neural crest-derived aortic arches. Deletion of both *Hand1* and *Hand2* results in a more dramatic phenotype where only one chamber is formed, which expresses atrial genes and lacks expression of ventricular genes. These results suggest that *Hand1* and *Hand2* genes are required for proper left and right ventricle formation respectively.

3.2 Epigenetic Modifications

Heart development is orchestrated by a network of transcription factors. Transcription factors bind to their DNA recognition elements located in enhancer and promoter regions of cardiac genes, which recruits coactivators and facilitates the formation of the preinitiation complex (PIC) containing RNA polymerase II (Green 2005). Accessibility of transcription factors to binding sites on chromosomes is modulated by conformational changes of nucleosomes, a basic unit of chromatin. Nucleosome is composed of ~147 base pairs of DNA packed in left-hand superhelical turns around the histone octamer, which consists of two copies each of the core histones H2A, H2B, H3, and H4 (Luger et al. 1997).

Epigenetic regulation is defined as genetic changes not caused by changes in DNA sequence. Chromatin regulations include DNA methylation, histone modifications, and ATP-dependent chromatin remodeling, and are fundamental in cell fate determination during embryonic development. Dynamic change of epigenetic signatures during mammalian cardiogenesis has been studied using embryonic stem (ES) cells (Paige et al. 2012; Wamstad et al. 2012). In ES cells, cardiomyocyte lineage genes are silent. A majority of cardiomyocyte transcription factor genes such as *Nkx2-5* and *Tbx5* in pluripotent cells are marked by high levels of the repressive histone marker H3K27me3, which gradually decreases during differentiation into cardiomyocyte lineage. Conversely, the active marker H3K4me3 and mRNA transcript level gradually increase during cardiac differentiation (Paige et al. 2012). Genes encoding cardiac structural proteins including Myh6 and cardiac α-actin are marked by neither H3K4me3 nor H3K27me3. During cardiac differentiation, H3K4me3 levels at these gene loci gradually increase (Paige et al. 2012; Wamstad et al. 2012) (Fig. 3.2). These studies suggest that epigenetic modifications are involved in cardiac gene expression, cardiomyocyte specification, and differentiation. In the following sections, we will discuss the roles of epigenetic regulators in cardiac development and disease.

3.2.1 DNA Methylation

DNA methylation is a well characterized process where a methyl group (-CH3) is added to the 5 position of the cytosine pyrimidine ring within cytosine-guanine (CpG) dinucleotides in the genome, leading to 5-methylcytosine (5mC). The 5mC

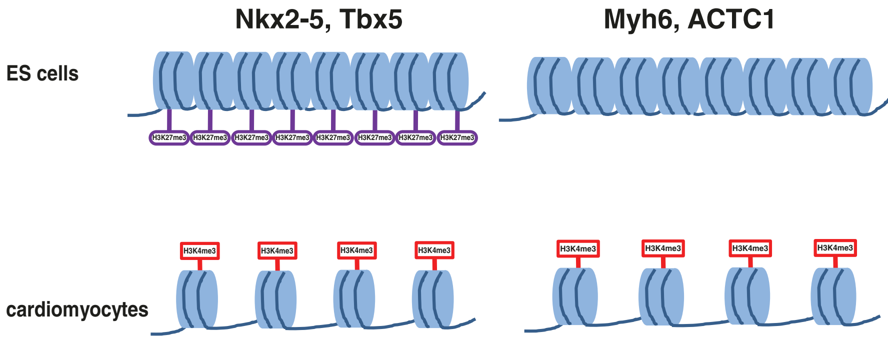

Fig. 3.2 Chromatin modifications at cardiac gene loci during differentiation of embryonic stem cells into cardiomyocytes. The levels of histone modifications H3K27me3 (repressive) and H3K4me3 (active) at regions around cardiac transcription factor genes *Nkx2-5* and *Tbx5* (*left*), and structure proteins *Myh6* and *ACTC1* (*right*) in embryonic stem cells (ES cells), and cardiomyocytes (Paige et al. (2012) and Wamstad et al. (2012))

is typically associated with repressed gene expression in examined vertebrates. In mammals, more than 60 % of all CpG are methylated (Ehrlich et al. 1982). The enzymes catalyzing DNA methylation belong to a family of DNA methyltransferase (DNMTs). DNMT1 is the most abundant and the key DNA methyltransferase in mammals. Deletion of Dnmt1 in mice leads to decreased 5mC levels and embryonic lethality (Li et al. 1992). DNMT3, composed of DNMT3a and DNMT3b, is able to methylate hemimethylated and unmethylated CpG at a rate much slower than DNMT1 (Goll and Bestor 2005). *Dnmt3a* and *Dnmt3b* knockout decreased de novo DNA methylation in ES cells, and resulted in embryonic lethality before E11.5 (Okano et al. 1999). The 5mC can be oxidized to 5-hydroxymethylcytosine (5hmC) by TET (ten-eleven translocation) family enzymes. The 5hmC is a key intermediate in DNA demethylation via either DNA replication or iterative oxidation and the thymine DNA glycosylase (TDG)-mediated base excision repair (BER) pathway (Fig. 3.3a) (Kohli and Zhang 2013). Depletion of TET family members, Tet1, Tet2, and Tet3 results in DNA hypermethylation and impairs mouse development (Dawlaty et al. 2014), which suggests a critical role of Tet-mediated 5hmC in DNA methylation and development.

3.2.2 Histone Modifications

Histones are subject to various posttranscriptional modifications including acetylation, methylation, and ADP-ribosylation of lysine, ubiquitylation/sumoylation, phosphorylation of serine and threonine, and citrullination of arginine (Bannister and Kouzarides 2011; Christophorou et al. 2014). These changes in histone structure are essential in the regulation of gene expression. The most studied histone modifications are lysine acetylation/deacetylation, catalyzed by histone acetyltransferases/histone deacetylases, and lysine methylation/demethylation, catalyzed by

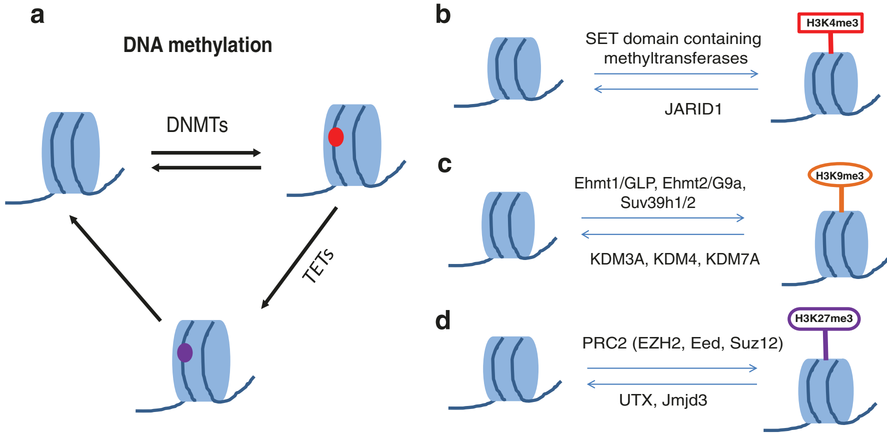

Fig. 3.3 Reversible chromatin modifications. (**a**) Methylation at 5-cytosine (5mC, in *Red*) of DNA is added by DNA methyltransferases (*DNMTs*). TET family members are involved in DNA demethylation by converting 5mC to 5-hydroxymethylcytosine (5hmC, in *Purple*). Histone methyltransferases and histone demethylases control the levels of active H3K4me3 (**b**), and repressive H3K9me3 (**c**) and H3k27me3 (**d**)

histone methyltransferases/histone demethylases (Mckinsey 2012; Klose and Zhang 2007). Effects of lysine acetylation and deacetylation on cardiovascular function have been broadly discussed in other chapters. Here, we focus on roles of histone methylation and demethylation in cardiac development.

3.2.2.1 H3K4me3

Trimethylation of lysine 4 on histone H3 (H3K4me3) is a histone code marking active transcription of the gene. In human ES cells, H3K4me3 marks two thirds of total genes (Zhao et al. 2007). Modification of H3K4me3 in promoters of pluripotent genes such as *OCT3/4*, *SOX2*, and *NANOG* correlates with activation of these genes, which indicates that H3K4me3 is a key histone code to maintain pluripotency of hESCs (Pan et al. 2007). Dynamics of H3K4 methylation is mediated by several SET (Su(var)3-9, Enhancer of zeste, Trithorax) domain containing methyltransferases such as mixed lineage leukemia 1-5 (MLL1-5), SET1A/B, SET7/9, and Smyd family (SET And MYND Domain-Containing Protein) (Martin and Zhang 2005). Jumonji C (JmjC)-domain-containing JARID1 protein family including RBP2/JARID1A, PLU1/JARID1B, and SMCX/JARID1C function as histone demethylases to remove methyl group from di- or tri-methylation of H3K4 (Christensen et al. 2007; Yamane et al. 2007) (Fig. 3.3b).

3.2.2.2 H3K9me3

Trimethylation of lysine 9 on histone H3 (H3K9me3) is a repressive marker associated with gene silencing. H3K9me3 is a constitutive heterochromatin mark in terminally differentiated cells. H3K9 methyltransferases including Ehmt1/GLP (euchromatic histone-Lysine N-methyltransferase 1), Ehmt2/G9a, Setdb1 (SET

Domain, Bifurcated 1), and Suv39h1/2 (Suppressor of Variegation 3-9 Homolog 1 (Drosophila)) regulate global levels of H3K9me3 (Sridharan et al. 2013). H3K9me3 level is critical to maintain differentiated cell fate of fibroblasts. Reduction of H3K9 methylation by knocking down H3K9 methyltransferases including Suv39H1/H2, Ehmt1, Ehmt2, and Setdb1 enhances reprogramming fibroblasts into pluripotent stem cells (Soufi et al. 2012; Sridharan et al. 2013). Demethylation of H3K9 is maintained by several members of the JmjC domain-containing histone demethylase family. For example, KDM3 (also known as JHDM2) or Jumonji domain containing 1 (JMJD1) members are involved in demethylation of mono- or di-methylation on H3K9 (Fodor et al. 2006; Wissmann et al. 2007). Demethylation of trimethylation on H3K9 is catalyzed by enzymes of the KDM4 subfamily (also known as JHDM3 or JMJD2) (Whetstine et al. 2006) (Fig. 3.3c).

3.2.2.3 H3K27me3

Polycomb repressor complex 2 (PRC2), is composed of three subunits: enhancer of zeste 2 (Ezh2), embryonic ectoderm development (Eed) and suppressor of zeste 12 (Suz12), and methylate lysine 27 on histone H3 (H3K27) to generate mono-, di-, and trimethylation of H3K27 (Simon and Kingston 2013). H3K27me3 functions as a docking site for Polycomb group (PcG) repressor complex 1 (PRC1) to repress gene expression (Simon and Kingston 2013). Removal of a methyl group from H3K27me3 is mediated by H3K27 demethylases including the JmjC domain-containing protein ubiquitously transcribed tetratricopeptide repeat X chromosome (UTX), as well as Jmjd3 (Hong et al. 2007; Agger et al. 2007) (Fig. 3.3d).

3.2.2.4 ATP-Dependent Chromatin Remodeling

ATP-dependent chromatin remodeling complexes control gene expression by moving, destabilizing, and reassembling nucleosomes. Energy produced by ATP hydrolysis is used to disrupt or alter interactions between histones and DNA, leading to altered accessibility of transcription factors to regulatory regions of genes. ATP-dependent chromatin remodeling members share a conserved ATPase domain, but bear unique flanking domains, which categorize them into four distinct families: SWI/SNF (switching defective/sucrose nonfermenting) family, ISWI (imitation switch) family, CHD (chromodomain, helicase, DNA binding) family, and INO80 (inositol requiring 80) family (Clapier and Cairns 2009; Ho and Crabtree 2010).

3.3 Epigenetic Regulators in Cardiac Development and Disease

3.3.1 H3K4 Methyltransferase

SmyD1a and SmyD1b, alternative splicing products of the *SmyD* gene in zebrafish function as H3K4 methyltransferases to generate mono-, di-, or trimethylation on H3K4 (Tan et al. 2006). SmyD1 is required for myofibril organization and cardiac contraction (Tan et al. 2006). Targeted deletion of *SmyD1* in mice leads to

embryonic lethality due to several cardiac defects including ventricular hypoplasia, enlarged ventricular chambers, defects in cardiomyocyte maturation, and malformation of right ventricle (Gottlieb et al. 2002). During right ventricle development, *SmyD1* is a downstream target of transcript factor MEF2C and an upstream regulator of the transcript factor Hand2 (Gottlieb et al. 2002; Phan et al. 2005).

Mll2 is a member of trithorax group (TrxG) family and functions as an H3K4 methyltransferase. Mutations in human *Mll2* gene have been linked to Kabuki syndrome (Ng et al. 2010). Half of examined Kabuki patients showed the cardiac defects ASD and VSD (Ng et al. 2010). Global deletion of *Mll2* in mice resulted in embryonic lethality before E11.5 (Glaser et al. 2006). Deletion of *Mll2* in ES cells did not block differentiation into cardiac lineage, but delay maturation of contractile cardiomyocytes (Lubitz et al. 2007). Specific deletion of *Mll2* in cardiac lineage cells will be helpful to further understand its roles in cardiomyogenesis.

3.3.2 H3K9 Methyltransferase

G9a and GLP are known H3K9 methyltransferases and contribute to gene silencing (Sridharan et al. 2013). Deletion of G9a or GLP by gene targeting led to embryonic lethality around E9.5 due to growth defects (Tachibana et al. 2002, 2005). In cardiac specific GLP knockout and G9a knockdown (*GLP*-KO/*G9a*-KD) mice, H3K9me2 greatly decreased in cardiomyocytes (Inagawa et al. 2013). Remarkably, *GLP*-KO/*G9a*-KD mice had severe atrioventricular septal defects (AVSD) and died just after birth (Inagawa et al. 2013). Gene expression profiling showed that a few non-cardiac genes were up-regulated in *GLP*-KO/*G9a*-KD mice, indicative of important roles of H3K9 methylation in cardiomyocyte specification.

3.3.3 H3K27 Demethylases

So far, UTX (also known as KDM6A) and JMJD3 (also known as KDM6B) are the only known demethylases to remove methyl groups from H3K27me3. UTX and JMJD3 remodel chromatin through either demethylation of H3K27me3 or demethylase-independent mechanisms (Hong et al. 2007; Lan et al. 2007; Agger et al. 2007; Miller et al. 2010; Shpargel et al. 2014; Wang et al. 2012).

JmjC-domain-containing protein, UTX, is able to remove methyl groups from H3K4me2 or H3K4me3 (Hong et al. 2007; Lan et al. 2007). UTX is highly expressed in the heart during mouse embryonic development (Lee et al. 2012), suggesting a potential role in cardiac development. UTX-null ES cells failed to differentiate into beating embryonic bodies (EB), and expression of cardiac specific genes was inhibited (Lee et al. 2012). UTX-null embryos died before E11 (Lee et al. 2012). At E9.0, the tubular heart in mouse embryos undergoes looping and forms separated atrial and ventricular chambers. However, UTX-null embryo hearts remained tubular structures and lacked atrial-ventricular chamber formation. In addition, the neural tube in UTX-null embryos could not close (Lee et al. 2012; Shpargel et al. 2014;

Wang et al. 2012). UTX physically interacts with cardiac transcription factors including Nkx2-5, Tbx5, GATA4, which recruits the UTX complex to enhancer regions and activates cardiac gene expression during cardiac differentiation (Lee et al. 2012; Shpargel et al. 2014). Physical interaction between UTX and Brg1 (Brahma Protein-Like 1, also known as SMARCA4), a component of the SWI/SNF ATP-dependent chromatin remodeling complex, raises a hypothesis that cross-talk between multiple classes of epigenetic regulators mediate cardiomyocyte specification and differentiation (Lee et al. 2012; Shpargel et al. 2014).

Inactivation of Jmjd3 by gene trap in mice leads to perinatal lethality due to respiratory failure. Interestingly, ES cells in this system expressed nearly half the amount of Jmjd3 as wild type control cells, indicative of a hypomorphic mutation (Burgold et al. 2012). Deletion of Jmjd3 by gene targeting completely abolished Jmjd3 expression in Jmjd3$^{-/-}$ ES cells (Ohtani et al. 2013). Jmjd3$^{-/-}$ embryos died before E6.5 (Ohtani et al. 2013). During ES cell differentiation, Jmjd3 is recruited to the promoter of *Brachyury*, a pan-mesoderm marker, to reduce H3K27me3 levels. Jmjd3 physically interacts with β-catenin, a key component of Wnt signaling pathway. Deletion of *Jmjd3* impairs differentiation of ES cells into mesoderm and cardiac lineages including endothelium and cardiomyocytes in vitro (Ohtani et al. 2013). Specific deletion of Jmjd3 in cardiomyocyte-lineage by Nkx2-5-cre or Myh6-cre is required to fully understand function of Jmjd3 in cardiomyogenesis.

3.3.4 ATP-Dependent Chromatin Remodeling Complexes

The BAF (Brg1/Brm-associated-factor) complex, composed of 12 subunits, is one of two types of SWI/SNF chromatin remodeling complex in vertebrates (Ho and Crabtree 2010). The other type is PBAF (polybromo-associated BAF).

In mammals, the BAF complex includes one of two ATPases, Brg1 and Brm. Brm is not necessary for mouse development (Reyes et al. 1998), whereas Brg1 is required for embryogenesis (Bultman et al. 2000). Deletion of *Brg1* in cardiomyocytes by Nkx2-5-cre resulted in embryonic lethality before E10.5. Those embryos showed small chamber size and impaired cardiac looping (Takeuchi et al. 2011). The few Brg1-null mice who survived through birth had dilated ventricular chambers and ventricular septation defects (VSD) (Takeuchi et al. 2011). Half of *Brg1* haploinsufficient mice *Brg1*$^{\pm}$ died before 3 weeks of age. These *Brg1*$^{\pm}$ mice had congenital heart defects (CHD) such as a dilated heart, VSD, and ASD. *Brg1* haploinsufficient mice that survived the neonatal period showed abnormalities in cardiac function such as impaired cardiac relaxation, slow and irregular heart conduction determined by atrioventricular block, mild prolongation of the QRS complex, and occasional sinus node dysfunction (Takeuchi et al. 2011). Brg1 genetically interacts with key cardiac transcription factors such as Tbx5 and Nkx2-5 to activate cardiac gene expression at least in part via Baf60c (Takeuchi et al. 2011).

Baf60c is specifically expressed in heart and somites during early mouse embryogenesis. Knockdown of Baf60c in mouse embryos led to lethality at E10–E11. These mutant embryos showed shortened OFT, hypoplastic atria and right ventricle,

and lack of the atrioventricular canal (Lickert et al. 2004). The SWI/SNF ATPase Brg1 weakly interacts with cardiac transcription factor GATA4, but no direct interaction between Brg1 and key cardiac transcription factors Tbx5 and Nkx2-5 have been observed. However, expression of Baf60c initiates and enhances association of Brg1 and Tbx5, Nkx2-5, and GATA4 (Lickert et al. 2004). Therefore, Baf60c functions as a mediator to promote interactions of cardiac transcription factors and BAF complexes. Forming complexes composed of cardiac transcription factors and chromatin remodeling regulators specifically potentiates activation of cardiac genes during heart development.

3.4 Perspective

Cardiac development is tightly regulated by networks of epigenetic regulators and transcription factors. More and more studies show that physiological interactions of epigenetic regulators and cardiac transcription factors orchestrate cardiac specification, differentiation, morphogenesis, and chamber formation. Malformations during cardiac development lead to congenital heart disease. Studies on the interaction networks of cardiac transcription factors and epigenetic regulators may lead to finding cures for congenital heart disease.

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DNA Methylation in Heart Failure

4

Justus Stenzig and Roger S-Y Foo

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Abstract

Methylation of cytosine residues in DNA is an epigenetic feature that plays an important role in gene transcription regulation. However, it is controversial whether DNA methylation is functionally relevant for the initiation and progression of heart disease. This chapter explains the basic mechanisms and functional consequences of DNA methylation. Its potential role in heart disease and its link to other epigenetic processes are examined and summarized, and opposing results from major studies in the field are discussed. The chapter concludes with an outlook on the field, including possible therapeutic and diagnostic applications.

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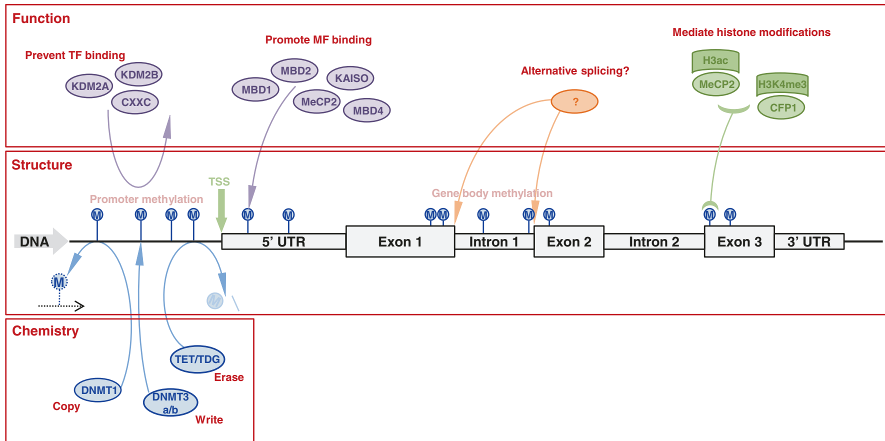

Fig. 4.1 Basic properties of DNA methylation. *UTR* untranslated region, *TSS* transcription start site, *M* methyl group on cytosine residue, *TF* transcription factor, *MF* methylated DNA associated factor, *DNMT* DNA methyl transferase, *TET* ten-eleven translocation enzyme, *TDG* thymine-DNA glycosylase, *KDM2A* histone lysine demethylase, *CXXC* cxxc domain containing proteins (binding demethylated DNA), *MBD2* methyl-CpG-binding domain protein 2, *KAISO* kaiso protein, *CFP1* cerebellar foliar pattern 1

4.1 Preface

DNA methylation is a form of chromatin modification that was discovered decades ago. Methylation of the nucleic acid cytosine in a palindromic CpG format is involved in gene expression regulation, can be inherited during cell division and alters chromatin structure but not DNA sequence. It fulfils all criteria to be called a classical epigenetic feature (Fig. 4.1).

When Conrad Waddington introduced the term 'epigenetics' in the late 1930s (Waddington 1939, 1942), underlying mechanisms were yet to be discovered. The notion that cytosine residues in the DNA were frequently modified by the addition of a methyl group at ring carbon-position 5 was discovered as early as in the 1950s (Doskocil and Sorm 1962; Wyatt 1951). Theoretical considerations and experimental evidence linking DNA methylation to the consolidation and maintenance of any given cellular differentiation state were soon to follow (Riggs 1975; Holliday and Pugh 1975). However, decades of research established a too narrow 'paradigm' of the properties of DNA methylation that has only recently been challenged. The most widely accepted concept is that the addition of a methyl group to cytosine residues in gene-promoter regions of the mammalian genome ultimately shuts off transcription of the corresponding gene (Stein et al. 1982; Jones 2012; Vardimon et al. 1982). This mechanism was believed to maintain the individual differentiated state of every cell while assisting other epigenetic marks like histone modifications and nucleosome positioning but also transcription factors in this role (Jones 2012). The advent of induced pluripotent stem cell (iPS) technology enabling cell reprogramming in vitro (Takahashi and Yamanaka 2006), recent evidence for surprisingly fast dynamics of cytosine methylation even in non-dividing cells (Gilsbach et al. 2014; Barres et al. 2012) as well as the discovery of active demethylation mechanisms have now reshaped our view of DNA methylation (Shen et al. 2013; Tahiliani et al. 2009). The idea that DNA methylation is a permanent un-erasable mark has been largely challenged over the recent years.

DNA methylation is much more complex than previously understood. On one hand, it can be stable over years or decades and can be transgenerationally inherited, as evident from studies on the 'Dutch Hunger Winter'. The offspring of women pregnant during this period of starvation in winter 1944–1945 were, many years later, more susceptible to diabetes and cardiovascular disease than those born shortly before or afterwards, implicating a mechanism of epigenetic inheritance (Heijmans et al. 2008). Moreover, while DNA methylation is clearly responsible for at least certain forms of long-term gene repression like X-chromosome inactivation (Venolia and Gartler 1983), transposon silencing (Feenstra et al. 1986; Yoder et al. 1997) and imprinting (Li et al. 1993), it is on the other hand able to respond to external stimuli and becomes altered in many complex diseases like cancer (Esteller 2008), neurological disorders (Jakovcevski and Akbarian 2012) and importantly also heart disease (Movassagh et al. 2011). DNA methylation thereby provides a unique mechanism integrating cellular memory and external signals. This new insight reaches beyond the mere switching on or off of gene transcription during development and has sparked research on the topic in many new fields of medicine.

Gene transcription in heart disease is clearly deregulated (Dorn et al. 2003). Epigenetics, in particular DNA methylation, represents an integrative memory storage for information, which guides, or at least reflects, gene transcriptional regulation (Schubeler 2015). Targeting this mechanism in a systems approach presents an attractive therapeutic option. Many common heart diseases such as heart failure and coronary artery disease are associated with slow but persistent risk factor-driven progression over years, as well as aberrant gene transcription. It is tempting to hypothesize that ageing and continuous exposure to risk factors may leave their mark on DNA methylation and in turn alter gene transcription, aggravating or even causing heart disease. Hence, understanding the precise nature of beneficial and deleterious DNA methylation changes may ultimately lead to the possibility of reverting DNA methylation marks, opening up new ways to treat heart disease. These are new avenues only just beginning to be explored.

4.2 Mechanisms of DNA Methylation and Demethylation

Despite our rapidly advancing and broadening view of DNA methylation, generally accepted mechanisms of how DNA methylation proceeds still apply (Schubeler 2015). DNA methyltransferase enzymes are responsible for cytosine methylation. In mammals, the main isoforms known to date are DNA methyltransferase 1, 3A and 3B, encoded by *DNMT1, DNMT3A* and *DNMT3B* genes. Whereas DNMT1 is believed to be responsible for maintenance methylation, the latter isoforms 3A and 3B are thought to be responsible for de novo methylation.

As DNA methylation is inheritable from parent cell to daughter cell, and also from parent organism to daughter organism, it has to be copied from one DNA strand to the other during cell division. Cytosine methylation mainly appears in the context of CG dinucleotides (also commonly referred to as CpG, taking the bridging phosphate group into account), enabling DNMT1 to copy the methylation mark from one cytosine to the complementary C of the neighbouring G, maintaining GpG methylation in a palindromic manner. DNMT1 is assisted in this role of the

so-called 'maintenance' methylation by adapter proteins that recognize hemi-methylated DNA and subsequently recruit DNMT1 to fill in the methylation gaps (Sharif et al. 2007), preventing passive demethylation through DNA replication. To maintain DNA methylation over several DNA replication rounds though, the assistance of DNMT3A or 3B is required (Liao et al. 2015; Schubeler 2015).

DNMT3A and 3B on the other hand are believed to be responsible for de novo methylation. They represent the DNMT enzymes that respond to external stimuli and play the crucial role of integrating heritability and DNA methylation plasticity. DNMT3A and 3B do not require hemi-methylation for their activity but can act on non-methylated DNA (Okano et al. 1999). All DNA methyltransferases use S-adenosyl-methionine as substrate. DNMT2 turned out to be a tRNA methyltransferase and was accordingly renamed TRDMT2 while DNMT3L shares sequence homology with the other DNMT3 enzymes (Aapola et al. 2000) and acts as a cofactor, but displays no DNMT activity itself (Bourc'his et al. 2001).

The process of DNA demethylation had been elusive for decades. Passive demethylation by dilution of methyl marks during cell division and DNA replication was previously believed to be the only means of DNA demethylation. Only recently has the process of active DNA demethylation been convincingly mapped out. The enzymatic moieties involved in the process are ten-eleven translocation enzymes 1–3, encoded by *TET1-3* and thymine DNA glycosylase, encoded by *TDG* (Kohli and Zhang 2013). Members of the TET family of enzymes oxidize methylcytosine in several iterative steps, first to hydroxymethylcytosine, then consequently to formylcytosine and finally to carboxycytosine (He et al. 2011; Ito et al. 2011). The latter is then removed and replaced with non-methylated cytosine by TDG via base excision repair (BER (Zhang et al. 2012)). The discovery of this mechanism has sparked more research in neurology and also in the cardiac field. Formerly, DNA methylation research in terminally differentiated cells seemed to be of little importance as it was deemed unlikely to gain therapeutic relevance. However, now that we know about the unexpected plasticity of DNA methylation in cardiomyocytes, it turns into a potentially attractive drug target.

In the classical scenario, DNA cytosine methylation inhibits gene transcription. This is achieved either by sterical prevention of transcription factor binding or by binding of specialized factors that recognize methylated DNA. The latter mechanism in fact only applies to a small subset of methylated regions and many differentially methylated CpG have functions beyond transcription repression as discussed below (Ziller et al. 2013). Gene transcription can be repressed either by strong methylation of CpG-rich regions associated with genomic features, the so-called CpG islands (Venolia and Gartler 1983; Yoder et al. 1997) or in gene promoter or enhancer regions with low CpG content (Stadler et al. 2011; Hon et al. 2013; Ziller et al. 2013). In this case, DNA methylation affects binding of different chromatin-binding factors that govern transcriptional repression. These include methylated DNA-binding domain containing proteins MBD1, 2, 4 and MeCP2 (Baubec et al. 2013; Hendrich and Bird 1998) as well as SET and ring-associated (SRA) and zinc-finger domain containing proteins (Buck-Koehntop and Defossez 2013). It is likely that many more of such chromatin-binding factors exist that are yet to be identified

and characterized (Vaquerizas et al. 2009). These reader proteins in turn interact with the DNA methylation and demethylation machinery. Interestingly, knockout mice for *Mbd1*, *Mbd2* and *Kaiso* only display neurological phenotypes and no overt cardiovascular symptoms (Zhao et al. 2003; Hendrich et al. 2001; Prokhortchouk et al. 2006). Even *Mecp2* knockout mice, which recapitulate the phenotype of Rett's syndrome, displayed no overt cardiovascular abnormalities despite the existence of mild cardiac symptoms in the respective human *MECP2*-related disease (Guy et al. 2001).

Many additional mechanisms of interplay between different epigenetic marks have been identified and add further complexity to the epigenetic landscape. Adapter proteins link DNA methylation to histone acetylation and methylation and vice versa. Examples are repressive marks such as H3K9me3 (histone 3 lysine 9 trimethylation), which is upregulated by DNA methylation via MeCP2 and various histone methyltransferases (Sarraf and Stancheva 2004), or H3K27me3, which is decreased by DNA methylation via polycomb-repressive complex 2 (PRC2 (Wu et al. 2010)). This means that DNA methylation can have opposite functions by increasing or decreasing repression. Activating marks are also influenced, among them H3ac, decreased by DNA methylation via MeCP2 and histone deacetylases (HDAC (Skene et al. 2010)) and H3K4me3, decreased via cerebellar foliar pattern 1 (CFP1, CXXC1 in humans (Thomson et al. 2010)).

Though many interactions between DNA methylation and the histone code are known, and an important role of histone modifications in heart disease is established, tissue-specific interactions between the two epigenetic features are only just beginning to be analysed.

4.3 Regulation of DNA Methylation and Demethylation

Though the basic principles of how DNA is methylated were discovered long ago, little is known about how DNA methylation is regulated in the heart. DNMT enzymes establish DNA methylation whereas the removal of methyl groups (demethylation) involves intermediates 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and possibly 5-carboxycytosine (5caC). However, how these processes are regulated in individual tissues remains largely unknown. To guide both mechanisms to their target structures, a complex interplay with other chromatin modifications and transcription factors is necessary. General principles of this network have been identified but tissue-specific data for the heart is still scarce.

The regulation of genome-wide DNA methylation marks seems to be increasingly complex, keeping pace with our increasing understanding of the regulation of other epigenetic events: DNA methylation is largely dependent on neighbouring histone modifications. Examples are the recruitment of DNMT3A and 3B to sites of H3K9me3 via heterochromatin protein 1 (HP1 (Lehnertz et al. 2003)), the co-targeting of histone and DNA methylation (Muramatsu et al. 2013; Chang et al. 2011) and a multitude of other known mechanisms through which DNA methylation may depend on histone modifications (Rose and Klose 2014). Regulation of

histone modification by conventional intracellular signalling in the heart has in part been known for years (Backs and Olson 2006) but is currently recognized to be additionally exerted by different species of non-coding RNA (Mathiyalagan et al. 2014). Further direct experimental evidence is required to show if similar links exist between non-coding RNA and DNA methylation. Mechanisms that link different RNA species to histone modifications and ultimately to DNA methylation might be more general than previously anticipated. LncRNA antisense transcripts like XIST, Hotair and Kcnq1ot1 establish the repressive histone methylation mark H3K27me3 on the inactive X-chromosome by guiding PRC2 to its target locus (Rinn et al. 2007; Pandey et al. 2008) or in case of Kcnq1ot1 by directly guiding DNMT1 (Mohammad et al. 2010). H3K27me3 in turn recruits DNMT enzymes to these loci (Schlesinger et al. 2007), ultimately leading to DNA methylation. Moreover, another small RNA species, so-called PIWI-interacting RNAs (piRNA) guide DNMTs to target loci in mice (Aravin et al. 2007).

4.4 The DNA Methylation Landscape

Much effort has been undertaken to map out the landscape of human DNA methylation in diverse tissues and diseases (Ziller et al. 2013; Stadler et al. 2011; Hon et al. 2013). Large projects such as ENCODE (The ENCODE (ENCyclopedia Of DNA Elements) Project 2004) have only been enabled by the recent advance in DNA methylation analysis techniques. The age of high-throughput sequencing (Koboldt et al. 2013) has opened up the possibility to analyse DNA methylation on a whole genome scale down to the resolution of a single base in a single cell (Guo et al. 2013; Smallwood et al. 2014) using techniques like whole genome bisulphite sequencing (BS-seq (Meissner et al. 2008)). This method combines next-generation sequencing with sodium bisulphite conversion, by which DNA is chemically modified to convert non-methylated cytosine residues to uracil, which is then represented by thymine in the following amplification steps (Frommer et al. 1992). By comparing BS-seq results to the reference genome, the methylation status of individual cytosines can be analysed as previously non-methylated cytosines would be represented as thymidines whereas methylated residues would remain unmodified.

This technological leap has provided deep and wide insight into the complex landscape of DNA methylation but also raised many questions and challenged the textbook view of DNA methylation as merely a means of transcriptional repression. About 70–80 % of all CpGs in differentiated human cells are methylated (Bird 2002; Ziller et al. 2013). Cytosine residues in non-CpG context usually display low methylation below 5 % but exhibit higher frequencies of methylation in embryonic stem (ES) cells and in brain tissue (Lister et al. 2009; Varley et al. 2013). The role of non-CpG methylation to date remains speculative, but it seems to be also dynamic (Lister et al. 2011) and to play a role in transcription factor binding (Malone et al. 2001; Inoue and Oishi 2005). Due to the limited data regarding the function of non-CpG methylation in general and the total absence of functional data regarding the heart, we will only focus on CpG methylation in the following sections.

CpGs are unevenly distributed among the genome. The majority of the genome is evolutionarily depleted of CpG content. CpG-dense regions of ~1-kb length are called CpG islands (CGI) although an exact definition is still lacking. About 70 % of human-annotated genes carry a CGI region near their transcription start site (TSS), but more than half of CGIs in the genome are not located near or in annotated genes (orphan CGIs). These orphan CGIs possibly represent regulatory features of non-coding RNA (Smith and Meissner 2013). Most gene-associated CGIs are non-methylated in the majority of cell types. This is likely due to transcription factor binding preventing de novo methylation (Stadler et al. 2011; Hon et al. 2013; Ziller et al. 2013). Interestingly, gene promoters associated with dense and large CGIs (high CpG promoter, HCP) seem to be less prone to regulation by DNA methylation than those with intermediate (ICP) or low CpG density (LCP) or enhancers with average CpG density (Brinkman et al. 2012; Bartke et al. 2010; Smith and Meissner 2013; Ziller et al. 2013). Promoter regions with low and intermediate CpG density consequently display the largest tissue-specific variation. About 20 % of all CpGs seem to have tissue-specific methylation and the differential methylation of the flanking regions of CpG-dense stretches of DNA – CGI shores – seems to be of greatest importance for expression regulation (Ziller et al. 2013). Regions with very high CpG content are only regulated by DNA methylation in rare but prominent exceptional cases such as imprinted regions, silencing of transposable elements and X-inactivation (Li et al. 1993; Schubeler 2015; Leung et al. 2011).

Additional regulatory information is conferred by differential methylation of exons and introns, the so-called gene body methylation. The role of gene body methylation is not yet completely understood. Recent evidence suggests mainly two roles of gene body methylation: the regulation of alternative splicing and the inhibition of alternative promoter usage. It is known that exons are usually methylated to a higher degree than introns, whereas intron–exon boundaries are differentially methylated, indeed suggesting a role for DNA methylation in alternative splicing (Maunakea et al. 2013; Chodavarapu et al. 2010; Maunakea et al. 2010).

4.5 The Advent of DNA Methylation Research in Heart Disease

By the time the first paper on DNA methylation in heart disease was published (Movassagh et al. 2010) research on the topic in other fields like neuroscience, stem cell technology and above all cancer had been considerably more advanced. This discrepancy still holds true. A simple Pubmed database search today yields 740 entries for ‘DNA methylation’ and ‘heart’, most of which are review articles and publications only peripherally touching the subject. The number of high-ranking publications on the topic containing original data is probably still below 30. A similar search using the keywords ‘DNA methylation’ and ‘cancer’ or ‘DNA methylation’ and ‘brain’ yields more than 25,000 or more than 3,500 publications, respectively. In 2010, at the time of the first publication on DNA methylation in the heart, the count was already more than 10,000 for methylation in cancer. What could explain this?

Both cancer and heart disease are associated with gene-transcriptional deregulation (Dorn et al. 2003; Meyerson et al. 2010). Studying mechanisms of gene expression regulation in these diseases is therefore attractive, but the point of departure is obviously different. A prominent feature of cancer cells is their ability to divide rapidly, whereas in vivo cardiomyocytes proliferate only marginally – if at all (Bergmann et al. 2009). DNA methylation was previously believed to be stable throughout the entire life cycle of a cell. For cardiomyocytes, this would have meant permanent DNA methylation marks throughout the entire life of an individual. In addition, in cancer the situation is rather unequivocal: mutations lead to aberrant transcription, which needs correction. As opposed to that, in heart disease the hypertrophic or foetal gene programme following myocardial stress comprises both causative and compensatory deregulation (Dorn et al. 2003). We believe that these two facts, stability and complexity, have initially rendered transcriptional regulation by DNA methylation plainly unattractive to study. Moreover, the heritability of DNA methylation is one of its most intriguing features – one that had no relevance for cardiomyocytes before the discovery of potential transdifferentiation (Yi et al. 2013) and the advent of stem cell therapy in general.

If DNA methylation had locked the gene transcription of a cardiomyocyte in its present situation, defining its differentiated state, never to be changed in vivo, what would be the point of accumulating deeper insight of DNA methylation in cardiomyocytes? The observation that DNA methylation might be altered in heart disease (Movassagh et al. 2011) is able to react rapidly to external stimuli like mechanical stress (Gilsbach et al. 2014) and moreover does not need dilution by cell division and passive demethylation to be erased (Zhang et al. 2012; Kohli and Zhang 2013) was unexpected. These new observations have brought DNA methylation into the centre of attention, highlighting its potential for a deeper understanding of heart function and pathology and making it a potentially attractive drug target.

4.6 The DNA Methylation Landscape in Healthy Heart

Individual tissues are characterized by specific DNA methylation signatures. Even without cell-type-specific analysis, the heart is clearly epigenetically distinguishable from other tissues, as numerous studies have shown (Kitamura et al. 2007; Xie et al. 2011; Pai et al. 2011). After almost complete erasure of DNA methylation in the female and male pronuclei shortly after fertilization, the common general DNA methylation signature of all tissues is re-established at an early stage of embryogenesis. The level of lowest methylation is reached in the early blastocyst, hours after fertilization (Santos et al. 2002). During the first days of development, most DNA methylation marks are re-established (Smith et al. 2012) with at least 70 % of all CpG methylation present by the time of implantation (Monk et al. 1987; Kafri et al. 1992). There is significant debate concerning the exact sequence of events (Smith et al. 2012), but it appears that a large portion of the DNA methylation map is laid out before organogenesis. Nevertheless, a small but significant fraction of

methylated cytosines is established later during organ development and postnatal growth as has been shown for many tissues, including explicitly the heart (Chamberlain et al. 2014; Sim et al. 2015; Gilsbach et al. 2014; Gu et al. 2013). These methylated CpGs confer, or at least mark tissue specificity. The situation in physiological embryonic development is different from ES cells. ES cells bear a distinct DNA methylation signature, which confers pluripotency and includes a higher-average CpG methylation than found in somatic cells (Lister et al. 2009; Ziller et al. 2013). As discussed above, it remains elusive to what extent DNA methylation itself is instructive for gene expression regulation. However, the role of DNA methylation is clearly not restricted to non-tissue-specific processes like X-inactivation or transposon silencing and imprinting (Schubeler 2015) and the aforementioned publications have established a role in tissue-specific regulation in the heart.

Though the exact number and location of the marks remain to be more firmly established, recent data argues for far less than half of all CpG methylation to be tissue specific. Using different-mapping tools, the DNA methylation marks that are established during embryonic heart development have been attributed to heart-specific regulatory pathways but also to more general embryogenesis-related-signalling pathways in recent studies (Sim et al. 2015; Chamberlain et al. 2014; Gilsbach et al. 2014). Our current understanding is that most differentially methylated regions (DMRs) in cardiomyocytes are usually less than 1 kb long (Ziller et al. 2013; Gilsbach et al. 2014). And though the number of DMRs will vary greatly depending on the cell type to which cardiomyocytes are compared, the fact that between ~2500 (Sim et al. 2015) and ~6500 (Gilsbach et al. 2014) regions are differentially methylated between neonatal and adult heart (in mice) conveys a rough impression of the extent of dynamic regulation.

In addition to studying DNA methylation in disease conditions, as discussed below, a clearer understanding of physiological DNA methylation functions in the heart might lead to further therapeutic options. One prominent and highly controversial field specifically with regard to DNA methylation is pluripotency, stem cell research and technology and the regulation of cell renewal by epigenetic mechanisms. The question if and to what extent cardiomyocytes proliferate in adult life, particularly following injury, remains unsolved. Nevertheless, there is good evidence for at least a small degree of myocyte renewal (Bergmann et al. 2009). A recent popular theory is based on the idea that cardiomyocytes or cardiac progenitor cells possess the general ability to proliferate upon certain stimuli such as injury but are prevented from this activity by inhibitory signalling from other cell types or by epigenetic regulation or an interplay between both (Senyo et al. 2013; Felician et al. 2014).

Cardiomyocyte renewal as a definitive solution for preventing or reversing contractility loss after myocardial injury would certainly revolutionize therapy options for heart disease. Apart from exogenous cell therapy, this could be achieved by either stimulating the proliferation of cardiomyocytes, or of putative endogenous stem cell populations, or by reprogramming resident non-cardiomyocytes to become cardiomyocytes (Sahara et al. 2015). DNA methylation is an important

feature of cell identity, and it is hence expected to be an important factor for the regulation of these processes in iPS cells (Sanchez-Freire et al. 2014), which represent a potential source for exogenous cell renewal, but also in endogenous cell populations of possible progenitor cell pools. However, the role of DNA methylation for endogenous cell proliferation to date has only been exemplarily studied in specific cases such as Notch signalling (Felician et al. 2014). An overview of current research on DNA methylation in ES cells and iPS cells would certainly go beyond the scope of this chapter. In a more specific regard though, and consistent with the general lack of knowledge regarding DNA methylation in cardiac disease, little organ-specific research has been conducted so far on the role of DNA methylation for cell renewal and forward reprogramming. The latter approach aims at reprogramming other cell types like mesenchymal stem cells, fibroblasts and others which do not necessarily need to be natural cardiac progenitors directly to cardiomyocytes and is increasingly favoured in tissue replacement research (Sahara et al. 2015).

In both cases of cell renewal using de novo-programmed cardiomyocytes, either endogenous or exogenous, epigenetic memory is likely to play a role in cellular functionality. In reprogrammed cells, DNA methylation signatures of former differentiation states may be retained, potentially impairing maturation and function of the derived cell type. This phenomenon has been shown for various cell types (Kim et al. 2010a) including cardiomyocytes (Sanchez-Freire et al. 2014). Information about the physiological DNA methylation signature of cardiomyocytes in the form of reference epigenomes is therefore indispensable for successful cell-based therapy. As fully differentiated, functional and mature cardiomyocytes are needed, future research will routinely include the assessment of a mature cardiomyocyte DNA methylation profile in any quality-control process for cells used in cell replacement therapy.

4.7 DNA Methylation in Heart Failure

4.7.1 Evidence from Studies in Humans

The current view of DNA methylation in heart failure has been shaped by only a handful of publications. These studies nevertheless firmly established the view that DNA methylation plays an essential role in disease-related gene regulation. However, the field is still in its infancy and a large knowledge gap remains to be filled, connecting the very general evidence from whole genome approaches in disease and in focused in vitro studies. Research on DNA methylation in heart failure remains exciting at present: on one hand, it is known that DNA methylation plays an important role in heart disease. On the other hand, there are countless fundamental questions that remain to be solved.

First evidence for a role for DNA methylation in heart failure stems from two studies in human disease, in which differential methylation in end-stage heart failure was detected, when compared to healthy controls (Movassagh et al. 2010;

Movassagh et al. 2011). The authors speculated that the hallmark reactivation of foetal genes in hypertrophy and heart failure, the so-called foetal gene programme (Dorn et al. 2003), would be associated with an analogous foetal or hypertrophic DNA methylation programme. In both publications, the authors enriched methylated DNA fragments and quantified them, firstly by microarray, secondly using next-generation sequencing. Both studies were performed using whole heart tissue, as was the case in a third relevant study from a different group, where DNA methylation was quantified by microarray (Haas et al. 2013). In all three publications, a unifying pathological DNA methylation signature was detected. Though the results cannot be directly compared due to different methodology and disease aetiology, the findings are similar. The first report, importantly, was able to identify differentially regulated genes by their methylation signature, which had formerly only been possible the other way round (Movassagh et al. 2010). Additional findings from all three studies underpin that what had been known before from other tissues also holds true for heart disease: the DNA methylation signature of the heart is surprisingly stable, despite the fact that the cellular composition slightly varies between health and disease. Only a subset of genomic features is differentially methylated between these two states. Hypomethylation in disease is more common than hypermethylation (Movassagh et al. 2011; Haas et al. 2013) and differential methylation, though it does occur in promoter regions, is not confined to the vicinity of TSSs. In fact, CGIs in several different contexts were significantly differentially methylated: promoters, gene bodies and putative promoters of non-coding transcripts, but not intergenic regions, untranslated regions (UTRs) or active enhancers, as marked by transcription factor p300 binding (Movassagh et al. 2011). All three studies additionally aimed at identifying candidate genes and correlating DNA methylation and transcription. While upregulation of transcription correlated with promoter demethylation, the opposite could not be detected (Movassagh et al. 2011). Even the analysis of some of the most DMRs initially suggested no obvious correlation with transcription. However, further analysis revealed a more complex mechanism by which DNA methylation might regulate transcription of some candidate genes such as *ADORA2A* (Haas et al. 2013) while for others, like *LY75* and *DUX4,* the classical ‘paradigm’ of repression by promoter methylation could be confirmed (Haas et al. 2013; Movassagh et al. 2011).

Apart from the identification of a specific DNA methylation signature of heart failure, we learned two more lessons from these studies. Firstly, when the authors performed pathway analysis on their set of genes that were associated with DMRs, they on one hand confirmed these genes to be indeed involved in heart disease-associated pathways but on the other hand also detected far less specific pathways (Haas et al. 2013). Secondly, the aforementioned candidate genes that could be identified through their differential methylation are doubtlessly interesting and worth deeper investigation. However, one would have expected a much closer relationship between methylation and expression. In fact, none of the classical heart failure-associated genes, such as *NPPA*, *NPPB*, *ACTA1*, *SERCA2A*, *MYH7* and many others, stand out by their DNA methylation signature. At present, one can

only speculate on the possible reasons for this observation, which will be discussed in detail below: (i) only a subset of cardiac genes might be directly regulated by DNA methylation, if at all, (ii) DNA methylation might play a crucial role for trans-regulation by distant elements and non-coding transcripts involved in heart failure, which are far less obviously identifiable and (iii) lastly analysis can be improved by cell-type specificity.

(i) The assumption that only a small subset of cardiac genes might be directly regulated by DNA methylation in a classical way is almost self-evident. Several studies have shown that only a subset of CpGs is methylated in a tissue-specific manner, strongly arguing for the fact that many genes might not be directly regulated by DNA methylation (Ziller et al. 2013; Chamberlain et al. 2014). (ii) Distal regulatory elements such as enhancers are increasingly recognized to be regulated by DNA methylation (Hon et al. 2013). As their influence on cardiac gene expression depends on chromatin secondary and tertiary structure and is far from being sufficiently understood, studies on DNA methylation could not yet have possibly been able to map those elements to cardiac gene expression regulation. Further insight into the nature of long-range chromatin interactions and nucleosome positioning, using techniques such as chromosome conformation capture (3C (Karbassi and Vondriska 2014; de Wit and de Laat 2012)) or linkage analysis (Johnson et al. 2014), will be necessary. Moreover, transcriptional regulation is relying on a variety of non-coding transcripts. Only a few with a known relevance for heart disease are identified yet, such as Myheart (*MHRT* (Han et al. 2014)) or Braveheart in mice (*Bvht* (Klattenhoff et al. 2013)), and details are reviewed in Chap. 5. These likely represent only the tip of the iceberg, as the number of long non-coding RNAs (lnRNA) encoded in the human genome is believed to be at least twice as high as the number of coding transcripts (Uchida and Dimmeler 2015). Regulatory microRNAs (miRNA) add to the complexity with at least 700 miRNAs expressed in the heart and more than 20 differentially regulated in pathological hypertrophy (Hirt et al. 2015). All these non-coding RNAs can potentially regulate heart disease-related transcription and might themselves be epigenetically regulated. (iii) Analysis of DNA methylation in the aforementioned publications was performed on whole bulk tissue. Cell composition in heart varies considerably during development and disease. Cardiomyocytes account for 20–60 % of all cell types in adult heart (Nag 1980; Banerjee et al. 2007; Gilsbach et al. 2014). Even if other cell types such as fibroblasts, blood and inflammatory cell types, endothelial cells and others do not change their DNA methylation signature in disease, the epigenetic readout will still be significantly diluted when whole tissue is studied rather than individual cell types. The studies performed on human heart would probably have yielded more clear-cut results if cell-type-specific analysis had been carried out, which is far more technologically challenging.

In summary, to fully understand the mechanisms of DNA methylation in heart disease, fundamental questions about RNA biology and trans-regulation by chromatin conformation have yet to be solved. Nonetheless, studies on human tissues have provided solid evidence for a specific DNA methylation signature in heart disease.

4.7.2 Evidence from Animal Studies

The number of animal studies on DNA methylation in cardiac hypertrophy and heart failure is equally limited. Whereas the current studies in human all used failing hearts, with disease either caused by mixed aetiology (Movassagh et al. 2011) or by DCM (Haas et al. 2013), the animal work published so far is mainly based on cardiac hypertrophy or heart failure evoked by pressure overload using the transversal aortic constriction (TAC) model (Gilsbach et al. 2014; Vujic et al. 2015) or on in vitro hypertrophy modelling (Stenzig et al. 2016). Results are nevertheless comparable as hypertrophy is known to be the single most influential risk factor for heart failure and represents its pre-failure stage (Levy et al. 1996).

In a recent comprehensive study, Gilsbach et al. were the first to provide cardiomyocyte-specific in vivo evidence for DNA methylation changes during development and heart disease (Gilsbach et al. 2014). Their work, also the first study to combine myocyte-specific analysis with whole genome bisulphite sequencing in the heart (WGBS), provides significant further insight. Presumably due to the advanced methodology, the authors were not only able to reproduce the findings of preceding studies in human heart failure but to provide many additional new findings. The authors compared DNA methylation in mouse ES cells, newborn, adult and failing mouse cardiomyocytes as well as in cardiomyocyte-specific Dnmt3a/3b double knockout (Cre-recombinase driven by *Mlc2a* promoter). Taken together, as in other studies, more CpGs appear to be hypomethylated during development and disease than hypermethylated. The associations between DNA methylation and gene regulation they observed were much tighter than previously anticipated. Many prominent cardiac genes were regulated at least in part by DNA methylation. Interestingly, differential DNA methylation additionally occurred at enhancers marked by binding motifs for cardiac transcription factors such as Mef2c, Gata4, Nkx2.5 and others. DNA methylation seemed to be closely associated with the regulation of genes like *Tnnt2*, *Tnni1* and *Tnni3*, *Atp2a2*, *Myh6* and *Myh7*. The most striking observations were made comparing ES cells and adult heart. Here, stem cell genes became hypermethylated and switched off whereas cardiac genes became hypomethylated and activated, in line with the traditional view of DNA methylation. Moreover, this process continued to be active in postnatal development. Strikingly though, the observations were largely independent of the exact location of DNA methylation, either promoter associated or genic. More specifically, the authors detected a DNA 'demethylation wave' that extended from upstream of the TSS into the first exons during development, paralleling increased expression.

In summary, the study shows that (i) genes active in ES cells but not in cardiomyocytes are progressively methylated and accordingly histone marks are established. (ii) In reverse, genes that are active in cardiomyocytes but not in ES cells are demethylated and respective histone marks are established. (iii) Genes that are transiently active in heart development (such as *Isl1*) are not re-methylated but instead they are repressed by histone modifications such as H3K27me2 in case of prenatally silenced genes or regulated by completely different means in case of postnatally regulated developmental genes. For these main findings, the authors provide

pathway-mapping data and hand-picked examples. Though one might ask for the epigenetic fate of other typical cardiac genes, the association between gene regulation and DNA methylation in heart development is obvious.

The authors performed additional experiments to assess the role of DNA methylation in pressure overload-induced heart failure and the role of Dnmt3a/b--mediated postnatal de novo methylation both in the abovementioned study and in an independent publication. Though the association seems to be less clear-cut, pressure overload led to a partial reactivation of the foetal DNA methylation signature, even after 4 weeks, arguing for previously unanticipated dynamics. This extent of short-term flexibility in striated muscle is likely to analogously exist in humans, as evidence for DNA methylation changes in human skeletal muscle even after hours of exercise suggests (Barres et al. 2012). Moreover, another group has recently described that DNA methylation in rat cardiomyocytes reacts to afterload enhancement even after 7 days (Stenzig et al. 2016). In addition, a second *Dnmt3a* and *Dnmt3b* double knockout mouse line was established by the group of Gilsbach and Hein, this time only knocking out the catalytic domains of the Dnmts (Cre-recombinase controlled by an *Myl7* promoter) and the results were published separately (Nuhrenberg et al. 2015). Curiously, the authors found great differences in gene expression and some differences in DNA methylation in their first publication, when comparing wild-type and knockout using WGBS, but less pronounced changes in their second publication using a microarray. In both studies, the authors described that cardiomyocyte-specific double knockout, either of the entire gene or of only the catalytic domain, prevents gene hypermethylation during development, leading to expression changes of cardiac genes (Gilsbach et al. 2014). However, they surprisingly found no functional effect of these expression changes when analysing mice with catalytic domain knockout – neither in health, nor in pathological hypertrophy.

Additional information stems from another study in which *Dnmt3b* was knocked out in a cardiomyocyte-specific manner. Vujic et al. also present a characterization of these mice in both healthy state and subjected to pressure overload (Vujic et al. 2015). *Dnmt3b* knockout mice initially displayed no overt phenotype. This changed with time or upon pressure overload. Knockout animals then displayed aggravated myocardial disarray and fibrosis. Interestingly, hypertrophic response to pressure overload was blocked in these mice but replaced by early dilatation and further reduced fractional shortening.

So far, the authors only present a restricted expression and methylation profiling of their knockout mice. Expression analysis of a limited number of cardiac- and fibrosis-related genes revealed only very few obvious differences, in line with Gilsbach et al. who only observed expression changes in a limited number of genes. Nevertheless, Vujic et al. were able to detect methylation-related aberrant splicing of *Myh7*. Since they based target choice for methylation analysis on differential expression, they present methylation data for *Myh7* only. It would be intriguing to analyse if aberrant splicing is frequently regulated by differential methylation, as has been reported previously in heart failure (Ames et al. 2013), and to what extent it contributes to heart failure pathophysiology.

To date, heart-specific *Dnmt3a* and *Dnmt3b* and double knockout mice are established in different laboratories, with conflicting results. There are several possible ways to explain the different findings. One possible confounder is strain variability. Methylation in rodents is known to considerably suffer from this confounder, to such an extent that strain-dependent variability can even be greater than cell-type variability (Johnson et al. 2014). An example for strain-specific susceptibility to hypertrophy induction caused by strain-specific DNA methylation differences has even been recently published. However, the authors only present a non-cardiomyocyte-specific DNA methylation analysis (Chen et al. 2016). Other explanations for the conflicting findings include (i) the different severity and duration of the hypertrophic intervention, (ii) the fact that Gilsbach et al. only knocked out the catalytic domain of both Dnmt isoforms in their second study, whereas Vujic et al. performed a complete Dnmt3b knockout and (iii) the use of different promoters to drive the Cre-recombinase (*Myl7* vs. *α-MHC*). To render the picture even more complicated, a recent study showed that the largest portion of de novo methylation targets is shared by Dnmt3a and 3b. Only a fraction of differentially methylated cytosines was non-redundantly targeted by either isoform (Liao et al. 2015). Thus, the informative value of studies on *Dnmt3a* or *3b* single knockout mice is potentially diminished, and these considerations make the observed more pronounced effect of *Dnmt3b* single knockout even more complicated to explain.

Several targeted studies contribute additional knowledge to our current understanding. Methylation of the promoter of *Acta1*, encoding alpha1 skeletal actin, was studied as early as 1999 (Warnecke and Clark 1999). In their pioneering study, the authors demonstrated that the *Acta1* promoter region is progressively methylated during heart development, though methylation does not directly correlate with gene expression – a finding that is compatible with recent studies 15 years later (Gilsbach et al. 2014). DNA methylation has also been shown to contribute to cardiac fibrosis and upregulation of collagen-1 expression in the state of hypoxia, in which *Dnmt1* and *3b* are upregulated through an HIF-1α-dependent mechanism. Though the exact pathways remain to be resolved, knockdown of DNMTs in human fibroblasts led to attenuated fibrosis and confirmed the role of DNA methylation for the pathophysiology, suggesting an indirect mechanism of *COL1A1* gene regulation (Watson et al. 2014). These findings were confirmed in vivo, where non-specific Dnmt inhibition using 5-aza-2’-deoxycytidine (5-aza) attenuated fibrosis in spontaneously hypertensive rats (Watson et al. 2016). However, these results are conflicting with another study on DNA methylation in fibrosis, where transforming growth factor (TGF)-β induced collagen synthesis by combined downregulation and inhibition of DNMT1 and 3A, subsequent promoter demethylation and direct upregulation of *Col1a1* (Pan et al. 2013). The role of DNA methylation in fibrosis therefore needs further investigation.

Direct evidence for the involvement of DNA methylation in gene expression regulation has furthermore been shown for the gene encoding protein kinase Cε (PKCε (Patterson et al. 2012)), the sulphonyl urea receptor genes *Abcc8* and *Abcc9* (Fatima et al. 2012) and *Atp2a2* encoding the sarcoplasmic reticulum calcium ATPase serca2a (Kao et al. 2010, 2011; Angrisano et al. 2014; Stenzig et al. 2016).

The promising results and the mechanistic insight from the aforementioned studies are sparking therapeutic potential. However, it will need much more time and effort for therapies targeting DNA methylation in heart disease to enter the clinics, as will be discussed in the following section.

4.8 Therapeutic Considerations

What would an epigenetic therapy for heart disease look like? Visions and options are unlimited at present. Therapeutic manipulation of DNA methylation has been investigated in a limited number of studies and will open up new possibilities but will also face obstacles, some of which will be discussed in the following section.

The basic idea is simple: Pathological hypertrophy and heart failure are marked by a hallmark transcriptome signature. Some of the changes are detrimental and some are compensatory (Dorn et al. 2003). Current heart failure therapy results in a restitution of aberrant gene expression (Lowes et al. 2002). Normalizing aberrant transcription itself might therefore be directly beneficial. As the transcriptional programme in heart disease appears to be partly governed by an underlying DNA methylation signature, normalizing this programme might in turn lead to normalized transcription and could thereby be useful in heart failure therapy.

Achieving this goal though would be more challenging than it initially appears. Firstly, when comparing failing hearts to healthy hearts there is hypomethylation as well as hypermethylation (Movassagh et al. 2011; Gilsbach et al. 2014), rendering inhibition of either process potentially unfeasible – though there might be exceptions. Secondly, the basic components of the DNA methylation machinery are identical throughout the whole organism, a fact that would presumably warrant inadmissible off-target effects of unspecific DNA methylation or demethylation blockade.

Unspecific blockade of DNA methylation or demethylation might nevertheless be useful for proof-of-principle research and to obtain general information on possible pitfalls and optimization strategies. Attempts so far have focused on hypertrophy and heart failure provoked by norepinephrine infusion (Xiao et al. 2013), on fibrosis (Pan et al. 2013; Watson et al. 2014), on in vitro hypertrophy induced by afterload and phenylephrine treatment (Stenzig et al. 2016) and on serca expression (Kao et al. 2011).

The authors of the first of these studies on DNA methylation manipulation present fairly optimistic data on rats subjected to norepinephrine-induced cardiac hypertrophy. The animals were treated with chronic norepinephrine infusion for 28 days and one group of rats received additional 5-aza treatment for the last 6 days. The authors provide data to support the point that this treatment reversed norepinephrine-induced hypertrophy and failure. In their hands, 5-aza treatment rescued contractility and normalized the expression of a panel of marker genes including *Nppa*, *Nppb* and *Myh7*. Methylation analysis was restricted to a marker locus, which was previously identified by the same group (Patterson et al. 2012), but indeed argues in favour of a DNA methylation-based action of 5-aza in the experiment. Lastly, explanted rat hearts were subjected to ex vivo ischaemia reperfusion injury and

infarct size was measured by triphenyltetrazolium (TTC) assay, though no images are supplied. Preceding in vivo 5-aza treatment rescued the norepinephrine-induced increased susceptibility to ischaemia reperfusion injury.

As discussed above, the impact of DNA methylation on fibrosis is currently under debate. In one study, DNMT inhibition led to increased fibrosis (Pan et al. 2013), compatible with results from *Dnmt3b* knockout mice which display increased fibrosis (Vujic et al. 2015). Conversely, two other studies by another showed DNMT inhibition to be beneficial in reducing fibrosis in vitro (Watson et al. 2014; Watson et al. 2016).

A role of DNA methylation in the regulation of serca expression, encoded by *Atp2a2*, has been postulated by several groups. Kao et al. used 5-aza to prevent the promoter methylation-mediated downregulation of serca upon TNF-α treatment in atrial like HL-1 cells (Kao et al. 2010). In a subsequent study, the same group used the putative DNMT inhibitor hydralazine to achieve this goal (Kao et al. 2011). Two other groups independently identified a role of DNA methylation in serca expression regulation (Angrisano et al. 2014; Stenzig et al. 2016). Stenzig et al. mechanically induced hypertrophy ex vivo in rat Engineered Heart Tissue (EHT). They observed genome-wide methylation changes as early as 7 days after the hypertrophic intervention and were able to attenuate these changes, including *Atp2a2*-promoter hypermethylation and hypertrophy-induced functional impairment, using the non-specific, non-nucleosidic DNMT inhibitor RG108.

Atp2a2 might indeed be an exemption. It is possible that the downregulation of serca might be of such outstanding importance for heart failure pathology that the prevention of such downregulation by DNA methylation inhibition might still be feasible in end-stage disease, even at the cost of significant side effects. Other important genes might be identified to which similar prerequisites apply. There is no doubt about the importance of serca for calcium cycling in heart failure pathophysiology (Kranias and Hajjar 2012). And yet clinical trials using serca-activating small molecules such as istaroxime (Shah et al. 2009) for which no clinical outcome trials have been published and recently planned trials have been stopped (NCT00838253, NCT00869115), as well as serca gene therapy have failed (Celladon press release on Mydicar, 26.04.2015). In view of this, preventing the downregulation of serca by epigenetic means may be particularly attractive.

Thus far, published data is encouraging but the field is still in its infancy. Several obstacles have to be overcome. The lack of tissue and process specificity probably represents the greatest challenge. Since the DNA methylation and demethylation machineries are ubiquitous, further understanding of their regulation in the heart will be necessary to confer specificity to interventions in the processes. Unless this is granted, it is unlikely that the field will advance significantly. This has been the case regarding histone modifications: Proof-of-principle evidence arguing for a therapeutic effect of HDAC inhibition was published a decade ago. Insight into the process since has further evolved. Yet, no further therapeutic strategies have been successful so far, presumably due to an analogous ubiquity of the underlying processes.

One can only speculate on the future of DNA methylation-based therapy as specific targets to be addressed by small molecules, antibodies, miRNAs, siRNA, Crispr-based gene editing and many other therapeutic agents remain to be identified. At

present, even addressing DNA methylation in a general manner is difficult. DNMT inhibitors in current clinical and experimental use can be divided in two classes: nucleosidic and non-nucleosidic inhibitors, both of which face disadvantages. Several nucleosidic DNMT inhibitors are Food and Drug Administration (FDA) and European Medicine Agency (EMA) approved, making them attractive as research compounds. These drugs, among them azacytidine and 5-aza-2-deoxycytidine (5-aza, decitabine), are thoroughly characterized and potentially repurposeable. However, nucleosidic compounds have to be integrated into DNA to be active, trapping DNMT enzymes to their integration sites (Stresemann and Lyko 2008). This mode of action to a certain extent excludes their applicability for heart disease, as most cardiomyocytes even in diseased states are non-dividing (Bergmann et al. 2009). In these cells, azanucleotides are therefore only integrated upon natural base repair and turnover, limiting their therapeutic usefulness considerably since they would be integrated to a much higher extent in rapidly dividing cells in other tissues at the same time. As the molecules can interfere with DNA repair and replication, they may moreover exhibit cytotoxic side effects (Murakami et al. 1995). These will be pronounced as high doses might be necessary to achieve meaningful activity in non-dividing cardiomyocytes. Non-nucleosidic DNMT inhibitors such as RG108 (Brueckner et al. 2005), nanaomycin A (Kuck et al. 2010), hydralazine (Singh et al. 2009) and procainamide (Lee et al. 2005) are often poorly characterized and sometimes speculative. But as these compounds may competitively inhibit cofactor binding (Kuck et al. 2010) rather than integrating into DNA, they display no cytotoxicity. Unfortunately, binding to the cofactor-binding pocket requires these compounds to be highly lipophilic. This unfavourable property is complicating, if not preventing their usefulness in vivo, though with RG108, for at least one compound in vivo pharmacokinetics have been published (Schneeberger et al. 2016). In addition, even the better characterized compounds have not been systematically analysed for effects other than DNMT inhibition. Given the high micromolar concentrations needed for DNMT inhibition (Kuck et al. 2010), other effects are likely to interfere in the in vivo setting.

It may have several implications that DNMT inhibition in vivo and in vitro displayed more consistent results than DNMT knockout, which were mostly beneficial and sometimes contradicting results from genetic manipulation studies. Pharmacological inhibition stretches to both non-cardiomyocytes and DNMT1, which suggests a role for both. Especially the effect of DNMT inhibition in cardiac fibroblasts and the role of DNMT1 both in fibroblasts and in cardiomyocytes remain to be elucidated.

4.9 Biomarker Considerations

Though no clear evidence for an association with DNA methylation in circulating cells has been published so far, DNA methylation might be additionally interesting as a biomarker for heart disease. Indeed, heart disease may correlate with certain methylation marks in circulating cells, sparking hope that DNA methylation might not only be useful as a therapeutic target but also as a diagnostic tool. Results so far

are sparse, not directly related to heart failure, and sometimes conflicting. Homocysteine plasma concentration is still under heavy debate as a biomarker for cardiovascular disease (Dzau 2004), despite considerable effort to identify its contribution to disease (Marti-Carvajal et al. 2015). Kim et al. investigated an association between plasma homocysteine concentration and peripheral blood leukocyte (PBL) gross DNA methylation, speculating that low homocysteine might represent cardiovascular disease via the intermediate of DNA methylation. As homocysteine is the product of the DNA methylation reaction, low levels might reflect low availability of the precursor methionine which might impair physiological DNA methylation (Kim et al. 2010b). However, the authors detected a correlation between hypermethylation rather than hypomethylation in PBL, which conflicts with data from a previous smaller study (Castro et al. 2003), arguing against a strong predictive role for DNA methylation in cardiovascular disease. Additional efforts have been undertaken by a group who reported a correlation between low LINE-1-repetitive DNA element methylation in PBL (Baccarelli et al. 2010), and high mitochondrial DNA methylation (Byun et al. 2015) with ischaemic heart disease and stroke. More evidence stems from further studies associating DNA methylation in circulating cells with vascular disease, leaving open the possibility for a similar association with heart failure (Borghini et al. 2013; Dunn et al. 2014).

4.10 Future Directions

In only 5 years time since the first comprehensive analysis of DNA methylation in heart disease, an exciting new field of research has evolved and sparked hopes of new avenues of heart failure therapies. The recent past has seen tremendous progress and increasing popularity, moving from previous neglect of the topic to promising bold visions. A definite role for DNA methylation in gene expression regulation in cardiomyocyte differentiation, renewal and disease is now established. Early differentiation, maturation, terminal differentiation, possible de-differentiation and pathological transformation each bear individual DNA methylation signatures. We have a rough impression of what DNA methylation landscapes in cardiomyocytes look like. However, at present most of the evidence for perturbed DNA methylation is understood as a static picture. What are the mechanisms that drive differential methylation in heart disease, what are the processes that lead there? What, in turn, might be the consequences of altered DNA methylation in cardiac pathology? How can we ultimately profit from the knowledge, target these processes and design novel therapies? To understand the complete meaning of DNA methylation in each sequence context and the context-specific mechanism of how methylation and demethylation are regulated as well as to identify suitable modifier compounds will be crucial if its therapeutic options are to be exploited. At present though, and despite a lack of these fundamental insights, the idea of epigenetic therapies for heart failure appears as a real possibility and is indeed exciting.

Conflict of Interest The authors declare that they have no conflict of interest.

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Chromatin Remodeling in Heart Failure

5

Pei Han, Jin Yang, Ching Shang, and Ching-Pin Chang

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Abstract

Chromatin provides a dynamic DNA scaffold that reacts to physiological and pathological signals to control the accessibility of DNA sequence and the genomic responses to environmental stimuli. Chromatin can be regulated by nucleosome remodeling, histone modification, and DNA methylation. Histone and DNA modifications occur by covalent alterations of the side chains of histone or bases of DNA, catalyzed by specific histone- and DNA-modifying enzymes, whereas nucleosome or chromatin-remodeling controls noncovalent changes of nucleosomes, including their position and histone composition, effected by adenosine triphosphate (ATP)-dependent chromatin-remodeling complexes. Within the nucleosome, the chromatin remodelers can replace canonical histones with variant forms of histones, which are involved in cardiac stress response. In addition, chromatin remodelers can interact with histone- and DNA-modifying enzymes to control chromatin structure and reprogram gene expression in pathologically stressed hearts. More recently, a chromatin-remodeling factor was found to interact with a cardiac-specific long noncoding RNA to control gene expression and maintain cardiac homeostasis. These functional aspects of chromatin remodelers are critical for the pathogenesis of cardiomyopathy and heart failure. This chapter is focused on the recent progress in understanding the roles of chromatin-remodeling factors in heart failure, new chromatin-based mechanisms, and potential therapeutic strategies for heart failure.

5.1 Introduction

The human genomic DNA within a cell is ~1.7 m long, whereas the nucleus is only ~6 μm in diameter. The cells have evolved chromatin that associates DNA with histone proteins, which provide a scaffold to package the DNA into a small nucleus. The basic packaging unit of chromatin is the nucleosome, consisting of ~147 bp DNA that wraps around an octamer core of histones (two histone H3, two histone H4, two histone H2A–H2B dimers) (Fig. 5.1a). In vertebrates, histone H1 binds to the nucleosomes to stabilize the core histone-bound DNA. Histone H1 also binds to 20–80 bp “linker DNA” between nucleosomes to facilitate the condensation of nucleosomes into 30-nm helical chromatin fibers to further package the genome.

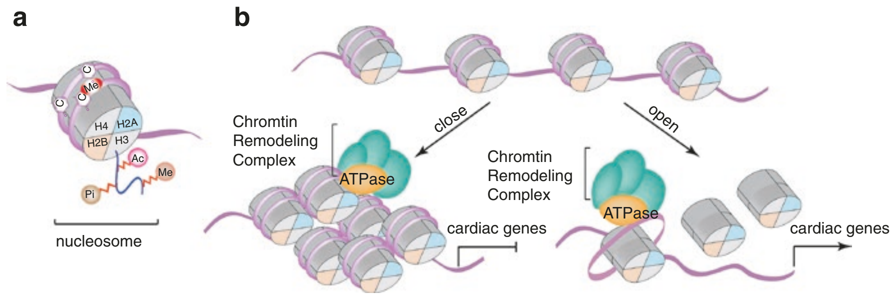

Fig. 5.1 Mechanisms of chromatin remodeling (**a**) Schematic illustration of the nucleosome structure. A typical nucleosome contains 147 base pairs of DNA (*purple line*) that wrap a histone core composed of two molecules of each of the canonical histone 2A, 2B, 3, and 4 proteins. The N-terminal tail (*blue line*) of a histone protein is subject to a variety of covalent modifications, including acetylation (*Ac*), methylation (*Me*), and phosphorylation (*Pi*). (**b**) The open and close state of chromatin. Chromatin-remodeling complex (*yellow ovals*) utilizes energy derived from ATP hydrolysis to package (*left*) or open (*right*) the chromatin to switch on (*right*) or off (*left*) downstream genes

The nucleosome structure is determined by chemical interactions between histones and DNA, which can be covalently modified by histone- or DNA-modifying enzymes. In addition, the genomic position and histone composition of nucleosomes can be altered noncovalently by the ATP-dependent nucleosome-/chromatin-remodeling complexes (Figs. 5.1b and 5.2a). Such covalent and noncovalent modifications of nucleosomes result in alterations of chromatin and changes of DNA sequence accessibility to transcription, recombination, or repair.

An essential component of the chromatin-remodeling complex is the ATPase subunit, which hydrolyzes ATP to provide the energy required for the complex to mobilize nucleosomes or change their histone composition. This ATPase subunit defines four families of chromatin-remodeling complexes: SWI/SNF (switch defective/sucrose nonfermenting), ISWI (imitation switch), CHD (chromodomain, helicase, DNA binding), and INO80 (inositol requiring 80) (reviewed in (Clapier and Cairns 2009; Wu et al. 2009; Ho and Crabtree 2010; Saha et al. 2006)) (Fig. 5.2a). The ATPase protein contains a central catalytic domain (ATPase) flanked by specific domains that uniquely distinguish the ATPase subunit of each family (Fig. 5.2b). The catalytic domain hydrolyzes ATP, whereas the other domains may recognize modified histones, regulate the catalytic activity, or mediate protein–protein interactions with other regulators of chromatin or transcription. The core catalytic domain consists of two RecA-like subdomains: a DEAD-like helicase superfamily C-terminal domain (DExx) and a helicase superfamily C-terminal domain (HELICc). Both the DExx and HELICc domain contain conserved motifs that are characteristic of the superfamily 2 (SF2) helicase (Fairman-Williams et al. 2010; Byrd and Raney 2012) (Fig. 5.2b). Hence, the catalytic domain that hydrolyzes ATP also shares the biochemical structure of SF2 helicases that participate in the

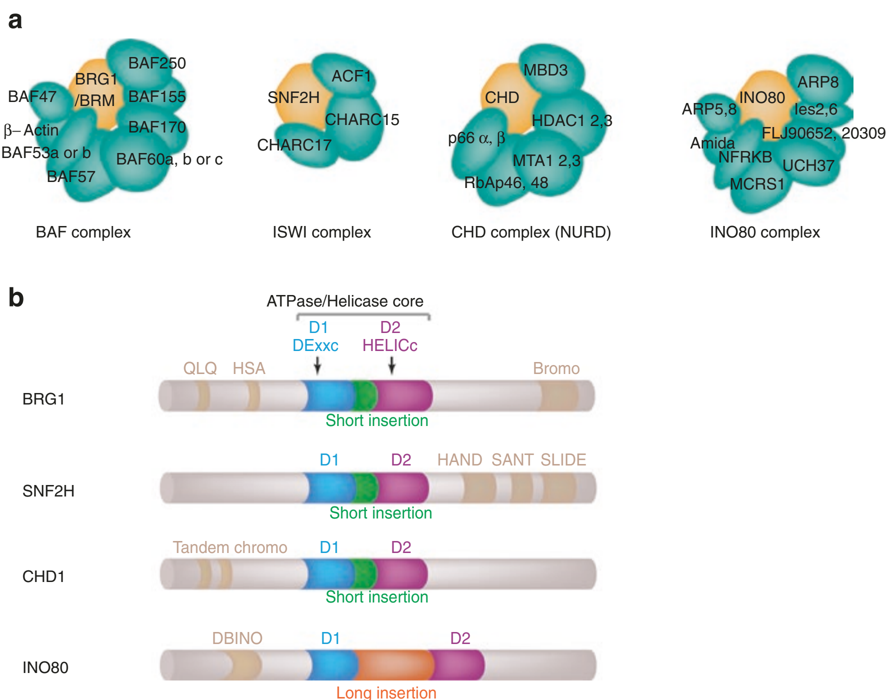

Fig. 5.2 Chromatin-remodeling complex and the ATPases (**a**) Composition of four major families of chromatin-remodeling complexes, defined by the ATPase protein subunit (Brg1/Brm, SNF2H/L, CHD, p400/INO80). ATPases are shown in *yellow*, and other subunits in *emerald*. (**b**) Major domains of the ATPase subunit are denoted in colors. The ATPase/helicase core domain is split into two parts: DExx (*blue*) and HELICc (*purple*), separated by short (*green*) or long (*orange*) insertion. Other flanking domains, which are distinct among four families of ATPases, are displayed in *light brown* color

unwinding and translocation of nucleic acids (Fairman-Williams et al. 2010; Byrd and Raney 2012). The presence of such ATPase/helicase domain in a chromatin-remodeling complex suggests that the activities of these complexes can be regulated by RNA or DNA. A recent work, indeed, showed that the ATPase/helicase domain of the chromatin-remodeling factor Brg1 is sequestered by a long noncoding RNA to prevent Brg1 from binding to its genomic target sites (Han et al. 2014).

Besides the ATPase subunit, the chromatin-remodeling complex comprises >6–10 other subunit proteins, which contain specific domains involved in diverse functions, such as histone binding, DNA binding, or protein–protein interactions. For example, the bromodomain recognizes acetylated lysine within the histone tail (Filippakopoulos et al. 2012), chromodomain binds to methylated histone (Eissenberg 2012), and plant homeodomain (PHD) recognizes methylated lysine, acetylated lysine, or unmethylated arginine of histone H3 (Sanchez and Zhou 2011). These functional domains help target the remodeling complex to specifically

modified histones on the genome. The complex's protein subunits may also contain DNA-binding domains that assist with the binding of the remodeling complex to DNA. For instance, the ARID/BRIGHT domain binds to DNA directly without known DNA sequence specificity (Dallas et al. 2000), the high motility group-box (HMG-b) domain recognizes the minor groove of DNA (Stros et al. 2007), and the RFX domain binds to single-stranded DNA (Emery et al. 1996; Masternak et al. 1998). Furthermore, the subunit domains can mediate the complex's interaction with other regulatory proteins. The N-terminal domain of Brg1, for example, can bind to zinc-finger proteins (Kadam and Emerson 2003), providing a mechanism for the remodeling complex to associate with specific partners. Importantly, many subunit proteins of the remodeling complex have different isoforms that are expressed broadly or in a tissue-specific manner. Such diversity of subunit proteins allows the assembly of distinct chromatin-remodeling complexes under different pathophysiological contexts, including stem cell self-renewal, cancer progression, and brain development (Wu et al. 2009; Ho and Crabtree 2010; Kadoch and Crabtree 2013; Kadoch et al. 2013; Wu and Arora 2015).

The interactions between chromatin remodelers, histone- and DNA-modifying enzymes are essential for chromatin regulation. The cross talk between chromatin regulators is partly mediated by their direct molecular interactions. In yeast, the SWI/SNF complex is recruited by GCN5 histone acetyltransferase to the target promoters to activate gene transcription (Natarajan et al. 1999). Moreover, the Snf2h- and Tip5-containing nucleolar remodeling complex (NoRC), a member of the ISWI family, represses the transcription of ribosomal RNA by recruiting histone deacetylase 1 (Hdac1), Dnmt1, and Dnmt3b to deacetylate histones and methylate the promoter of ribosomal DNA (Santoro et al. 2002; Zhou et al. 2002), thus creating repressive chromatin for gene silencing. Histone modification status can also influence the targeting of chromatin remodelers to specific genomic loci. The chromatin remodeler RSC (remodeler of structure of chromatin), for example, is recruited to the 5′ ends of genes through its bromodomain-mediated recognition of tandem H3K14ac (Kasten et al. 2004).

Most of the fundamental mechanisms underlying the interactions among different chromatin-regulating factors were conducted in the yeast or cells. The implications of those interactions in cardiac pathophysiology are largely undefined, despite that chromatin remodeling is critical for heart development and disease (Han et al. 2011; Chang and Bruneau 2012). Also largely unknown is how chromatin-remodeling factors work with variant forms of histone and long noncoding RNAs to regulate chromatin and control gene expression in the heart. Here, we focus on the recent progress of chromatin-remodeling research in heart failure.

5.2 ATP-Dependent Chromatin Remodeler and Cardiomyopathy: Lessons from Brg1

Brg1 is an ATPase subunit of the BAF (brahma-associated factor) chromatin-remodeling complex, a vertebrate homolog of the yeast SWI/SNF complex. In mammals, the BAF complex contains ~12 protein components, including an ATPase subunit encoded by either Brm (brahma) or Brg1 (brahma-related gene 1). These

two ATPase subunits, although highly homologous, exhibit nonredundant functions *in vivo*. Brg1-null mutation is peri-implantationally lethal (Bultman et al. 2000), whereas Brm-null mice are viable and develop normally with modest increase in body mass (Reyes et al. 1998). Recent studies showed that Brg1 plays essential roles in heart development and disease (Han et al. 2014; Chang et al. 2011; Hang et al. 2010; Stankunas et al. 2008; Takeuchi et al. 2011; Li et al. 2013).

5.2.1 Brg1 and Myocardial Morphogenesis

In developing mouse embryos, Brg1 functions in the endocardium to control myocardial trabeculation that is required for forming cardiac muscle mass (Stankunas et al. 2008; Zhang et al. 2013). Abnormal myocardial trabeculation causes cardiomyopathy and heart failure in humans (Jenni et al. 1999). The developing myocardial cells respond to signals released from the adjacent endocardial layer to form a network of finger-like projections (trabeculae) that grow into the ventricular chambers (Stankunas et al. 2008; Zhang et al. 2013). Between the endocardium and myocardium of early embryos is a thick layer of extracellular matrix (termed cardiac jelly) that coordinates endocardial–myocardial signals and growth of the myocardial trabeculae. In early development, endocardial Brg1 represses the expression of a secreted matrix metalloprotease (*Adamts1*) to maintain cardiac jelly composition so as to support myocardial trabecular growth. Later in development, *Adamts1* is derepressed in the endocardium, resulting in normal degradation of cardiac jelly and thus physiological cessation of trabecular growth. Disruption of Brg1 in the mouse endocardium causes premature expression of *Adamts1*, leading to dissipation of cardiac jelly and premature termination of myocardial trabeculation (Stankunas et al. 2008). This chromatin-based repression of endocardial *Adamts1* allows the establishment of extracellular microenvironment to support myocardial morphogenesis.

5.2.2 Brg1 Function in Fetal and Adult Cardiomyocytes

Brg1 also plays critical roles in fetal cardiomyocytes to control gene expression, cardiac growth, and differentiation (Hang et al. 2010). Mouse embryos lacking myocardial Brg1 die around embryonic day 11.5 (E11.5) due to thin compact myocardium and absent interventricular septum (Hang et al. 2010). Brg1 promotes fetal cardiomyocyte proliferation by maintaining Bmp10 and suppressing p57kip2 expression (Hang et al. 2010). Bmp10 is a myocardial growth factor, whereas p57kip2 is a cyclin-dependent kinase inhibitor that suppresses cell-cycle progression (Chen et al. 2004). The myocardial defects observed in the mutants are caused by severely reduced myocardial proliferation as a result of Bmp10 deficiency and ectopic p57kip2 expression. In parallel to proliferation control, Brg1 partners with HDAC and poly (ADP ribose) polymerases (PARP) on the promoters of myosin heavy chain (MHC) genes to repress α-MHC (*Myh6*) and activate β-MHC (*Myh7*)

expression in embryonic heart ventricles (Hang et al. 2010). In mice, Myh6 is the dominant MHC isoform expressed in adult hearts, while Myh7 is the primary isoform in embryonic hearts. The Brg1/HDAC/PARP protein complex thus maintains an expression of the fetal isoform of MHC in developing cardiomyocytes to preserve the fetal state of myocyte differentiation. Therefore, Brg1 governs two independent pathways to separately control fetal cardiomyocyte proliferation and differentiation (Hang et al. 2010).

Brg1, although highly expressed in fetal hearts, is downregulated in the neonatal period, coinciding with the physiological switch of *Myh7* to *Myh6* expression and with the maturation of fetal cardiomyocytes into postmitotic, well-differentiated adult cardiomyocytes (Hang et al. 2010). However, when the mature heart is pathologically stressed, Brg1 expression is reactivated in the stressed cardiomyocytes (Hang et al. 2010). Once activated, Brg1 forms a protein complex with its embryonic partners, HDAC and PARP1, on the promoters of *Myh* genes to repress *Myh6* and activate *Myh7* expression, representing a return to the fetal state of Brg1-mediated *Myh* expression control (Hang et al. 2010) (Fig. 5.3a). Such pathological switch of *Myh* expression contributes to the development of cardiac hypertrophy and failure (Herron and McDonald 2002; Krenz and Robbins 2004; James et al.

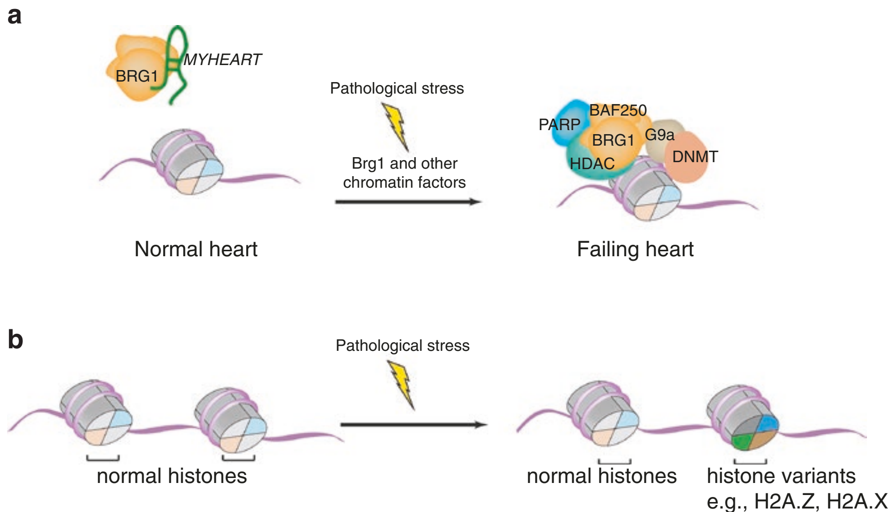

Fig. 5.3 Chromatin remodeling underlies heart failure (**a**) In response to pathologic cardiac stress, BRG1 (ATPase of BAF complex) expression is activated, and once activated, BRG1 forms a multimeric protein complex with G9a, DNMT3, HDAC, and PARP to alter the chromatin structure and expression of cardiac genes, such as *MYH6*. The long noncoding RNA *MYHEART* (*MHRT*, *green*) sequesters BRG1 from its genomic target sites by directly binding to the helicase domain of BRG1. (**b**) Histone variants, such as H2A.X and H2A.Z, can replace canonical histone proteins in the nucleosome to alter chromatin structure and regulate gene expression in the heart

2005; Lompre et al. 1979; Swynghedauw 1999; Miyata et al. 2000; Abraham et al. 2002; Lowes et al. 2002; Blaxall et al. 2003). Besides *Myh*, Brg1 directly activates the expression of osteopontin (*Opn*), a pro-fibrotic factor secreted by cardiomyocytes to induce fibrosis (Han et al. 2014). Therefore, Brg1 is a stress-activated chromatin remodeler that reprograms cardiac gene expression and triggers hypertrophy and heart failure (Hang et al. 2010). The stress-induced assembly of three classes of chromatin-regulating factors—Brg1, HDAC, and PARP—on the *Myh* gene promoters suggests that chromatin is a place where stress-response signals converge to reprogram gene expression. In patients with hypertrophy cardiomyopathy, BRG1 expression is also activated in the hearts, and its level of expression correlates strongly with the degree of *Myh* switch and the severity of hypertrophy (Hang et al. 2010), suggesting a conservation of Brg1-mediated hypertrophy mechanism.

5.3 Other BAF Subunits and Heart Function

Many BAF subunits are involved in the regulation of heart function or development (reviewed previously (Han et al. 2011; Chang and Bruneau 2012)). Notably, they appear to control different pathways in the heart, as evidenced by a spectrum of cardiac defects caused by the genetic mutation of individual subunits. This raises an intriguing question of how these subunits control specific cardiac functions. One possibility is that each BAF subunit can interact with different partners and form a distinct chromatin-remodeling complex that has specific cardiac functions. Another scenario is that specific BAF subunits may have functions independent of the BAF chromatin-remodeling complex; for example, besides being a component of the BAF complex, BAF180 is also present in the kinetochore complex of neural cells (Lessard et al. 2007).

During heart development, Baf180 controls cardiac chamber maturation partly by controlling promoters of genes implicated in the retinoid acid pathway, including RARβ2 and CRABPII (Wang et al. 2004). Furthermore, Baf180 is required for expression of genes essential for coronary vessel formation (Huang et al. 2008). The other BAF subunit Baf60c is essential for heart morphogenesis partly through its regulation of *Hand2*, *Bmp10*, and *Irx3* (Lickert et al. 2004), and this Baf60c subunit also controls cardiac looping by activating Notch signaling and Nodal expression (Takeuchi et al. 2007). Baf200 is required for heart morphogenesis, and Baf200-null mutant embryos display thin myocardium, ventricular septal defect, common atrioventricular valve, double outlet right ventricle, and reduced coronary arteries (He et al. 2014). Since mouse embryos lacking these BAF subunits are embryonically lethal, their roles in adult heart disease remain to be determined. More recently, Baf250a is implicated in controlling cardiomyocyte function. Baf250a depletion in cultured cardiomyocytes results in arrhythmic contraction of these cells (Singh and Archer 2014; Wu et al. 2014). Although the details remain elusive, Baf250a may interact with the nucleosome remodeling and histone deacetylase (NURD/HDAC) complex to inhibit H3K27Ac formation and repress the expression of *Myocardin*, *Gata4*, *Myl3*, and *cTnnt* (Singh and Archer 2014). In addition, Baf250a is critical for

the proper functioning of sinoatrial node, which controls the rhythmic cardiac beating (Wu et al. 2014). Mutant mice with sinoatrial node-specific Baf250a deletion exhibit sinus bradycardia and irregular sinus rhythm. Mechanistically, Baf250a activates *Tbx3* expression and coordinates with Hdac3 and Tbx3 to repress *Nkx2.5* expression. Disruption of Baf250a causes an ectopic expression of Nkx2.5, which in return stimulates the expression of cardiac transcription factors, such as Gata4 and Tbx5, and activates improperly the contractile program in the sinoatrial node. Such regulation of cardiac gene programming by the chromatin remodeler (Baf250a), histone modifier (Hdac3), and transcription factors (Tbx3, Nkx2.5), is essential for the heart to beat steadily.

5.4 Other ATP-Dependent Chromatin-Remodeling Factors and Heart Function

The CHD7 chromatin remodeler is essential for mammalian heart development and function. *Chd7*-null mice die before birth and show defects in multiple tissues. In mice, Chd7 controls aortic arch development through its interaction with Tbx1 (Hurd et al. 2007; Randall et al. 2009). Chd7 interacts with BMP and SMADs to control *Nkx2.5* expression and cardiogenesis (Liu et al. 2014a). In mouse neural crest cells, Chd7 complexes with Brg1 on the *PlexinA2* promoter to activate *PlexinA2*, which encodes a receptor that responds to Semaphorin signals to navigate neural crest cells into the pharyngeal arch and cardiac outflow tract to pattern their morphogenesis (Li et al. 2013). Besides, Chd2 and Snf2h of the ISWI family are required for early embryo development and survival (Stopka and Skoultchi 2003; Marfella et al. 2006); however, their roles in cardiovascular development are unknown (reviewed previously (Han et al. 2011; Chang and Bruneau 2012)).

5.5 Interplay Among Nucleosome Remodeling, Histone Modifications, and DNA Methylation in Heart Failure

5.5.1 A Brg1–G9a–Dnmt3-Mediated Sequential Assembly of Repressive Chromatin in Pathologically Stressed Hearts

Although chromatin-based gene reprogramming is a crucial part of heart failure, it remains obscure how chromatin-regulating factors act in concert with each other to establish, maintain, and modulate chromatin structure in the heart. Of special interest are the cardiac-specific interplays between three different classes of chromatin regulators: ATP-dependent chromatin-remodeling factors, histone-modifying enzymes, and DNA-modifying enzymes. Defining how these regulators interact to regulate chromatin and gene expression in the pathologically stressed heart will enhance our understanding of the epigenetic basis of heart failure.

Myh6 and *Myh7* are antithetically regulated under different pathophysiological conditions to regulate heart function. Interestingly, embedded within the *Myh6* and

Myh7 gene loci is crucial information of cardiac epigenetic regulation. These loci include genomic sites that encode the epigenetic regulators *miR208a* (within *Myh6* loci) (Montgomery et al. 2011; Callis et al. 2009; van Rooij et al. 2007), *miR208b* (within *Myh7* loci) (van Rooij et al. 2009), and *Myheart* (within *Myh7* loci) (Han et al. 2014). Further analysis of the chromatin scaffold of *Myh6* at different pathophysiological states revealed two new classes of chromatin modifiers—G9a/GLP histone methyltransferase and DNA methyltransferase (DNMT)—that are necessary for stress-induced cardiac hypertrophy and failure (Han et al. 2016). In mice, G9a/Glp and Dnmt3 are highly expressed in fetal hearts, but their expression is downregulated in healthy adult hearts. Expression of G9a/Glp and Dnmt3, however, is reactivated in cardiomyocytes of adult mouse hearts that are pressure-overloaded by transaortic constriction. The activation of G9a/Glp and Dnmt3 establishes a repressive chromatin scaffold composed of methylated histone 3 lysine 9 (H3K9) and CpG dinucleotides on the loci of *Myh6* to repress its expression in stressed hearts. Cardiomyocyte-specific deletion of G9a/Glp or Dnmt3a reduces the repressive chromatin marks, lessens cardiac hypertrophy, and prevents heart failure in pathologically stressed mice. Pharmacological inhibition of their enzyme activities by small molecule inhibitors of G9a/Glp or Dnmt can also improve stress-induced cardiac hypertrophy and failure. In hypertrophic human hearts, G9a/GLP and DNMT are also activated, and their activation correlates strongly with the severity of cardiomyopathy as well as with H3K9 and CpG methylation of the human *MYH6* promoter. These observations show new roles of G9a/GLP and DNMT in the pathogenesis of cardiomyopathy and suggest a conserved chromatin mechanism in human cardiomyopathy. Furthermore, the effectiveness of small molecule inhibitors BIX01294 and AZA that inhibit G9a/GLP and DNMT, respectively, in reducing heart failure suggests a feasible pharmacological approach to deactivate chromatin methylation and augment *Myh6* and other genes to improve ventricular function of the failing heart (Han et al. 2016).

Our studies showed that Brg1 is required for the targeting of G9a and Dnmt3 to *Myh6* genomic sites and the subsequent H3K9 and DNA methylation of *Myh6* promoter. Brg1, G9a/Glp, and Dnmt3 form a physical complex in the stressed hearts (Han et al. 2016). In hearts that lack Brg1 in cardiomyocytes, G9a/Glp and Dnmt3 expression remains activated by cardiac stress, but the proteins fail to recognize *Myh6* promoters and catalyze H3K9/DNA methylation on *Myh6* sites, indicating that Brg1 is essential for the recruitment of G9a/Glp and Dnmt3 enzymes to *Myh6* promoter. Moreover, G9a is necessary for Dnmt3 to target and catalyze CpG methylation of *Myh6* sites, whereas G9a does not require Dnmt3 to target and methylate histones of *Myh6* promoters. The findings reveal a Brg1-governed sequential recruitment of chromatin-modifying enzymes to *Myh6* loci and demonstrate a new mechanistic link from chromatin-remodeling factor to H3K9 and CpG methylation. This provides a mechanism by which pathological stress triggers a successive assembly of repressive chromatin, leading to DNA methylation and repression of a key molecular motor gene, essential for cardiac myopathy (Fig. 5.3a).

These studies show that how a repressive chromatin environment on *Myh6* is created by five classes of chromatin regulators: Brg1, G9a/Glp, Dnmt3, Hdac, and

Parp. Among these chromatin factors, however, only the expression of Brg1, G9a/Glp, and Dnmt3—but not Hdac (Hdac1, 2, 3) or Parp1—is stress induced in mouse and human hearts. Further investigations will be crucial to elucidate the biochemical basis of how such stress-induced chromatin factors integrate with constitutive ones to modify chromatin substrates for gene silencing. It is also critical to address whether such coordination occurs only on specific genomic loci or it can be generalized to other genes that are repressed in the stressed hearts.

5.6 Long Noncoding RNA and Chromatin Remodeling in the Heart

Although ATP-dependent chromatin-remodeling factors are capable of localizing to specific regions of the genome (Clapier and Cairns 2009), it remains largely unknown how chromatin-remodeling factors target the genome to effect chromatin regulation. Defining the targeting mechanism is essential for understanding the specificity of chromatin and gene regulation by these chromatin regulators. Recent studies revealed a new targeting mechanism of the ATPase subunit of chromatin-remodeling complexes and showed that a long noncoding RNAs (lncRNA) is crucial for controlling such genomic targeting of chromatin remodelers (Han et al. 2014).

LncRNAs are differentially expressed in human hearts with ischemic and non-ischemic cardiomyopathy (Yang et al. 2014; Devaux et al. 2015), suggesting the involvement of lncRNAs in human heart disease. Also, more than 1000 lncRNAs show expression changes in the developing mouse heart (Zhu et al. 2014), but their functions are largely unknown. Recent studies suggest essential roles of lncRNAs in mouse heart development. The lncRNA *Braveheart* (*Bvht*) can activate the expression of *Mesp1* and other cardiac regulatory genes to maintain cardiac lineage (Klattenhoff et al. 2013), possibly by sequestering the SUZ12 component of polycomb repressor complex 2 (PRC2) from cardiac gene promoters (Klattenhoff et al. 2013). The mechanism of *Bvht*, however, was mostly determined using cultured cells; it remains unclear how *Bvht* functions *in vivo*, which requires a mouse model with genetic disruption of *Bvht* transcript. In addition, no homolog of *Bvht* has been identified in humans or other species, raising an issue of whether *Bvht* provides a conserved mechanism for human cardiac lineage determination. On the other hand, *Fendrr* is a lateral mesoderm-specific lncRNA that controls development of tissues derived from the lateral mesoderm (Grote et al. 2013). Mouse embryos lacking *Fendrr* show reduced ventral body wall and hypoplasia of the heart ventricles (Grote et al. 2013). In these mutant embryos, *Gata6* and *Nkx2.5*, two important regulators of heart development (Olson 2006), are activated in the heart field, accompanied by an increase of active histone mark H3K4me3. Though detailed mechanism remains unknown, *Fendrr* may reduce the binding of TrxG/MLL histone-modifying complex to gene promoters, thus reducing the H3K4me3 gene activation mark (Schuettengruber et al. 2011). Meanwhile, *Fendrr* can bind to PRC2 to enhance PRC2's occupancy and H3K27me3 formation on a subset of gene promoters, leading to gene repression.

Our studies showed that lncRNA can regulate chromatin-remodeling factors to maintain heart function. A cardiac-specific lncRNA (*Myheart*) inhibits the chromatin remodeler Brg1 to protect the adult heart from pathological hypertrophy and failure (Han et al. 2014) (Fig. 5.3a). *Myheart (Mhrt)* represents a cluster of alternatively spliced nuclear lncRNA transcripts that are transcribed in an antisense direction from the promoter of *Myh6* into the genomic site of *Myh7*. In fetal cardiomyocytes, *Mhrt* transcripts are expressed at low levels, but the abundance of *Mhrt* increases as the heart matures from the fetal stage to the neonatal period and to adulthood. However, when the mature adult heart is stressed by pathological stress such as pressure overload, *Mhrt* expression is suppressed, leading to cardiomyopathy. Transgenic restoration of *Mhrt779* (the most abundant *Mhrt* species with 779 nucleotides) in cardiomyocytes to its prestress level prevents the heart from developing pathological hypertrophy and failure. Mechanistically, *Mhrt* directly binds to the helicase core of Brg1, which has dual nucleotide-binding specificities: it binds to lncRNA (*Mhrt*) and chromatinized DNA promoter (*Myh*) (Han et al. 2014). This dual binding of Brg1 helicase not only enables Brg1 to target its downstream promoter DNA but also allows *Mhrt* to competitively inhibit Brg1's promoter targeting and thus prevent Brg1 from controlling its downstream gene activities (Han et al. 2014). Brg1's target genes in the heart include *Myh6*, *Myh7*, and *Opn* that play critical roles during hypertrophy and fibrosis (Han et al. 2014).

Mhrt prevents Brg1 helicase from recognizing its chromatin targets in the heart, whereas Brg1 can bind to *Mhrt* promoter to repress cardiac *Mhrt* transcription (Han et al. 2014). This reciprocal inhibition demonstrates a feedback *Mhrt*–Brg1 circuit critical for the maintenance of cardiac function. Perturbation of the *Mhrt*–Brg1 circuit results in pathological hypertrophy of the mature adult heart (Han et al. 2014) and reduced proliferation of neonatal cardiomyocytes (Chang lab, unpublished results). The *Mhrt*–Brg1 interaction exemplifies a new mechanism by which lncRNA controls chromatin structure and sheds light on a new way in which Brg1/BAF recognizes its genomic targets (Han et al. 2014; Devaux et al. 2015; Han and Chang 2015). The specificity of Brg1 chromatin targeting, however, remains unclear. At a given genetic locus, the distinct DNA sequence and specifically modified histones may establish a precise nucleosomal conformation that binds strongly to Brg1 helicase, thereby conferring to the Brg1-chromatin complex both specificity (DNA sequence–histone conformation) and stability (binding of Brg1 helicase to histone 3) (Han et al. 2014). The targeting specificity of Brg1 helicase can also be enhanced by the other chromatin-binding domains present in the remodeling complex, such as Brg1 bromodomain (Lee et al. 2010; Shen et al. 2007) that recognizes acetylated histones, and PHD domain of Baf45 subunit (Zeng et al. 2010; Lange et al. 2008) that binds to acetylated and methylated histones. Further studies of whether and how *Mhrt*, by binding to Brg1, alters the assembly of BAF complex may uncover new BAF chromatin-targeting mechanisms, just as the studies of how *Mhrt* inhibits Brg1 revealed an unexpected targeting mechanism of Brg1 (Han et al. 2014). In addition, it will be essential to define additional interactions between the helicase, DNA sequence, histones, and other BAF subunits to decipher the specificity of

chromatin targeting. A solution of *Mhrt*–helicase crystal structure may provide deep insights into the chemical basis of this new lncRNA regulation and chromatin remodeling.

5.7 Histone Variants, Chromatin Remodeling, and Heart Failure

Chromatin-remodeling complexes can exchange canonical histones with variant forms of histones within the nucleosome. Canonical histones include histone 1 (H1) present at the linker region and the core histones (H2A, H2B, H3, and H4) that compose the nucleosome. These major histone proteins are encoded by multiple copies of the histone genes to provide the bulk of cellular histones, whereas the nonallelic histone variants are usually encoded by single-copy genes to provide specific regulation. Histone variants, which contain distinct amino acids and physicochemical properties from those of canonical histones, are localized to specific genomic regions, thus providing an additional way of controlling chromatin architecture (Kamakaka and Biggins 2005; Henikoff and Ahmad 2005; Sarma and Reinberg 2005).

Histone H1 has many sequence variants, including $H1^0$ and H5. Most of the H1 variants differ from H1 in the N- and C-terminal tail domains, and their abundance fluctuates in different cell types and under different physiological conditions (Terme et al. 2011). Among the core histones, histone H2A has the largest number of variants, which include H2A.Z, H2A.X, macroH2A, and H2A-Bbd. These variants differ from H2A in the length and sequence of C-terminal tails and in the genomic distribution. H2A.Z is deposited at specific genomic loci by the chromatin-remodeling complex SWR1 (a SWI/SNF-like ATPase) to prevent the spread of silent heterochromatins and to poise genes for transcriptional activation (Mizuguchi et al. 2004). Conversely, H2A.Z can be evicted from the genome by INO80 chromatin-remodeling complex to maintain genomic stability (Papamichos-Chronakis et al. 2011; Billon and Cote 2013). On the other hand, the H2A.X variant participates in DNA repair by binding to genomic regions with double-strand DNA breaks (Paull et al. 2000). Histone H3 also has a number of variants: centromere-associated CENP-A and noncentromere-associated H3.1, H3.2, and H3.3 variants (Kamakaka and Biggins 2005). CENP-A assists gene transcription in the centromere (Henikoff and Ahmad 2005; Foltz et al. 2006), whereas H3.3 is associated with the gene body of active genes in non-pluripotent cells (Ahmad and Henikoff 2002) but localized to both active and repressed genes in embryonic stem cells and neuronal precursor cells (Goldberg et al. 2010). Unlike the other major histone subtypes, histone H2B and H4 are deficient in variant forms.

The roles of histone variants in heart failure are largely unknown. Analyses of chromatin purified from the cardiac nuclei revealed a wide spectrum of proteins associated with chromatin, with histone variants constituting 54 proteins out of the 1046 identified (Franklin et al. 2011). These findings suggest that histone variants are potential regulators of heart function (Fig. 5.3b). Indeed, H2A.Z was found to

be upregulated in the hypertrophic heart, and RNAi-mediated H2A.Z knockdown in cultured cardiomyocytes resulted in the reduction of cardiac hypertrophy and downregulation of growth-related genes (Chen et al. 2006). Another example is the H2A.X variant involved in doxorubicin-induced cardiac damage (Pang et al. 2013). In many types of cancers, doxorubicin is effective in inducing double-stranded DNA breaks to inhibit cancer cell growth; however, the use of doxorubicin in cancer treatment is limited by its severe side effects, particularly cardiotoxicity (Von Hoff et al. 1979). Part of the chemotherapeutic efficacy of doxorubicin comes from its ability to evict H2A.X from the chromatin to inhibit the repair of double-stranded DNA breaks (Pang et al. 2013). However, such H2A.X eviction also contributes to the development of cardiomyopathy (Pang et al. 2013). To further define the roles of histone variants in heart failure and their interactions with chromatin remodelers, functional and mechanistic studies involving animal models will be critical. The role of each histone variant can be defined by examining the heart function of animal models that have cardiac-specific disruption of histone variants, and their cardiac genomic localization determined by ChIP-seq analysis in the presence or absence of chromatin remodelers, such as the pro-hypertrophic Brg1 (Hang et al. 2010). A more complete picture of chromatin regulation in cardiac pathophysiology can be obtained by the genomic distribution of histone variants in combination with the landscape of nucleosome positioning and histone/DNA modifications.

5.8 Targeting Chromatin Remodeling and Associated Epigenetic Events for Heart Failure Therapy

In the United States alone, heart failure afflicts five million people, causes 300,000 deaths a year, and costs >30 billion dollars per year in treatment (Mozaffarian et al. 2015). The mortality rate of heart failure, however, remains high at ~50 % within 5 years of diagnosis. It is therefore important to define new mechanisms and develop new therapeutic strategies for heart failure. Genomewide studies showed the essential roles of chromatin modifications in cardiac gene reprograming in the hypertrophic hearts (Papait et al. 2013). Moreover, the plasticity of cardiac epigenome is demonstrated by the dynamic changes of DNA methylation pattern in stressed mouse hearts and in the hearts of patients with dilated cardiomyopathy or end-stage heart failure (Haas et al. 2013; Gilsbach et al. 2014; Movassagh et al. 2011). However, current heart failure therapy does not include drugs that specifically target chromatin regulators, despite the necessity of chromatin-based regulation for the development of heart failure and the plasticity of cardiac epigenome. Many drugs that target chromatin-modifying enzymes have been tested in clinical trials and show promise for cancer treatment. These drugs include HDAC inhibitors (for multiple myeloma, neuroblastoma, and T-cell lymphoma), EZH2 inhibitor (for solid tumors or B-cell lymphomas), and DNMT inhibitors (for myelodysplastic syndrome) (Mack 2010; Finley and Copeland 2014), and the FDA has approved the use of HDAC inhibitors for cutaneous T-cell lymphoma and DNMT inhibitors for

myelodysplastic syndrome therapy (Mack 2010). The success of chromatin-based therapy for cancers provides a premise for the feasibility of chromatin-based therapy of human heart failure.

5.8.1 Targeting Histone/DNA-Modifying Enzymes Recruited by Chromatin Remodelers to Trigger Heart Failure

Our studies reveal that the chromatin remodeler Brg1 can recruit histone- or DNA-modifying enzymes to reprogram gene expression in stressed hearts, leading to pathological hypertrophy and heart failure (Han et al. 2016). Such concerted covalent and noncovalent modifications of cardiac epigenome provide a foundation for targeting specific components of the epigenetic machinery for heart failure therapy. Chromatin remodelers, such as Brg1, can be inhibited by chemical inhibitors or by RNAs. In addition, chemical inhibitors of many histone/DNA-modifying enzymes are effective in reducing cardiomyocyte hypertrophy in cell culture or in animals (McKinsey and Kass 2007). These inhibitors, which include PARP inhibitors (PJ-34 (Szabo et al. 2002), AG-690/11026014 (Liu et al. 2014b)), HDAC inhibitors (TSA and analogs (Olson et al. 2006; Backs and Olson 2006; McKinsey and Olson 2004)), G9a/GLP inhibitors (BIX01294 (Han et al. 2016)), and DNMT inhibitors (AZA (Han et al. 2016)), are potential drug candidates for heart failure treatment. Besides these prototypic drugs for heart failure, a wide spectrum of chemical inhibitors—that target histone methyltransferases, demethylase, acetyltransferase, and deacetylase—is available for testing chromatin-based mechanisms and new therapies of heart failure.

5.8.2 Targeting Chromatin Readers That Participate in Chromatin Remodeling and Modifications

Chromatin regulation requires proteins that read and interpret the epigenetic information. Chromatin readers contain domains that recognize specific chromatin modifications and are recruited to the corresponding genomic loci to assist with gene regulation. A popular drug target is the bromodomain (Garnier et al. 2014; Filippakopoulos et al. 2010; Filippakopoulos and Knapp 2014; Delmore et al. 2011), which is a chromatin-recognition domain that enables a protein to bind to acetylated histones to facilitate transcriptional gene activation. The bromodomain and extraterminal (BET) family, including BRD2, BRD3, BRD4, and BRDT, are bromodomain-containing readers. Among them, BRD4 is activated during cardiac stress to induce pathological gene reprograming and is crucial for the development of cardiac hypertrophy and failure (Spiltoir et al. 2013; Anand et al. 2013). BRD4 is highly enriched at the promoter regions of actively transcribed genes and a subset of cardiac enhancer elements (Wei et al. 2008). When BRD4 proteins are displaced from the chromatin by a selective BET bromodomain inhibitor, the elongation of RNA polymerase II during gene transcription is hindered, resulting in transcriptional pause (Anand et al. 2013). Inhibition of the BET bromodomain blocks

hypertrophic growth of cardiomyocytes *in vitro* and attenuates hypertrophy and heart failure *in vivo* (Anand et al. 2013). Such inhibition of BET readers provides a means to change the interpretation of chromatin information in the stressed hearts without changing chromatin conformation, as achieved by direct inhibition of chromatin remodelers or modifiers.

5.8.3 Targeting the Helicase/ATPase Domain of Brg1

Brg1 contains an evolutionarily conserved helicase domain, which belongs to the Dead box RNA helicase subfamily of the superfamily 2 (SF2) helicase (Clapier and Cairns 2009; Jankowsky 2011; Byrd et al. 2012; Tang et al. 2010). A SF2 helicase consists of two homologous "RecA-like" domains (D1 and D2), which fold upon each other to from a cleft structure that binds ATP on one side and DNA/RNA nucleotides on the other, enabling ATP hydrolysis and DNA/RNA unwinding (Linder and Jankowsky 2011; Mallam et al. 2012). The helicase core of Brg1 is crucial for Brg1 to bind to its genomic target sites to control cardiac gene expression, hypertrophy, and failure, and this domain can be inhibited by the lncRNA *Mhrt* to mitigate heart failure (Han et al. 2014). Accordingly, the helicase of Brg1 is targetable for heart failure treatment. Many viral or endogenous RNA helicases have been targeted by antiviral drugs, with assays developed to screen for specific chemical inhibitors that abolish helicase functions of binding and hydrolysis of NTP or unwinding of the DNA/RNA substrates (Kwong et al. 2005; Frick 2003). The search for helicases inhibitors have led to many successful drug candidates for treating infections caused by herpes simplex or hepatitis C virus (Kwong et al. 2005; Frick 2003). Given the pathogenic role of Brg1 helicase (Han et al. 2014; Hang et al. 2010), identifying Brg1-specific helicase inhibitors may facilitate drug discovery for heart failure therapy.

5.8.4 RNA Drugs for Heart Failure

The discovery of cardioprotective lncRNA *Mhrt* that inhibits Brg1-mediated chromatin remodeling provides a new avenue for lncRNA-based heart failure therapy (Han et al. 2014; Wu and Arora 2015; Gupta et al. 2015; Liu and Wang 2014; Wang and Wang 2015). RNAs, with their special biochemical properties and versatile functions, have emerged as an important class of potential therapeutic drugs (Burnett and Rossi 2012). Dozens of RNA-based drugs have been tested in clinical trials for a variety of human diseases, which range from rare genetic disorders and viral infections to different types of cancers. Although the therapeutic use of RNAs could be challenging partly due to their intrinsic instability and potential immunogenicity, RNAs can be chemically modified and delivered successfully to the tissue targets. The Federal Drug Administration (FDA) has recently approved the use of an antisense RNA (Kynamro), which inhibits the hepatic synthesis of apolipoprotein B-100, for treating familial hypercholesterolemia. Besides antisense RNAs that

inhibit mRNA translation, other types of RNAs can be exploited for therapeutic purposes: these RNAs have different mechanisms of action and include RNAs involved in RNA interference (RNAi), catalytic RNAs (ribozymes), RNAs partnering with proteins and other ligands (aptamers), antisense RNAs, and lncRNAs (such as *Mhrt*) that participate in epigenetic regulation of gene expression.

5.9 Future Perspective

Although the essential role of Brg1/BAF in heart failure has been defined, it remains unclear whether the other families of chromatin-remodeling complexes—CHD, ISWI, and INO80—are involved in the pathogenesis of heart failure. The studies of Brg1 establish a paradigm of how chromatin remodelers are activated by cardiac stress and how they recruit histone- and DNA-modifying enzymes to restructure chromatin and reprogram gene expression, triggering adverse tissue remodeling and heart failure. It will be important to define whether other families of chromatin-remodeling complexes act with or against the Brg1/BAF family in the process of pathological hypertrophy and how these remodeler families program gene expression under different pathophysiological conditions. Another important question is how pathological stress alters protein subunits of the remodeling complex to modulate the chromatin function and gene regulation of the entire complex. How do the protein subunits influence the complex's interactions with other chromatin and transcription regulators to effect chromatin reorganization and gene reprogramming in the stressed hearts? Answering these fundamental questions will not only deepen our understanding of chromatin remodeling mechanism, but also help us identify new targets for heart failure therapy. Moreover, in light of the recent discovery of lncRNA *Myheart* that inhibits Brg1's chromatin function to protect the heart from hypertrophy (Han et al. 2014), further efforts to identify new lncRNA that interacts with different families of chromatin remodelers or different components of the remodeling complex will provide new mechanisms of chromatin regulation and inspire new strategies for heart failure therapy.

Compliance with Ethical Standards

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Histone Methylation in Heart Development and Cardiovascular Disease

6

Zhi-Ping Liu

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Abstract

Cardiovascular development and homeostasis are regulated by a group of core cardiac transcription factors in a coordinated temporal and spatial manner. Histone methylation is an emerging epigenetic mechanism for regulating gene transcription. Interplay among transcription factors and histone lysine modifiers plays important role in cardiovascular development and diseases. Aberrant expression and mutation of the histone lysine modifiers during development and in adult life can cause either embryonic lethality or congenital heart diseases, and influences the response of adult hearts to pathological stresses. In this review, we describe basics of histone methylation, and current body of literature on the role of several common histone methylations and their modifying enzymes in cardiovascular development, congenital and adult heart diseases.

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6.1 Introduction

Histone methylation is one of many post-translational modifications (PTMs) that can occur at the amino-terminus of each subunit of histone octamer (two copies of four histone protein H2A/H2B/H3/H4). Histone PTMs include lysine (K) and arginine (R) methylation, serine and threonine phosphorylation, lysine acetylation, ubiquitination, sumoylation, and adenosine diphosphate (ADP)-ribosylation. Histone methylation does not directly affect chromatin structure, rather it functions as a signaling platform to recruit other chromatin-remodeling complexes that directly regulate chromatin structure. Consequently, chromatin structures can be altered between a permissive euchromatin state, which allows the binding of transcription factors and coactivators to the DNA, and a nonpermissive heterochromatin state, which precludes active transcription of genes. Histone methylation not only influences gene transcription but also other variety of genomic activities such as DNA replication, chromosome segregation, and maintenance (Alabert and Groth 2012). The disruption of such changes underlies a wide variety of pathologies, including cardiac diseases (Tingare et al. 2013; Black and Whetstine 2013; Rozek et al. 2014).

Methylation of histone was originally identified in the 1960s (Allfrey et al. 1964). Histone methylation is most commonly observed and well studied on N-terminal lysine residues of H3 and H4. Many of the arginine methylations have yet to be characterized and the enzymes that methylate/demethylate arginine residues have yet to be elucidated. Well-characterized histone methylation residues include K4, K9, K27, K36, and K79 on histone H3 and K20 of histone H4 (Fig. 6.1a). The first histone lysine methyl transferase (KMT), human and mouse SUV39H1/KMT1A, was discovered by Thomas Jenuwein et al. in 2000 (Rea et al. 2000). SUV39H1 is H3-specific histone methyltransferase that selectively methylates H3K9. SUV39H1 contains a catalytic SET domain that is approximately 130 amino acid residues long and was initially found in *Drosophila* Su(var) 3–9, Enhancer of Zeste, and Trithorax proteins. The SET domain is evolutionally conserved from yeast to human. So far, there are 33 KMTs identified in humans that all have SET domain, with the exception being KMT4/DOT1L (Table 6.1). Both classes of KMTs use S-adenosyl-L-methionie (SAM) as the methyl group donor (Fig. 6.1b) (Dillon et al. 2005; Nguyen and Zhang 2011). Lysine residues can be methylated to different degrees to include mono(me1), di(me2), or trimethyl(me3) groups (Fig. 6.1b). The sequential addition of methyl moieties can occur. However, mono-methylation is not a prerequisite for di-methylation, and di-methylation is not required for trimethylation, since some KMTs have the capacities to mono-, di-, and tri-methylate unmodified lysine residues.

The first histone lysine demethylase (KDM), LSD1 (lysine-specific demethylase 1) was identified by Yang Shi et al. (Shi et al. 2004). Discovery of LSD1 changed the perception that histone methylation is a permanent post-translational modification. Twenty-one KDMs are identified in humans and are classified into two families (Table 6.1) (Shi and Tsukada 2013), the KDM1/LSD demethylase family, which contains a flavin adenine dinucleotide (FAD)-dependent amine oxidase domain, and the KDM2-8 demethylase family, which contains a Jumonji (Jmj) domain (Fig. 6.1c). Jmj-proteins rely on α-ketoglutarate, molecular oxygen, and Fe(II) as cofactor for demethylation (Fig. 6.1d). Some of the Jmj-proteins have two jmj domains (JmjN and JmjC). KMTs and KDMs have a high degree of specificity for specific lysine residues and the degree of methylation (i.e., mono, di-, or tri-methylation) (Table 6.1). However, the

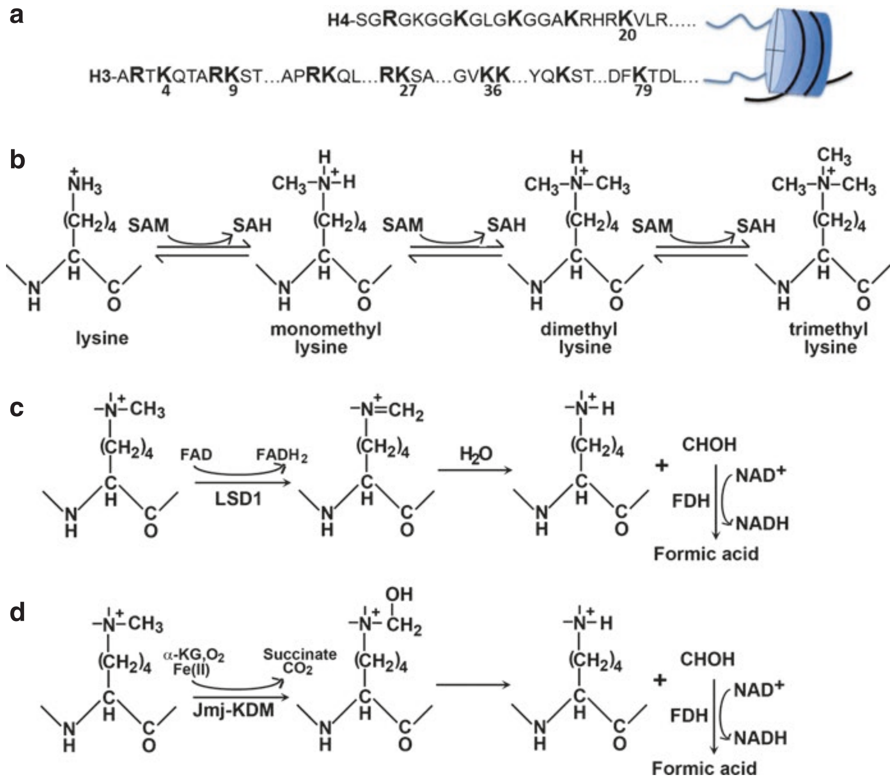

Fig. 6.1 Histone methylations (**a**) N-terminal amino acid sequences of of H3 and H4. Lysine residues that can be methylated are highlighted in *bold*. (**b**) Scheme showing lysine mono-, di-, and tri-methylation by histone lysine methyltransferases. (**c**) Mechanism of demethylation of lysine, catalyzed by enzymes of the LSD1/KDM1 family. (**d**) Mechanism of demethylation of lysine, catalyzed by enzymes of the jmj family

specificity of some KMT and KDMs can be context-dependent and affected by their interactive partners. For example, LSD1 has dual substrate specificity (H3K4me2/1 and H3K9me2/1). LSD1 demethylates H3K4me1/2 and generally represses transcription when it is a part of transcriptional corepressor complexes that contain CoREST and/or HDAC1/2 (Yang et al. 2006). When bound to androgen receptor (AR) in prostate cancer cells, LSD1 collaborates with Jmj demethylases to remove the repressive H3K9me2/1 marks and activates AR-dependent gene transcription (Metzger et al. 2005). In addition to modifying methylation on histone octamer, some of the KMTs and KDMs can also modify linker histone H1 (Table 6.1). Histone methylation patterns can provide specialized binding surfaces that recruit protein complexes containing chromatin remodeling and transcriptional activation/repression activity. While lysine methylation at H3K9, H3K27, and H4K20 is, in general, associated with regions of transcriptionally silenced heterochromatin, methylation at H3K4, H3K36, and H3K79 is normally associated with transcriptionally active euchromatin (Klose et al. 2006). However, it is important to note that since histone methylation occurs in the context of other surrounding PTMs, the ultimate functional output of a specific histone lysine methylation event will depend on the collective abundance, regulation, and context of all surrounding PTMs (the so-called histone code) (Strahl and Allis 2000).

Table 6.1 KMTs, KDMs, and their substrates (adopted from Black and Whetstine 2013)

			H3K4ME			H3K9me			H3K27me			H3K36me			H3K79me			H4K20me			H1K26me		
	Nomenclature	Official symbol	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
KMTs	KMT1A	SUV39H1					x	x															
	KMT1B	SUV39H2					x	x															
	KMT1C	G9a/EHMT2				x	x																
	KMT1D	GLP/EHMT1				x	x																
	KMT1E	SETDB1					x	x															
	KMT1F	SETDB2					x	x															
	KMT2A	MLL	x	x	x																		
	KMT2B	MLL2	x	x	x																		
	KMT2C	MLL3	x	x	x																		
	KMT2D	MLL4	x	x	x																		
	KMT2E	MLL5	x	x	x																		
	KMT2F	SETD1A	x	x	x																		
	KMT2G	SETD1B	x	x	x																		
	KMT2H	ASH1L	x	x	x	x	x	x															
	KMT3A	SETD2												x									
	KMT3B	NSD1										x	x										
	KMT3C	SMYD2										x	x										
	KMT3D	SMYD1																					
	KMT3E	SMYD3		x	x																		
	KMT3F	WHSC1L1/NSD3	x	x						x	x												
	KMT3G	WHSC1/NSD2	x	x					x	x	x	x	x					x	x				
	KMT5A	SET8																x					
	KMT5B	SUV420H1																	x	x			
	KMT5C	SUV420H2																	x	x			
	KMT6	EZH2							x	x	x											x	x
	KMT7	SET7/SET9/SETD7		x																			

	KMT8A	PRDM2/RIZ1				x	x	x															
	KMT8B	PRDM9			x																		
	KMT8C	PRDM6																					
	KMT8D	PRDM8					x																
	KMT8E	PRDM3																					
	KMT8F	PRDM16																					
	KMT4	DOT1L													x	x	x						
KDMs	KDM1A	LSD1	x	x																			
	KDM1B	LSD2	x	x																			
	KDM2A	FBXL11/JHDM1A										x	x										
	KDM2B	FBXL10/JHDM1B										x	x										
	KDM3A	JHDM2A				x	x																
	KDM3B	JHDM2B				x	x																
	KDM3C	JMJD1C				x	x																
	KDM4A	JMJD2A					x	x															
	KDM4B	JMJD2B					x	x															
	KDM4C	JMJD2C					x	x															
	KDM4D	JMJD2D					x	x															
	KDM5A	JARID1A		x	x																		
	KDM5B	JARID1B/PLU1	x	x	x																		
	KDM5C	JARID1C/SMCX		x	x																		
	KDM5D	JARID1D		x	x																		
	KDM6A	UTX								x	x												
	KDM6B	JMJD3								x	x												
	KDM7A	JHDM1D				x	x		x	x													
	KDM7B	PHF8				x	x		x	x													
	KDM7C	PHF2					x																
	KDM8	JMJD5											x										

6.2 Histone Methylation in Heart Development and Cardiovascular Diseases

The development of vertebrate heart is a complex process that requires concurrent differentiation of multiple cell types including endothelial cells (ECs), smooth muscle cells (SMCs), and cardiomyocytes. Formation of the heart can be considered as a sequential addition of modular elements with distinct genetic and epigenetic control (Olson 2006; Vincent and Buckingham 2010; McCulley and Black 2012; Bruneau 2013). Heart development consists of five major stages; formation of cardiac crescent at E7.75, linear heart tube formation at E8.0, chamber initiation and formation from E9.5, and chamber maturation and septation from E12.5, and valve formation from E12 to birth. Two populations of cardiac progenitor cells have been identified at the cardiac crescent stage, first heart field (FHF), and a more medial region called second heart field (SHF) that lie adjacent to each other (Vincent and Buckingham 2010). The two heart fields are spatially and molecularly distinct. The FHF gives rise to the initial linear heart tube that ultimately becomes the myocardium of the left ventricle. Progenitor cells in the SHF remain undifferentiated until they are subsequently added to the heart tube to give rise to the right ventricle and the outflow tract (OFT). Cardiac neural crest cells control SHF development and form endocardial cushions in the OFT. Heart development is controlled by a group of core cardiac transcription factors (Isl1, Nkx2.5, Mef2c, Tbx1, Gata4, Foxa2/c1/c2/h1, Hand2, and Mesp1) and growth factors (Fgf8/10, Wnt3a/5a/11, BMP2/4/7, Shh, and others) in a temporal and spatial-dependent manner. Mesp1 is the earliest marker of cardiovascular progenitors, including derivatives of the first and second heart fields (Vincent and Buckingham 2010). Mesp1 and Mesp2 are required for establishing embryonic mesoderm that gives rise to heart, somites, and gut. Mesp1 cells are converted further into cardiac progenitors by Isl1, Nkx2.5, Gata4, Mef2C, and Tbx5. Mutation and abnormal expression of these factors during heart development have been shown to lead to inherited forms of congenital heart diseases (CHD) such as septal defects, atrioventricular malformations or developmental absence of right or left heart (Bruneau 2013; Fahed et al. 2013; Yuan et al. 2013). As we will describe in the following sections, histone KMTs and KDMs can interact with these core cardiac transcription factors and affect cardiac homeostasis by regulating cardiac transcription factor-targeted gene expression. Methylation at the promoter/enhancer of these transcription factors can also affect their expression and thus influence the outcomes of cardiac phenotype.

Histone methylation also plays a role in pathogenesis of adult heart disease, as there is a switch of gene expression program between fetal and adult forms during cardiac hypertrophy and heart failure. Adult cardiomyocytes are terminally differentiated with limited proliferative capacity. In response to physiological stimuli such as excise and pregnancy, the heart undergoes adaptive and reversible hypertrophy via an increase in cardiomyocyte size such that sufficient blood can be pumped through the circulation to meet increased hemodynamic requirement (Hill and Olson 2008). Adaptive cardiac hypertrophy becomes mal-adaptive and irreversible under pathological conditions, including chronic hypertension, myocardial infarction,

atherosclerosis, obesity, diabetes, aging, and genetic mutations. Pathological cardiac hypertrophy loses its plasticity with diminished ability to mount an adaptive response to meet systemic demands and is a primary risk factor for heart failure and an adverse prognosis. At the molecular level, pathological cardiac hypertrophy is marked by dramatic alterations of gene expression associated with contractile apparatus and metabolism (Song et al. 2012). Many "fetal genes" such as βMHC that are silenced in adult cardiomyocytes are reactivated, whereas genes that are expressed in differentiated adult cardiomyocyte such as αMHC are repressed. The structural and gene expressional change during pathological cardiac hypertrophy are collectively termed cardiac hypertrophic remodeling. Alterations of gene expression have been proposed to play a causative role in pathological cardiac hypertrophy and heart failure (Hill and Olson 2008).

It is now well-established that histone methylation plays a critical role in stem-cell renewal and cell differentiation, maintaining genome integrity, gene regulation, and cancer evasion (Black and Whetstine 2013). Histone methylation also plays important roles in cardiac lineage specification and differentiation (Wamstad et al. 2012; Cattaneo et al. 2014), heart development (He et al. 2012; Delgado-Olguín et al. 2012), and pathogenesis of congenital and adult heart diseases (Fahed et al. 2013; Zhang et al. 2011). Stage-specific gene expression programs were found to be associated with distinct histone methylation profiles during cardiomyocyte differentiation *in vitro* in which mouse ES cells were induced to become mesodermal cells, then cardiac precursors, and finally differentiated cardiomyocytes (Wamstad et al. 2012). It was shown that while multiple dynamic chromatin patterns were observed during the differentiation process, a specific chromatin pattern was found for most mesodermal-specific genes. Genes with metabolic functions also found to share a similar chromatin pattern, suggesting that epigenetic regulation may play an important role to ensure that functional gene modules are synchronized to produce robust and coordinated expression. Furthermore, H3K4me1 was found to mark the transcriptional start sites of a group of genes at early differentiation stages that are committed to cardiac lineage and activated later via acquisition of H3K4me3 (Wamstad et al. 2012).

Barker (1999) proposed a "fetal programming of cardiovascular disease hypothesis" that prenatal exposure to environmental stresses will result in an increased cardiovascular disease risk in adulthood. In support of this hypothesis, it was shown that prenatal and fetal malnutrition can induce changes in the DNA methylation pattern of several genes involved in metabolism (Tobi et al. 2014), including *Igf2, ABCA1*, and *LEP* (Heijmans et al. 2008). *ABCA1* hypermethylation is associated with coronary artery disease and aging in men. In older men, *ABCA1* also correlates with increased total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride levels (Guay et al. 2014). Based on its role in heart development, subtle alteration of histone methylation *in utero* may, like DNA methylation, increase the cardiovascular disease risk in adulthood as well.

The biological functions of histone methyl marks are derived from studies of their KMTs (writers), KDMs (erasers), and proteins that bind them (readers). Genomewide association studies, genomic sequencing and mapping identified mutations, copy number variation, and aberrant gene regulation of KMTs and KDMs that are

associated with embryonic development, aging, cancer, neurological disorders, and congenital heart diseases (Black and Whetstine 2013; Young and Hendzel 2013; Mummaneni and Shord 2014). In the following sections, we will review the five most characterized histone methylation marks, and the evidence suggesting that dysregulation of these lysine methylation events due to mutations in genes encoding KMTs/KDMs/readers may play a causative role in promoting congenital and adult cardiac diseases (Table 6.2).

6.3 H3K4 Methylation

H3K4 methylation is the most studied methylation mark. It is generally associated with genes that are transcriptionally active. Trimethylated H3K4 (H3K4me3) at the transcription start site (TSS) of an active gene correlates strongly with the histone acetylation, transcription rate, and active RNA polymerase II binding. H3K4me3 at TSS may play a functional role in transcription initiation as H3K4me3 can bind the PhD domain of TAF3, facilitating the recruitment of RNA polymerase II (Vermeulen et al. 2007). H3K4me3 is also found together with H3K27me3 at promoters and enhancers of poised key developmental genes in pluripotent ES cells (Bernstein et al. 2006; Voigt et al. 2013). In vertebrate, the majority of H3K4me2/1 colocalize with H3K4me3 at the TSS. H3K4me2/1 can also be found in distal enhancer region. Although the functional consequence of H3K4me2/1 at TSS and enhancer regions remains elusive, it may mark lineage-specific genes at early differentiation stages such that they are poised to be activated via acquisition of H3K4me3 at later differentiation stages (Wamstad et al. 2012). H3K4 methylation may contribute to the genetic program that drives juvenile body growth deceleration, as there is an extensive genomewide decrease in H3K4me3 at promoter regions of genes involved in cell cycle and cell proliferation when organs approach adult sizes (Lui et al. 2014).

H3K4 methylation along with H3K9 and H4K12 acetylation was found on the promoter of the gene for endothelial nitric oxide synthase (eNOS) in vascular ECs but not in vascular SMCs (Ho et al. 2012). eNOS is abundantly expressed in vascular ECs but repressed in SMCs. eNOS produces nitric oxide (NO) that regulates vascular tone and protects against atherosclerosis development. These histone modifications in ECs are lost in response to hypoxia, resulting in transcriptional repression of eNOS (Fish et al. 2010), suggesting a potential role of H3K4 methylation in atherosclerosis and coronary heart diseases. Alteration of the level of histone H3K4me3 was also found to correlate with heart failure in a hypertensive rat model (Kaneda et al. 2009).

6.3.1 H3K4 Methyltransferase

H3K4 methylation is catalyzed by methyltransferase KMT2A-H, KMT3E-G, KMT7, and KMT8B (Table 6.1) (Ruthenburg et al. 2007). Mammalian H3K4 methyltransferases can be classified into two groups: SET-domain-containing proteins

Table 6.2 Selected histone methylations and their modifying enzymes involved in heart development, CHD, and cardiac diseases

Methylation	Modifier	Function and mechanism of the modifier	Cardiac phenotype	References
H3K4	MLL2	Methylates H3K4me3 at "bivalent" loci and also active gene promoters	Mutation of *Mll2* may cause Kabuki syndrome in patients that have atrial and ventricular septal defects and aortic coarctation *Mll2*-null mice are embryonic lethal Adult *Mll2M2628K/+* mice have type 2 diabetes	Ng et al. 2010 Bokinni 2012 Glaser et al. 2006, 2009 Goldsworthy et al. 2013
	Symd1	Contains SET domain, interacts with skNAC and regulates Hand2 and Irx4	*Symd1*-null mice have hypoplastic right ventricle, knockdown in zebra fish resulted in disrupted myofibril formation	Abu-Farha et al. 2008 Hamamoto et al. 2004
	PTIP	Part of MLL3/4 complexes may recruit MLL3/4 complexes to target genes such as Kcnip2	Deletion of *Ptip* in adult cardiomyocyte resulted in defective conduction system and greater susceptibility to Ca^{2+}-based ventricular arrhythmia	Randall et al. 2009
	LSD1	Demethylates H3K4me1/2	*Lsd1+/−* mice have salt-sensitive hypertension	Diehl et al. 2010
H3K27	Ezh2	Establishes H3K27me3 at noncardiac loci to repress their expression during cardiomyocyte differentiation	Deletion of *Ezh2* in cardiac progenitor cells leads to thinned myocardial walls and ventricular septation defects Deletion of *Ezh2* in SHF, a more restricted pool of cardiac progenitor cells, resulted in enlarged heart after birth	He et al. 2012 Delgado-Olguín et al. 2012
	UTX	Removes H3K27me3 at cardiac-specific enhancers to activate cardiac gene expression during heart development	*Utx*-null mice are embryonic lethal at E9.0 with defective loop and chamber morphogenesis	Lee et al. 2012

(continued)

Table 6.2 (continued)

Methylation	Modifier	Function and mechanism of the modifier	Cardiac phenotype	References
H3K9	JMJD2A	Removes H3K9me3 at FHL1 and other cardiac fetal gene loci	Deletion of *Jmjd2A* in adult cardiomyocyte blunts pressure-overload induced cardiac hypertrophy	Zhang et al. 2011
H3K36	WHSC1	Establishes K3K36me3 at Pdgfra locus and repress Pdgfra expression together with Nkx2.5	*Whsc1*-null mice are perinatal lethal with variety of atrial and ventricular septal defects	Wagner and Carpenter 2012 Nimura et al. 2009
H3K79	Dot1L	Methylates H3K79, regulates telomere elongation and cell proliferation	Cardiac dilatation, yolk sac angiogenesis defects, embryonic lethal at E9.5–E10.5	Nguyen et al. 2011

that include mixed-lineage leukemia family (MLL1-4) and Set1A/B, and other H3K4 methyltransferases – those unrelated yet able to methylate H3K4 (ASH1, SMYD, SET7/9, and PRDM9). MLL proteins are part of COMPASS-like complexes that contain three additional core subunits (WDR5, RbPB5, and ASH2). COMPASS-like complexes are capable of mono-, di-, and trimethylating H3K4 (Shilatifard 2012). Several other proteins are found in biochemically purified COMPASS-like complexes, including MEN1, UTX, PTIP, NCOA6, PA1, Dpy30, and/or HCF1. The precise biological function of the noncatalytic subunits copurified with COMPASS-like complexes remains elusive as the four-component minimal complex is sufficient to catalyze H3K4 methylation. The possible functions of these noncatalytic subunits include context-dependent recruitment of the complex to target genes, and substrate recognition and presentation. Many of the subunits are H3K4 methyl readers that are coupled to other chromatin modification mechanisms necessary to execute specific biological functions, as described below.

De novo mutations (present in the probands but absent in both parents) of genes that encode subunits of COMPASS-like complexes were found in patients with CHD through exome sequencing of parent-offspring trios (Zaidi et al. 2013). These genes include *MLL2/KMT2D, CHD7, WDR5, RNF20, UBE2B*, and *USP44*. CHD7 and WDR5 are H3K4 methylation readers that bind H3K4me1/me2. RNF20 and UBE2B are subunits of H2BK120 ubiquitination complex. USP44 is H2BK120 deubiquitinase. Ubiquitination at H2BK120 is required for H3K4 methylation (Shilatifard 2012). MLL2 mutations were previously found in majority of patients with Kubaki syndrome that includes atrial and ventricular septal defects and aortic coarctation (Bokinni 2012; Ng et al. 2010). Studies using mouse models indicated that Mll2 plays an important role during early embryogenesis as mice are embryonic lethal prior to E11.5 with wide spread apoptosis when *Mll2* is deleted germline and are normal when deleted after E11.5 (Glaser et al. 2006, 2009). Mice with

mutant *Mll2M2628K* were generated through N-ethyl-N-nitrosourea mutagenesis screens (Goldsworthy et al. 2013). *Mll2M2628K/M2628K* mice are embryonic lethal between E8.5 and E11.5 with abnormal heart looping, pericardial effusion, exencephaly, and various head abnormalities and anterior truncation defects. *Mll2M2628K/+* mice are viable and have diabetes in adulthood.

CHARGE syndrome is a congenital disease characterized by coloboma of the retina, heart defects, atresia of the choanae, retarded growth and development genital and ear abnormalities (Aramaki et al. 2006). The majority of children clinically diagnosed with CHARGE syndrome have mutations in *CHD7* gene. Most cardiovascular anomalies of CHARGE syndrome are related to pharyngeal arch artery (PAA) defects or cardiac outflow tract malformations. Mechanistic studies that link the mutations to cardiac gene expression are lacking at present. However, it was suggested that Chd7 may regulate Tbx1-targeted genes. Some of the cardiovascular defects of CHARGE syndrome are recapitulated in *Chd7*$^{+/-}$ mice (Randall et al. 2009). Both *Tbx1* and *Chd7* heterozygous mice had hypoplasia of PAA. *Chd7*$^{+/-}$*;Tbx1*$^{+/-}$ compound heterozygote showed greater defect in PAA than either *Chd7* or *Tbx1* heterozygote alone, suggesting that *Chd7* and *Tbx1* may interact genetically.

PTIP is a subunit in MLL3/4(KMT2C/D) complexes. PTIP promotes assembly of the MLL3/4 complexes and H3K4 methylation at a Pax2 DNA-binding site via binding to Pax2 transcription factor (Patel et al. 2007). Deletion of *Ptip* in mice resulted in developmental arrest at the gastrulation stage (Patel et al. 2007), with reduced levels of H3K4me3 in the whole embryo (Randall et al. 2009) and in embryonic stem cells (Kim et al. 2009). Conditional deletion of *Ptip* in differentiated cardiomyocytes in adult mouse heart resulted in reduced expression of *Kcnip2* and H3K4me3 occupancy at the *Kcnip2* promoter. *Ptip*-mutant hearts had conduction defects and greater susceptibility to calcium-based ventricular arrhythmias (Stein et al. 2011).

SMYD (SET-MYND domain) family proteins (SMYD1-5/KMT3) are H3K4 methyl transferases that contain SET domains required for histone methyltransferases activity and MYND domains mediating interactions with proteins involved in transcriptional regulation and signal transduction. Smyd2 has been shown to monomethylate H3K4 and H3K36 (Brown et al. 2006; Abu-Farha et al. 2008). Smyd3 di- and trimethylates H3K4 (Hamamoto et al. 2004). Smyd2 is also shown to methylate nonhistone proteins including p53, Rb, and Hsp90. The substrate specificity for Smyd1, 4, and 5 proteins remains elusive. Studies with animal models indicated that Smyd proteins are involved in skeletal and cardiac muscle development. Smyd1/Bop is mainly expressed in skeletal and cardiac muscles and is a striated muscle-specific regulator of myogenic differentiation and myosin assembly (Gottlieb et al. 2002). Deletion of Smyd1 in mice disrupted cardiac differentiation and morphogenesis, resulting in embryonic lethality. *Smyd1*-null embryonic hearts had a hypoplastic right ventricle with reduced expression of Hand1 and Irx4, two genes require for myocardial growth and differentiation (Gottlieb et al. 2002). Conditional deletion of *Smyd1* in the heart indicated that the early embryonic heart phenotype is caused by the cardiomyocyte-autonomous function of Smyd1 in SHF (Rasmussen et al. 2015). Smyd1 was shown to interact with transcription factor skNAC to regulate

downstream gene expression. *skNAC* knockout mice have similar embryonic phenotype to that of *Smyd1*-null mice (Park et al. 2010). Smyd1 also binds myosin and is localized at the M-band region in the center of the sarcomere. Mutation in *Smyd1* in zebrafish causes disturbed sarcomere assembly in the heart and fast-twitch skeletal muscle (Just et al. 2011). Smyd2 methylates Hsp90, promoting interaction of a Smyd2–methyl–Hsp90 complex with the N2A domain of titin (Voelkel et al. 2013). Although no cardiac phenotypes are observed in *Smyd2*-null mice at baseline (Diehl et al. 2010), it may be required for protection of the sarcomeric I-band region and myocyte organization under conditions of mechanical or biochemical stress (Voelkel et al. 2013). A role for Smyd3 in the development of cardiac and skeletal muscle was shown in zebrafish (Fujii et al. 2011).

6.3.2 H3K4 Demethylase

H3K4 methylation can be removed by LSD/KDM1 (from K4me2/1 to K4me0) and/or members of JARID1/KDM5 (from K4me3/2 to K4me1) family of histone demethylases (Table 6.1). H3K4 demethylases can be recruited to specific genomic loci through their intrinsic reader domains or gene-specific transcriptional factors. Polymorphisms in the *LSD1* gene have been associated with salt-sensitive hypertension in human (Pojoga et al. 2011). *Lsd1*$^{+/-}$ mice had higher blood pressure, enhanced vascular contraction, and reduced relaxation via NO-cGMP pathway compared to wild-type control mice during high salt diet (Pojoga et al. 2011). Lsd1 has also been shown to be involved in the inflammatory phenotype of vascular SMCs relevant for atherosclerosis. vSMCs isolated from diabetic *db/db* mice have elevated expression of monocyte chemotatic protein (MCP)-1 and interleukin (IL)-6; this is associated with both increased H3K4 methylation and decreased LSD1 expression and recruitment to the promoters of MCP-1 and IL-6. The expression of MCP-1 and IL-6 is increased in Lsd1 knockdown vSMCs and decreased in Lsd1-overexpressing vSMCs (Reddy et al. 2008). *De novo* mutations in KDM5A/5B and KDM6A were identified in human patients with CHD (Zaidi et al. 2013), although mechanisms linking mutations of these demethylases to histone H3K4 methylation and to target gene expression remain unknown.

6.4 H3K27 Methylation

H3K27 methylation is usually associated with gene repression and appears mostly in Polycomb-associated repressive chromatin. Polycomb-group proteins were first discovered in fruit flies that silence gene expression. In mammals, there are two principal Polycomb-repressive complexes (PRC), PRC1 and PRC2. H3K27me3 is catalyzed by the catalytic subunit Ezh2 in PRC2 (Nestorov et al. 2013). H3K27me3 can in turn recruit PRC1 that monoubiquitinlates H2A at K119 (H2AK119ub1). H2AK119ub1 can inhibit RNA polymerase II elongation (Stock et al. 2007). H3K27me3 is demethylated by KDM6A/UTX.

H3K27me3 represses many developmental genes in self-renewing ES cells and in differentiated adult organs (Bernstein et al. 2006; Voigt et al. 2013). In ES cells, H3K27me3 can form “bivalent” marks with H3K4me3 on promoters and enhancers of key developmental genes that are poised to undergo transcriptional activation/inactivation upon developmental cues and/or environmental stimuli. When the silencing H3K27me3 mark is removed and the activating H3K4me3 is retained, “posed” genes are activated and ES cells are committed to a particular lineage. Conversely, bivalent domains of other genes required for the commitment of ES cells to different lineages remain silenced with H3K27me3 retained along with other repressive marks including H3K9me3, and/or 5mC DNA methylation. The function of bivalent marks and their associated protein complexes may help maintaining bivalent loci in a state that is both responsive to developmental cues and at the same time refractory to subthreshold noises (Voigt et al. 2013). A genomewide upregulation of H3K27me3 at the promoters of genes involved in cell cycle and cell proliferation has been observed in tissues, including lung and kidney during fetal to adulthood transition (Lui et al. 2014).

H3K27 methylation plays a dynamic role in heart development and is implicated in the pathogenesis of CHD. During heart development, cardiac progenitor cell commitment and subsequent growth and differentiation require accurate activation of gene programs in a precise temporal and spatial manner. H3K27me3 marks promoters of cardiac-specific genes in ES cells (Bernstein et al. 2006; Wamstad et al. 2012; Lee et al. 2012). It is necessary to remove H3K27me3 in order for ES cells to differentiate to a cardiac lineage as ES cells lacking H3K27 demethylase UTX failed to express cardiac-specific genes under cardiac differentiation conditions *in vitro* (Lee et al. 2012). *Utx*-null mice are embryonic lethal with failed cardiac looping morphogenesis and chamber development at E9. Utx was shown to interact with and functions as a co-activator of core cardiac transcription factors including SRF, Tbx5, Nkx2.5, and GATA4. Furthermore, Utx interacts with ATPase Brg1 and facilitates the recruitment of Brg1-containing Swi/Snf chromatin remodeling complex to the cardiac genes. The Swi/Snf complex was shown to play important role in heart development (Han et al. 2011). Partial or complete deletion of *UTX* is also implicated in human CHD (Zaidi et al. 2013).

Failure to remove H3K27me3 during development has been implicated in the pathogenesis of CHD. In iPS cell-derived cardiomyocytes from hypoplastic left heart syndrome patients, transcription of NKX2.5, HAND1, and NOTCH1 was found to be significantly repressed compared with control iPS-derived cardiomyocytes from individuals with biventricle hearts. Downregulation of NKX2.5 transcription was associated with increased promoter occupancy of H3K27me3 and reduced H3K4me2 and H3 acetylation levels (Kobayashi et al. 2014), suggesting a potential role of H3K27 and H3K4 methylation in the regulating expression of cardiac core transcription factors. DiGeorge and Velocardiofacial syndromes are CHD caused by a chromosomal deletion that includes TBX1 and many other genes. *Tbx*$^{+/-}$ mouse recapitulates most of the disease phenotypes including hypoplasia or aplasia of the fourth pharyngeal arch arteries (PAAs) (Papangeli and Scambler 2013). Gbx2 is a downstream effector of Tbx1 and p53. Gbx2 is

positively regulated by Tbx1 and negatively regulated by p53. Gbx2 is downregulated in *Tbx1*$^{+/-}$ embryos. Inhibition of p53 by genetic deletion (*Trp53*$^{+/-}$) or pharmacologically (p53 inhibitors) restored Gbx2 expression and rescued the fourth PAAs phenotype in *Tbx1*$^{+/-}$ embryos. Upregulation of Gbx2 in *Tbx1*$^{+/-}$ *Trp53*$^{+/-}$ embryos is associated with downregulation of H3K27me3 at an enhancer region that binds both Tbx1 and p53 (Caprio and Baldini 2014). It was shown that p53 can recruit Ezh2 and establish H3K27me3 mark in this enhancer region. Suppression of p53 can thus reduce H3K27me3 at Gbx2 gene and thereby facilitates transcriptional activation of Gbx2 by Tbx1.

While removal of H3K27me3 is required for the activation of cardiac-specific genes during cardiac cell differentiation, maintaining H3K27 methylation at noncardiac genes to repress their expression is also essential for proper heart development, as inactivation of H3K27 methyltransferase *Ezh2* in differentiating cardiomyocytes in mice causes CHD (Delgado-Olguín et al. 2012; He et al. 2012). When *Ezh2* was deleted in cardiac progenitor cells in the SHF, using *Mef2cAHF*-cre at E7.5, mice developed hypertrophic and fibrotic hearts after birth (Delgado-Olguín et al. 2012). Deletion of *Ezh2* in differentiating ventricular cardiomyocytes using Nkx2.5-cre resulted in perinatal lethality. Mutant mouse hearts had persistent hypertrabeculation, right ventricular hypoplasia, atrial and ventricular septal defects, and myocardial fibrosis (He et al. 2012). In both studies, noncardiac gene transcriptions, including those of *Ink4a, Six1, Isl1, and Pax6,* were upregulated. Levels of H3K27me3 at the promoters of these genes were downregulated.

Repression of fetal gene expression by H3K27me3 may be important for long-term maintenance of ventricular function. Transcription factor Asxl2 has been shown to recruit Ezh2 to the promoter of fetal gene β-MHC and represses β-MHC expression (Lai et al. 2012). Loss of *Asxl2* resulted in the downregulation of H3K27me3 at β-MHC promoter and de-repression of β-MHC. *Asxl2*$^{-/-}$ mouse hearts displayed progressive deterioration of ventricular function with ~37 % reduction in fractional shortening by 10 months of age compared to wild-type mice (Lai et al. 2012), suggesting that Asxl2 is required for long-term maintenance of ventricular function. ASXL2 is down-regulated in the hearts of patients with ischemic or idiopathic dilated cardiomyopathy.

6.5 H3K9 Methylation

Unlike H3K27 methylation, which appears mostly in Polycomb-associated repressive chromatin in gene-rich regions, H3K9 methylation is enriched in transcriptionally inert heterochromatin, which includes satellite repeats in telomeres and pericentromeres. Trimethylated H3K9 (H3K9me3) can bind to heterochromatin protein-1 (HP1), which further recruits other proteins to assemble chromatin into compact or "closed" heterochromatic structures, leading to gene silencing (Bannister et al. 2001; Lachner et al. 2001). H3K9 methylation is also found to participate in the transcriptional repression of genes in euchromatic regions through epigenetic modification of promoters (Zhang et al. 2011) and in the repression of illegitimate

initiations of transcription inside of actively transcribed loci (Krauss 2008). H3K9me3 can coexist with other repressive histone methylation marks, including H3K27me3 and H4K20me3 on silenced chromatin (Bilodeau et al. 2009; Magklara et al. 2011). These marks are often found on macrodomains that have characteristics of a facultative heterochromatin and can lose condensed structure and become transcriptional permissive in response to specific developmental and environmental cues.

Methylation of H3K9 is maintained by methyl transferases (KMT1, KMT2H, and KMT8A/D) and demethylases (KDM3, KDM4, and KDM7). Imbalance of H3K9 methylation due to mis-regulation of either KMTs or KDMs is associated with numerous malignancies, including B-cell lymphoma, medulloblastoma, acute myeloid leukemia (AML), breast, lung, and prostate cancers, and neurological disorders such as mental retardation, epileptic seizures, and autism. H3K9 KMT and KDM are involved in cardiac diseases and development as well. The H3K9me3-specific demethylase, JMJD2A/KDM4A, is upregulated in human patients with hypertrophic cardiomyopathy (Zhang et al. 2011). Many fetal gene expressions are repressed in normal adult hearts and re-expressed again in response to hypertrophic stress signals. Since H3K9me3 is associated with gene silencing, its level may be downregulated in hypertrophic heart as the fetal gene programs are reactivated. This hypothesis was tested in mouse model of cardiac hypertrophy where the transverse aorta was constricted (TAC) to induce pressure overload in the left ventricle (Zhang et al. 2011; Fig. 6.2). The global H3K9me3 level was downregulated in hypertrophic mouse hearts after 3 weeks of TAC (authors' unpublished results). Jmjd2A promoted TAC-induced cardiac hypertrophy when overexpressed in postnatal cardiomyocytes and blunted the hypertrophic response when deleted from the heart. Mechanistically, Jmjd2A was shown to bind promoters of fetal genes (ANP and BNP) and FHL1 in response to hypertrophic stimuli and activated their transcription through binding to transcription factors SRF/myocardin. FHL1 is a key component of the mechanotransducer machinery in the heart. *Fhl1* KO mouse has blunted hypertrophic response to TAC-induced injury (Sheikh et al. 2008). The demethylase activity of Jmjd2A is required to promote transcription of fetal genes and Fhl1. Activation of Fhl1 in TAC-induced hypertrophic heart by Jmjd2A is associated with the downregulation of H3K9me3 at Fhl1 promoter. It is interesting to note that although global H3K9me3 was altered in response to hypertrophic stress signal, transcription of only a limited number of genes was changed due to *Jmjd2A* deletion (Zhang et al. 2011). It is possible that other Jmjd2 proteins are involved in H3K9me3 regulation, and Jmjd2A only regulates specific subset of genes. Alternatively, Jmjd2A may regulate global H3K9me3 methylation, but only a subset of the targets has altered gene transcription due to the effect of other PTMs. Genomewide profiling of Jmjd2A-binding sites in the heart during hypertrophic remodeling is needed to test these hypotheses.

Jmjd2A may play an important role in embryonic heart development as well. Knockdown of Jmjd2A in chick embryos by Jmjd2A-specific antisense morpholino oligonucleotides was shown to dramatically downregulate the expression of neural crest-specific genes including Sox10, leading to defects in neural crest derivatives at

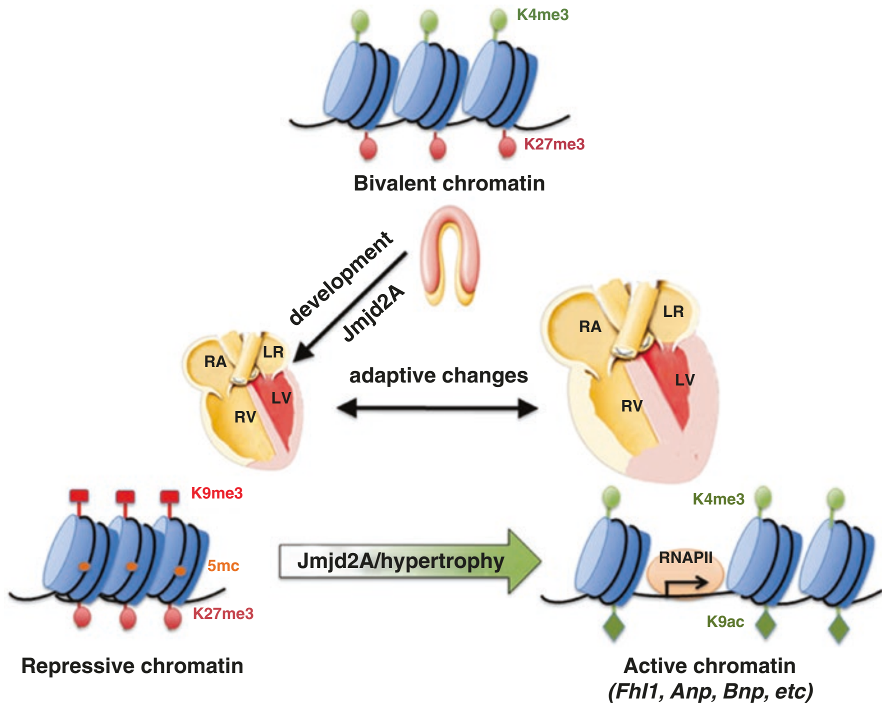

Fig. 6.2 A model of Jmjd2A involvement in heart development and cardiac hypertrophy Jmjd2A demethylates H3K9me3, which promotes the formation of active chromatin and gene transcription. Jmjd2A may play an important role in embryonic heart development, although the precise mechanism remains elusive. Jmjd2A promotes cardiac hypertrophy by synergistically activating the transcription of *fhl1, Anp,* and *Bnp* with core transcription factors, including SRF/myocardin

later stages of embryonic development (Strobl-Mazzulla et al. 2010). We observed no embryonic defects in global deletion of *Jmjd2A* in mice of mix genetic background (C57/Bl6:129). However, a partial embryonic lethality was observed in pure C57/Bl6 mouse. Some of the *Jmjd2A*-null embryos at E10.5 had abnormal hearts with various cardiac defects, including thin myocardium and underdeveloped right ventricle (authors' unpublished results). Since development of right ventricle is derived from SHF that is controlled by cardiac neural crest cells, the embryonic phenotype of *Jmjd2A*-null mouse may be due to the defects in neural crest cells, a hypothesis needed to be tested in the future (Fig. 6.2). The wide spectrum of embryonic phenotypes of *Jmjd2A*-null embryos and the dependency on genetic background may implicate genomic context, maternal–fetal environment, and other factors that were proposed to explain why an identical gene mutation can give rise to a variety of distinct malformations (Fahed et al. 2013). Incomplete penetrance may be caused by the variability of gene expression (Raj et al. 2010), as in some embryos, the alteration of Jmjd2A target gene expression may not be sufficient to reach a threshold that causes a phenotypic change.

6.6 H3K36 Methylation

H3K36 methylation is normally found in the open-reading frames of protein-encoding genes. H3K36me3 has a positive correlation with transcriptional rate (Pokholok et al. 2005) and may promote transcriptional elongation and prevent aberrant transcriptional initiation within coding sequences (Carrozza et al. 2005). H3K36 methylation has also been implicated in transcriptional repression, alternative splicing, and many other biological processes (Marango et al. 2008; Wagner and Carpenter 2012). Whether H3K36me3 acts as a transcriptional activating or inhibitory signal may depend on the context of additional PTM marks and their corresponding reader proteins (Wagner and Carpenter 2012). H3K36 can be mono- and di-methylated by WHSC1. Deletion of a critical region of human chromosome 4q16.3-containing *WSHC1* gene is associated with Wolf-Hirschhorn syndrome that is characterized by craniofacial malformations, growth retardation, learning disability, and congenital heart defects. *Whsc1*$^{-/-}$ mice are perinatal lethal with marked growth retardation and die within 10 days after birth. *Whsc1*-null mice display a variety of atrial and ventricular septal defects that manifest those in Wolf-Hirschhorn patients. Whsc1 can interact with transcription factors such as Sal4 and Nanog in ES cells. In embryonic heart, Whsc1 was found to interact with Nkx2.5 to repress Pdgfra expression. Loss of *Whsc1* resulted in the reduction of H3K36me3 at the Pdgfra locus and upregulation of Pdgfra (Nimura et al. 2009). H3K36me3 is required for vasculogenesis/angiogenesis (Hu et al. 2010). HYPB/SETD2/KMT3A tri-methylates H3K36. Mice with homozygous deletion of *Hypb* are embryonic lethal at E10.5-E11.5 with severe defects in blood vessel development (Hu et al. 2010). *Hypb*$^{-/-}$ embryos and yolk sacs have abnormally dilated capillaries that could not be remodeled into large blood vessels or intricate networks. *Hypb*$^{-/-}$ embryonic stem cell-derived embryonic bodies and human endothelial cells with siRNA-knocked down *HYPB* showed defects in cell migration and invasion during vessel formation.

6.7 H3K79 Methylation

The function of H3K79 methylation is inferred from studies of its methyltransferase Dot1, and its mammalian homolog DOT1L/KMT4, since knock out of *Dot1* in yeast, flies, and mice results in complete loss of H3K79 methylation (Nguyen et al. 2011). Dot1/DOT1L-mediated H3K79 methylation has been linked to telomeric silencing in yeast and active transcription. Dot1L is differentially expressed during *in vitro* differentiation and maturation of mouse cardiomyocytes (Cattaneo et al. 2014). Dot1L-mediated H3K79me2 has been shown to be necessary for establishing the epigenetic signature of cardiomyocyte differentiation and maturation *in vitro*. H3K79me2 may be involved in preactivating mechanism in which the histone modification is used during early lineage commitment to mark the genes for subsequent activation during maturation phase. H3K79me2 may also participate the bivalent domain H3K4/H3K27 methylation in regulation of the expression of key

cardiomyocyte differentiation genes. Cardiac-specific knockout of *Dot1L* caused dilated cardiomyopathy (DCM) with chamber dilation and systolic dysfunction (Nguyen et al. 2011). Mechanistically, Dot1L was shown to activate the transcription of dystrophin and Dot1L-mediated H3K79 methylation is required for dystrophin expression. It was also shown that dystrophin is the major downstream target of Dot1L, since ectopic expression of a functionally truncated form of dystrophin can rescue the DCM phenotype observed in *Dot1L*-cardiac-specific knockout mice. DOTL1 may also play an important role in human DCM, as *DOT1L* is significantly lower in human idiopathic DCM heart tissues compared with normal ones (Nguyen et al. 2011). Unlike other histone lysine methylation, H3K79-specific lysine demethylases have yet to be discovered.

6.8 Conclusions and Future Perspectives

Aberrant histone methylation due to dysregulation of KMTs and KDMs, as well as defective methyl-histone readers, can lead to aberrant cardiac development and congenital and adult heart diseases. Growing number of mutations for KMTs, KDMs, and histone methylation readers are being identified with the advancement of modern genomic technologies. Mechanistic studies linking association of defects in lysine methylation to downstream gene expression using transgenic animal models will be needed in order to understand which, and how, alterations in histone methylations cause, rather than correlate with, developmental defects and/or heart diseases. Unlike most of the cell types that were profiled for histone methylation, adult cardiomyocytes are lineage determined and "terminally" differentiated, with little cell-cycle activity. Thus, the epigenetic regulatory mechanisms in cardiomyocytes are most likely to differ from those identified so far in pluripotent ES cells or lineage determined cycling cells. Analysis of genomewide changes in histone methylation in differentiated adult cardiomyocytes in response to hypertrophic stimuli is lacking and needs to be explored in order to discover key epigenetic events underlying adult cardiac hypertrophy and heart failure. Studies in humans and animals indicate that early exposure to nutrients at the time of conception and during pregnancy can lead to lasting epigenetic changes in their off-spring, with increased risk of disease in adult life (Wolff et al. 1998; Barker 1999; Dolinoy et al. 2007; Dominguez-Salas et al. 2012). The activities of many epigenetic enzymes require cofactors and are hence potentially susceptible to changes in nutrient intake and metabolism (Kaelin Jr. and McKnight 2013). How enzymatic activity of epigenetic regulators senses cellular metabolism and alters the epigenetic mark in response are largely uncharted areas and worthy of investigation in the future. Although epigenetic therapy for heart disease has yet to be achieved, the reversible nature of histone modifications, and the development of effective small molecules able to inhibit KMTs and KDMs, will likely shed light on new perspectives of clinical intervention for heart diseases.

Conflict of Interest The authors declare that they have no conflict of interest.

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The Lysine Acetyltransferases in Cardiovascular Disease

7

Nanette H. Bishopric

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Abstract

The lysine acetyltransferases (KATs) are enzymes that catalyze the reversible acetylation of lysine residues within histones, transcription factors, and other proteins. These dynamic and signal-responsive enzymes allow a single pool of nuclear DNA to be interpreted in multiple ways, to create many different cell types, and to carry out a broad range of specialized cell functions. KATs play a particularly important role in adapting the long-lived cells of the cardiovascular system to environmental challenges and changing metabolic states. This chapter will provide an overview of the major classes of mammalian KATs: GNAT, p300/CBP, MYST, and the nuclear receptor coactivators, including CLOCK. Several additional KATs that to date have not been structurally analyzed will also be considered. Special attention will be paid to the role of the KATs in human genetic disorders and in processes important to cardiac and vascular biology.

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7.1 Introduction

In all multicellular organisms, the same starting DNA can be reprogrammed to form different cell types or enable new cell functions. The science of epigenetics seeks to understand this process, one which is particularly relevant to cardiovascular biology. The cardiovascular system is made up of many cell types, such as cardiac myocytes and endothelial cells, that have long lifespans and turn over very slowly. These cells cannot be easily sacrificed and replaced in response to injury, nor are they able to mutate, so that challenges from the environment must be met using a fixed set of genetic tools. This review will focus on a family of enzymes, the lysine acetyltransferases (KATs), which allow each cell to equip itself for both normal and extraordinary situations by selecting different tools from the same chest of DNA. A discussion of the major mammalian KAT families, using (as much as possible) the nomenclature proposed by Allis et al. (2007), will focus on human clinical genetic information, as well as issues of potential significance in cardiac and vascular biology, and will serve as an introduction to some of the many excellent studies in this area.

7.1.1 Accessing the Nuclear Warehouse

Every mammalian cell contains roughly 1 m of double-stranded DNA packed into a roughly 140 μm^3 nucleus. This feat is achieved by supercoiling and condensing the acidic DNA molecule around basic proteins, the histones, in ordered structures called nucleosomes. The process of DNA compaction has been described elsewhere in this text, but in brief, nucleosomes are repeating units of 147 DNA nucleotides wrapped around a double tetramer of histones 2A, 2B, 3, and 4, connected by histone 1 linkers. These nucleosomes are further condensed into higher order structures, strictly limiting access of the transcriptional machinery and other DNA regulatory proteins to the genome. Each cell accesses this vast storage locker of DNA in a dynamic and spatially restricted way that defines its identity, form, and function. The discovery of enzymes that control the accessioning and de-accessioning of genes was therefore a defining event in genome biology, moving beyond DNA sequence and into the study of how gene activity is controlled.

7.1.2 The KAT Superfamily

The first histone-modifying enzymes to be identified and characterized were members of the superfamily of KATs (see Verdin and Ott 2015 for an excellent overview). KATs catalyze the transfer of an acetyl group from acetyl-coenzyme A to the epsilon-amino group of susceptible protein lysine residues. In biological systems, these acetyl groups are highly dynamic and are rapidly hydrolyzed by multiple histone deacetylases (Olson et al. 2006; Weeks and Avkiran 2015). Like phosphorylation, acetylation changes both the size and charge of a protein and can substantially

alter its function and interactions; thus, these enzymes exert potent regulatory control that mirrors that of the protein kinases. KATs alter the DNA–histone interaction by acetylating clusters of lysines in core histones; this generally promotes transcription, particularly in the case of lysines 5 and 8 in H4 and 9 and 14 in H3 (Dancy and Cole 2015). The scope and biological importance of the nuclear acetyltransferases is now known to expand considerably beyond histone modification, to acetylation of an array of other DNA-associated and structural proteins, and accordingly they have been renamed KATs. Indeed, lysine acetylation rivals phosphorylation in importance as a regulatory modification (Zhao et al. 2010; Chen et al. 2012a; Norris et al. 2009).

The number of KATs varies considerably across species and phyla, and the family has branched significantly in relatively recent evolution. KATs include members that are largely cytosolic and members that are exclusively nuclear. Most function as parts of multi-subunit complexes, for example, Elongator (Okada et al. 2010; Close et al. 2012) and Mediator (Malik and Roeder 2000). Their activity is distinct from the non-enzymatic lysine acylation that occurs in some organelles and provides substrates for the sirtuins, for example, in metabolism (reviewed in Ghanta et al. 2013; Wagner and Hirschey 2014).

7.1.3 Discovery of the KATs

In the early 1960s, it was known that histones interfered with the transcription of RNA from genomic DNA, but chromatin was generally considered little more than inert packaging material. In 1963, while analyzing the amino acid composition of calf thymus histones, Phillips detected a subpopulation of lysine-rich histones with N-terminal acetyl modifications (Phillips 1963). Vincent Allfrey (1921–2002) subsequently showed that these modifications occurred after histones were synthesized, reducing their ability to inhibit RNA transcription. He considered that acetylation would neutralize the charge of histones and thus profoundly affect their interaction with DNA. In a remarkably prescient conclusion, he foresaw that "… specificity in DNA-histone binding, alterable by acetylation of the histone, can influence the rate of RNA synthesis. This would allow a means of switching-on or -off RNA synthesis at different times, and at different loci of the chromosomes" (Allfrey et al. 1964).

Confirmation of this hypothesis – that histone acetylation regulates transcriptional activation – proceeded slowly at first. Pogo et al. (1966) and others found that growth signals induce a rapid increase in histone acetylation that precedes other changes in the cell, including RNA and protein synthesis. This observation was initially made in lymphocytes exposed to phytohemagglutinin and was quickly replicated in regenerating liver, cortisol-treated thymus from adrenalectomized rats, erythropoietin-stimulated spleens, aldosterone-treated kidneys, and uteri stimulated with estradiol (Pogo et al. 1968; Libby 1972) (and references therein). Acetylated histones were physically localized to sites of active chromatin by autoradiography of insect chromosome squashes (Fig. 7.1) (Allfrey et al. 1968).

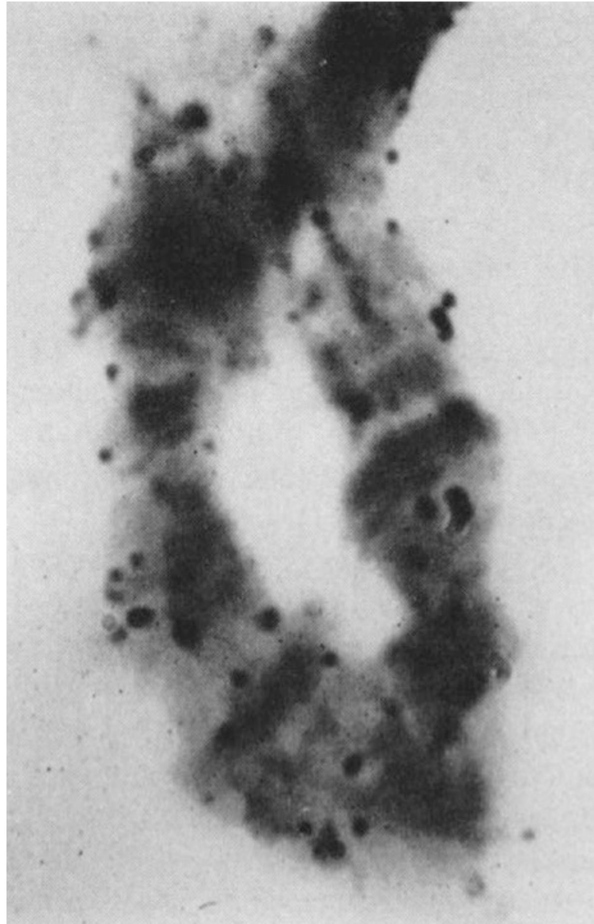

Fig. 7.1 Vincent Allfrey's 1967 autoradiograph of insect chromatin labeled with 3H-AcCoA (Allfrey et al. 1968)

Evidence for an acetyltransferase with nuclear localization and selectivity for histones was reported in the late 1960s by Gallwitz (1968) and a similar activity was subsequently identified in the nuclei of rat liver and brain (Bondy et al. 1970). Studies in the laboratory of Bruce Alberts showed that this activity was guided by association with the nuclear matrix, and they concluded that structural features or nuclear factors were responsible for directing it to specific DNA locations (Garcea and Alberts 1980). Collectively, these studies established (1) the dynamic, stress-responsive nature of histone acetylation; (2) the spatial localization of acetyltransferase activity along the genome; and (3) the control of acetyltransferase activity and specificity by interaction with other factors in multimolecular complexes.

Finding the enzymes responsible for histone acetylation took another decade and a half. Two types of activities were recognized: type A, acting on chromatin-associated histones, and type B, active only on isolated histones. In 1995, Brownell et al. isolated a 55-kd nuclear Type A HAT activity from *Tetrahymena thermophila* (Brownell and

Allis 1995). Subsequent cloning and sequencing of the enzyme, HAT A, revealed that it was a homologue of a yeast protein, general control non-repressed protein 5 (Gcn5), a transcription adapter, which then proved to have HAT activity also. In addition to a domain common to Type B HATs, both proteins harbored a bromodomain, a motif previously associated with yeast, fly, and human transcriptional regulatory proteins (Tamkun et al. 1992). Yeast GCN5 was later shown to exert Type A HAT activity in complex with the site-specific and general transcriptional activators Ada and Spt (Grant et al. 1997). These observations established a definitive biochemical link between transcriptional activation and histone lysine acetylation.

7.2 The Mammalian KAT Families

Most mammalian KATs fall into one of four major groups. The KAT2 or GNAT family comprises homologues of yeast GCN5, including mammalian KAT2A, KAT2B, and alpha-tubulin acetyltransferase (ATAT1). The MYST group shares homology with the yeast acetyltransferases Mst1/2, Sas2, and Ybf2/Sas3 and includes mammalian KAT5 (TIP60, PLIP), KAT6A and KAT6B, KAT7, and KAT8. The KAT13 group contains the nuclear steroid receptor coactivators NCOA1, NCOA2, and NCOA3 and the circadian regulator CLOCK (Verdin and Ott 2015; Olson et al. 2006; Weeks and Avkiran 2015). The fourth and smallest family, KAT3, contains the EP300 (E1A-associated 300 kDa protein, p300) and CREBBP (CREB-binding protein, CBP) paralogues, which have some structural features in common with the yeast KAT Rtt109 (regulator of Ty1 transposition gene product 109) (Tang et al. 2008), but otherwise have no homologues in single-celled organisms. The structural relationship of the remaining KATs with these larger groups has not yet been definitively established, but sequence comparison provides some insight (Fig. 7.2). Table 7.1 contains a summary of information about the function, genetics, and model organism phenotypes of known mammalian KATs.

7.2.1 The GNAT Family

This group includes KAT2A (hGCN5), KAT2B (p300–CBP-associated factor, PCAF), and the structurally related ATAT1. KAT2 proteins commonly participate in complexes with other KAT species (see below). Their contribution may be to add to the total available enzymatic activity at target promoters or to serve to acetylate and activate the other KATs, since hypoacetylated p300 is relatively weak (Thompson et al. 2004). KAT2B itself is regulated by autoacetylation and accumulates in the cytosol when deacetylated (Blanco-Garcia et al. 2009).

7.2.1.1 KAT2A

KAT2A (GCN5) and its paralogue, KAT2B (P/CAF), are the two vertebrate homologues of the yeast acetyltransferase Gcn5 (General Control Non-derepressible 5). In both yeast and vertebrates, these enzymes constitute the working end of

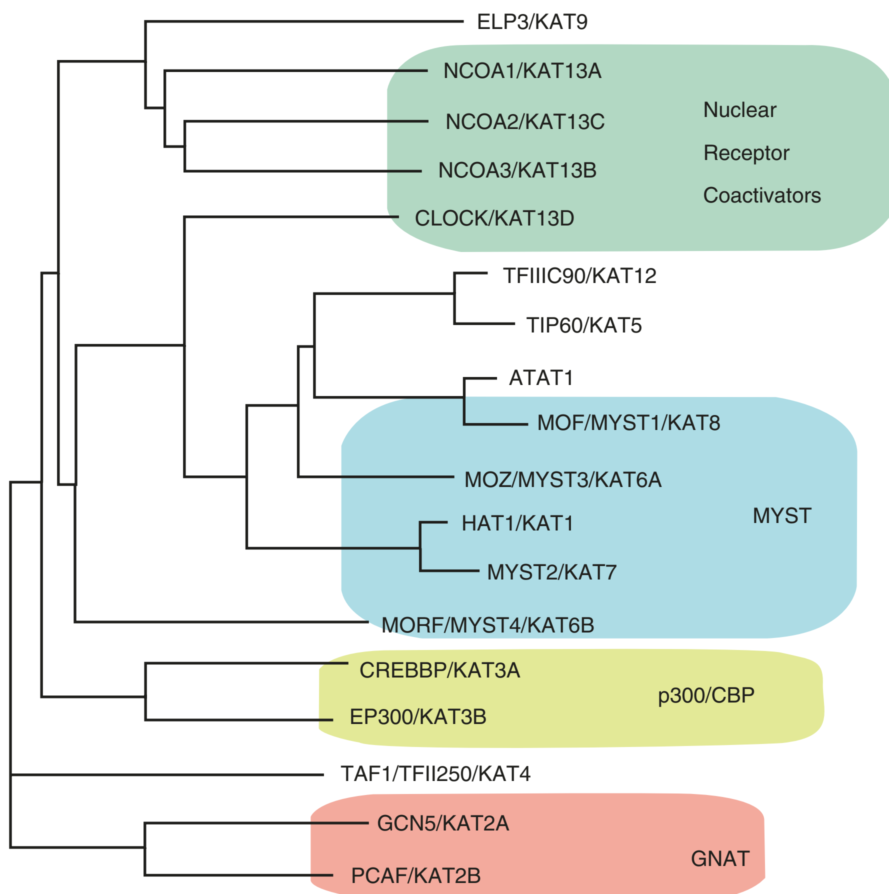

Fig. 7.2 KAT family phylogeny. Major mRNA transcript sequences for each gene were used to construct a phylogenetic tree using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalw2/). *Shaded circles* enclose members of the 4 major mammalian KAT families

multiprotein complexes that facilitate basal transcription, nucleosome sliding, nucleotide excision repair, and other DNA-based processes. Two such KAT2A/B complexes have been identified in humans, SPT3-TAF9-GCN5/PCAF acetylase and Ada-Two-A-containing. Each contains more than 20 subunits, including components and regulators of the basal transcriptional machinery, several histone-like proteins, DNA replication proteins, and one or more proteins in the MAPK signal pathway (Ogryzko et al. 1998; Wang et al. 2008a). In composition and function, these complexes resemble the basic transcription factor complex TFIID (Lee et al. 2000; Struhl and Moqtaderi 1998). Loss of KAT2A from these complexes induces early embryonic lethality (Wang and Dent 2014).

KAT2B (P/CAF) was originally identified as a protein associated with the KAT3 members p300 and CBP. It was shown to be an important acetyltransferase for

Table 7.1 Genetics, functions, and model organism phenotypes of known mammalian KATs

Class	Name	Mouse knockout phenotypes	Locus	Human disease	Pathways	Substrate specificity	Homologues	References
KAT1	HAT1	Neonatal lethality, defective lung development, craniofacial defects	2q31.1	–	Replication-dependent nucleosome assembly, DNA repair	H4 (5, 12), H2 (5), H3 (9, 18, and 27)	(D) CG2051, (S.c) Hat1, (S.p.) Hat1/Hag603	(Nagarajan et al. 2013)
KAT2A	hGCN5 GCN5L2	Failure of dorsal mesoderm formation	17q21.2	–	Inhibition of gluconeogenesis	H3 (9, 14, 18)/H2B, PGC1a	(D) KAT2, dGCN5/PCAF, (S.c., S.p.) GCN5	(Xu et al. 2000a; Jin et al. 2011)
KAT2B	PCAF	None	3p24.3	–	Myogenesis, viral replication, apoptosis	H3 (9, 14, 18)/H2B, MYOD (99, 102, 104), PTEN (125, 128), p53 (K320), HIF1A, E2A (34), TRIM50 (372)		(Xu et al. 2000a; Sartorelli et al. 1999; Xenaki et al. 2008; Fusco et al. 2014)
–	ATAT1	Defective sperm motility, male fertility	6p21.33	–	Locomotion, chemotaxis, DNA repair	a-tubulin (40)	(C.e., D.r.) Mec17	(Kalebic et al. 2013)

(continued)

Table 6.2 (continued)

Class	Name	Mouse knockout phenotypes	Locus	Human disease	Pathways	Substrate specificity	Homologues	References
KAT3A	CREBBP	Embryonic lethality, craniofacial, hematopoietic, long-term memory defects, tumorigenesis, white adipose lipodystrophy	16p13.3	Rubinstein–Taybi Syndrome 1, (gene fusion) acute myeloid leukemia, myelodyspastic syndrome	Gluconeogenesis, myogenesis, CREB-dependent gene expression, apoptosis	H2A (5), H2B (12, 15), H3 (9, 14, 18, 23, 27, 56), H4, CREB (136), E2A (34), multiple transcription factors	(D) dCBP/NEJ, KAT3, no yeast homologues	(Jin et al. 2011; Kung et al. 2000; Bedford et al. 2011; Kasper and Brindle 2006; Kasper et al. 2011; Zhou et al. 2004; Polesskaya et al. 2001; Bartsch et al. 2010; Kalkhoven et al. 2003; Alarcon et al. 2004; Yamauchi et al. 2002)
KAT3B	EP300	Embryonic lethality, cardiac, craniofacial, vasculogenic, growth/size retardation	22q13.2	Rubinstein-Taybi Syndrome 2, (somatic) colorectal cancer, (gene fusion) leukemia	Megakaryocytopoiesis, erythropoiesis, and lymphopoiesis; myogenesis; smooth muscle differentiation, cardiac development, and hypertrophy	H2A (5), H2B (12, 15), H3 (9, 14, 18, 23, 18, 27, 56), H4; E2A (34), HDAC1, HDAC6, TRIM50 (372), multiple transcription factors		(Fusco et al. 2014; Kasper and Brindle 2006; Kasper et al. 2002; Kauppi et al. 2008; Eckner et al. 1996; Roth et al. 2003; Yao et al. 1998; Wei et al. 2008; Spin et al. 2010; Xiao et al. 2000; Sartorelli et al. 1997; Bartholdi et al. 2007; Pentz et al. 2012)

KAT4	TAF1 TAFII250 CCG1 BA2R	–	Xq13.1	X-linked dystonia-Parkinsonism (XDP)	RNA Pol II-mediated transcription, transcription-dependent reprogramming, bookmarking active genes	H3 > H4	(D) dTAF1, dTAF(II) 230, (S.c., S.p.) Taf1, yTAF(II) 130	(Lee et al. 2000; Mizzen et al. 1996; Pijnappel et al. 2013; Makino et al. 2007)
KAT5	TIP60a HTATIP ESA1 TIP60b/ PLIP	(−/−) embryonic lethality at gastrulation, (−/+) reduced apoptosis under stress	11q13.1	Haploid loss in breast cancer	Homologous recombination, double-stranded DNA break repair, apoptosis, autophagy, tumor suppression	H4 (5, 8, 12, 16), H2A (chicken 5, 9, 13, 15), p53 (120), ATM, androgen receptor	dTIP60, s.c. Esa1, s.p. Mst1	(Hu et al. 2009; Kamine et al. 1996; Col et al. 2005; Sykes et al. 2006; Sun et al. 2010; Tang et al. 2013; Fisher et al. 2012; Gorrini et al. 2007)
KAT6A	MOZ MYST3 ZNF220	Ventricular septal defects, interrupted aortic arch	8p11.21	Developmental delay syndrome with ventricular and atrial septal defects, (t(8;16)(p11;p13)) AML	Transcription activation and elongation, DNA replication, cardiac, pharyngeal, and facial development	H3 (9, 18), p53, TBX1	(D) CG1894, ENOK (S.c.) Sas3 (S.p.) Mst2	(Tham et al. 2015; Arboleda Valerie et al. 2015; Voss et al. 2012; Vanyai et al. 2015)
KAT6B	MORF MYST4 MORF GTPTS		10q22.2	Genitopatellar syndrome, Ohdo/ Say–Barber–Biesecker–Young–Simpson Syndrome, (gene fusion) AML	Transcription activation	H3 (14)		

(continued)

Table 6.2 (continued)

Class	Name	Mouse knockout phenotypes	Locus	Human disease	Pathways	Substrate specificity	Homologues	References
KAT7	HBO1 MYST2	Growth arrest and lethality at 10-somite stage	17q21.33	–	Transcription activation, mesenchymal development	H3 (14)>>H4 (5, 8, 12)	(S.c.) Sas2p (S.p.) Mst2 CHM	(Iizuka and Stillman 1999; Kueh et al. 2011; Sharma et al. 2000)
KAT8	MOF HMOF MYST1	(Homozygotes) early embryonic lethality	16p11.2	–	DNA repair, cell cycle progression, proliferation, oncogenesis, dosage compensation (Drosophila)	H4 (16)	(D) dMOF (CG1894) (S.c.) Sas2 (S.p.) Mst2	(Neal et al. 2000; Gupta et al. 2005; Smith et al. 2005; Gupta et al. 2008)
KAT9	ELP3	Required for paternal zygote DNA demethylation	8p21.1	Amyotropic lateral sclerosis	Transcript elongation, paternal demethylation, motility, motor neuron branching and migration	H3, alpha-tubulin	D) 5433 dELP3/CG1 (S.c.) Elp3 (S.p.) Elp3	(Okada et al. 2010; Close et al. 2012) (Simpson et al. 2009)
KAT10	–	Yeast only	–	–	–	H3 (14); H4	(S.c.) Hap2	
KAT11	–	Yeast only	–	–	Genome stability, transcription elongation	H3 (56)	(S.c.) Rtt109	
KAT12	GTF3C4 TFIIIC90	–	9q34.13	–	Pol III transcription	H3 (9, 14, 18)	No yeast homologues	(Hsieh et al. 1999)
KAT13A	NCOA1 SRC1	(–/–) viable, fertile, smaller reproductive organs, osteopenia	2p23.3	–	Transcription activation, steroid hormone signaling	H3/H4	No yeast homologues	(Onate et al. 1995; Hayashi et al. 1997; Zhang et al. 2007; Li and Shang 2007; Xu et al. 1998; Yamada et al. 2004; Qi et al. 1999)

Class	Name	Mouse knockout phenotypes	Locus	Human disease	Pathways	Substrate specificity	Homologues	References
KAT13B	NCOA3 SRC-3 ACTR AIB1 TRAM1 RAC3 p/CIP	(−/−): reduced white adipose tissue, B cell lymphomas, thrombocytopenia; enhanced inflammatory cytokine production	20q13.12	Amplified in breast cancer	Transcription activation, vascular smooth muscle and adipose differentiation, steroid hormone signaling	H3/H4		(Li and Shang 2007; Chen et al. 1997; Anzick et al. 1997; Takeshita et al. 1997; Reiter et al. 2001; York et al. 2010; Louet et al. 2006; Coste et al. 2006; Yu et al. 2007; Wang et al. 2006)
KAT13C	NCOA2 TIF2 GRIP1 SRC2 P160	(−/−) Defective spermatogenesis, placental development, and hepatic gluconeogenesis and glycolysis	8q13.3	MOZ gene fusion: AML	Transcription activation, myogenic transcription	H3/H4		(Hong et al. 1997; Voegel et al. 1996; Chen et al. 2000; Troke et al. 2006; Deguchi et al. 2003; Chopra et al. 2008)
KAT13D	CLOCK	(−/+) Prolongation of circadian cycle, (pancreas-targeted) diabetes	4q12		Transcription activation, circadian rhythm	H3/H4, BMAL1 (537)		(Marcheva et al. 2010; King et al. 1997; Antoch et al. 1997; Doi et al. 2006; Vitaterna et al. 1994)

MyoD and a driver of myogenic differentiation in cultured myoblasts (Puri et al. 1997). KAT2B is interchangeable with KAT2A for most or all of its functions in basal and signal-activated transcription, and mice with homozygous deletion of P/Caf have no apparent phenotype, suggesting that its functions are largely those of gene dosage compensation.

7.2.1.2 ATAT1: Tubulin Acetyltransferase

Cell polarization and migration require the accumulation of microtubules at the leading edge of the cell, a process that involves acetylation of alpha-tubulin. ATAT1 encodes the major cellular tubulin acetyltransferase (Kalebic et al. 2013). Tubulin acetylation is also required for cells to progress through mitosis. Changes in tubulin acetylation thus have significant consequences for tissue repair and fibrosis. KAT2A and P/CAF can also directly acetylate tubulin (Kalebic et al. 2013; Liu et al. 2013). Microtubule hyperacetylation occurs in response to several cell stressors, including reactive oxygen species and nutrient deprivation, and results from activation of ATAT1 by AMP kinase (Mackeh et al. 2014). Impairment of microtubule acetyltransferase function has been observed in diabetes and may contribute to defective wound healing in this condition (Spallotta et al. 2013).

7.2.2 The KAT3 Family: CBP/p300

The two members of this family, encoded by EP300 and CREBBP, are large multidomain proteins able to acetylate lysines in each of the four core histones. They also acetylate many other proteins, especially transcription factors, and can auto-acetylate (Thompson et al. 2004; Lee and La Thangue 1999; Wang et al. 2005a); more than 70 proteins are reported to be KAT3 substrates (Wang et al. 2008b). CBP and p300 exhibit >60 % protein sequence homology overall, and their HAT domains are >90 % identical. As a result, they are often referred to by a hybrid term (e.g., p300/CBP). However, they are encoded by different genes on different chromosomes, and the two distinct genes have been retained and conserved during mammalian evolution.

CBP (CREBBP, KAT3A) was originally identified as a coactivator recruited by cyclic AMP response element binding protein (CREB) and required for cyclic AMP-dependent transcription (Kwok et al. 1994; Chrivia et al. 1993). Its activity was found to be stimulated not only by protein kinase A but also by growth factors, CaM kinase and MAPK/ERK kinase pathways (Hu et al. 1999; Liu et al. 1998; Gusterson et al. 2002), placing it at a nodal position for relaying extracellular signals to the nuclear transcriptional machinery.

p300 (EP300, KAT3B) was originally identified as a 300 kd cellular protein associated with the E1A oncoprotein in adenovirus-infected cells (Stein et al. 1990). An E1A amino-terminal domain efficiently represses specialized, but not basal, gene expression, and this transcriptional repression can be reversed by restoring EP300 (Hen et al. 1985; Lillie et al. 1986; Bishopric et al. 1997; Hasegawa et al. 1997). Cloning of the p300 protein revealed it to be an acetyltransferase,

homologous to CBP. p300 was subsequently established as an activator of gene expression through its ability to interact with a large number of signal-responsive and tissue-specific transcription factors (reviewed in Vo and Goodman 2001).

CBP and p300 are co-expressed ubiquitously during early embryogenesis, but in progressively restricted patterns during later development. For example, in the mouse heart, both proteins are present and co-localized in atrial and ventricular myocytes at or before d7.5, but are no longer detectable after d14.5 (Partanen et al. 1999; Phan et al. 2005). After birth, myocardial levels of EP300 rise progressively during the first two months of life and then fall again after puberty, subject to stress-induced re-expression (Wei et al. 2008; Sharma et al. 2012). The HAT activities of both proteins generally parallel their expression patterns in adulthood, with evidence of decline in aging muscle, liver, and testis (Li et al. 2002).

The KAT3s have evolved in parallel with the appearance and radiation of multicellular life. No EP300 homologue can be found in single-cell organisms. Only one isoform is present in *C. elegans*, Drosophila, and primitive chordates. Most vertebrates have both p300 and CBP, and there are at least five KAT3 family members in multicellular plants (Pandey et al. 2002). There are at least eight other paralogue pairs on chromosomes 16p and 22q, for example, hemoxygenases HMOX2 and HMOX1; phosphomannomutases PMM2 and PMM1; myosins MYH11 and MYH9, suggesting that the entire chromosomal regions encoding p300 and CBP emerged from a relatively recent gene duplication during vertebrate evolution (Giles et al. 1998a). The implication is that the KAT3 family has undergone recent evolutionary expansion to meet the requirements of animals with highly specialized tissues, living in complex and changing environments.

7.2.2.1 The KAT3s Drive Specialized, Signal-Responsive Transcription

In addition to their acetyltransferase activities, the KAT3 proteins have in common a large number of modular protein-binding regions, most of which are not found in other coactivators (Dancy and Cole 2015; Kasper and and Brindle 2006; Goodman and Smolik 2000). These include a steroid receptor interaction domain, a glutamine-rich site, three cysteine–histidine-rich (C/H1-3) sites, a KIX domain (the binding site for CREB), four zinc finger motifs, and a bromodomain (Fig. 7.3). The bromodomain is an evolutionarily conserved feature of proteins that facilitate signal-activated but not basal transcription (Tamkun et al. 1992); its identification in p300 and CBP was an early sign that these proteins are important for communicating extracellular or environmental signals to the transcriptional apparatus, rather than driving basal transcription. Consistent with this, both genes can be entirely deleted without impairing basal cell function or viability (Kasper et al. 2010, 2011).

A very large number of transcription factors, as well as components of the basal transcriptional machinery, interact physically and functionally with the KAT3s (Kasper and Brindle 2006; Abraham et al. 1993). More than 300 different transcription factor partners have been identified for CBP and p300, and dozens of these are direct substrates (Goodman and Smolik 2000). Extensive redundancy between p300 and CBP exists for these interactions and probably between the KAT3 proteins and other coactivators, including the KAT2 family (GCN5/PCAF) (Phan et al. 2005).

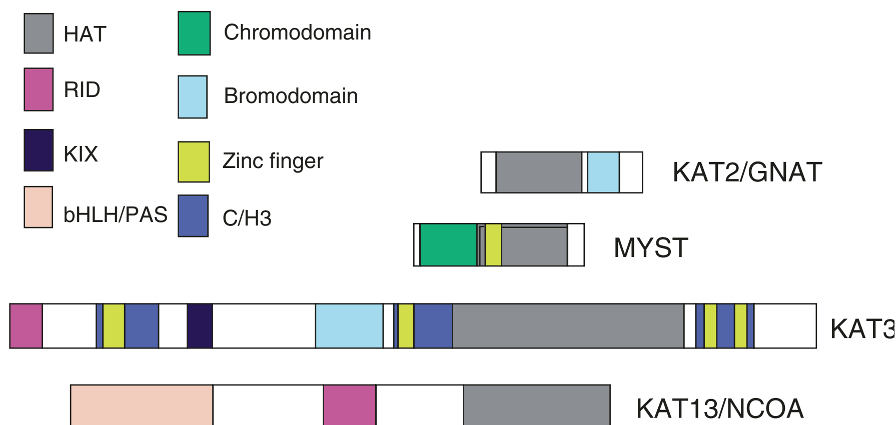

Fig. 7.3 Comparative functional domains of KAT families. *Drawing* indicates the major functional domains and relative size of representative proteins from each family, from approximately 440 aa for KAT2 to 2441aa for KAT3A (shown). Adapted from Marmorstein (Marmorstein 2001)

The transcription programs for which the KAT3 proteins are indispensable are specialized and cell type-specific, such as inflammation, long-term memory formation (Alarcon et al. 2004), and hepatic gluconeogenesis (Zhou et al. 2004). KAT3s are also required for the transcriptional activation of immediate-early genes, through their rapid, dynamic acetylation of trimethylated lysine 4 on histone 3 (H3K4me3). This chromatin mark is associated with RNA polymerase II recruitment and transcriptional activation (Crump et al. 2011). Thus, rather than acting as general transcriptional integrators, the KAT3 proteins enable differentiated cell and organ functions and link extracellular signals to a rapid, complex transcriptional response (Lee et al. 2009).

7.2.2.2 The KAT3s Are Gene Dose Limiting

Genetic studies show that CBP and p300 are present in limited supply in the cell. Heterozygous mutations in either of the human genes CREBBP and EP300 result in the Rubinstein–Taybi Syndrome (RTS) types 1 and 2, respectively. RTS is a very rare, multisystem disorder associated with bone malformations, hematological malignancies, mental retardation, and cardiac defects (Marion et al. 1993; Roelfsema et al. 2005; Zimmermann et al. 2007; Stevens and Bhakta 1995). Over half of the patients with RTS have mutations in CREBBP (Bartsch et al. 2005); EP300 is involved less frequently, representing about 3.8 % of all RTS cases, although interestingly the normal gene is more polymorphic than CREBBP (Zimmermann et al. 2007). Both deletions and missense mutations affecting the HAT domain have been reported (Kalkhoven et al. 2003; Woods et al. 2014; Wincent J et al. 2016; Hamilton MJ et al. 2016). Although about 10 % of mutations may express a truncated protein, in most cases RTS is a haploinsufficiency disorder, associated with ≥50 % reduction in cellular HAT activity (Petrij et al. 2000; Blough et al. 2000; Lopez-Atalaya et al. 2012). These findings reveal that KAT3 gene dosage is critical and that there is no significant dose compensation between the two proteins.

Surveys of RTS patients demonstrate a high 32.6% (45/138) rate of cardiovascular abnormalities, including atrial and ventricular septal defects, coarctation of the aorta, pulmonic stenosis, bicuspid aortic valve, and patent ductus arteriosus (Stevens and Bhakta 1995).

Mouse genetic studies indicate that complete loss of either EP300 or CBP is embryonic lethal. Surprisingly, heterozygous loss of both proteins is also embryonic lethal, indicating both functional overlap and a lack of dosage compensation. Thus, at least three intact KAT3 alleles are required for normal development (Yao et al. 1998; Tanaka et al. 1997).

Functional Divergence of EP300 and CREBBP

Since the recognition that both CREBBP and EP300 mutations can lead to RTS, phenotypic differences have begun to emerge. EP300 loss induces a milder intellectual deficit and does not appear to promote the same rate of cardiac malformations (Zimmermann et al. 2007; Spena et al. 2014; Solomon et al. 2015). Wider use of clinical sequencing in these syndromes will provide further insight.

In the meantime, mouse genetic studies support functional differences between p300 and CBP during development, consistent with a large body of in vitro data (reviewed in Kalkhoven 2004). Ep300 −/− mice die between d9.5 and 11, with abnormalities of cell proliferation in the ventricular myocardium and elsewhere, neural tube defects, and defective vasculogenesis. Although the heart forms, the ventricular myocardium does not progress beyond a few cells in thickness and fails to express cardiac sarcomeric genes, leading to heart failure and death in utero (Yao et al. 1998). An Ep300 allele with inactivation of the acetyltransferase domain conferred a similar phenotype (Yao et al. 1998; Shikama et al. 2003). Mice with Crebbp loss die later in embryogenesis, exhibiting skeletal abnormalities reminiscent of RTS, but without cardiac abnormalities (Kung et al. 2000; Tanaka et al. 2000; Oike et al. 1999). Thus, p300 appears to be uniquely indispensable for proliferation, development, and sarcomerogenesis in the fetal mouse heart. Interestingly, mice expressing a truncated form of Cbp had cardiac malformations, abnormal vasculogenesis, and earlier (d9.5–11) embryonic demise, likely through a dominant negative mechanism (Oike et al. 1999).

Adult heterozygous phenotypes for Ep300 and Crebbp loss also appear to differ, although few direct comparisons have been performed (see later). Rebel et al. showed that both copies of Crebbp were required for hematopoietic stem cell self-renewal, while p300 was required for hematopoietic differentiation. Mice heterozygous for Crebbp loss have significant deficits in long-term memory and fear conditioning, which are not seen in Ep300-deficient mice (Alarcon et al. 2004; Viosca et al. 2010). Remarkably, these deficits were reversible by treatment with a histone deacetylase inhibitor, implying that they were due to active acetylation deficits rather than to irreversible errors of development. In another study, Crebbp−/+ mice were lean, with selective reduction of white adipose tissue, increased insulin sensitivity, and heightened glucose tolerance, associated with increased levels of adiponectin and leptin. Mice with partial loss of Crebbp were also resistant to weight gain on a high fat diet (Yamauchi et al. 2002). It is not yet known whether these metabolic effects are mimicked by Ep300 loss.

Mechanistic Aspects of the EP300-CBP Divergence

The molecular basis for the distinct phenotypes of p300 and CBP deficiency has not yet been established. Multiple studies have shown that certain transcription factors preferentially use one of the two acetyltransferases (see, for example, Korzus et al. 1998; Ianculescu et al. 2012). This preference is unlikely to be due to selective recruitment, since both p300 and CBP can be found in the same promoter-bound complexes (Bedford et al. 2010). Instead, intrinsic structural and biochemical differences may determine their selective use from within the coactivator complex (Bedford et al. 2010). Differences in expression, trafficking, or stability may affect the relative availability of the two coactivators. In some studies, p300 and CBP were shown to have distinct tissue- and stage-specific patterns of expression and cellular localization (Partanen et al. 1999; Kwok et al. 2006; Chen et al. 2012b). Using in situ hybridization and a series of CBP- and p300-specific antibodies, Partanen et al. provided evidence that some fraction of p300 and CBP may be cytosolic and that the subcellular distribution of the two proteins may be differentially regulated (Partanen et al. 1999). Some KAT3 preferences may be determined by differing posttranslational modifications (see below). Others have reported structural divergence in the AT domains of p300 and CBP (Bordoli et al. 2001), and the two proteins exhibit different selectivity and specificity for their lysine targets on H3 and H4 in vitro (Henry et al. 2013, 2015). These observations indicate that there are subtle but important differences in substrate interactions and protein partner recruitment that underlie the distinct properties of p300 and CBP. Further work will be required to elucidate the basis of these differences and to identify their unique targets.

7.2.2.3 EP300 as a Stress Response Gene

EP300 and CBP are widely viewed as "housekeeping genes," perhaps because they encode proteins that interact with an extraordinary number of other transcriptional regulators and participate in an equally broad range of basic cell functions. However, in tissues of the adult cardiovascular and elsewhere, expression of these genes is strongly upregulated in response to stress signals, implying additional roles in the response to environmental challenge. Both p300 and CBP were upregulated in murine myocardium following an experimental coronary occlusion (Miyamoto et al. 2006). We observed similar marked increases in p300 after transverse aortic coarctation, a model of pressure overload; p300 protein content rose by more than 20-fold and remained elevated for at least 6 weeks (Wei et al. 2008). Both content and activity of p300 can be directly induced in cultured myocytes by hypertrophic and oxidative stressors and in endothelial cells and kidney by hyperglycemic stress (Wang et al. 2012; Chen et al. 2010; Emani et al. 2009). Since the KAT3s are present in limiting numbers in the cell (Giles et al. 1998b), increases in availability can be assumed to have important biological consequences.

We tested the significance of graded changes in p300 in a series of 11 mouse lines with varying copy numbers of a human EP300 transgene targeted to the myocardium. All mice developed classic maladaptive hypertrophy as defined by myocyte enlargement, re-expression of embryonic sarcomeric genes, and partly compensatory angiogenesis (Wei et al. 2008; Shehadeh et al. 2013). Timing and

frequency of progression from hypertrophy to heart failure and death was directly related to p300 content. Correspondingly, Ep300 −/+ haploinsufficient mice displayed reductions in hypertrophy and heart failure (Wei et al. 2008). Thus, p300 levels determine cardiac growth potential in response to hemodynamic stress. Targeting p300 and/or CBP has been shown to be effective in preventing pathological hypertrophy in several other models: a HAT-defective EP300 transgene blocked post-infarction remodeling (Miyamoto et al. 2006), and curcumin, a polyphenol blocker of CBP/p300 HAT activity (Balasubramanyam et al. 2004; Marcu et al. 2006), successfully prevented experimental hypertrophy in rats and mice (Ghosh et al. 2010; Morimoto et al. 2008; Feng et al. 2008). It will be important to determine whether CBP and p300 play similar or diverging roles in heart failure, since to date no pharmacological inhibitors selective for either KAT3 have emerged (Dancy and Cole 2015; Bowers et al. 2010).

7.2.2.4 KAT3 Regulation by Posttranslational Modifications

The ability of p300 and CBP to respond to environmental stress is primarily conferred by post-transcriptional mechanisms, some of which may be tissue-specific, including phosphorylation, acetylation, sumoylation, and methylation (Lee et al. 2011).

p300 was originally isolated as a phosphoprotein, and phosphorylation plays an important role in regulating KAT3 acetyltransferase activity (Gusterson et al. 2002, 2004; Kitabayashi et al. 1995; Ait-Si-Ali et al. 1998; Liu et al. 1999a) . Both proteins are substrates for calcium-calmodulin kinase, protein kinase A, p38 and MAP/ERK kinases, and protein kinase D. Phosphorylation may differentially regulate the properties of CBP and p300: phenylephrine-stimulated MAP kinase activates CBP and p300 through phosphorylation of serine residues at opposite ends of the two proteins (S2015 and S89, respectively) (Gusterson et al. 2004). Hepatic gluconeogenesis requires CBP to undergo insulin-stimulated phosphorylation at a site (S436) that is not present in p300 (Zhou et al. 2004).

Ubiquitination is a critical regulator of p300 action in the myocardium, because dynamic changes in p300 levels are largely established through changes in stability rather than changes in transcription. Myocardial p300, like many immediate-early gene products (e.g., Myc, p53, Fos, Jun, and E2F), has a very short half-life enforced by the ubiquitin–proteasome system (Varshavsky 1997; Mora et al. 1982; Oren et al. 1982; Treier et al. 1994; Hateboer et al. 1996; Campanero and Flemington 1997; Jain et al. 2012). This brisk turnover allows p300 to respond rapidly and dynamically to extracellular signals that reduce or increase its ubiquitination. Phosphorylation of p300 by p38MAPK is reported to increase p300 ubiquitination and degradation (Poizat et al. 2005).

The E3 ubiquitin ligases specific to p300/CBP are not known. In some cases, different signals may target p300 and CBP for destruction. CBP, but not p300, is targeted for degradation by the mutant protein responsible for Huntington's disease (Cong et al. 2005). In F9 cells, retinoic acid induced proteasomal degradation of p300, but not CBP (Brouillard and Cremisi 2003; Sanchez-Molina et al. 2006). Signal- or tissue-specific proteasomal degradation of these KATs could thus account for some of their functional specificities. p300 itself has intrinsic

ubiquitin ligase activity that can be inhibited by E1A, resulting in the stabilization of p53 (Grossman et al. 2003) and potentially other p300 target proteins. This feature of p300 may important for its interaction with other acetyltransferase substrates.

Autoacetylation of p300 has been shown to affect its ability to acetylate other substrates and to alter the kinetics of its association with other components of the transcriptional machinery (Black et al. 2006; Stiehl et al. 2007). In myocytes exposed to doxorubicin, a potent oxidative stress, autoacetylation rendered p300 resistant to proteasomal degradation, allowing it to accumulate to high levels in a positively reinforcing manner (Jain et al. 2012). Thus, autoacetylation represents a positively reinforcing loop in which increased activity of p300 results in increased p300 content.

7.2.2.5 KAT3 Substrates

In addition to their histone substrates, the KAT3s may acetylate at least 70 other proteins, most of them transcription factors (reviewed in Wang et al. 2008b). Many transcription factors important in cardiac development and disease are coactivated by the KAT3s, and a few of these are documented KAT3 substrates. Noteworthy in this group are E2F1, p53, MyoD, STAT3, FOXO1, beta-catenin, the insulin regulator BETA2, NFkB, and two transcription factors, GATA4 and Myocyte Enhancer Factor 2 (MEF2), which have been implicated as drivers of pathological hypertrophy (Liu et al. 1999b; Martinez-Balbas et al. 2000; Polesskaya et al. 2000; Levy et al. 2004; Qiu et al. 2004; Ma et al. 2005; Perrot and Rechler 2005; Wang et al. 2005b; Yuan et al. 2005; Zhang et al. 2005; Kiernan et al. 2003). Both GATA4 and MEF2 become extensively acetylated during experimental pressure overload, associated with increasing p300 levels (Wei et al. 2008). Takaya et al. identified a cluster of lysines in GATA4 that, when mutated, prevented p300-dependent acetylation and simultaneously blocked phenylephrine-induced hypertrophy in cultured myocytes (Takaya et al. 2008), indicating that this modification is critical to activation of the hypertrophic program by p300.

The MEF2 transcription factors are strictly controlled by acetylation (Ma et al. 2005; Miska et al. 1999; Zhao et al. 2005). MEF2 proteins are maintained in an inactive state by association with class II histone deacetylases (HDAC4, 5, 7, and 9) (Ornatsky and McDermott 1996; Buchberger et al. 1994; Youn and Liu 2000); the signal-dependent dissociation of HDACs from MEF2 has been covered elsewhere in this volume (Lu et al. 2000; McKinsey et al. 2001; Vega et al. 2004; Backs et al. 2009). However, dissociation of HDACs is not sufficient for MEF2 activation; this requires a second step of acetylation by p300 and/or P/CAF (Sartorelli et al. 1997; Eckner et al. 1996).

CBP has also been reported to acetylate structural components of the cell, including collagen (Choi et al. 2015), and the nuclear import proteins importin-alpha and importin-alpha7 (Bannister et al. 2000). p300 indirectly promotes tubulin acetylation by acetylating and inhibiting HDAC6, a tubulin deacetylase, and also by acetylating TRIM50, an E3 ubiquitin ligase regulating HDAC6 stability (Fusco et al. 2014).

7.2.3 KAT4

KAT4 (TAFI, TAFII250) does not fall within any of the major mammalian KAT families. It is a member of the basal transcription complex TFIID and is required for RNA polymerase II-dependent transcription. The TFIID complex is thought to be the earliest of the basal transcription factors to be recruited to the promoter following gene activation, and forms a scaffold for recruitment of additional components of the RNA polymerase complex.

Like its yeast and Drosophila homologues, KAT4/TAF1 is a type A histone acetyltransferase that is relatively selective for H3 and H4 (Mizzen et al. 1996) (KAT4 should not be confused with HAT4, a recently identified type B acetyltransferase (Yang et al. 2011)). KAT4 contains 2 tandem bromodomains that coordinate binding to acetyllysine residues and permit it to recognize promoters bearing histone acetylation marks (Jacobson et al. 2000). KAT also has serine/threonine kinase activity and can phosphorylate the basal TFIIF complex, as well as autophosphorylating itself (Dikstein et al. 1996).

The KAT4-containing TFIID complex is retained on active gene promoters, allowing for rapid cycling of transcription. Interestingly, this complex is retained on promoters during cell division, persisting through chromatin compaction. This permits the bookmarking of active genes and transmission of parental transcription profiles to daughter cells following mitosis (Christova and Oelgeschlager 2002). The TFIID complex appears to be required for preservation of the pluripotency program in cycling embryonic stem cells; overexpression of TFIID components was recently reported to greatly enhance reprogramming of fibroblasts by the combination of Oct4, Sox2, Klf4, and Myc (Pijnappel et al. 2013).

7.2.4 The MYST Family

Five mammalian KATs are included in the MYST family: KAT5, KAT6A, KAT6B, KAT7, and KAT8. All of these proteins share a MYST domain, a 240-amino acid sequence with a characteristic binding site for acetyl-CoA, and a C2HC-type zinc finger motif. The MYST proteins also have a chromodomain that mediates interaction with other proteins, especially methylated histones. Two (KAT5 and KAT8) are involved in DNA damage repair. Three (KAT5, KAT7, and KAT8) are required for very early stages in embryogenesis. The other two (KAT6A and KAT6B) appear to be redundant for critical basal functions, while having individually specific requirements in certain organ systems.

7.2.4.1 KAT5/TIP60

KAT5 was originally cloned as a 60-kd HIV TAT-interacting protein, TIP60, and has since been shown to be critical for DNA damage repair. KAT5 acetylates and recruits components of the homologous recombination repair pathway, including ATM and BRCA1, to sites of DNA damage. KAT5 acetylates p53 on Lys120, which is required for induction of apoptosis (Tang et al. 2006). Broad-spectrum histone

deacetylase inhibitors, which activate the DNA damage pathway, augment TIP60 activity (Tang et al. 2013). TIP60 acts as a haploinsufficient tumor suppressor in several cancer types and in mouse models of oncogenesis (Gorrini et al. 2007).

During the DNA damage response, KAT5 participates in a complex with p400, a SWI/SNF-related ATPase, to open and remodel chromatin in the vicinity of the double-stranded break (Xu et al. 2010; Chan et al. 2005; Samuelson et al. 2005). Fazzio et al. identified the KAT4–p400 complex as a key determinant of Nanog-dependent transcription, which was required for the maintenance of gene expression programs ensuring pluripotency and self-renewal (2008). The selective use of this complex for ES-specific transcription is interesting and perhaps reflects the importance of tight genome surveillance and vigilant DNA repair mechanisms in progenitor cells.

A shorter 50-kd splice variant of KAT5 (a.k.a. TIP60beta, PLIP) was identified in a human heart library, lacking residues encoded by exon 5. The region omitted is proline-rich and important for protein–protein interactions. Unlike TIP60alpha, which is exclusively nuclear, TIP60beta is both nuclear and cytosolic (Ran and Pereira-Smith 2000; Sheridan et al. 2001). TIP60beta appears to be the predominant isoform expressed in the adult heart, while TIP60alpha is more abundant in the embryonic heart (Fisher et al. 2012).

The DNA damage repair pathway was recently shown to be activated during ischemia-reperfusion injury in the heart (Shukla et al. 2011), suggesting that components of this pathway may participate in cardiac myocyte pathophysiology in an unexpected manner. Consistent with this, cardiac myocyte apoptosis induced by pressure overload was significantly diminished in mice haploinsufficient for TIP60 (Fisher et al. 2012). In the same TIP60 −/+ background, overexpression of c-Myc was associated with increased cardiomyocyte cell cycle activity; the authors postulated that TIP60 enforces replicative senescence in these cells in a dose-dependent manner (Fisher et al. 2012).

KAT5 and Autophagy Autophagy is a mechanism for the breakdown and recycling of cellular components under conditions of stress, particularly (but not exclusively) nutrient deprivation, and is increasingly recognized as an important contributor to myocardial pathophysiology (Zhang et al. 2015; Ren and Taegtmeyer 2015; Delbridge et al. 2015; Lavandero et al. 2015). In the absence of nutrients, autophagy permits the re-use of unnecessary cell proteins as fuel for survival. Unlike single-cell organisms, in multicellular organisms nutrient transporters are under signal-dependent transcriptional control and require growth factors for induction. When growth factors are available and nutrients are plentiful, autophagy is suppressed. Nutrient or growth factor deprivation activates a group of autophagy-related genes, including mammalian ULK1 and ULK2, which encode kinases that are required for the formation of autophagosomes. In the absence of growth factors, the growth-inhibitory glycogen synthase kinase-3 is de-repressed and phosphorylates KAT5 on Ser86. This then potentiates an interaction between KAT5 and its substrate ULK1. Acetylation of ULK1 by KAT5 is required for its kinase activity and induction of autophagy (Lin et al. 2012).

7.2.4.2 KAT6

The two KAT6 paralogues in mammals, KAT6A and KAT6B, are large multidomain proteins belonging to the MYST KAT family. Like the paralogues KAT3A and KAT3B, they are encoded by separate genes and have evolved distinct functions during mammalian evolution, but retain considerable sequence homology (60 % overall amino-acid sequence identity). Both were identified through their participation in leukemia-associated chromosome breakpoints.

KAT6A

KAT6A, also known as MYST3 and MOZ (*mo*nocytic leukemia *z*inc finger protein), is widely expressed both during development and in adulthood, indicating a potentially broad range of regulatory activities (Voss et al. 2009). KAT6A has a particularly important role in the regulation of HOX genes and in p53-dependent transcription. It was originally identified by sequencing of a chromosomal translocation recurring in a severe form of acute myelogenous leukemia, in which the fusion of 8:16 creates an oncogene encoding parts of KAT6A and KAT3A (CREBBP, see above) (Giles et al. 1997). Similar fusions have occurred with p300 (Yang 2004).

Recently, Tham et al. and Arboleda et al. independently reported identification of a syndrome of developmental delay associated with mutations in KAT6A, totaling ten cases (Tham et al. 2015; Arboleda Valerie et al. 2015). In the Tham study, four individuals were identified through exome sequencing of proband and parents (trio sequencing) and exclusion of other known recessive and autosomal dominant disease genes. Most were heterozygous nonsense mutations truncating the C-terminal acidic transactivation domain of KAT6A, retaining the acetyltransferase domain. Interestingly, five similar nonsense variants were found in single individuals among the nearly 65,000 putatively normal exomes represented in the Exome Aggregation Consortium (http://exac.broadinstitute.org), but no clinical information was available on these subjects (Tham et al. 2015). In the Arboleda study, six individuals from five unrelated families were shown to have similar dominant C-terminal truncating mutations. The syndrome of KAT6A deficiency includes craniosynostosis, thin upper lip and other midline facial abnormalities, absent speech and motor delay, and cardiac abnormalities. Altogether seven of the eight probands examined were found to have cardiac malformations, including patent ductus arteriosus and atrial and/or ventricular septal defects.

Similar nonsense mutations in the mouse Kat6a gene phenocopy the cardiac defects found in human KAT6A deficiency. These mice exhibit a variety of defects reminiscent of DiGeorge/velo-cardio-facial syndrome and also resembled mice with haploinsufficiency of the causal DiGeorge gene Tbx1 in having a high frequency of ventricular septal defects (Voss et al. 2012). Voss et al. demonstrated that Kat6a specifically occupies the Tbx1 site and causes local H3K9 acetylation. Loss of Kat6a led to deficiency of Tbx1, and the phenotype of Kat6a loss was partially rescued by Tbx1 expression. Thus, KAT6A has a remarkably specific role in the regulation of genes critical for cardiac septal formation, as well as in correct patterning of the aortic arches and pharyngeal structure (Voss et al. 2012).

KAT6B

KAT6B, also known as MORF, was identified as a part of an oncogenic fusion with CBP, which drives some cases of acute myelogenous leukemia. Mutations in the normal, non-translocated gene cause two human genetic disorders: genito-patellar syndrome (GPS) and the Say–Barber–Biesecker–Young–Simpson variant of Ohdo syndrome (Clayton-Smith et al. 2011; Campeau et al. 2012). The latter is a rare complex disorder of mental and physical developmental delay, with blepharophimosis and characteristic long thumbs and great toes. Cardiac defects have been reported in about half; one series of eight patients included two with ventricular septal defects, one with atrial septal defect and mild pulmonic stenosis, and one with pulmonary atresia and hypoplastic right ventricle (Day et al. 2008). GPS is a congenital intellectual disability associated with agenesis of the corpus callosum, genital malformations, and absence of the patella; pulmonary agenesis and pulmonary insufficiency have been reported (Cormier-Daire et al. 2000). There is considerable overlap between the two syndromes. Most KAT6B mutations in SBBYS (89 %) truncate the protein in a similar C-terminal location to those reported in KAT6A, also sparing the HAT domain (Campeau et al. 2012).

7.2.4.3 KAT7

KAT7 (MYST2, HBO1) is a ubiquitously expressed protein that was originally identified as a component of the human origin recognition complex (ORC) (Iizuka and Stillman 1999). KAT7 interacts directly with two proteins, MCM2 and ORC1, which are involved in the initiation of DNA replication (Iizuka and Stillman 1999; Burke et al. 2001). KAT7 was shown to interact with and co-regulate the androgen receptor and is expressed at particularly high levels in ovary, testis, and prostate cancer cell line (Iizuka and Stillman 1999; Sharma et al. 2000). Mice homozygous for Myst2 loss fail to develop beyond gastrulation and degenerate at the 10-somite stage. Loss of KAT7 caused a general reduction in gene expression and particularly affected the development of mesenchymal tissues. Histone 3 lysine 14 (H3K14) acetylation was specifically reduced by >90 %. However, mouse embryo fibroblasts lacking Myst2 were able to replicate DNA and proliferate normally (Kueh et al. 2011).

7.2.4.4 KAT1

KAT1 (HAT1) clusters with KAT7/HBO1 (Fig. 7.2) and so is mentioned here, but it differs from the rest of the KATs in this chapter in that it is a Type B histone acetyltransferase. KAT1 preferentially interacts with free histones and carries out the diacetylation of newly synthesized H4 on lysines 5 and 12. These marks are thought to be permissive for incorporation of H4 into chromatin and are subsequently removed. Deletion of KAT1 in mouse lines has a phenotype reminiscent of mutations in members of the MYST family, with craniofacial abnormalities and failure of pulmonary development, and enhanced sensitivity to DNA-damaging agents in mouse embryo fibroblasts lacking KAT1 (Nagarajan et al. 2013). No mutations have yet been identified in human disease.

7.2.4.5 KAT8

Originally identified as a homologue of the Drosophila protein Males absent On First (MOF), KAT8 (Neal et al. 2000) is ubiquitously expressed in all tissues and appears to be critically important to basic processes promoting cell proliferation and viability. It has strong specificity for acetylation of H4 lysine 16, which is required for cell cycle progression, and appears to be the primary H4K16 acetyltransferase in mammalian cells. Consistent with the role in most fundamental cell functions, homozygous loss of Kat8 leads to early embryonic lethality in mice, after the maternal contribution of the protein has been depleted and before gastrulation (Gupta et al. 2008; Thomas et al. 2008). KAT8 interacts with both p53 and ATM and is required for multiple ATM-dependent events in the context of DNA damage. Cells deficient in KAT8 are defective for DNA repair in response to ionizing radiation (Gupta et al. 2005).

7.2.4.6 KAT9

KAT9, or ELP3, is a poorly studied human homologue of the yeast and Drosophila elongator complex protein Elp3, where it is involved in protein translation. It may have a more specialized role in motor neuron branching, and single nucleotide polymorphisms in ELP3 have been associated with amyotrophic lateral sclerosis (Simpson et al. 2009). In mice, KAT9 was found to be required for paternal zygote demethylation during meiosis, but this activity was independent of its acetyltransferase domain (Okada et al. 2010).

7.2.5 KAT12

KAT12 (TFIIIC90) is the largest subunit of a general transcriptional coactivator complex required for RNA Polymerase III transcription (Hsieh et al. 1999). Although similar complexes are present in multiple organisms, no specific homologue to this protein has been found in yeast or Drosophila. Its functions are unknown. Unsupervised hierarchical clustering based on sequence homology suggests that its closest relative in the KAT superfamily is TIP60 (KAT5, Fig. 7.2), although the three-dimensional structure of the protein has not been established.

7.2.6 The KAT13: Nuclear Receptor Coactivator Family

Nuclear receptor-dependent hormone transcription relies on one of the three closely related 160 kd proteins, the steroid receptor coactivator proteins NCOA1, NCOA3, and NCOA2 (KATs 13A, 13B, and 13C). In the absence of these factors, basal transcription of steroid hormone-dependent genes is unaffected, but the hormone fails to induce them. Like the KAT3s, they are present in limiting amounts in the cell. The three NCOA proteins have overall ~40 % sequence homology and share a number of structural features: N-terminal basic helix-loop-helix and Per-Arnt-Sim (PAS) domains that are involved in heterodimerization with other bHLH–PAS

proteins and binding to DNA, three nuclear receptor interaction motifs in the center, and at the C-terminus, two activation domains and a HAT domain (Fondell et al. 1996; Yuan et al. 1998). The C-terminal activation domains are required to recruit KAT2 and KAT3 acetyltransferases and arginine methyltransferases, respectively, to the promoters of steroid hormone-regulated genes ((Chen et al. 1997), reviewed in (Li and Shang 2007)). All three are subject to alternative splicing, which generates some transcriptional specificity to individual isoforms. Although best characterized as nuclear receptor coactivators, the p160 acetyltransferases are known to coactivate transcription through a number of other transcription factors, including NFkB, STAT3, ETS, E2F, and AP1.

The relative cellular abundance and availability of the NCOA proteins are primary determinants of their impact on the final transcriptional output. NCOAs are nucleocytoplasmic shuttling proteins; their location is regulated by phosphorylation and by association with steroid receptors (Amazit et al. 2007). In the absence of ligand, steroid receptor-dependent promoters are silenced by a repressor complex (Fondell et al. 1996), and NCOAs are retained in the cytosol through interaction with an ankyrin repeat-containing protein, SIP. In the presence of ligand, NCOA interacts with cytosolic steroid receptor, and SIP undergoes phosphorylation by casein kinase II, which allows NCOA to move together with its partner from the cytosol to the nucleus (Zhang et al. 2007). An NCOA-containing activator complex then replaces the repressor complex on the target promoter.

7.2.6.1 NCOA1 (KAT13A)

NCOA1, also called SRC-1, acts as a limiting coactivator for multiple steroid hormone receptors, including progesterone, estrogen, thyroid hormone, glucocorticoid, and retinoid X receptors (Onate et al. 1995). NCOA1 undergoes alternative splicing and is expressed as three different transcript variants of 5.5 and 7.5 kb in multiple tissue types. These in turn produce three different protein products, one of which has some degree of selectivity for thyroid hormone receptor coactivation (Hayashi et al. 1997). NCOA1 has been found to coactivate a small number of non-steroid hormone transcription factors, including E2A and MEF2, suggesting a wider range of gene targets than previously thought (Li and Shang 2007). In three different studies, the phenotype of NCOA1 knockout mice was remarkably benign; they are viable and fertile, but have smaller ovaries, testes, and other sex-hormone-response tissues, including trabecular bone (Xu et al. 1998; Yamada et al. 2004; Qi et al. 1999). Higher levels of NCOA2 in these mice suggest that there is significant dosage compensation between the two proteins (Xu et al. 1998).

7.2.6.2 NCOA2 (KAT13C)

This protein was first identified as a 160-kd protein interacting in a ligand-dependent manner with the retinoic acid and estrogen receptors (TIF2, Voegel et al. 1996) and again in a screen for glucocorticoid receptor coactivators (GRIP-1); it was subsequently found to activate a broad range of steroid and nuclear receptors (Hong et al.

1997). NCOA2 may have a specialized role in skeletal muscle differentiation. Chen et al. reported that it is the most abundant of the p160 proteins in skeletal myoblasts, it rises during differentiation, and it interacts directly with myogenic bHLH proteins through its bHLH domain and with MEF2C through its C-terminal activation domain (Chen et al. 2000). Functionally, NCO2 was required both for MEF2-dependent transcription and myogenic differentiation in culture.

Although viable, mice deficient in NCOA2 were hypofertile and exhibited defects in spermatogenesis and aspects of placental development (Qi et al. 1999). Careful metabolic analysis of these mice also revealed a defect in hepatic glycogenolysis and gluconeogenesis due to reduced expression of the rate-limiting enzyme glucose-6-phosphatase, leading to features of a glycogen storage disorder (Chopra et al. 2008).

7.2.6.3 NCOA3 (KAT13B)

NCOA3, also referred to as SRC3, AIB1, TRAM-1, ACTR, and p/CIP, was independently cloned by three groups as a novel steroid receptor-interacting protein and as one of several genes on the long arm of chromosome 20 that are overexpressed or amplified in human breast cancer (Chen et al. 1997; Anzick et al. 1997; Takeshita et al. 1997). Although it is expressed widely, its tissue distribution is more restricted than the other NCOA family members; it is abundant in skeletal muscle, placenta, and heart, but scarce in brain, liver, lung, and kidney (Chen et al. 1997). A splice variant of NCOA3 that lacks a portion of the N-terminal BHLH domain is highly expressed in breast tumors and exhibits enhanced transcriptional activation in response to both E2 and epidermal growth factor (Reiter et al. 2001). Overexpression of NCOA3 is oncogenic (reviewed in Lydon and O'Malley 2011).

In common with other transcription coactivators, NCOA3 is largely regulated at the posttranslational level, with transcription mechanisms being less important. Work from O'Malley's laboratory (Li et al. 2008) showed that the protein phosphatases PDXP, PP1, and PP2A inhibit SRC-3 transcriptional regulation by dephosphorylating it and inhibiting its association with estrogen receptor in the presence of estrogen. At the same time, dephosphorylation of serine residues 101 and 102 by protein phosphatase-1 (PP1) blocks SRC3 proteasomal degradation. Thus, while dephospho-NCOA3 protein is more stable, it is less catalytically active. Phosphorylation-coupled ubiquitination allows for precise control of the activation and termination of SRC activity in a transcription factor-specific manner (Wu et al. 2007).

In mice in vivo, NCOA3 is required for normal growth, female reproductive function, and mammary gland development. All of these are diminished but not eliminated in NCOA3 knockout mice, indicating that there is limited redundancy for estrogen and growth hormone receptor signaling through the p160 proteins (Xu et al. 2000b).

NCOA3 has been linked to a number of specialized functions beyond its canonical roles in endocrine tissues. NCOA3 is a significant regulator of white adipocyte development and adipocyte differentiation (Louet et al. 2006). Mice with homozygous loss of NCOA3 are lean and deficient in the adipogenesis regulator PPAR-γ2, while mice with knock-in of an NCOA3 allele with four mutated phosphorylation

sites are fat and insulin-resistant (York et al. 2010; Louet et al. 2006), phenocopying IGF1BP transgenic mice. Mice doubly deficient in NCOA1 and NCOA3 ate more, but were resistant to obesity both on regular chow and a high fat diet. This was attributable both to increased metabolic rate and increased physical activity (Wang et al. 2006).

Vascular function is strongly influenced by estrogen, and NCOA3 may also help to mediate the protective effects of estrogen in various forms of vascular disease. In vascular smooth muscle cells, NCOA3 is a coactivator for the critical transcription factor myocardin, which is required for converting these cells from a proliferative to a quiescent, contractile state after wound healing (Li et al. 2007). In endothelial cells, NCOA3 promotes migration, proliferation, and tube formation by mediating the induction of VEGFR2 by ERK3 (Wang et al. 2014).

7.2.6.4 KAT13D/CLOCK

CLOCK, the last of the KATs on this list, is a central organizer of circadian rhythms in mammals. CLOCK was originally identified as an autosomal dominant gene controlling the length of the activity cycle in mice, following an ENU mutagenesis screen (Vitaterna et al. 1994). Two laboratories isolated the gene by positional cloning and genetic complementation of the phenotype using bacterial artificial chromosomes (King et al. 1997; Antoch et al. 1997). In humans, CLOCK encodes two major RNA transcripts of 8 and 10 kb, which are expressed at high levels in the suprachiasmatic nuclei, the part of the brain that controls light–dark cycles (Steeves et al. 1999). CLOCK has high sequence homology with NCOA3 and shares important features with the NCOA proteins, including a bHLH domain, two PAS domains, a nuclear receptor interaction domain, and a C-terminal transcriptional activation domain (Fig. 7.3) (Steeves et al. 1999). In addition, it has a HAT domain similar to those found in the MYST proteins and similar enzymatic specificity for H3 and H4 (Doi et al. 2006). Analogous with the NCOA proteins, CLOCK is regulated by phosphorylation and interaction with proteins that control its cytoplasmic-nuclear shuttling (Kondratov et al. 2003). In addition, similar to the NCOA proteins, CLOCK recruits KAT2 and KAT3 proteins to activate transcription (Curtis et al. 2004).

The circadian cycle is established by a positive, transcriptional loop and a negative, posttranslationally controlled loop. In the first loop, CLOCK heterodimerizes with the bHLH protein BMAL1 and, together with CBP/p300, binds to E box elements in the promoters of circadian regulator genes. These include the period genes (PER1, PER2, PER3), which drive the positive half of the cycle, the Cryptochrome genes (Cry1, Cry2), and the recently described CHRON (Anafi et al. 2014). Both Cry and CHRONO inhibit the CLOCK-BMAL1 heterodimer by competing with KAT3 for binding to the C-terminal activation domain (Kondratov et al. 2003; Curtis et al. 2004; Xu et al. 2015). CBP and/or p300 are required in both positive and negative halves of the cycle: acetylation of BMAL1 by CLOCK is required to allow Cry to bind and the cycle to complete (Hirayama et al. 2007).

The amplitude, phase, and periodicity of CLOCK are further tuned by cyclic AMP signaling (O'Neill et al. 2008) and by association with other factors (Gustafson

and Partch 2015), including an RNA binding protein, CIRP, which potentiates its levels and oscillatory cycle (Morf et al. 2012).

CLOCK regulates many aspects of physiology that show diurnal variation. Pancreatic islet cell secretion of insulin, for example, cycles to match periods of food intake and is under circadian regulation by CLOCK (Marcheva et al. 2010). Mice with targeted disruption of CLOCK develop diabetes mellitus (Marcheva et al. 2010). Evidence is emerging that nuclear hormone signaling is circadian and that CLOCK is regulated in turn by nuclear hormones (reviewed in (Zhao et al. 2014)).

Many tissues and cultured cells exhibit circadian variation in gene expression, and the "peripheral clocks" controlling these cycles are subject to entrainment by the central CLOCK. McNamara et al. provided evidence for a vascular CLOCK, regulated by CLOCK and a CLOCK homolog, MOP4, which could potentially explain the known periodicity of blood pressure as well as the diurnal cycles seen with heart attack and stroke (McNamara et al. 2001). Consistent with this, the gene encoding Kcnh2, the major subunit of the rapidly activating delayed-rectifier channel (IKr), is subject to robust diurnal variation. Targeted deletion of CLOCK partner Bmal1 from mouse hearts disrupted this pattern, impaired IKr current, and prolonged the QT interval during the resting phase of the diurnal cycle (Schroder et al. 2015). Metabolic abnormalities affecting heart function have also been reported with Bmal1 deletion (Young et al. 2014). These and other recent insights into the circadian nature of cardiac biology and function suggest that the KAT-dependent CLOCK could have a significant influence on human cardiovascular health (Richards et al. 2014; Podobed et al. 2014).

Conclusions

After 50 years of study, the KATs are at last recognized as critical and potentially approachable therapeutic targets in their own right, as well as links to other key pathways. As we continue to unravel the importance of specific acetyltransferases and acetylation substrates in cardiovascular disease, entirely new avenues for drug development are likely to emerge. The reader is encouraged to explore a number of excellent recent articles and reviews that have covered the biochemical properties of the KATs and their potential as drug targets (Dancy and Cole 2015; Henry et al. 2015; Uttarkar et al. 2015; Gee et al. 2015; Milite et al. 2015; Gajer et al. 2015; Wang et al. 2015; Castellano et al. 2015; Oike et al. 2014; Mai et al. 2009).

The KATs have evolved considerably and recently; thus, it will be particularly important to characterize the functions of these enzymes in our own species. One important approach to this task is through the study of human genetic disorders. Increasingly, successful drug development is being guided by observations from human genetics (Roden 2015; Evans and Davey Smith 2015; Wadhawan et al. 2015). Unfortunately, most, if not all, of the KAT genes reviewed in this chapter are involved in cell functions critical to live birth and survival thereafter. The lethality of KAT loss means that, to date, reported mutations in KAT genes are rare. In addition, almost all cases have been sporadic, which, until recently, limited identification of the responsible genes. Fortunately, the use of next-generation

sequencing technology now brings unprecedented power to identify rare survivable mutations that occur de novo and/or have mosaic distribution. Recently, using next-generation sequencing, Zaidi et al. showed that de novo mutations accounted for about 10 % of severe congenital heart disease cases, and these mutations disproportionately affected genes encoding writers, readers, and erasers of epigenetic marks (2013). Another example mentioned earlier in this chapter (Tham et al. 2015; Arboleda Valerie et al. 2015) shows that NGS can also help to identify rare syndromes involving the KATs. It is exciting to speculate that new KAT family members and their cardiovascular functions will be discovered by wider application of clinical exome sequencing. The use of human inducible progenitor stem cells holds further potential to elucidate functions and disease mechanisms involving these powerful and vital enzymes.

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HDAC Signaling Networks in Heart Failure

8

Mariya Kronlage, Hugo A. Katus, and Johannes Backs

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Abstract

Histone modifications are mediated by multiple chromatin-modifying enzymes such as histone methyltransferases and demethylases or histone acetyltransferases and histone deacetylases (HDACs). Because of their involvement in heart muscle disease, HDACs have gained a lot of attention over the last 15 years. HDACs are divided into different classes. Among them, class IIa HDACs undergo many posttranslational modification and bind to signaling molecules and other transcriptional regulators. Thereby, class IIa HDACs play a central role in cardiac signaling networks. Understanding the specific signaling events may allow the development of pharmacological approaches other than inhibiting their enzymatic activity. Thus, targeting HDAC signaling may provide more specificity towards disease- and stress-related mechanisms, potentially leading to fewer side effects than global inhibition of HDACs.

8.1 Introduction

Heart failure (HF) is a chronic progressive disease with unfavorable long-term survival that could be compared with the grim prognosis of many aggressive cancer diagnoses: 5-year mortality rate following the first admission for HF is over 40 %, and costs of re-hospitalization rates are steadily increasing (McMurray et al. 1998; Mosterd and Hoes 2007; Murray-Thomas and Cowie 2003). HF is not only the leading cause of death in the developed countries but also a major public health burden worldwide with far-reaching economic and political consequences (Cook et al. 2014; McMurray et al. 1998). Modern treatment of HF already covers a broad spectrum of known maladaptive remodeling mechanisms in the heart, involving neurohumoral and sympathetic inhibitors (angiotensin and β-adrenergic receptor antagonists) (Hunt et al. 2005). Ongoing therapeutic efforts are directed towards signaling factors downstream of receptors, as this would have the advantage of increasing effectiveness and minimizing the risk of adverse drug reactions (McKinsey 2012).

In the search to identify such targets, a substantial amount of evidence has been gathered in support of the importance of histone acetyltransferases and histone deacetylases (HDACs) in the physiology and pathophysiology of the heart. HDACs represent a family of enzymes that catalyze the removal of acetyl groups from lysine residues in a variety of proteins (such as histones), thus leading to chromatin condensation, which impairs binding of certain transcriptional factors (TFs) to their specific targets on the DNA (Backs and Olson 2006; Chen et al. 2015; Haberland et al. 2009; McKinsey and Olson 2004). HDACs can control gene expression through their ability to deacetylate nucleosomal histones, change chromatin, and in effect repress gene expression (Wang et al. 2009).

Apart from this, HDAC downstream effects extend far beyond their histone substrates and encompass a huge group of non-histone targets that regulate remodeling, contractility, autophagy, or metabolism (Gut and Verdin 2013; Karpac and Jasper 2011; Mihaylova et al. 2011; Xie and Hill 2013). In the heart, HDACs function as

important links between upstream neurohumoral, adrenergic, and metabolic signaling pathways and downstream gene targets (Lehmann et al. 2014b; McKinsey 2011a; Weeks and Avkiran 2015). Thus, HDACs are critically involved in the differential response of the cardiomyocyte to different physiological and pathological environmental signals.

8.2 Classification of HDACs and Basic Principles

A total of 18 mammalian HDACs are distributed into four classes, based on structural and functional characteristics (Backs and Olson 2006; Landry et al. 2000). Class I, II, and IV HDACs are zinc-dependent enzymes, whereas class III HDACs (sirtuins) are nicotinamide adenine dinucleotide (NAD)-dependent enzymes (Gut and Verdin 2013; Landry et al. 2000). HDACs in general catalyze the removal of acetyl groups from the ε-amino group of acetyl lysine residues within their targets (Gong and Miller 2013; Landry et al. 2000). Deacetylation of histones in nucleosomes induces chromatin condensation, which represses transcription by preventing binding of transcription factors (TFs) to gene promoter and enhancer regions. In contrast, acetylation of histones by histone acetyltransferases (HATs) induces chromatin relaxation, resulting in increased gene transcription (Gong and Miller 2013; Wang et al. 2009; Yang and Seto 2007). Thus, HDACs and HATs are involved in an intense cross talk and serve as antagonistic epigenetic regulators of gene expression. Class IIa HDACs have gained a lot of attention in the field of cardiovascular health and disease. The class IIa family of HDACs consists of HDAC4, HDAC5, HDAC7, and HDAC9. The latter is also expressed as the splicing variant (myocyte enhancer factor-2 (MEF2)-interacting transcriptional repressor (MITR)) that lacks the actual deacetalyse domain. Expression of HDAC4, HDAC5, and HDAC9 is the highest in heart, brain, and skeletal muscles (Grozinger et al. 1999; Kao et al. 2000; Lehmann et al. 2014b), whereas HDAC7 is abundant in endothelial cells within the heart, lung, and skeletal muscles (Chang et al. 2006; Kao et al. 2000). In the human heart, HDAC5 is thought to be the member of class IIa HDACs with the strongest expression (de Ruijter et al. 2003) (Table 8.1).

Class IIa HDACs consist of an N-terminal regulatory domain and a C-terminal domain with a predicted enzymatic function (Lehmann et al. 2014b; McKinsey et al. 2001; Montgomery et al. 2007; Weeks and Avkiran 2015). The N-terminal regulatory domain of these HDACs plays important roles in the recruitment of various cofactors and contains a binding domain for the TF MEF2, a nuclear localization signal (NLS), and several conserved serine residues that act as docking sites for 14-3-3 chaperone proteins when phosphorylated by different kinases. Further structural features that mediate signal responsiveness are discussed below. Figure 8.1 summarizes structural domains that mediate (a) signal responsiveness, (b) their localization to specific chromatin regions through binding to TFs, and (c) the recruitment of other chromatin-modifying enzymes, which ultimately mediate transcriptional repression. Work by the Verdin group and others described that class IIa HDACs possess only low deacetylase activity but that the recruitment of class I

Table 8.1 Summary of the expression patterns of different members of the class IIa HDACs in a variety of tissues. This table also provides a short description of the reported phenotypes of HDAC-deficient mice

Member	Localization	Expressed in	KO Phenotype	Embryonic/ early lethality	References
HDAC4	Nucleus/ cytoplasm	Heart, skeletal muscle, brain	Premature ossification	YES	Hohl et al. 2013 Vega et al. 2004b
HDAC5	Nucleus/ cytoplasm	Heart, skeletal muscle, brain	Cardiac defects	NO	Chang et al. 2005
HDAC7	Nucleus/ cytoplasm/ Mitochondria	Heart, skeletal muscle, pancreas, placenta	Defects in endothelium	Conditional: NO Global: YES	Chang et al. 2006
HDAC9	Nucleus/ cytoplasm	Brain, skeletal muscle	Cardiac defects	NO	Chang et al. 2004

HDACs ultimately mediates HDAC activity (Fischle et al. 2002; Lahm et al. 2007; Lehmann et al. 2014b). Similarly, class IIa HDACs can recruit other types of epigenetic enzymes such as histone methyltransferases that in turn may mediate transcriptional repression by adding methyl groups to chromatin regions where class IIa HDACs localize due to their binding of certain TFs such as MEF2 (Hohl et al. 2013; Zhang et al. 2002b). Class IIa HDACs antagonize adverse cardiac remodeling in the nucleus by suppressing TFs, such as the MEF2 family (Lu et al. 2000; Zhang et al. 2000). MEF2 is not the only TF that HDACs interact with; it has also been shown that the N-terminal domain of class IIa HDACs also represses the activity of serum response factor (SRF), nuclear factor of activated T-cells (NFAT), calmodulin-binding transcriptional activators (CAMTA), and GATA TFs (Backs et al. 2011; Dai et al. 2005; Davis et al. 2003; Lehmann et al. 2014b; Song et al. 2006). Association with 14-3-3 chaperone proteins upon phosphorylation leads to cytosolic accumulation of HDACs (McKinsey et al. 2001; Vega et al. 2004a; Weeks and Avkiran 2015), resulting in adverse cardiac remodeling by disinhibition of TFs and consequent increased expression of downstream target genes. Thus, nucleo-cytoplasmic shuttling has been demonstrated to be a key mechanism-regulating class IIa HDAC function (Backs et al. 2006; Brehm et al. 1999; Lu et al. 2000; McKinsey 2007; Vega et al. 2004a).

8.3 Class IIa HDACs and Adverse Cardiac Remodeling

The role of class IIa HDACs in maintaining cardiac homeostasis has been intensively investigated in the past 15 years in a number of different experimental models. Importantly, global deficiency of either HDAC5 or HDAC9 was compatible with life. However, global HDAC5 and HDAC9-knockout (KO) mice were characterized by abnormal cardiac growth (Chang et al. 2004; Zhang et al. 2002a). The simultaneous deletion of HDAC5 and HDAC9 was not compatible with life since these mice suffered from associated growth retardation, ventricular defects, and

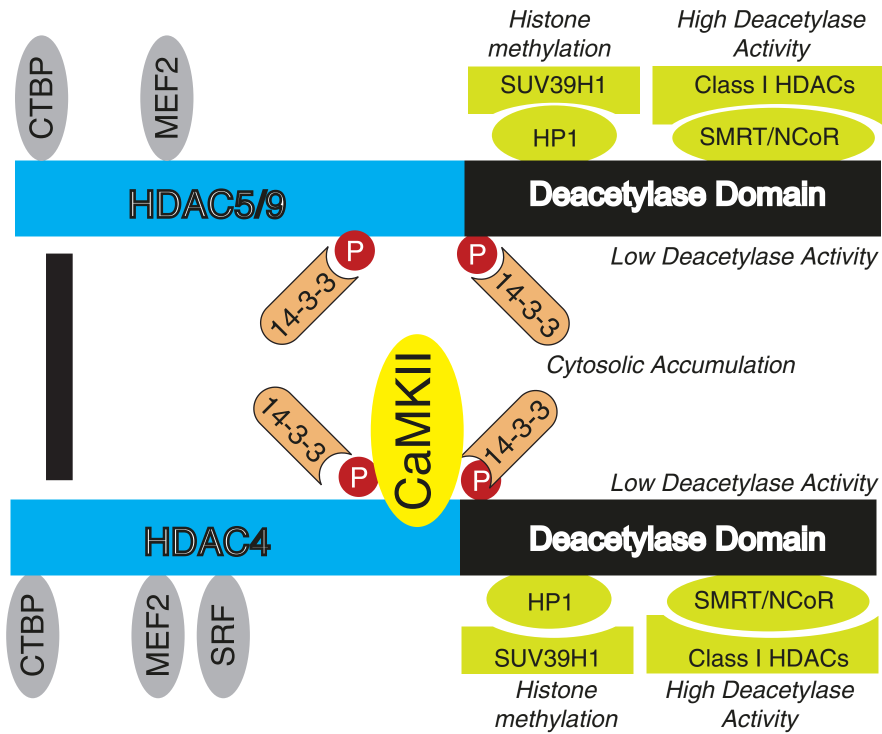

Fig. 8.1 A diagram highlighting the interactions of class IIa HDACs with (**a**) the transcriptional regulators C-terminal binding protein (CTBP), myocyte enhancer factor 2 (MEF2) and the serum response factor (SRF) that direct these HDACs to specific genomic regions, with (**b**) other chromatin-modifying enzymes such as class I HDACs that contain high deacetylase activity (in contrast to low deacetylase activity of HDAC4, HDAC5 and HDAC9) through binding to SMRT/NCoR, and the histone methyltransferase SUV39H1 through binding to heterochromatin protein 1 (HP1) that mediate histone deacetylation or methylation, and with (**c**) CaMKII that binds to HDAC4 but not HDAC5 or HDAC9. (**d**) Oligomerization between HDAC4 and HDAC5/HDAC9 (as indicated by the black bar) confers CaMKII responsiveness from HDAC4 to HDAC5 and HDAC9. (**e**) Phosphorylation by CaMKII creates then 14-3-3 binding sites on HDAC4 and HDAC5/9 resulting in 14-3-3 mediated cytosolic accumulation

bleeding, resulting eventually in premature death (Chang et al. 2004). A limitation of these studies regarding cardiomyocyte autonomous effects was, however, that gene deletion occurred globally, implying that the observed phenotype could also be influenced by the deletion of HDAC5 and/or HDAC9 in non-myocytes. Exaggerated cardiac hypertrophy could be observed upon crossing HDAC9 KO with a transgenic model with constitutively activated calcineurin (Molkentin et al. 1998; Zhang et al. 2002a). However, recent evidence suggested that calcineurin signaling might also mediate adaptive signaling as opposed to class IIa HDAC inactivation (Chung et al. 2013; Heineke et al. 2010; Kreusser et al. 2014). Mice lacking HDAC4 globally die before birth due to chondrocyte hypertrophy, leading to premature ossification of developing bones, which excluded HDAC4 until very recently to be investigated

more specifically regarding its role for the adult heart (Vega et al. 2004b). In order to better understand the specific role of HDAC4 in the heart, mice with cardiomyocyte-specific deletion of HDAC4 have been generated in the Olson lab and its role has been studied so far in ex vivo models, showing that HDAC4 might act as a nexus between calcium-dependent signaling and histone methylation upon elevated cardiac load (Hohl et al. 2013). However, the effects of stress on HDAC4-deficient mice in vivo have not been described yet. Because HDAC4 was implied to respond specifically to β-adrenergic signaling (Backs et al. 2006; Backs et al. 2011; Lehmann et al. 2014b; McKinsey 2007; Weeks and Avkiran 2015), an important stress pathway in the heart that drives the progression of HF, it will be interesting in the future to investigate whether HDAC4 exerts a distinct function in the heart as compared with HDAC5 and HDAC9. Mice with global deletion of HDAC7 die also during early embryogenetic development due to cardiovascular defects (Chang et al. 2006). Conditional deletion of HDAC7 endothelial cells showed a similar phenotype to the global deletion one (Chang et al. 2006). An essential role of HDAC7 in the heart has not been described so far, most likely because of its low expression in cardiac myocytes. Thus, although many genetic mouse models were generated and characterzized, it is still somewhat unclear, which HDAC in what cell type is responsible for cardiac hypertrophy and cardiac dysfunction. It will also be interesting to investigate whether there are distinct functions of the different class IIa HDACs regarding cardiac growth and function and, in particular, to identify critical downstream gene programs and cellular processes that cause hypertrophy and dysfunction.

8.4 Class IIa HDACs are Signal Responsive Through Posttranslational Modifications (PTMs)

PTMs may change the structure, function, as well as intracellular compartmentalization of affected proteins. The following sections describe prominent PTMs of class IIa HDACs because they provide an extraordinary level of changing and fine-tuning HDAC functions.

8.4.1 Phosphorylation

Class IIa HDACs are substrates of a number of different protein kinases including protein kinase D (PKD); Ca^{2+}/calmodulin-dependent protein kinase I, II, and IV (CaMKI, CaMKII, and CaMKIV); cAMP-dependent protein kinase (PKA); microtubule affinity-regulating kinase; salt-induced kinase 1; and G-protein-coupled receptor kinase-5 (Berdeaux et al. 2007; Chang et al. 2013; Chang et al. 2005; Dequiedt et al. 2006; Ha et al. 2010; Martini et al. 2008; Passier et al. 2000; Vega et al. 2004a; Walkinshaw et al. 2013). Phosphorylation of class IIa HDACs has been intensely studied and has first been identified as a mechanism that affects nucleocytoplasmatic shuttling of class IIa HDACs. Prominent phosphorylation sites of HDAC5 are, for instance, Ser-259 and Ser-498, which are localized close to the

NLS, and Ser-279, which lies within the NLS. Phosphorylation at Ser-259 and Ser-498 of HDAC5 and of the corresponding serine residues of the other class IIa HDACs profoundly affects their subcellular localization. In particular, phosphorylation of class IIa HDACs by e.g., CaMKI, CaMKIV, and PKD induces nuclear export of HDAC5 by creating binding sites for the chaperone 14-3-3 that escorts class IIa HDACs via a CRM1-dependent nuclear export to the cytosol (Harrison et al. 2004; Lehmann et al. 2014b; McKinsey et al. 2000a; McKinsey et al. 2000b; Passier et al. 2000; Vega et al. 2004a). In contrast, phosphorylation of Ser-279 by PKA has been suggested to trigger nuclear retention by disrupting the binding of 14-3-3 proteins (Chang et al. 2013; Greco et al. 2011; Ha et al. 2010).

Of note, whereas PKD, CaMKI, or CaMKIV phosphorylate all members of the class IIa HDAC family, CaMKII selectively binds to HDAC4 (Backs et al. 2006; Little et al. 2007). This is an important observation because CaMKII is implied by multiple studies to play a critical role in the development of HF (Kreusser and Backs 2014; Westenbrink et al. 2013). This selectivity is mediated through a specific CaMKII docking site in HDAC4 that is missing in HDAC5, 7, or 9 (Backs et al. 2006). But importantly, it was also shown—as illustrated in Fig. 8.1—that formation of a protein complex by oligomerization between HDAC4 and HDAC5 allows CaMKII to phosphorylate HDAC5 and thereby also facilitate cytosolic accumulation of HDAC5 (Backs et al. 2008; Metrich et al. 2010). Oligomerization of class IIa HDACs represents therefore a point of convergence and a mechanism for coupling specific upstream signaling molecules with downstream transcriptional targets. In an attempt to better understand the mechanistic implications of the CaMKII–HDAC4 interaction, different in vivo models have been developed and established so far. Transgenic mice with cardiac-specific overexpression of CaMKIIδ—the predominant CaMKII isoform in the heart—show adverse cardiac remodeling, elevated MEF2 activity, and increased expression of genes that are normally only expressed in the fetal stage (so-called "fetal gene program"). These findings were associated with increased phosphorylation and inactivation of HDAC4 (Zhang et al. 2007). CaMKIIδ-deficient mice show no obvious abnormalities under unstressed conditions but less adverse cardiac remodeling in response to pathological pressure overload and lower levels of phosphorylated HDAC4 (Backs et al. 2009; Ling et al. 2009). However, the single deletion of CaMKIIδ alone led to compensatory activation of the other cardiac CaMKII isoform, namely CaMKIIγ. In an attempt to completely abolish CaMKII activity in the heart, mice with a cardiac-specific simultaneous deletion of CaMKIIδ and CaMKIIγ have been generated (Kreusser et al. 2014; Weinreuter et al. 2014), lacking the two major cardiac CaMKII genes δ and γ specifically only in cardiac myocytes. Under unstressed conditions, these mutant mice develop normally and display neither abnormal Ca^{2+} handling nor functional and structural abnormalities. However, in response to pathological pressure overload and β-adrenergic stimulation, these mice are protected from cardiac dysfunction and interstitial fibrosis and in this model phosphorylation of HDAC4 at the CaMKII phosphorylation and 14-3-3 binding sites (Backs et al. 2006; Little et al. 2007) Ser-632 was decreased, indicating that CaMKII is the endogenous kinase of HDAC4. Interestingly, CaMKII double KO mice did develop cardiac

hypertrophy driven by excessive activation of endogenous calcineurin, which is associated with a lack of phosphorylation at the auto-inhibitory calcineurin A site Ser-411 (Kreusser et al. 2014). These results point into the direction that CaMKII–HDAC4 signaling regulates specifically cardiac function rather than hypertrophic growth. It will be important to analyze the relative contribution of the specific downstream mechanisms that control cardiac function.

In addition to the abovementioned mechanisms, some recent evidence suggests that increased HDAC4 and HDAC5 phosphorylation and consequent MEF2 activation following endothelin-1 stimulation may occur primarily through stimulation of α_1- and β_1-adrenergic receptors on cardiomyocytes, following activation of presynaptic ET_A receptors and subsequent inhibition of noradrenaline reuptake into sympathetic nerve terminals (Lehmann et al. 2014a). This observation suggests that there is a significant cross talk between different neurohumoral stimuli in the regulation of class IIa HDACs and that this cross talk also affects cardiac integrity. This also implies that HDAC signaling builds up a tight network not only within the cardiomyocyte downstream of β-adrenergic receptors but also between cardiomyocytes and the sympathetic nervous system at the neuromuscular junction.

Apart from CaMKII, another major downstream kinase of *β*-adrenergic receptors, namely PKA, is known to interact at least with two members of the class IIa HDAC family. On the one hand, and as mentioned above, PKA was proposed to phosphorylate HDAC5 at Ser-279, blocking 14-3-3 binding and nuclear export (Ha et al. 2010). On the other hand, PKA also inhibits PKD-dependent phosphorylation of HDAC5, thus preventing depression of MEF2 transcription factor (Haworth et al. 2007; Sucharov et al. 2011). However, recently we could also demonstrate that PKA signaling influences specifically the HDAC4 function. In the setting of acute β-adrenergic stimulation, PKA activates a so far unknown serine protease, which in turn cleaves HDAC4 and produces an N-terminal proteolytic cleavage polypeptide (HDAC4-NT) (Backs et al. 2011). The cleavage site, located between amino acids 201 and 202, is highly specific for HDAC4 and not present in other class IIa HDACs. HDAC4-NT accumulates in the nucleus and suppresses MEF2-dependent gene transcription (Backs et al. 2011). This pathway is highlighted in Fig. 8.2 and described in more detail below.

8.4.2 Proteolysis of HDAC4

PKA activation leads to proteolysis of HDAC4 by a serine protease at Tyr201. This site is not present in other members of the class IIa HDAC family (Backs et al. 2011). The resulting N-terminal polypeptide of HDAC4 (HDAC4-NT) is stable whereas the C-terminal polypeptide seems to be unstable. HDAC4-NT enters the nucleus where it inhibits selectively the pro-remodeling transcription factor MEF2 but not the essential transcription factor SRF, because the SRF binding sites extend to a part C-terminal of Tyr-201. Thereby, HDAC4-NT may counteract adverse cardiac remodeling. Indeed, in vivo experiments showed that HDAC4-NT could be found in hearts of wild-type mice upon ß-adrenergic stimulation, but not in hearts of

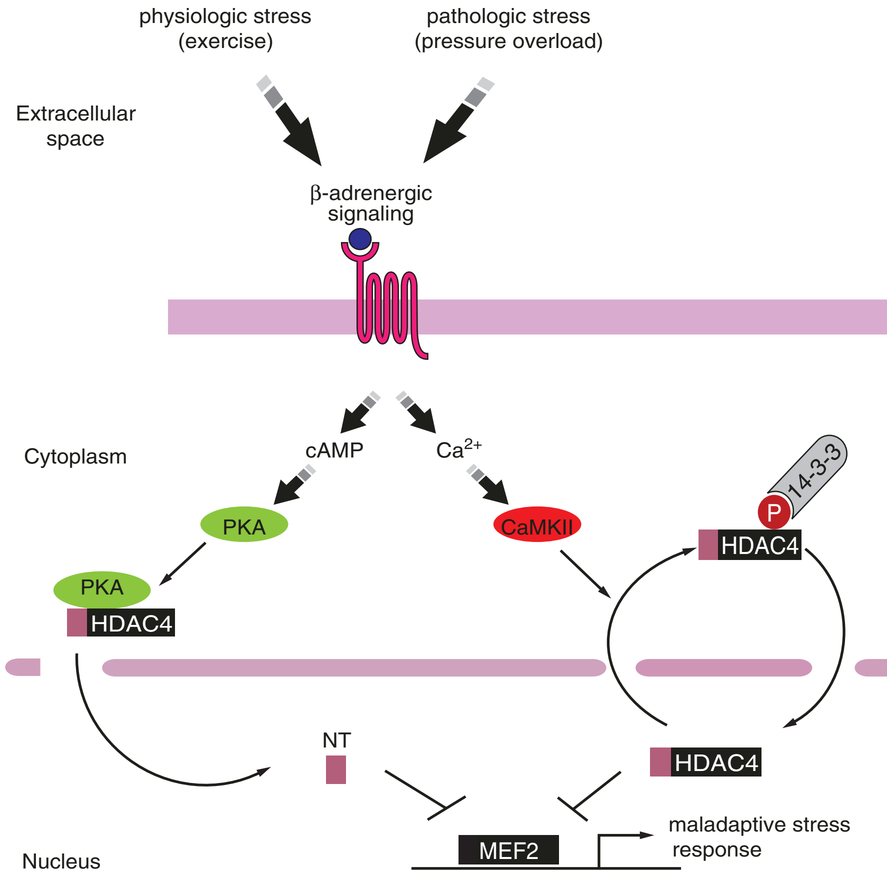

Fig. 8.2 Working model highlighting two opposing signaling cascades downstream of β-adrenergic receptors. Short-term β-adrenergic stimulation as it occurs under physiologic conditions such as exercise training preferentially activates PKA. PKA in turn binds to HDAC4 and leads to recruitment of a protease that cleaves off an N-terminal fragment of HDAC4. HDAC4-NT inhibits the transcription factor MEF2 in a CaMKII-resistant manner because it does not contain the CaMKII binding site or phosphorylation sites. Sustained β-adrenergic stimulation leads to CaMKII activation as it occurs under pathologic conditions such as pressure overload. CaMKII binds and then phosphorylates full-length HDAC4 leading to 14-3-3 binding, which in turn triggers cytosolic accumulation and consequently MEF2 activation, which is left uninhibited in the nucleus

mice lacking the catalytic α-subunit of PKA (Backs et al. 2011). Based on this model, it has been suggested that this mechanism of proteolytic cleavage may enable differential responses to hypertrophic stimuli depending on the type of stress. It would be also intriguing to explore whether and to what extent endogenous HDAC4-NT is expressed in the heart under normal conditions and how this expression changes in situations of acute and chronic stress. Moreover, HDAC4-NT lacks the CaMKII-dependent phosphorylation sites of HDAC4 (Ser-246 and Ser-467) and thus would resist from being inactivated due to CaMKII activation in pathological

conditions such as HF. Thus, a gain of function approach mimicking HDAC4-NT might be a promising approach to combat HF.

Moreover, it has also been reported that HDAC4 can be proteolytically processed by caspase-2 and caspase-3 at Asp-289 (Paroni et al. 2004). The resulting N-terminal fragment was suggested to induce apoptosis via the repression of SRF and Runt-related transcription factor Runx2 (Paroni et al. 2007; Paroni et al. 2004). Interestingly, the additional amino acids of HDAC4 between 201 and 289 represent a binding site for SRF (Backs et al. 2011). Thereby, proteolysis is a biological process, by which the specificity of HDACs towards different TFs can be modified. Moreover, a most recent report demonstrated that an even shorter N-terminal fragment of HDAC4 (shorter than HDAC4-NT) plays a critical protective role for photoreceptors in a model of retinitis pigmentosa by suppressing cellular death in the eye (Guo et al. 2015). The presence and the function of this proteolytic product in the heart have not been described yet.

8.4.3 Oxidation

Oxidation represents a phosphorylation-independent PTM regulating subcellular localization and nuclear export of HDAC4 in cardiomyocytes (Ago et al. 2008; Haworth et al. 2012; Matsushima et al. 2013). Formation of a disulphide bond between two cysteine residues (Cys-667 and Cys-669) in HDAC4 leads to a conformational change that enhances the exposure of the nuclear export signal to CRM1 (Ago et al. 2008). Of note and other than the abovementioned unique responsiveness of HDAC4 to CaMKII and PKA, the cysteine residues and thus the disulphide bond occur in all class IIa HDACs, indicating that oxidation is rather a general mechanism and not an HDAC4-selective phenomenon. Likewise, under oxidative stress, HDAC5 appears to be regulated in a similar manner to HDAC4, as catecholamine-induced nuclear export of HDAC5 was blocked by overexpression of the disulphide oxidoreductase protein thioredoxin-1 or by treatment with the antioxidant N-acetylcysteine (Haworth et al. 2012).

8.4.4 Sumoylation

Sumoylation is a process in which the small ubiquitine-like modifier SUMO is added to lysine residues. HDAC4 has been shown to be sumoylated at Lys-559 by the SUMO E3 ligase RanBP2, which is attenuated by CaMK signaling and occurs at the nuclear core complex (Kirsh et al. 2002; Weeks and Avkiran 2015). Similar modifications were also found in the case of HDAC9, indicating that class IIa HDACs are generally modified by sumoylation. Most recently, sumoylation of HDAC4 has been studied in isolated cardiac myocytes and has been suggested to be cardioprotective upon hypoxia/reoxygenation injury (Du et al. 2015). These data call for more studies that investigate the functional relevance of HDAC sumoylation in vivo in the adult heart.

8.4.5 Ubiquitination

Ubiquitination of proteins might serve as a signal for proteasome degradation, but might also change the intracellular compartmentalization and affect the proteins. In cardiovascular pathogenesis, it has a profound effect on gene expression (Portbury et al. 2012). Although HDAC ubiquitination has not been intensively studied in the heart, a study on skeletal muscle suggests that it is an important posttranslational modification that regulates class IIa HDAC function, activation of MEF2, and an increased expression of slow-twitch fiber-specific genes (Potthoff et al. 2007). Based on these observations, further studies are warranted to investigate whether ubiquitination could also play a role in altering HDAC function in the heart.

8.4.6 Other PTMs

To the best of our knowledge, other PTMs such as nitrosylation or glycosylation of HDACs and their possible functional implications have not been described yet in the heart. Recently, evidence about HDAC4's critical involvement in metabolic processes such as defective insulin signaling in diabetes in the liver or its involvement in the podocyte injury in diabetic nephropathy (Mihaylova et al. 2011; Ozcan et al. 2016; Wang et al. 2011; Wang et al. 2014) has been growing. This involvement also implies a role of HDACs in the regulation of metabolic processes and makes a further investigation of responsiveness to metabolic stress such as hyperglycemia or nutritional changes via previously-not-investigated PTMs intriguingly. This is in particular interesting because epigenetic processes have been demonstrated to act as a nexus between metabolic factors and gene expression (Gut and Verdin 2013).

8.5 Outlook: Epigenetic Therapies

The HDAC family opens new potentials for development of next-generation therapeutic strategies against cardiac disease as it acts as an important regulator of both maladaptive and adaptive signaling and could contribute to normalizing gene expression in the failing heart (Lehmann et al. 2014b; McKinsey 2011a, 2012). Administration of small molecule HDAC inhibitors, such as trichostatin A (TSA), suberanilohydroxamic acid (SAHA), and valproic acid, has already been tested under experimental conditions and has been implied to play a cardioprotective role. Administration of TSA upon pressure overload attenuated cardiac hypertrophy in mice (Kee et al. 2006; Kong et al. 2006) and SAHA-treatment was associated with a reduced infarct size and functional improvement in a rabbit model of ischemia-reperfusion injury (Xie et al. 2014). Valproic acid was in addition demonstrated to reduce right ventricular hypertrophy upon pulmonary hypertension (Cavasin et al. 2015). Furthermore, HDAC inhibitors have been argued to play an advantageous role in modulating the pro-inflammatory response upon cardiac injury. In spontaneously hypertensive rats, a 20-week treatment with valproic acid led to normalization

of IL-1ß and TNFα-levels, which correlated well with a reduction in the LV-wall thickness and fibrosis (Cardinale et al. 2010). Four weeks' treatment with SAHA led also to a significant reduction in the circulating levels of IL-1ß, IL-6, and TNF-α (McKinsey 2011b). HDAC inhibitors have also profound effects on cardiac fibrosis; for example, TSA blocks transforming growth factor ß-mediated induction of collagen synthesis in ventricular fibroblasts (McKinsey 2011b). Based on these insights in the mechanisms of action and the potential off-target effects of HDAC inhibitors, further studies are warranted to reveal not only the short- and long-term advantages but also the potentials and hazards of HDAC inhibitor treatment. The effects of HDAC inhibitors are also discussed in other chapters of this book. It is important to emphasize that these effects are mainly explained by inhibition of class I HDACs rather than class IIa HDACs because the deacetylase activity of class HDACs is higher than the deacetylase activity of class IIa HDACs (Fischle et al. 2002; Lahm et al. 2007). Moreover, the aforementioned mouse genetic studies suggested that promoting the gain of function of class IIa HDACs in the nucleus is rather beneficial as compared with their inhibition. Following this line, the remarkable signal responsiveness and our gained understanding of specific signaling events that mediate for instance cytosolic accumulation or proteolysis of HDAC4 may open new therapeutic avenues. Some of these signaling events seem to be specific in disease situations. Thus, inhibiting a signaling event may be a more specific approach to inhibit an HDAC action only in this situation, but not generally as enzymatic inhibitors do. For instance, it might be feasible to develop disrupting compounds or peptides that block binding of CaMKII to HDAC4 (Backs et al. 2006). Because HDAC5 regulation by CaMKII depends on HDAC4 (Backs et al. 2008), such an approach would be specific to increase in HDAC4 and HDAC5 activity in the nucleus only in a CaMKII-dependent stress situation. Another feasible approach may be an activation of the HDAC4 proteolysis pathway (Backs et al. 2011) by a gene therapy approach using HDAC4-NT or the upstream protease. A deeper understanding of the regulatory mechanisms and the molecular basis of the regulation of chromatin-modifying enzymes therefore may open new translational avenues.

8.6 Summary

The class IIa HDAC family has been identified as an important molecular nexus linking both acute and chronic stress signaling to activation of different transcriptional programs that affect cardiac growth, hypertrophy, and both pathological and physiological remodeling. The unique role of HDACs in the fine-orchestrated epigenetic regulation of cardiac gene expression originates from nucleo-cytoplasmic shuttling in response to signal-driven (β-adrenergic, neurohumoral) PTMs. Due to their direct interactions with several potent transcriptional regulators, such as MEF2, HDACs represent interesting potential targets for development of novel pharmacological strategies to treat HF. Some of the possible pathways that are currently under discussion comprise direct inhibition of HDACs, prevention of nuclear export and/or enhancing nuclear import, and stimulation of proteolytic cleavage formation.

However, many of the mechanisms of cardioprotective HDAC function are still only insufficiently known and there is a need for future investigations.

Due to the high epidemiologic relevance, HF has turned into a heavy burden not only for patients and their families but also for the health care system in our modern society. Defeating the disease and substantially improving duration and quality of life rather than simply living with HF is hence one of the biggest challenges in modern cardiology. An important prerequisite for the development of new, specific, and effective pharmacological therapies against HF is the profound understanding of the underlying mechanisms and signaling cascades transmitting pathological stress to effector molecules in the cardiomyocyte that ultimately lead to maladaptive remodeling. The HDAC family offers critical mechanisms that directly link to adverse remodeling responses and ultimately HF.

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Conflict of Interest J.B. holds a patent on epigenetic therapies, in particular, disrupting the CaMKII–HDAC4 interaction and gene therapy with HDAC4-NT and the upstream protease.

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9 Epigenetic and Nongenomic Roles for Histone Deacetylases in Heart Failure

Weston W. Blakeslee, Philip D. Tatman, and Timothy A. McKinsey

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Abstract

Heart failure due to systolic and/or diastolic ventricular dysfunction afflicts millions of people worldwide. Most heart failure drugs target signaling cascades that emanate from the cardiomyocyte cell surface. However, given the ability of numerous

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receptors to trigger adverse ventricular remodeling, it has been hypothesized that more efficacious therapeutic strategies would be based on inhibition of downstream "nodal" points that integrate upstream signals to convey a common pathway for heart failure pathogenesis. This chapter highlights data suggesting that a family of epigenetic regulatory enzymes, histone deacetylases (HDACs), represent such nodal points. We discuss recent findings that illustrate how HDACs not only control cardiac gene expression via epigenetic regulation, but also serve crucial non-genomic functions in the heart through deacetylation of non-histone proteins.

9.1 Introduction

Heart failure due to systolic and/or diastolic ventricular dysfunction currently afflicts approximately six million adults in the United States, and this number is projected to rise to eight million by 2030 (Mozaffarian et al. 2015). Research efforts over the past two decades have led to enormous progress in our understanding of the molecular mechanisms that control heart failure (Burchfield et al. 2013). The ultimate goal of this work has been to facilitate development of novel therapies for the treatment of this devastating condition. With the exception of the dual angiotensin receptor/neprilysin inhibitor, LCZ696 (McMurray et al. 2014), innovative new therapies for heart failure have not emerged, and the 5-year mortality rate for individuals with heart failure still approaches 50 % (Mozaffarian et al. 2015).

Most heart failure drugs target signaling cascades that emanate from the cardiomyocyte cell surface (e.g., β-adrenergic and angiotensin receptor blockers) (Xie et al. 2013). However, given the ability of numerous receptors to trigger adverse ventricular remodeling, it has been hypothesized that more efficacious therapeutic strategies would be based on inhibition of downstream "nodal" points that integrate upstream signals to convey a common pathway for heart failure pathogenesis (Haldar and McKinsey 2014). This chapter highlights data suggesting that a family of epigenetic regulatory enzymes, histone deacetylases (HDACs), represent such nodal points. These enzymes are highly amenable to pharmacological manipulation. Two HDAC inhibitors, SAHA (vorinostat) and FK-228 (romidepsin), are approved for use in humans with cutaneous T-cell lymphoma, and the drugs are generally well tolerated, establishing the feasibility of targeting these, and other epigenetic regulators, to treat human disease (West and Johnstone 2014). We describe recent findings that illustrate how HDACs not only control epigenetic processes in the heart but also serve crucial nongenomic functions in the regulation of cardiac contraction/relaxation and intracellular signaling.

9.2 HDACs: Erasers of the Histone Code

The basic unit of chromatin is the nucleosome, which is a histone octamer wrapped in a 147-bp stretch of DNA. Modifications of nucleosomal DNA or protein, without changes to the underlying nucleotide sequence, can have a profound effect on gene

expression. This mode of gene regulation is referred to as epigenetics. One of the most widely studied epigenetic events is histone tail acetylation on ε-amino groups of lysine residues. Acetyl groups are coupled to lysines by histone acetyltransferases (HATs) and removed by HDACs. HATs and HDACs are commonly referred to as "writers" and "erasers" of the epigenetic code, respectively. In addition to influencing transcription by altering the electrostatic properties of histones, lysine acetylation often creates docking sites for proteins such as bromodomain-containing BET proteins, which "read" the histone code and alter the gene expression by

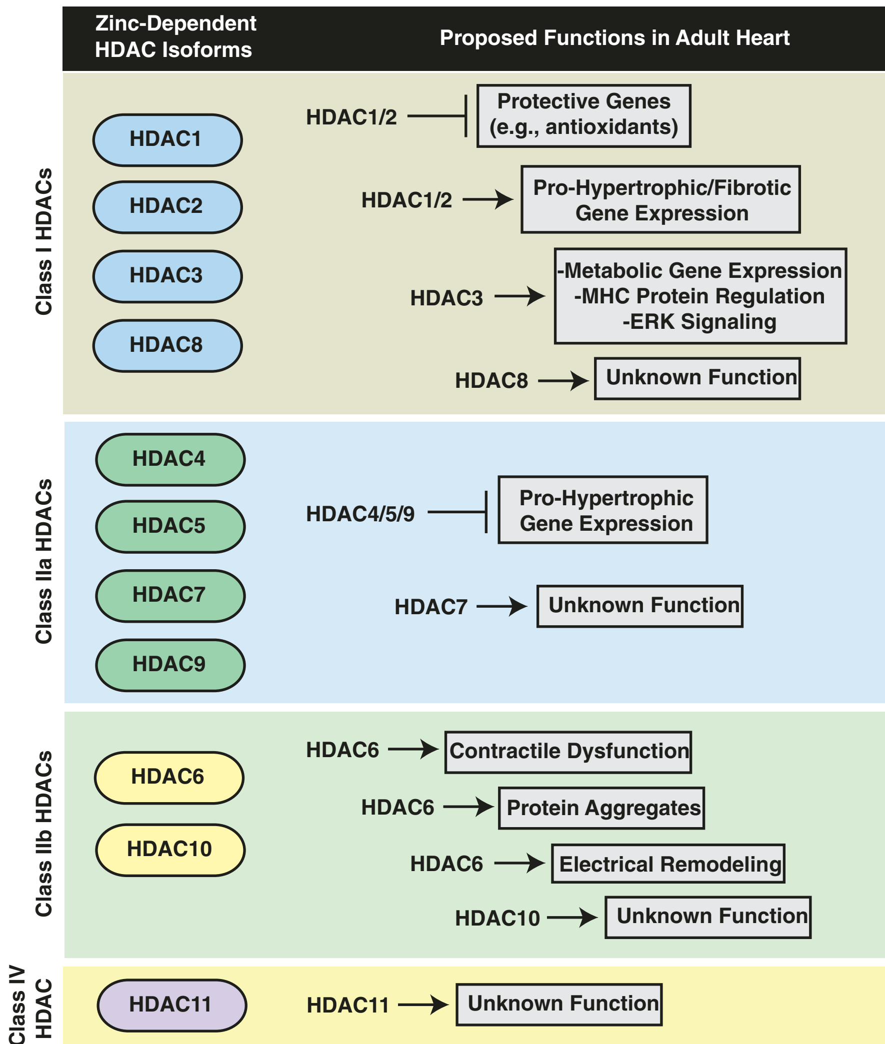

Fig. 9.1 HDAC isoform functions in the heart. The 11 zinc-dependent HDACs fall into four classes. Proposed functions for specific HDAC isoforms are indicated

recruiting factors that influence RNA polymerase II (Pol II) dynamics (Haldar and McKinsey 2014; Anand et al. 2013; Jenuwein and Allis 2001; Spiltoir et al. 2013; Wang et al. 2004).

The 18 mammalian HDACs are encoded by distinct genes and are grouped into four classes: class I (HDACs 1, 2, 3, and 8), class II (HDACs 4, 5, 6, 9, and 10), class III (SirT1–7), and class IV (HDAC11) (Gregoretti et al. 2004). Class II HDACs are further divided into two subclasses, IIa (HDACs 4, 5, 7, and 9) and IIb (HDACs 6 and 10). Coordination of a zinc atom in the catalytic domains of class I, II, and IV HDACs is required for catalysis, while class III HDACs (sirtuins) utilize nicotinamide adenine dinucleotide (NAD^+) as a co-factor for catalytic activity. This chapter focuses on zinc-dependent HDACs (Fig. 9.1).

9.3 Cardiac Phenotypes in Class I HDAC Knockout Mice

Class I HDACs, particularly HDACs −1, −2, and −3, are canonical epigenetic regulators. These HDACs are present in the nucleus in large multi-protein complexes referred to as Sin3, NuRD, CoREST, and NCoR/SMRT, which are recruited to gene-regulatory elements by sequence-specific DNA binding transcription factors (Cunliffe 2008; Kelly and Cowley 2013). HDAC1 and HDAC2 tend to be present together in Sin3, NuRD, and CoREST complexes, while HDAC3 is a component of the NCoR/SMRT complex. However, as discussed below, class I HDACs can be detected outside of the nucleus and likely also serve diverse nongenomic functions in the heart by deacetylating proteins other than histones.

Conditional cardiac deletions of either HDAC1 or HDAC2 produce no phenotype, while homozygous loss of both genes in the heart results in dilated cardiomyopathy, arrhythmia, and neonatal lethality (Montgomery et al. 2007). HDAC1 or HDAC2 conditional null mice remain susceptible to isoproterenol- or pressure overload-induced cardiac hypertrophy, suggesting that these isoforms may serve redundant functions in controlling growth of the adult heart. Alternatively, it is possible that these HDACs do not regulate cardiac hypertrophy.

Mice with cardiac-specific deletion of HDAC3 succumb to metabolic cataclysm in the heart. These animals develop massive cardiac hypertrophy and excessive myocardial lipid accumulation due to constitutive activation of peroxisome proliferator-activated receptor-mediated gene expression (Montgomery et al. 2008). Cardiac deletion of HDAC3 in this study was driven by Cre recombinase expressed via the α-myosin heavy chain (MHC) promoter, which is active during embryonic development. Deletion of HDAC3 from the mouse heart at a later point, using an MCK-Cre transgene, did not yield an overt phenotype until animals were fed a high-fat diet, whereupon they developed severe cardiomyopathy with concomitant down-regulation of genes involved in lipid metabolism (Sun et al. 2011).

In contrast to the injurious effects of knocking out HDAC3 in the heart, knockdown of HDAC3 expression using a morpholino oligonucleotide led to reduced left ventricular (LV) hypertrophy and fibrosis in a mouse pressure overload model (Sharifi-Sanjani et al. 2014). It is possible that these discrepant findings are due to incomplete knockdown of HDAC3 expression with the oligonucleotide.

9.4 Class I HDAC Inhibitors in Heart Failure Models

Several studies have shown that HDAC inhibitors such as trichostatin A (TSA) are able to block cardiac hypertrophy in rodent models. TSA is a "pan" inhibitor that targets all zinc-dependent HDACs (Bradner et al. 2010). It has long been speculated that the anti-hypertrophic action of pan-HDAC inhibitors is due to blockade of class I HDAC activity (McKinsey and Olson 2004). Indeed, experiments with cultured cardiomyocytes have implicated class I HDACs in the control of cardiac hypertrophy (Antos et al. 2003; Cao et al. 2011; Eom et al. 2014; Ferguson et al. 2013; Kee et al. 2008). However, data that definitively illustrates a pro-hypertrophic role for these HDACs *in vivo* are still lacking. Using a rat model having right ventricular (RV) hypertrophy, we demonstrated that a highly selective HDAC1/2/3 inhibitor, Mocetinostat, blocked adverse signaling events in the heart (e.g., caspase activation), while only modestly blunting hypertrophic growth of the RV (Cavasin et al. 2012). Likewise, a derivative of apicidin, which is a fungal metabolite that selectively inhibits class I HDACs, incompletely blocked hypertrophy in a long-term mouse pressure overload model (Gallo et al. 2008). SK-7041 is an HDAC inhibitor that is reportedly specific for class I HDACs and was shown to effectively block pressure overload-induced hypertrophy in mice (Kee et al. 2006). However, we have evaluated SK-7041 in vitro and found that the compound is a pan-HDAC inhibitor (unpublished observations).

While the role of class I HDACs in cardiac hypertrophy remains incompletely understood, it is clear that inhibiting the catalytic activity of these HDACs is beneficial in the context of heart failure. Selective class I HDAC inhibition with Mocetinostat blocked cardiac fibrosis in response to chronic angiotension II infusion (Williams et al. 2014). This class I HDAC inhibitor also blunted progression of fibrosis in a chronic rat MI model (with no impact on scar size), resulting in a profound reduction in LV end diastolic pressure (Nural-Guvener et al. 2014). In this model, Mocetinostat was delivered 3 weeks post-MI and continued for 3 additional weeks, illustrating the potential of class I HDAC inhibition to improve the preexisting cardiac disease. In a 7-day model of mouse MI, induced by left anterior descending coronary artery ligation, administration of the class I HDAC inhibitor, PD-106, post-MI resulted in reduced LV remodeling and improved cardiac function at the study endpoint, with concomitant suppression of matrix metalloproteinase-2 and 9 expression (Mani et al. 2015). In an *ex vivo* model of ischemia/reperfusion injury in the rat heart, pretreatment with MS-275, another class I HDAC inhibitor, led to reduced infarct area and significant improvements in LV contractile function (Aune et al. 2014). Efficacy of MS-275 was associated with FOXO3a-mediated induction of the antioxidant genes, catalase, and mitochondrial superoxide dismutase. Mocetinostat, PD-106, and MS-275 are structurally related HDAC inhibitors that contain a benzamide warhead that confers a high degree of specificity for inhibition of class I HDACs 1, 2, and 3 over other HDAC isoforms (Wagner et al. 2013).

Valproic acid (VPA) is a weak, albeit selective (Fass et al. 2010), inhibitor of class I HDACs that has been shown to block cardiac hypertrophy in various rodent models (Cardinale et al. 2010; Cho et al. 2010; Kang et al. 2015; Kee et al. 2006, 2013; Zhao et al. 2012). VPA has been used in the clinic for decades as a safe

anti-convulsant and mood stabilizer. Thus, it is not unreasonable to speculate that VPA could be repurposed for other indications, such as heart failure. Nonetheless, it remains unclear whether the efficacy of VPA observed in preclinical models of heart failure is actually due to class I HDAC inhibition, since VPA can also alter the activity of ion channels, kinases, and other biochemical pathways (Terbach and Williams 2009). Furthermore, VPA is able to reduce systemic blood pressure, as seen in spontaneously hypertensive rats (Cardinale et al. 2010), and thus some of the beneficial effects of VPA on the heart could be due to a reduction in the afterload.

Data from studies with small molecule inhibitors are often compared with results of genetic loss-of-function experiments to ascertain whether the given action of a compound is due to on-target or off-target action; the notion is that knockdown/knockout of a target should recapitulate the phenotype seen with pharmacological inhibition of the target. However, HDAC knockout/knockdown does not always mimic effects of HDAC inhibitors, due to noncatalytic functions of HDACs. This is exemplified by HDAC3. Cardiac-specific deletion of HDAC3 causes cardiomyopathy in mice; yet TSA, Mocetinostat, or other potent HDAC3 inhibitors have a positive impact on the heart. The noncatalytic function of HDAC3 was clearly demonstrated in the studies of mouse liver, where HDAC3 deletion caused hepatosteatosis, which was rescued by catalytically inactive mutants of HDAC3 (Sun et al. 2013).

A more relevant strategy for assessing on-/off-target effects of HDAC inhibitors is to employ numerous, structurally dissimilar compounds, because it is improbable that all of the compounds will interfere with the action of a common off-target effector. In preclinical models of heart failure, HDAC inhibitors from four broad chemical classes are efficacious: short chain fatty acids (VPA), hydroxamic acids (TSA), aminobenzamides (Mocetinostat), and cyclic peptides (apicidin) (Bush and McKinsey 2010). These findings strongly suggest that HDAC inhibition is beneficial in the setting of heart failure.

There is one HDAC-independent action of hydroxamic acid HDAC inhibitors that could contribute to the ability of this compound class to suppress adverse cardiac remodeling. Some hydroxamic acids were recently found to bind to free cellular iron and form complexes catalyzing the conversion of hydrogen peroxide into water and O_2 (Olson et al. 2015). This catalase-like antioxidant activity would be expected to benefit the heart, since oxidative stress contributes to cardiac hypertrophy, cardiac fibrosis, and contractile dysfunction (Ferguson and McKinsey 2015).

9.5 Pharmacological Manipulation of Class IIa HDACs in Heart Failure Models

The roles of class IIa HDACs as suppressors of cardiac hypertrophy are well described (McKinsey 2012), particularly for HDAC5 and HDAC9 (Chang et al. 2004; Zhang et al. 2002). Class IIa HDACs have extended (~500 amino acid) amino-terminal domains that govern recruitment to pro-hypertrophic genes through transcription factors such as myocyte enhancer factor 2 (MEF2). In response to signals for cardiac

hypertrophy, serine residues within these domains are phosphorylated, leading to nuclear export of the HDACs and derepression of downstream target genes. It has been hypothesized that blockade of HDAC5/9 nuclear export would block cardiac hypertrophy (Harrison et al. 2004) and that the most selective way to achieve this goal would be to inhibit the kinase(s) that phosphorylates the amino-terminal serine residues in class IIa HDACs. To this end, several class IIa HDAC kinases were discovered, most of which fall into the calcium/calmodulin-dependent kinase (CaM Kinase) superfamily (McKinsey 2007), including protein kinase D (PKD) and CaM Kinase II (Backs et al. 2006; Vega et al. 2004). While highly selective PKD inhibitors have been shown to block hypertrophy of cultured cardiac myocytes (Monovich et al. 2010), these compounds have thus far failed to suppress adverse cardiac remodeling *in vivo* (Meredith et al. 2010a, b). It is likely that functionally redundant class IIa HDAC kinases (e.g., CaM Kinase II) are able to compensate for the lack of PKD activity in inhibitor-treated animals and that simultaneous inhibition of multiple class IIa HDAC kinases will be required to block cardiac hypertrophy.

The designation of class IIa HDACs as HDAC enzymes is misleading since, despite having conserved catalytic domains, these proteins are unable to deacetylate histones. Class IIa HDAC catalytic activity is only detectable using an artificial substrate (Jones et al. 2008; Lahm et al. 2007). Research aimed at discovering endogenous substrates of class IIa HDACs is ongoing and will likely be aided by recently described small molecules that have a high degree of specificity for class IIa HDAC catalytic domains over other HDAC isoforms (Lobera et al. 2013); these compounds were discovered based on their ability to block class IIa HDAC-mediated deacetylation of the artificial substrate. A small molecule termed MC1568 has long been touted as a class IIa HDAC inhibitor (Mai et al. 2005). However, we and others have found that MC1568 lacks the ability to inhibit class IIa HDAC catalytic activity, and thus caution is warranted when interpreting data obtained with this compound (Fleming et al. 2014; Lemon et al. 2015).

9.6 Emerging Roles for Class IIb HDAC6 in Heart Failure

Although HDAC6 has been shown to enter the nucleus (Liu et al. 2012), where it presumably regulates gene expression, this class IIb HDAC primarily resides in the cytoplasmic compartment. HDAC6 is the predominant tubulin deacetylase (Matsuyama et al. 2002) and has also clearly been shown to deacetylate heat shock protein 90 (HSP90) and cortactin, which is F-actin binding protein (Valenzuela-Fernandez et al. 2008). Studies of HDAC6 in the heart are just beginning. HDAC6 catalytic activity was shown to be elevated in multiple models of cardiac dysfunction (Lemon et al. 2011), and subsequent findings suggest a role for this HDAC isoform in the pathogenesis of cardiac disease. In a dog pacing model, HDAC6 contributes to structural and electrical remodeling of atrial myocytes and has been proposed to trigger atrial fibrillation by deacetylating tubulin and disrupting microtubule structure (Zhang et al. 2014). HDAC6-deficient mice and wild-type mice treated with Tubastatin A, which is a selective

HDAC6 inhibitor (Butler et al. 2010), exhibited improved LV systolic function in models of pressure overload or chronic angiotensin II signaling, compared with controls (Demos-Davies et al. 2014). HDAC6 deletion did not block cardiac hypertrophy or fibrosis. Rather, cardiac myofibrils were found to have greater force-generating capacity in the absence of HDAC6. HDAC6 was shown to co-purify with cardiac myofibrils, suggesting that sarcomeric proteins are substrates of this deacetylase and that HDAC6-mediated deacetylation of one or more sarcomeric proteins leads to perturbation of systolic function of the heart (Demos-Davies et al. 2014).

HDAC6 was also recently shown to contribute to pathogenesis in a model of desmin-related cardiomyopathy (McLendon et al. 2014). Expression of a mutant αB-crystallin (R120G) in the mouse heart leads to accumulation of misfolded protein aggregates, which trigger pathological cardiac remodeling and heart failure (Wang et al. 2001). Tubastatin A reduced aggregate formation in cultured cardiomyocytes expressing αB-crystallin (R120G) and pan-HDAC inhibition with Vorinostat, which is a hydroxamic acid-containing compound, blunted cardiac aggregate formation, and improved ventricular function in αB-crystallin (R120G) transgenic mice (McLendon et al. 2014). Efficacy of SAHA in the model was associated with increased autophagy in the heart. Together, the early studies of HDAC6 reveal unique functions for this cytoplasmic deacetylase in the pathogenesis of heart failure. Intriguingly, HDAC6 null mice do not exhibit overt abnormalities, suggesting that pharmacological inhibition of this enzyme will be well tolerated in humans.

9.7 HDAC8, HDAC10, and HDAC11: Cardiac Functions Unknown

The functions of HDAC8, HDAC10, and HDAC11 in the heart remain completely unknown. HDAC8 catalytic activity was shown to be elevated in the heart in a rat model of hypertension-induced cardiac hypertrophy (Kee et al. 2013). Several substrates of HDAC8, in addition to histones, have been identified in noncardiac cells, including cortactin and estrogen-related receptor (Chakrabarti et al. 2015; Li et al. 2014; Wilson et al. 2010), and thus there is potential for this HDAC isoform to have diverse nuclear and cytoplasmic functions in the heart. The availability of selective small molecule inhibitors of HDAC8 provides an opportunity to use a chemical biological approach to dissect the role of this HDAC in normal heart and in the pathogenesis of heart failure (Chakrabarti et al. 2015).

HDAC10 is most closely related to HDAC6, and thus it is designated as a class IIb HDAC. In cultured cardiomyocytes, knockdown of HDAC10 leads to upregulation of HDAC6, and vice versa (Lemon et al. 2011), suggesting that these proteins serve redundant functions. In cancer cells, knockdown of HDAC10 expression leads to suppression of autophagy (Oehme et al. 2013a, b). It will be interesting to determine if HDAC6 and HDAC10 function within a common pathway to control autophagy in the heart.

The sole member of class IV HDACs is HDAC11, which remains poorly characterized due to the inability to effectively monitor its catalytic activity using in vitro assays. The original description of HDAC11 revealed high levels of HDAC11 mRNA in human cardiac tissue (Gao et al. 2002); yet a function for this HDAC isoform in the heart has not been described.

9.8 Regulation of the Cardiac Epigenetics by HDACs

Acetylated-lysine residues from all components of the nucleosome have been found, with acetyl marks on the tails of histone H3 (H3) and histone H4 (H4) being the best characterized and most abundant (Shahbazian and Grunstein 2007). In mammalian cells, acetylation of eight lysine residues on H3 and five lysines on H4 are readily detected by mass spectrometry (Tan et al. 2011). Adding further complexity, histone lysines can be methylated, sumoylated, ubiquitinated, crotonylated, succinylated, and malonylated (Tan et al. 2011; Bradbury 1992; Shiio and Eisenman 2003; Xie et al. 2012). Histone acetylation is typically associated with active transcription (Schubeler et al. 2004). However, whether a specific acetylation event has a positive, negative, or neutral effect on gene expression is dependent on the interplay with other histone modifications and is promoter context dependent (Felsenfeld and Groudine 2003).

An example of HDAC-mediated alterations in histone acetylation leading to changes in cardiac gene transcription was provided by studies of MHC expression. A hallmark of heart failure is the differential regulation of the two cardiac MHC isoforms, α and β, which has a profound effect on cardiac function (Braunwald and Bristow 2000). α-MHC, which is upregulated in the heart after birth, has high ATPase activity, whereas β-MHC has low ATPase activity. In the failing heart, β-MHC expression is upregulated and α-MHC is downregulated, with a consequent reduction in myofibrillar ATPase activity and reduced shortening velocity of cardiac myofibrils. It has long been recognized that HDAC inhibition can stimulate α-MHC expression in cardiomyocytes (Davis et al. 2005). In a mouse transverse aortic constriction (TAC) model, TSA was shown to enhance acetylation of H3K9/K14 within the α-MHC promoter, leading to increased expression of this "fast" myosin isoform (Chang et al. 2011). In response to pressure overload, HDAC2 was recruited to the α-MHC promoter, leading to decreased H3K9/K14 acetylation and consequent suppression of α-MHC expression. Improved contractile function in HDAC inhibitor-treated animals may be linked, in part, to the ability of these compounds to stimulate α-MHC expression.

Evaluation of epigenetic modifications in the heart has classically been in the context of specific gene promoters (e.g., α-MHC) using chromatin immunoprecipitation (ChIP) followed by polymerase chain reaction (PCR) of the relevant promoter sequences. While this approach has provided insight into the role of HDACs with respect to an isolated set of genes, studying single gene promoters does not address the functions of HDACs in the regulation of epigenetics throughout the cardiomyocyte genome. Given that histones are responsible for maintaining

chromatin structure and are bound throughout the genome, it is likely that HDACs influence vast regions of the genome in the context of heart failure.

Whole transcriptome sequencing (RNA-Seq) and ChIP sequencing (CHIP-Seq) technologies have begun to be applied to genome-wide investigations of epigenetics in the heart. RNA-Seq allows for the study of global change of all RNA transcripts in relation to HDAC gene alterations, or HDAC pharmacologic inhibition; while ChIP-Seq is a method used to determine the exact locations of specific transcription factors or histone marks throughout the genome. ChIP-Seq differs from established ChIP protocols in that, instead of using PCR to amplify specific regions of the genome, all isolated DNA fragments are sequenced and then aligned to a reference genome. Processing data on this scale can be computationally demanding; however, in recent years it has become possible to overlay RNA-Seq data sets with ChIP-Seq data sets to look at quantitative changes in global transcription in relation to global changes in chromatin modifications (Hughes et al. 2014; Wang et al. 2013).

Antibodies specific for distinct H3 acetylation and methylation marks were used to map genome-wide changes that occur in mouse hearts subjected to 1 week of pressure overload due to TAC (Papait et al. 2013); this study was performed with isolated cardiomyocytes from these hearts. By combining the ChIP-Seq datasets with RNA-Seq data obtained from the same cells, it was found that the combined presence of acetyl-H3K9, acetyl-H3K27, methyl-H3K4, and dimethyl-H3K79 within promoter sequences was associated with enhanced gene transcription during hypertrophy. In a separate study performed with whole hearts of mice subjected to TAC for 4 days, it was found that RNA polymerase II (Pol II) co-localized with acetyl-H3K9 in hypertrophic hearts (Sayed et al. 2013). All promoters where Pol II was bound, acetylated-H3K9 was also bound, although Pol II was not found at all acetyl-H3K9 sites. The findings establish that dynamic changes in H3K9 acetylation and Pol II recruitment or mobilization occur during pathological cardiac hypertrophy.

A genome-wide evaluation of the impact of HDAC inhibition on cardiomyocyte epigenetics in heart failure was recently described (Ooi et al. 2015). Mice underwent TAC surgery and were administered TSA or vehicle control for 4 weeks. Whole heart homogenates were analyzed by ChIP-Seq with an anti-acetyl-H3K9/K14 antibody. Pressure overload was shown to cause both increases and decreases in H3K9/14 acetylation throughout the genome, and these changes were reversed by TSA. A paradoxical finding from this study was the profound ability of TSA to reduce, rather than increase, H3 acetylation at many loci. Follow-up studies evaluating potential cross talk between HDACs and HATs in the heart should provide further insight into this intriguing finding.

Our understanding of the genome-wide functions of HDACs in the control of cardiac epigenetics in heart failure is still limited. These early studies suggest that HDACs regulate specific areas of the cardiomyocyte genome, perhaps through interactions with specific transcription factors. Use of isoform-selective HDAC inhibitors, as opposed to TSA, should simplify interpretation of the data and will provide a unique opportunity to address the mechanism(s) by which distinct HDAC

isoforms influence the histone code in the heart. In this regard, a recent, powerful study employed mass spectrometry to quantify changes in histone and nonhistone proteins in HeLa and MV4-11 cells exposed to pan and isoform-selective HDAC inhibitors (Scholz et al. 2015). The findings demonstrated HDAC isoform-specific substrates and revealed complexly orchestrated regulation of cellular function through specific and nonspecific protein deacetylation that extends beyond the nucleus.

9.9 Beyond Epigenetics: Nongenomic Targets of Acetylation in the Heart

The first "acetylome" was reported 6 years ago (Choudhary et al. 2009), and together with subsequent studies, thousands of acetylation sites on thousands of proteins have been identified in mammalian cells (Choudhary et al. 2014), illustrating that the reach of acetylation in biological control extends far beyond epigenetics. Two papers have described efforts to quantify acetylation of proteins in the heart (Foster et al. 2013; Lundby et al. 2012). Both studies employed immunoprecipitation of proteins from normal, unstressed hearts (rat or guinea pig) using "pan" anti-acetyl-lysine antibodies prior to mass spectrometry and label-free identification of acetylated peptides; the study using guinea pig hearts employed proteins that were fractionated into myofilament, mitochondrial, and cytosolic pools prior to immunoprecipitation. We overlaid the common orthologues from each dataset that were found to be acetylated, resulting in a total of 160 common nonhistone acetyl-proteins (Fig. 9.2a). Many of the common acetylated proteins are regulators of metabolism (not shown). Acetylation of mitochondrial proteins involved in metabolism is well appreciated, and deacetylation of these proteins is generally governed by class III HDACs (sirtuins) (Newman et al. 2012). The analysis also revealed acetylation of many cardiac proteins involved in intra- and extracellular signaling, gene regulation, cell–cell communication, and contraction (Fig. 9.2b). The potential role of reversible lysine acetylation in the regulation of cardiac contraction and relaxation is further highlighted by the numerous components of the sarcomere that were shown to be acetylated (Fig. 9.2c). It is intriguing to speculate that the ability of HDAC inhibitors to improve contractile function in animal models of heart failure is related to enhanced acetylation of sarcomeric proteins. In this regard, *in vitro* studies showed that acetylation of α-MHC and β-MHC increased the actin-sliding velocity of both myosin isoforms, and HDAC3 was implicated as the MHC deacetylase (Samant et al. 2015).

The difference in total acetylated proteins between the guinea pig and rat studies (Fig. 9.2a) could be due to variations in the fractionation steps prior to MS/MS. By increasing the degree of fractionation, the overall sample resolution increases, allowing for the identification of many more acetyl sites. Future investigations into the cardiac acetylome should, in part, also focus on refinement and standardization of a mass spectroscopy acetylome protocol to maximize the potential of this powerful technique.

a
Acetylated Proteins in Heart
71
160
833
Foster et al.
Lundby et al.
b
PROTEASOME
PEROXISOME
GLUTATHIONE METABOLISM
GNRH SIGNALING PATHWAY
VASOPRESSIN REGULATED WATER REABSORPTION
NEUROTROPHIN SIGNALING PATHWAY
SNARE INTERACTIONS IN VESICULAR TRANSPORT
PROTEIN EXPORT
PPAR SIGNALING PATHWAY
ABC TRANSPORTERS
HEDGEHOG SIGNALING PATHWAY
ERBB SIGNALING PATHWAY
COMPLEMENT AND COAGULATION CASCADES
OXIDATIVE PHOSPHORYLATION
RENIN ANGIOTENSIN SYSTEM
ECM RECEPTOR INTERACTION
TGF BETA SIGNALING PATHWAY
VEGF SIGNALING PATHWAY
ADIPOCYTOKINE SIGNALING PATHWAY
SPLICEOSOME
GAP JUNCTION
TIGHT JUNCTION
CALCIUM SIGNALING PATHWAY
RNA POLYMERASE
JAK STAT SIGNALING PATHWAY
ANTIGEN PROCESSING AND PRESENTATION
ENDOCYTOSIS
FOCAL ADHESION
CARDIAC MUSCLE CONTRACTION
BASAL TRANSCRIPTION FACTORS
CELL CYCLE
NOD LIKE RECEPTOR SIGNALING PATHWAY
REGULATION OF ACTIN CYTOSKELETON
ADHERENS JUNCTION
WNT SIGNALING PATHWAY
MAPK SIGNALING PATHWAY
LEUKOCYTE TRANSENDOTHELIAL MIGRATION
NOTCH SIGNALING PATHWAY
RIG I LIKE RECEPTOR SIGNALING PATHWAY
MTOR SIGNALING PATHWAY
CHEMOKINE SIGNALING PATHWAY
PHOSPHATIDYLINOSITOL SIGNALING SYSTEM
CYTOSOLIC DNA SENSING PATHWAY
NEUROACTIVE LIGAND RECEPTOR INTERACTION
VASCULAR SMOOTH MUSCLE CONTRACTION
CYTOKINE CYTOKINE RECEPTOR INTERACTION
NUCLEOTIDE EXCISION REPAIR
DNA REPLICATION
P53 SIGNALING PATHWAY
TOLL LIKE RECEPTOR SIGNALING PATHWAY
FC EPSILON RI SIGNALING PATHWAY
c
Cardiac Sarcomere
Ac Titin
Troponin T *
MyBP-C
Ac Troponin C
Ac Troponin I
Ac Actin
Tropomodulin
Ac β-MHC
α-MHC *
Ac MLC2
Ac MLC3
* Identified as acetyl-protein in rat but not guinea pig heart

Fig. 9.2 Nonhistone targets of acetylation in the heart. (**a**) Acetylated peptides identified from normal, unstressed rat hearts (Lundby et al. 2012) and guinea pig hearts (Foster et al. 2013) were overlaid and analyzed to identify acetylated targets found in both studies. (**b**) The 160 acetylated targets identified from both studies were subjected to the Kyoto encyclopedia of genes and genomes analysis. Results are displayed as a network via Cytoscape and grouped by specific cellular functions. *Gray* boxes indicate a function associated with an acetylated target. *Red* lines between boxes indicate that an acetylated target belongs to both cellular signaling pathways. (**c**) Of the 160 acetylated target found in both studies, many proteins within the cardiac sarcomere were found to be acetylated, as indicated by *green* boxes and *yellow* Ac labels. *Myosin binding protein C (MyBP-C), α-MHC, troponin T, and tropomodulin were identified as acetylated proteins in rat hearts but not guinea pig hearts

Investigation of the function of nonhistone protein acetylation in the heart is in its infancy. The cardiac acetylome represents a rich reservoir for discovery of novel regulatory pathways in normal and diseased hearts and has the potential to yield innovative therapeutic targets for heart failure. We hypothesize that beneficial effects of HDAC inhibitors in heart failure models are due to actions on multiple cell types (e.g., myocytes, fibroblasts, inflammatory cells) and due to influences of HDAC inhibition on acetylation of both histone and nonhistone targets. To address this hypothesis, it will be necessary to perform quantitative analyses of changes in nonhistone protein acetylation that occurs in the setting of heart failure and to evaluate the impact of isoform-selective HDAC inhibitors on nonhistone protein acetylation in the heart.

9.10 Cross Talk Between Acetylation and SUMOylation

Lysine is a highly versatile amino acid target for posttranslational modifications, which include methylation, acyl modifications (e.g., acetylation, crotonylation, succinylation), small protein isopeptide bonds (i.e., ubiquitination, NEDDylation, SUMOylation), and biotinylation (Yang and Seto 2008). A particular lysine residue may only be subject to one posttranslational modification at a given time; an exception to this is that lysine can be simultaneously conjugated to multiple methyl groups (i.e., mono-, di-, tri-methyl). Given the mutually exclusive nature of lysine modifications, we hypothesized that increasing nonhistone protein acetylation, through the use of HDAC inhibitors, would result in suppression of SUMO-1 conjugation in cardiac cells. Paradoxically, treatment of cardiomyocytes or cardiac fibroblasts with HDAC inhibitors such as TSA and Mocetinostat led to a global *increase* in protein SUMO-1-ylation, indicating cross talk between the two modifications, albeit in a direction converse to what was previously hypothesized (Blakeslee et al. 2014). Increased SUMO-1-ylation occurred in the absence of new protein synthesis, suggesting the possibility that HDAC inhibitor-mediated acetylation of a component(s) of the SUMO conjugation machinery enhances protein SUMO-1-ylation in cardiac cells.

It is intriguing to speculate that HDAC inhibitor efficacy in heart failure models is due, at least in part, to the ability of these compounds to promote SUMO-1-ylation. SUMO-1 is downregulated in human heart failure patients, as well as in mice subjected to TAC-induced pressure overload (Kho et al. 2011). Furthermore, overexpression of SUMO-1 using recombinant adeno-associated virus (rAAV) was shown to improve cardiac function in a mouse model of pressure overload model, as well as in a pig model of ischemia/reperfusion injury, in which animals received coronary infusion of rAAV-SUMO-1 1 month after I/R injury (Kho et al. 2011; Tilemann et al. 2013). Consistent with the idea of defective SUMOylation being maladaptive for the heart, cardiac-specific transgenic overexpression of the SUMO protease, SENP2, resulted in elevated levels of free SUMO-1 and dysfunctional hemodynamics and gross malformations of the myocardium (Kim et al. 2012). Additionally, SENP1 expression was elevated in failing mouse and human hearts, and rAAV-SENP1 overexpression *in vivo* was sufficient to cause dilated cardiomyopathy in mice (Cai et al. 2015). Collectively, these results favor the concept of

defective SUMOylation contributing to heart failure. Experiments designed to test whether concomitant HDAC inhibitor therapy and SUMO-1 gene transfer provide synergistic efficacy in heart failure models should be enlightening.

Conclusions

Despite significant advances in our understanding of the functions of HDACs in the heart, major knowledge gaps still exist (Fig. 9.1). The ability to couple state-of-the-art epigenomic technologies (e.g., high-resolution ChIP-Seq) with pharmacological tool compounds that selectively inhibit the catalytic activity of class I, IIa, or IIb HDACs should reveal novel mechanisms for epigenetic control of cardiac hypertrophy, fibrosis, and dysfunction. Furthermore, mass spectrometry-based global analysis of lysine acetylation in the heart has highlighted the fact that this posttranslational modification extends far beyond nucleus and likely plays a central role in the regulation of all processes in the cardiomyocyte. We are in an extremely unique and exciting era of research on HDACs in the heart, with the outcome of experiments conducted over the next few years having tremendous potential to provide novel insights into the molecular underpinnings of heart failure; and to reveal previously unappreciated targets for innovative therapeutic approaches aimed at combating the emerging global health crisis of heart failure.

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Conflict of Interest The authors declare that they have no conflict of interest.

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Cardiac Autophagy and Its Regulation by Reversible Protein Acetylation

10

Min Xie and Joseph A. Hill

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Abstract

Autophagy is an evolutionarily conserved and strictly regulated process implicated in the pathogenesis of multiple cardiovascular diseases. Autophagy is a multi-step process regulated by an orchestrated profile of molecular signaling pathways. Post-translational protein modifications, including reversible acetylation, regulate autophagy at the levels of signaling cascades, autophagy-related protein expression, and direct acetylation and trafficking of autophagic components. Autophagy has been implicated in cardiac hypertrophy, heart failure, ischemia/reperfusion injury, anticancer drug-induced cardiomyopathy and glycogen storage diseases. Manipulations of autophagy by HDAC inhibition is a novel strategy worthy of consideration in the treatment of these common cardiovascular diseases.

10.1 Introduction

Autophagy is an evolutionarily conserved process of intracellular recycling of defective proteins and organelles (Klionsky 2007). Autophagy was initially described as lysosome-dependent degradation of cytoplasmic components in the setting of starvation, serving to maintain intracellular energy homeostasis when external energy resources are limited (Klionsky 2007). Now, over the past 10–15 years, autophagy has been associated with multiple physiological processes, including the response to a wide range of extracellular or intracellular stress factors, including growth factor deprivation, ER stress, presence of damaged organelles and aggregated proteins, and cellular and tissue remodeling during development (Choi et al. 2013).

In addition, this evolutionarily ancient cellular process is associated with many human diseases, including heart disease (Lavandero et al. 2015). Further, autophagy is central to cellular defense against infection by various pathogens, as well as antigen processing (Choi et al. 2013). Alterations in autophagic activity have been linked to a variety of pathological conditions and can be either adaptive or maladaptive, depending on the context (Mizushima et al. 2008; Kroemer and Levine 2008). In the heart, autophagy has been implicated in the pathogenesis of heart failure, ischemia/reperfusion injury, glycogen storage disease, and atherosclerosis (Lavandero et al. 2015; Xie et al. 2011). Elucidation of the molecular underpinnings of the autophagy pathway, as well as their regulation, is essential to delineate precise mechanisms underlying cardiovascular disease and to devise novel therapies.

Autophagy can be triggered by pathological stimuli relevant to heart disease, including ischemia (Xie et al. 2014), mechanical stress (Cao et al. 2011), and metabolic derangement (Hariharan et al. 2010, 2011; Wang et al. 2013). These triggers provoke changes at transcriptional, translational, and posttranslational levels which culminate in altered autophagic flux (Banreti et al. 2013).

Among the posttranslational modifications governing autophagy are phosphorylation, ubiquitination, sumoylation, and acetylation. In fact, posttranslational

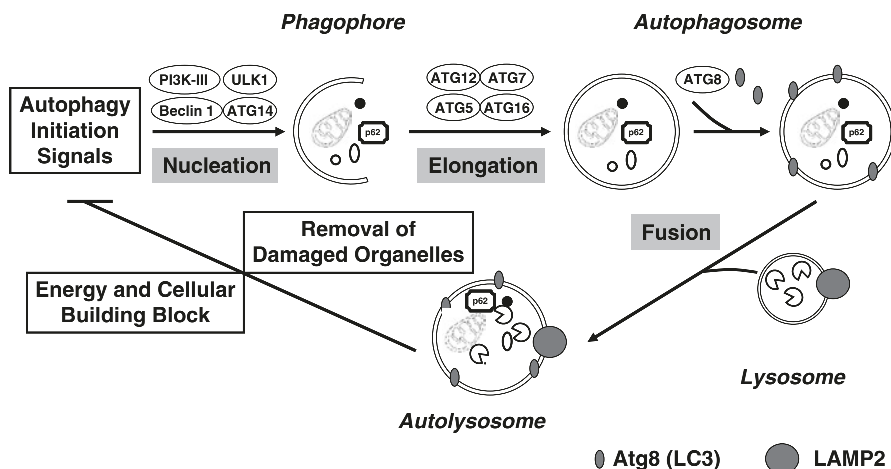

Fig. 10.1 Diagram of simplified autophagy pathway. The first step of the autophagic pathway is termed *nucleation*, a process regulated by PI3K and ULK1. The *elongation* step (phagophore expansion, autophagosome maturation and cargo recruitment) is accomplished by activation of E1-like (ATG7) and E2-like (ATG3). The final step is accomplished by the *fusion* of the autophagosome with a lysosome forming an autolysosome. The proteolytic products are recycled for energy production or providing building blocks for new organelles (Adapted from reference Xie et al. (2011))

regulation of autophagy has garnered attention recently for potential therapeutic implications. Arguably chief among them is reversible protein acetylation (Banreti et al. 2013). The emergence of histone deacetylase (HDAC) inhibitors as efficacious anticancer agents has heightened interest in potential translation within the realm of cardiovascular disease.

10.2 Autophagy

Autophagy is a ubiquitous catabolic process. The name derives from the Greek *auto-* "self" and *phagein* "to eat," which is an apt description of this process of catabolism of unnecessary or dysfunctional cellular components (Klionsky 2007). Degradation of these components promotes cell survival during starvation by maintaining cellular energy levels and, at the same time, eliminating otherwise toxic elements within the cytoplasm (Klionsky 2007). During the process of autophagic flux, targeted cytoplasmic constituents are isolated from the rest of the cell within a double-membrane vesicle known as an autophagosome. Next, the autophagosome fuses with a lysosome, releasing into the now autolysosome acidic pH and acid hydrolases. As a result, the sequestered cargo is degraded and repurposed for new functions within the cell (Klionsky 2007) (Fig. 10.1). Three different forms of autophagy have been described: macroautophagy, microautophagy, and chaperone-mediated autophagy (Lavandero et al. 2013). In the context of disease, autophagy can serve as an adaptive response to stress, promoting survival, whereas in other

cases, it appears to promote disease progression (Lavandero et al. 2015; Mizushima et al. 2008; Kroemer and Levine 2008; Rifki and Hill 2012).

Macroautophagy is the best characterized pathway, serving mainly to clear damaged organelles and damaged or aggregated proteins (Klionsky 2007). Molecular mechanisms of macroautophagy are well delineated and will be detailed below.

Microautophagy, in contrast, involves the engulfment of cytoplasmic material directly into the lysosome via invagination of the lysosomal membrane (Mijaljica et al. 2011).

Chaperone-mediated autophagy (CMA) involves the recognition of a specific group of proteins by the heat shock cognate 70 (hsc70)-containing complex to form a CMA-substrate/chaperone complex (Bandyopadhyay et al. 2008). This complex is transported to a lysosomal membrane-bound protein that binds the CMA receptor. Then, the substrate protein is unfolded and translocated across the lysosome membrane with the assistance of the lysosomal hsc70 chaperone (Bandyopadhyay et al. 2008). CMA is unique in that it translocates protein material in a one-by-one manner, and it is selective in terms of the proteins transported across the lysosomal barrier.

10.3 Molecular Mechanisms of Autophagy

10.3.1 Molecular Processes of Autophagy

Macroautophagy, hereafter termed autophagy, involves the formation of a double membrane that engulfs cytoplasmic substrates, resulting in an organelle known as the autophagosome. Overall, the process has been divided into specific steps, *viz.* induction, cargo recognition and selection, vesicle formation, autophagosome-vacuole fusion, breakdown of cargo, and release of the degradation products into the cytosol (Fig. 10.1). The core autophagic machinery consists of evolutionarily conserved signaling modules encoded by *autophagy-related* (*ATG*) genes (Mizushima et al. 2008; He and Klionsky 2009; Nakatogawa et al. 2009).

The first step of the autophagic pathway is termed *nucleation*, a process promoted by class III PI3K (phosphatidylinositol-3-kinase) activity. The PI3K complex comprises vacuolar protein sorting 34 (VPS34)-VPS15-VPS30/ATG6-ATG14 and the mammalian counterpart (including PIK3C3/VPS34-PIK3R4/VPS15-BECN1-ATG14), which promotes formation of the membrane compartment phagophore from intracellular membrane sources or via *de novo* membrane synthesis.

Next, the *elongation* step (phagophore expansion, autophagosome maturation, and cargo engulfment) is accomplished by activation of two parallel pathways: in one, the E1-like enzyme ATG7 mediates the conjugation of ATG12 to ATG5, which then couples with ATG16. This complex is required for autophagosome formation and ultimately dissociates from the vacuole upon maturation. In the other cascade, microtubule-associated protein 1 light chain 3 (LC3/ATG8) is cleaved by ATG4, producing the so-called LC3-I isoform. LC3-I is subsequently activated by conjugation to phosphatidylethanolamine (PE) by means of its interaction with ATG7 and ATG3 (E2-like enzymes), resulting in LC3-II (Kroemer et al. 2010; Ravikumar et al. 2010). The ubiquitin-like protein conjugation cascades involve ATG3, ATG4, ATG5, ATG7,

ATG8 (and the mammalian LC3 and GABA-A receptor-associated protein (GABARAP) subfamilies), ATG10, ATG12, and ATG16 (mammalian ATG16L1). LC3-II levels are commonly tracked markers of autophagy, as the molecule remains attached to the autophagosome membrane until degradation is completed (Mizushima and Klionsky 2007). LC3-II can also be detected as discrete puncta on immunofluorescence analysis, indicating autophagosome abundance (Choi et al. 2013).

The final step is accomplished by the *fusion* of the autophagosome with a lysosome, forming an autolysosome. Degradation of the autolysosomal cargo is accomplished by several lysosomal hydrolases, and the catabolized products are released into the cytosol and recycled for nutrient and/or structural needs (Ravikumar et al. 2010). This step also involves recycling of components between the phagophore, the phagophore assembly sites, and membrane donor sites. A recycling protein complex consists of ATG2, ATG9, ATG18, and ATG21 and the mammalian homologs, including WD repeat domain phosphoinositide-interacting protein1/2 (WIPI1/2).

10.3.2 Molecular Signaling Pathways Governing Autophagy

Environmental stimuli signal to the autophagic machinery via several regulatory factors. In the fed state, the abundance of nutrients, circulating insulin, and growth factors triggers activation of class I phosphatidylinositol-3-kinases (PI3K-I). This, in turn, leads to phosphorylation and activation of AKT, a nexus of metabolic signaling in many cell types. AKT activates mTOR (mechanistic target of rapamycin), including the isoform mTORC1, which negatively regulates a complex consisting of UNC-51-like kinase 1 (ULK1), ATG13, ATG101, and FIP200. This, in turn, suppresses autophagy by inhibiting nucleation of a macromolecular complex comprising class III PI3K/vacuolar protein sorting 34 (VPS34) (Kroemer et al. 2010; Ravikumar et al. 2010) (Fig. 10.2).

In states of nutrient deprivation, by contrast, mTOR is inhibited, and the autophagic flux pathway is released from repression (Levine and Kroemer 2008). This permits activation of ULK1, a crucial initiating step in autophagy. Consistent with this, the class III PI3K-selective inhibitor 3-methyladenine (3-MA) blocks autophagy (Mizushima and Klionsky 2007). The Beclin 1-interacting complex, a regulatory platform, consists of Beclin 1, B-cell lymphoma 2 (BCL-2) family proteins (inhibiting autophagy), and the class III phosphatidylinositol 3-kinase and ATG14L (required for autophagy). Activation of this complex generates phosphatidylinositol-3-phosphate, which promotes autophagosomal membrane nucleation (Mizushima and Klionsky 2007).

Apart from mTOR, AMP-activated protein kinase (AMPK) is activated by depletion of intracellular nutrient stores, activating ULK1 and up-regulating autophagy (Gottlieb and Mentzer 2010). Alternatively, in the setting of pathologic stress (e.g., starvation, ischemia, hypoxia, oxidative stress, mitochondrial damage, chemotherapeutic drugs), a Beclin 1/PI3K-III complex is formed promoting initiation of autophagy (Kroemer et al. 2010). Disrupting the interaction of Beclin 1 with the Vps34 complex, or depleting Beclin 1 by genetic silencing, can significantly diminish progression of the autophagic cascade (Yang and Klionsky 2010).

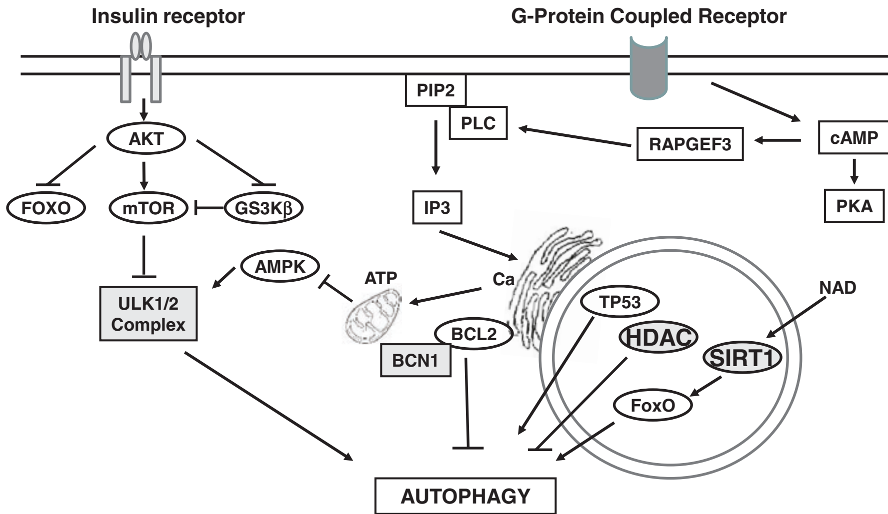

Fig. 10.2 Upstream signals regulating autophagy. Autophagy is regulated by mTOR-ULK1, 1,4,5-inositol trisphosphate (IP3) and ITPR (inositol 1,4,5-trisphosphate receptor), transcription factor TP53, cyclic AMP-dependent protein kinase A (PKA), glycogen synthase kinase 3B (GSK3B), nicotinamide adenine dinucleotide (NAD, through sirtuins (SIRT)), and also histone acetyltransferases (HATs) or histone deacetylases (HDACs)

Autophagy can also be regulated by 1,4,5-trisphosphate (IP3) and ITPR (inositol 1,4,5-trisphosphate receptor), the transcription factor TP53, cyclic AMP-dependent protein kinase A (PKA), and glycogen synthase kinase 3B (GSK3B). Reversible protein acetylation is a more recently recognized mechanism governing autophagy, mediated by nicotinamide adenine dinucleotide (NAD), sirtuins (SIRT), histone acetyltransferases (HATs), and histone deacetylases (HDACs) (Lavandero et al. 2013) (Fig. 10.2).

10.4 Regulation of Autophagy by Protein Acetylation

10.4.1 Epigenetic Regulation of Autophagy

Epigenetic control has emerged as a major mechanism governing a wide range of heart diseases (Gillette and Hill 2015). As part of this, posttranslational modifications (PTMs) are central to the regulation of eukaryotic proteins. Among the major PTMs are tyrosine or serine/threonine phosphorylation, lysine and N-terminal acetylation, lysine/arginine methylation, sumoylation, and ubiquitination (Banreti et al. 2013). Recently, a number of examples have emerged of the regulation of autophagy by PTMs other than acetylation. As noted above, it is well known that phosphorylation events have essential roles in the initiation of autophagy. For example, the ATG1-ATG13 complex is actively regulated by phosphorylation mediated by mTOR and PKA.

PTMs that are analogous to ubiquitination are also crucial for autophagy. Two major ubiquitin-like conjugation systems are involved in the elongation of the phagophore. First, ATG12 is conjugated to ATG5 by the E2-like protein ATG10, whereas LC3 is conjugated to phosphatidylethanolamine (PE) by ATG3 (Ravikumar et al. 2010; McEwan and Dikic 2011). Lysine side chains of proteins involved in autophagy can be targeted by multiple, mutually exclusive PTMs targeting the same lysine and providing an opportunity for cross-regulation (Yang and Seto 2008).

That being said, the importance of acetylation in autophagy control has been recognized only recently. While initially identified in histones 40 years ago, lysine acetylation also regulates many nonhistone proteins, including transcription factors and cytoplasmic proteins regulating cytoskeleton dynamics, energy metabolism, and endocytosis (Yang and Seto 2008; Glozak et al. 2005). Recent studies highlight the importance of protein acetylation in autophagy control (Lavandero et al. 2015; Xie et al. 2014; Cao et al. 2011; Banreti et al. 2013). Here, we summarize the emerging evidence for acetylation-mediated control of autophagy (mostly in yeast and cancer cells) and discuss the implications in the context of human and animal models of cardiovascular disease.

10.4.2 HATs and HDACs Operate at Multiple Levels Within the Autophagy Process

Protein acetylation and deacetylation are accomplished by HATs or HDACs, targeting a wide range of proteins within the cell (Yang and Seto 2008). HATs catalyze the transfer an acetyl-group from AcCoA (acetyl-coenzyme A) to the ε-amino group of a lysine residue on a protein; they are sometimes termed "writers." Conversely, HDACs, sometimes called "erasers," remove acetyl groups from proteins. As the PTMs occur on many proteins, not just histones, it is expected that with time the terms lysine acetyltransferase (KAT) and lysine deacetylases (KDAC) will emerge in common use.

There are three major classes of HATs: the E1A binding protein p300 (EP300/CREBBP (cAMP-response element binding protein)) family, the MYST family, and the KAT2/GCN5 (general control nonderepressible 5)-related N-acetyltransferases (GNAT) family. As mentioned in other chapters, HDACs are classified into four groups. The class I, IIa, IIb, and IV enzymes are zinc dependent, whereas the class III family comprises sirtuins which use NAD^+ as a cofactor to catalyze deacetylation reactions (Sadoul et al. 2011).

Data from yeast and mammalian cells have shown that HATs and HDACs are involved in the regulation of autophagic activity. Using gain- and loss-of-function strategies, HATs and HDACs have been divided into those inducing autophagy or inhibiting autophagy.

Another line of evidence emerges from work with small molecule HDAC inhibitors, which have been studied extensively for possible therapeutic uses. Among the HATs, CREBBP/KAT3A is reported to induce autophagy, (Steffan et al. 2000; Nucifora et al. 2001; Jeong et al. 2009; McCampbell et al. 2000) whereas EP300/KAT3B inhibits autophagy (Lee and Finkel 2009; Lin et al. 2012). KATs/TIP in the MYST family induces autophagy (Yang and Klionsky 2010; Yi et al. 2012).

Three classes of HDACs – class I, IIa/b, and III – have been reported to regulate autophagy. Among the class I HDACs, HDAC1 inhibits autophagy (Oh et al. 2008; Lamothe et al. 2012), HDAC2 induces autophagy (Cao et al. 2011), and HDAC3 inhibits autophagy through P21 (Rikiishi 2011). Among the class IIa HDACs, HDAC4 inhibits autophagy through CDKN1A (Rikiishi 2011; Roccaro et al. 2010). HDAC5 (Steffan et al. 2000) and HDAC7 (Hrzenjak et al. 2008) inhibit autophagy. Among the class IIb HDACs, HDAC6 induces autophagy through cortactin, tubulin, and dyenein, which are important for selective autophagy (Matthias et al. 2008; Lee and Yao 2010; Iwata et al. 2005a, b). In hepatic cancer cells, HDACs generally induce autophagy through BECN1-MAPK/JNK and the increase in autophagy in these cells is associated with cell death and limited tumor growth (Jung et al. 2012). Among the sirtuins (class III HDACs), SIRT1 induces autophagy through deacetylation of ATGs and FoxO1/3 (Hariharan et al. 2010; Lee et al. 2008; Jeong et al. 2013). SIRT2 inhibits autophagy by regulating FoxO1 (Kuo et al. 2010), and SIRT3 induces autophagy (Hrzenjak et al. 2008). For sake of completeness, we note that N-acetyltransferases (NATs) also have some role; NAT10 induces autophagy by repressing the TSC1-TSC2 complex (Kuo and Hung 2010). NAT9 inhibits autophagy and knockdown of NAT9 increases autophagy (Lipinski et al. 2010).

The most studied HDAC inhibitor, SAHA (suberoylanilide hydroxamic acid), is an inhibitor of class I, II, and IV HDACs. SAHA induces autophagy through inhibition of HDAC7 in endometrial stromal cells (Hrzenjak et al. 2008) or through hyperacetylation of tubulin in HeLa cells (Oh et al. 2008) and mouse neuronal cells(Dompierre et al. 2007). Similarly, the class I-II HDAC inhibitors valproic acid (VPA) (Fu et al. 2010) and butyrate (Donohoe et al. 2011; Shao et al. 2004) induce autophagy in cancer cells. Short-term exposure to SAHA has also been shown to induce autophagy in cardiomyocytes, returning flux suppressed by ischemia/reperfusion back to control levels (Xie et al. 2014). The HDAC 1, 2, 3-specific inhibitor MS-275 induces autophagy in malignant peripheral nerve sheath tumor (MPNST) cell lines (Lopez et al. 2011). The HDAC1-specific inhibitor, FK228, induces autophagy in HeLa cells (Oh et al. 2008). LBH589, an HDAC4-5 inhibitor, induces autophagy in Waldenström macroglubulinemia lymphoma cells (Roccaro et al. 2010). However, tubacin, an HDAC6-specific inhibitor, reduces autophagy in MEF cells (Lee et al. 2010).

Inhibition of class III HDACs generally induces autophagy. Longevinex, a specific inhibitor of SIRT1 and 3, induces autophagy in isolated neonatal rat ventricular myocytes (NRVM) (Mukherjee et al. 2010). The SIRT1-specific inhibitor, sirtinol, augments cigarette smoke-induced autophagy in lung epithelial cells (Hwang et al. 2010). Interestingly, resveratol, a Sirt1 inducer, also induces autophagy in lung epithelial cells, fibroblasts, colon cancer cells, C. elegans, and yeast (Hwang et al. 2010; Morselli et al. 2011). HDAC inhibitors are emerging as potent anticancer agents that can activate gene expression and enable elimination of malignant cells by apoptotic or autophagic cell death (Rikiishi 2011). Two potent HDAC inhibitors, OSU-HDAC42 and SAHA, induce autophagy in hepatocellular carcinoma cells through downregulation of AKT1-mTOR signaling and induction of an ER stress response (Liu et al. 2010). It was also reported that the HDAC inhibitor SAHA acts on HDAC7 and induces autophagic cell death in endometrial stromal sarcoma cells by influencing the mTOR pathway (Hrzenjak et al. 2008). SAHA also induces autophagy in chondrosarcoma and HeLa cells (Mizushima 2011; Yamamoto et al. 2008).

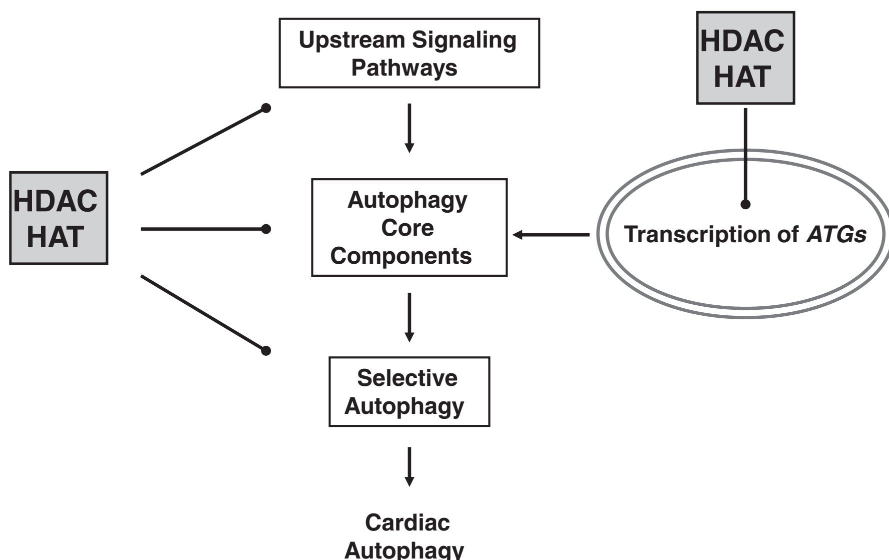

Fig. 10.3 Acetylation regulates autophagy at multiple levels. First, reversible protein acetylation governs upstream signaling pathways that regulate autophagy. Second, it regulates the activity of essential autophagy related proteins. Third, acetylation regulates the expression of autophagy-related proteins through acetylation of transcription factors, including FoxO proteins. Finally, reversible acetylation of structural proteins regulates the trafficking of autophagic structural components. Line with ball end denotes regulation (activation or inhibition in various contexts)

In the heart, chronic therapy with HDAC inhibitors suppresses pathological cardiac remodeling by inhibiting stress-induced autophagy, and this autophagic response is required for much of the pathological growth response (Cao et al. 2011; Kong et al. 2006; Berry et al. 2008; Cao and Hill 2011). On the other hand, during ischemia/reperfusion injury, short-term therapy with SAHA induces autophagy, returning flux to basal levels in the infarct border zone and protecting cardiomyocytes to reduce infarct size (Xie et al. 2014). Silencing the class IIB HDAC (HDAC6) leads to failure of autophagosomal maturation in the context of basal autophagy, thereby disrupting mitophagy and protein aggregate removal (Lee et al. 2010). Class III HDACs (sirtuins) are required for the autophagic response to nutrient deprivation but not for autophagy triggered by other signals, such as TP53 activation or mTOR inhibition (Lee et al. 2008).

In aggregate, considerable evidence supports the idea that HATs and HDACs play a pivotal role in governing autophagic flux. Accompanying mechanistic studies have revealed that HATs and HDACs regulate autophagy at multiple levels. One is through interacting with the upstream signaling pathways that trigger autophagy. Another is by regulating expression of autophagy-related proteins through acetylation of transcription factors, notably FoxO (forkhead box, subfamily O) proteins. Another is through regulation of the activity of essential autophagy-related proteins themselves. Finally, acetylation of structural proteins regulates the process of trafficking of autophagic structural components (Fig. 10.3).

10.4.3 Level I, Regulating Upstream Signaling Pathways: Acetylation of Proteins in the mTOR Pathway

In hepatocellular carcinoma (HCC) cells, two potent HDAC inhibitors, OSU-HDAC42 and SAHA, induce autophagy. SAHA and OSU-HDAC42 induce autophagy through downregulation of AKT/mTOR signaling and induction of the ER stress response (Liu et al. 2010). Inhibition of autophagy by 3-MA or ATG5 silencing reduces SAHA-induced cytotoxicity, suggesting that SAHA-induced autophagy contributes to cell death (Liu et al. 2010). In lymphoma cells, co-treatment with VPA and temsirolimus (mTOR inhibitor) synergistically inhibits tumor cell growth and triggers autophagy and cell death. Indeed, cell death may be caused by excessive autophagy (Dong et al. 2013). The class I HDAC inhibitor, VPA, potentiates the effect of temsirolimus on autophagy through inhibiting HDAC1. Loss of function of HDAC1 attenuates VPA-mediated regulation of CDKN1A (cyclin dependent kinase inhibitor 1A), CDKN1B, and LC3-I/II (Dong et al. 2013). However, loss of HDAC1 function induces regression of tumor cell growth and activation of autophagy. Furthermore, VPA antagonizes temsirolimus-induced AKT activation via HDAC3 inhibition (Dong et al. 2013). Loss of function of HDAC3 abolishes the ability of VPA to modulate AKT phosphorylation, suppressing tumor cell growth and inducing autophagy (Dong et al. 2013). These strong antitumor effects by HDAC and mTOR inhibition may be tumor cell-specific, sparing normal hematopoiesis *ex vivo* (Dong et al. 2013). In NRVMs, the induction of autophagy is likely through inhibiting HDAC1 and up-regulation of TSC2 (tuberous sclerosis complex 2). Autophagy induced by HDAC1 inhibition abrogates pressure overload-induced hypertrophy in mouse heart (Morales et al. 2016) (Fig. 10.4).

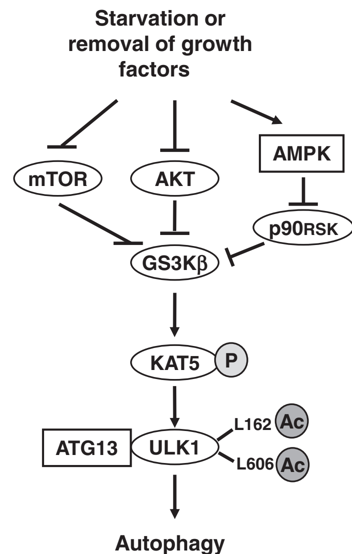

Fig. 10.4 Level I: Acetylation regulates autophagy through the mTOR-ULK1 cascade. In the setting of deprivation of growth factors or nutrients, GSK3-dependent inhibition by mTOR, AKT, and AMPK (through p90) is relieved. Subsequently, activated GSK3 phosphorylates the KAT5 acetyltransferase, which activates ULK1 kinase through acetylation. Acetylated ULK1 then binds to ATG13 and activates the autophagy cascade

10.4.4 Level II, Regulation of ATG Proteins at the Transcriptional Level: Acetylation of FoxO Transcription Factors

FoxO (Forkhead box-containing, family O) proteins are evolutionarily conserved transcription factors involved in a wide range of biological processes. There is only a single, unique dFoxO in Drosophila, whereas several mammalian orthologs exist, including FoxO1, FoxO3, FoxO4, and FoxO6 (Van Der Heide et al. 2004; Juhasz et al. 2007). FoxO transcription factors regulate cell cycle, proliferation, cell death, DNA repair, metabolism, protection from oxidative stress, and autophagy (Greer and Brunet 2005; Yang and Hung 2011).

Autophagy is tightly regulated by FoxO-dependent gene transcription. During fasting, dFoxO induces the transcription of ATGs, thereby inducing autophagy. In mammalian cardiac and skeletal muscles, overexpression of FoxO1 or FoxO3 results in expression of ATGs and induction of autophagy (Mammucari et al. 2007; Zhao et al. 2007a; Sengupta et al. 2009). FoxO factors are governed themselves by multiple posttranslational modifications regulating subcellular localization, DNA binding, and transcriptional properties (Van Der Heide et al. 2004; Boccitto and Kalb 2011). In fed conditions, EP300-CREBBP acetyltransferases promote FoxO1 and FoxO3 acetylation, which results in decreased DNA binding activity and increased sensitivity to phosphorylation. This results in the downregulation of FoxO1-mediated transcriptional regulation of ATGs (Matsuzaki et al. 2005). Consistent with this, overexpression of the HAT, EP300, significantly increases FoxO1 acetylation and inhibits autophagy (Hariharan et al. 2010). This acetylation event decreases the DNA binding efficiency of FoxO1 and promotes its subsequent phosphorylation by AKT1 (Matsuzaki et al. 2005). Stimulation by growth factors or insulin triggers FoxO1 and FoxO3 phosphorylation via AKT1. Subsequent acetylation leads to FoxO dissociation from DNA and transport from the nucleus into the cytoplasm through exportin 1/chromosomal maintenance 1 (XPO1/CRM1)-mediated transport. This, in turn, diminishes transcription of ATGs and autophagic activity (Tzivion et al. 1813).

The acetylation status of FoxO proteins is also regulated by class III HDACs, so-called sirtuin deacetylases. SIRT1 controls the subcellular localization and activity of FoxO1. Upon starvation, SIRT1 deacetylates at the LXXLL motif, which increases the transcription of the *PNPLA2/ATGL* (patatin-like phospholipase domain containing 2/adipose triglyceride lipase) gene encoding a rate-limiting lipolytic enzyme (Chakrabarti et al. 2011). The production of PNPLA2, a major lipase for fat mobilization from lipid droplets in mammals and in Drosophila, results in increased usage of fat to generate energy (Zimmermann et al. 2004; Gronke et al. 2005). Similarly, SIRT2 also deacetylates FoxO1 and FoxO3 in the setting of caloric restriction (Wang and Tong 2009; Wang et al. 2007). Of note, the expression of SIRT2 is upregulated in starved conditions, which highlights its important role in FoxO deacetylation and in promoting lipid remobilization (Wang and Tong 2009). At the same time, autophagic flux is also activated. In skeletal muscle, SIRT1 can deacetylate FoxO3, leading to transcription of genes involved in autophagosome formation, including *MAP1LC3*, *PIK3C3/VPS34*, *GABARAPL1*,

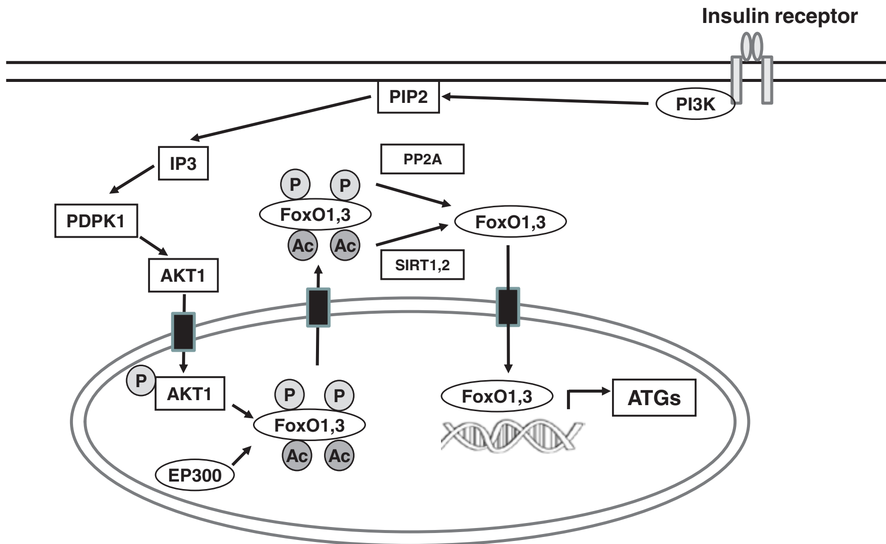

Fig. 10.5 Level II: Acetylation regulates expression of core autophagy proteins through FoxO transcription factors. FoxO1 and FoxO3 are required for the transcription of essential autophagy-related proteins. They are regulated by both acetylation and phosphorylation. Under fed conditions, EP300-CREBBP acetyltransferase increases FoxO1/3 acetylation, which results in decreasing their DNA binding activity and in increasing their sensitivity to phosphorylation. At the same time, insulin signaling activates AKT1 leading to phosphorylation of FoxO1/3. This results in the dissociation of FoxO1/3 from DNA and subsequent export from the nucleus. Upon starvation, SIRT1 induces autophagy through the deacetylation of FoxO1/3. The dephosphorylation of FoxO1/3 is carried out by protein phosphatase 2A (PP2A)

ATG12, *ATG4*, *BECN1*, *ULK1*, and *BNIP3* (Mammucari et al. 2007; Kume et al. 2010) (Fig. 10.5).

An additional mechanism of autophagy regulation by FoxO1 is transcription independent. In cancer cells exposed to serum starvation, FoxO1 is dissociated from SIRT2, resulting in the acetylation of FoxO1. Hyperacetylated FoxO1, in turn, interacts with ATG7 directly and induces autophagy (Kuo et al. 2010). In so doing, FoxO1 couples cellular metabolic status with autophagy regulation. Under conditions of starvation, acetylation-activated FoxO1 and FoxO3 not only increase lipase expression but also increase autophagy. This is achieved by transcriptional upregulation of core autophagy genes and/or by direct protein-protein interaction with ATG7 (Kuo et al. 2010).

10.4.5 Level III, Direct Regulation of ATG Activity by Modification of the Acetylation Status of Core Components

As discussed above, the ATG proteins work in concert to carry out the autophagic process through phagophore formation and elongation. There are four complexes

involved: (1) the kinase complex ATG1–ATG13; (2) the phosphatidylinositol-3-kinase complex I (containing BECN1, ATG14, PIK3C3/VPS34, and PIK3R4/VPS15); (3) two ubiquitin-like protein conjugation complexes (ATG12–ATG5-ATG16L1, along with ATG7 and ATG10); and (4) LC3–PE, with ATG4, ATG7 and ATG3) (Yang and Klionsky 2010). There are also two highly characterized HAT-HDAC pairs, EP300-sirtuins and MYST-HDAC3/RPD3, which regulate the acetylation status and activities of multiple ATG proteins.

10.4.5.1 MYST and HDAC3

These proteins are best characterized in macroautophagy occurring in yeast. MYST acetyltransferases are defined by a conserved histone acetyltransferase domain called MYST which harbors a C_2HC zinc finger and an AcCoA binding domain (Sapountzi and Cote 2011). MYST acetyltransferase includes KAT5/TIP60 and the yeast ortholog, ESA1. Mammalian HDAC3 and the yeast ortholog RPD3 catalyze the reverse reaction. Under conditions of growth factor deprivation or starvation, mTOR, AKT1 and the mitogen-activated protein kinase (MAPK) pathways are suppressed and GSK3 is activated. Then GSK3 phosphorylates KAT5/TIP60 acetyltransferase, resulting in its activation, which in turn acetylates and activates the autophagy-initiation kinase ULK1, an event essential to starvation-induced autophagy (Lin et al. 2012). In *S. cerevisiae*, ESA1, the ortholog of KAT5, acetylates ATG3 at K19 and K48 under conditions of starvation. Next, the activated ATG3 interacts with ATG8, promoting lipidation of ATG8. The reverse reaction is accomplished by the deacetylase RPD3, the ortholog of HDAC3 (Yi et al. 2012). Interestingly, an antagonistic function of the class I family member RPD3 (deacetylase) and of another MYST protein, chm/chapeau (acetyltransferase), has been reported (Grienenberger et al. 2002; Miotto et al. 2006) (Fig. 10.6).

10.4.5.2 EP300-CREBBP and Sirtuins

In mammalian cells, the HAT, EP300 acetyltransferase physically co-localizes with ATG7 during starvation and promotes hyperacetylation of ATG7 (Lee and Finkel 2009). Hyperacetylated ATG7 is inactive, which suppresses phagophore formation. The converse reaction is carried out by the sirtuin SIRT1, which can deacetylate ATG7, as well as ATG5, LC3, and ATG12, to promote autophagic vesicle formation (Lee et al. 2008). Other lines of evidence derive from studies employing the specific SIRT1 inducer, resveratrol, and an acetyltransferase inhibitor, spermidine, which together promote deacetylation of ATG5 and LC3 (ATG8), synergizing to induce autophagy (Morselli et al. 2011). Additional work has shown that resveratrol and spermidine induce changes in the acetylation status of 170 proteins whose activity is connected to autophagy control (Marino et al. 2011). Interestingly, HAT and HDAC proteins harbor lysine sites that may be regulated internally. For example, SIRT2 controls the self-acetylation of EP300, which can also acetylate SIRT2 to inhibit its enzymatic activity (Black et al. 2008; Han et al. 2008) (Fig. 10.6).

AcCoA is a major integrator of nutritional status at the crossroads of lipid, carbohydrate, and protein catabolism. Reversible protein acetylation is an elegant means of coupling the regulation of autophagy to the cellular metabolic state. The

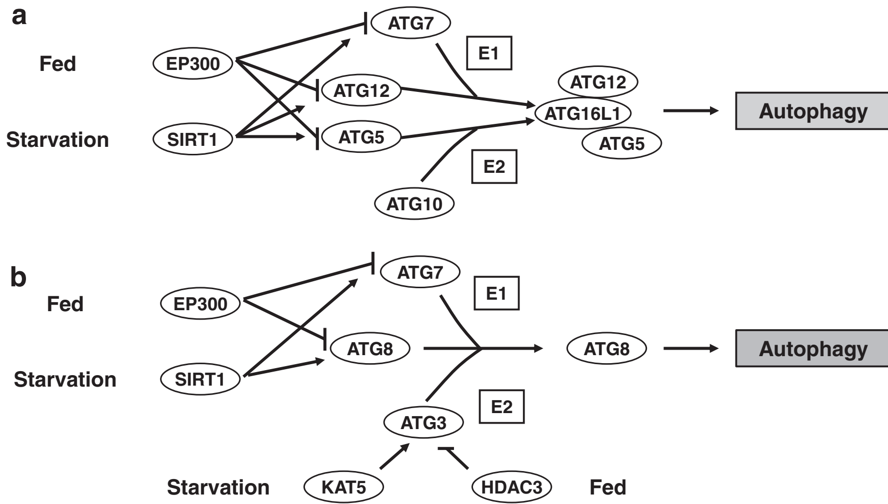

Fig. 10.6 Level III: Acetylation regulates the activity of core autophagy proteins. (**a**) In mammalian cells, the HAT, EP300, deactivates ATG5, ATG7, ATG8, and ATG12 by acetylation to inhibit the elongation step of autophagy. Conversely, upon starvation, SIRT1 deacetylates these ATG proteins and induces autophagy. (**b**) Results in yeast support the antagonistic roles of HATs and HDACs. Starvation induces acetylation of ATG3 by Esa1 (KAT5) increasing its interaction with ATG8 (LC3) and ATG8 lipidation, which induces autophagy. Rpd3 (HDAC3) deacetylates ATG3 leading to the inhibition of autophagy

maintenance of cellular energy homeostasis requires the intracellular storage and usage of lipids, and autophagy can play an important role in regulating intracellular lipid mobilization (macrolipophagy) (Singh et al. 2009; Dong and Czaja 2011; Singh and Cuervo 2012). Starvation triggers rapid depletion of AcCoA, which, in turn, reduces the activity of the acetyltransferase EP300, along with reduction in the overall acetylation of cytoplasmic proteins and induction of autophagy (Marino et al. 2014). However, lipid mobilization enhances β-oxidation of fatty acids in mitochondria which leads to the production of NADH and AcCoA, which are required for EP300 enzymatic activity, leading to reduced autophagic activity. Multiple distinct manipulations designed to increase or reduce cytosolic AcCoA lead to suppression or induction of autophagy, respectively, both in cultured human cells and in mice (Marino et al. 2014). Moreover, maintenance of high AcCoA levels inhibited maladaptive autophagy in a model of ventricular pressure overload (Marino et al. 2014). High levels of AcCoA induce EP300 activity leading to suppression of autophagy (Marino et al. 2014).

Cytosolic AcCoA functions as a central metabolic regulator of autophagy, and as such, it is of interest to delineate AcCoA-centered pharmacological strategies that allow for the therapeutic manipulation of autophagy. This is particularly the case in cardiomyocyte autophagy, a process pivotal to stress-dependent responses in heart (Lavandero et al. 2015).

10.4.6 Level IV, Regulation of Autophagosome Trafficking and Selective Autophagy

10.4.6.1 Autophagosome Trafficking and Lysosome Fusion Governed by Acetylation of Tubulin

Trafficking of autophagic vesicles is regulated by cytoskeletal structures, and reversible protein acetylation is critically involved here, as well. Microtubule stability and function are regulated by the reversible acetylation of α-tubulin, the first acetylated cytosolic protein described (L'Hernault and Rosenbaum 1985; Piperno and Fuller 1985). The acetylation status of microtubules is coordinated by the HAT, ELP3/KAT9 (Creppe et al. 2009) and by the HDACs, HDAC6 (Hubbert et al. 2002), and SIRT2 (North et al. 2003). These enzymes also regulate actin dynamics, stress signaling and exocytosis (Holmberg et al. 2002; Rahl et al. 2005). Upon starvation, acetylation of tubulin at Lys40 increases both in labile and stable microtubule fractions, which, in turn, enhances MAPK/JNK phosphorylation and activation via kinesin family member 1 (KIF1)-dependent mechanisms to promote autophagy. Specifically, MAPK/JNK signaling increases the dissociation of BECN1 (ATG6) from BCL2, releasing it from the repressive effects of BCL2 binding and thereby promoting its association with factors such as microtubules required for initiating autophagosome formation. While the markers of phagophore/autophagosome formation (BECN1, class III PI3K, WIPI1, ATG12–ATG5 and LC3-II) are recruited to labile microtubules, mature autophagosomes (marked with LC3-II) are transported along stable microtubules (Geeraert et al. 2010). Tubulin acetylation is not only important in the trafficking of autophagosomes but is also essential for fusion of autophagosomes to lysosomes to deliver cargo for degradation (Kochl et al. 2006; Xie et al. 2010).

Organelle movement is accomplished by cellular motor proteins functioning along microtubule tracks. Dynein moves toward the slow growing or “minus” ends of microtubules and is responsible for centripetal transport, whereas centrifugal movements are driven by kinesins. Tubulin acetylation at Lys40 increases the recruitment and mobility of KIF1 and dynein (Dompierre et al. 2007; Reed et al. 2006). Fully formed autophagosomes are delivered centripetally by dyneins along the microtubule tracks in the direction of the centrosomes (Geeraert et al. 2010; Ravikumar et al. 2005; Jahreiss et al. 2008; Kimura et al. 2008).

The dynein motor machinery also plays a role in autophagosome-lysosome fusion (Rubinsztein et al. 2005). As a consequence, mutations that influence the dynein motor machinery reduce the efficiency of autophagic clearance of protein aggregates and increase levels of LC3-II (Ravikumar et al. 2005). KIF1 is involved in autophagosome trafficking under basal nutritional conditions. In contrast with its recruitment to microtubules to activate MAPK/JNK, KIF1 is not involved in transporting autophagosomes upon nutrient deprivation. In parallel, dynein participates in transporting autophagosomes both in nutrient-rich and nutrient-depleted conditions (Geeraert et al. 2010). These data collectively establish the importance of tubulin acetylation in autophagic dynamics. Further work is required, however, to

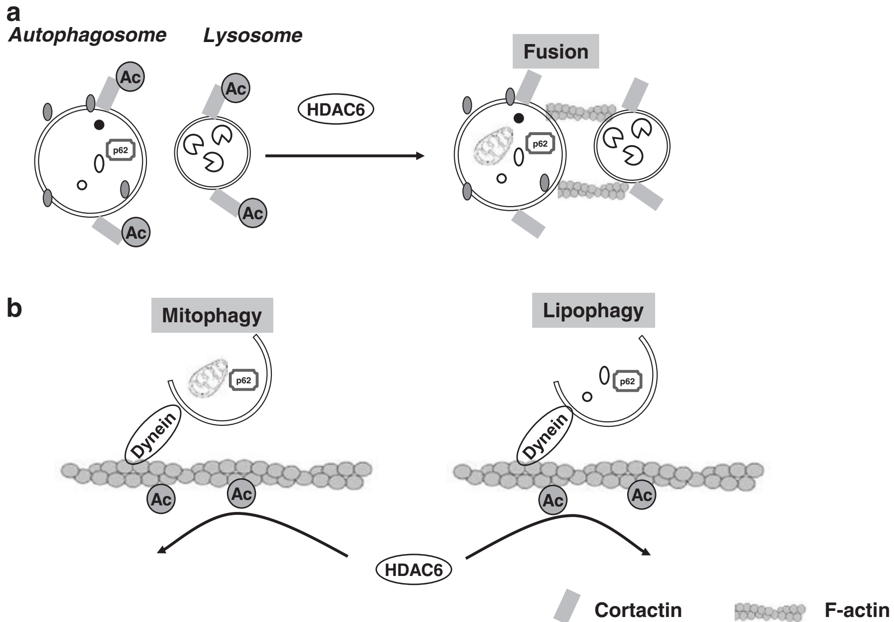

Fig. 10.7 Level IV: Acetylation regulates trafficking and selectivity of autophagy. (**a**) In the fusion step of autophagy, HDAC6 recruits cortactin to the ubiquitinated organelles or protein aggregates. HDAC6 mediates F-actin network assembly, promoting autophagosome-lysosome fusion by deacetylation of cortactin. (**b**) HDAC6 is also responsible for dynein-mediated transport and perinuclear concentration of altered mitochondria and lipid droplets, leading to mitophagy and lipophagy respectively

uncover direct links between HDAC6, SIRT2, and ELP3 activity and acetylated tubulin-controlled autophagy induction.

10.4.6.2 Selective Autophagy Governed by Actin Acetylation

HDAC6 has emerged as a central regulator of special types of autophagy, those which selectively eliminate specific cellular components, such as mitochondria (mitophagy), ER (reticulophagy), portions of the nucleus (nucleophagy), peroxisomes (pexophagy), microorganisms (xenophagy), ribosomes (ribophagy), lipid droplets (lipophagy), or protein aggregates (aggrephagy) (Fig. 10.7) (Lavandero et al. 2013; Lee et al. 2010; Reed et al. 2006; Reggiori and Klionsky 2005). A recent study suggests that a specific form of autophagy, termed quality control (QC) autophagy, selectively disassembles altered, nonfunctioning organelles and protein aggregates (Lee and Yao 2010).

HDAC6 controls selective autophagy by recruiting the actin and tubulin networks. And similar to tubulin, cytoskeletal actin is targeted by acetylation. Ubiquitinated substrates are specifically bound by the ubiquitin-associated

(UBA) domain of autophagy receptors SQSTM1/p62 and NBR1, while damaged mitochondria are specifically recognized by BNIP3L/NIP3-like protein X (NIX) receptors. All autophagy receptors have an LC3-interacting region (LIR) domain, which recruits the membrane to the cargo by binding LC3. HDAC6 recruits cortactin to ubiquitinated protein aggregates or mitochondria and, through cortactin deacetylation, mediates F-actin network assembly to promote autophagosome-lysosome fusion. HDAC6 is also responsible for dynein-mediated transport and perinuclear concentration of altered mitochondria and protein aggregates.

In yeast, actin microfilaments are dispensable for bulk autophagy but required for selective autophagy, such as pexophagy (the selective degradation of peroxisomes). The yeast actin-related protein (Arp)2/3 complex, which is necessary for actin nucleation and F-actin formation, directly regulates the dynamics of ATG9 (Monastyrska et al. 2008). In mammalian cell lines, the actin network also has an important role in selective autophagy. Cortactin, known to interact with F-actin in promoting polymerization and branching, was recently identified as a substrate of HDAC6 (Zhang et al. 2007). HDAC6 induces F-actin network formation around cytosolic aggregated proteins in a cortactin-dependent manner and promotes autophagosome-lysosome fusion. Thus, cortactin-dependent actin and HDAC6 distinguish QC autophagy from starvation-induced autophagy for which these components are dispensable (Lee and Yao 2010; Lee et al. 2010) (Fig. 10.7).

10.5 Autophagy in Cardiovascular Disease and Its Regulation by Acetylation

Autophagy is essential to the homeostasis of cardiomyocytes by governing energy generation, substrate recycling, organelle self-renewal, and degradation of misfolded or dysfunctional proteins (Lavandero et al. 2015). Numerous studies have shown that too much or too little autophagy is detrimental to cardiomyocytes. Maintaining basal levels of autophagy is vital for cardiomyocyte function and viability and yet overactivated autophagic flux can be harmful, too (Lavandero et al. 2013).

Due to limitations of tissue availability and detection methods, little is known about cardiomyocyte autophagy in human disease. Most evidence to date has been obtained from myocardial biopsy samples obtained from patients with heart failure (including patients treated with a left ventricular assist device (LVAD) and cardiac transplants), Danon disease, ischemic heart disease, and cancer chemotherapy cardiomyopathy. In these patients, increases in steady-state autophagy have been documented (Shimomura et al. 2001). However, whether this catabolic process is adaptive or maladaptive remains unknown. Animal models of these diseases have been developed to decipher the role of autophagy in disease pathogenesis and define the effects of novel therapeutic agents, including HDAC inhibitors (Xie and Hill 2013).

10.5.1 Autophagy in the Transition from Hypertrophy to Heart Failure

10.5.1.1 Autophagy in Human Heart Failure

Evidence for autophagy in human heart disease emerged first from tissue samples of dilated cardiomyopathy obtained at the time of cardiac transplantation (Shimomura et al. 2001). Autophagic vacuoles containing cytoplasmic material and organelles were detected within degenerated cardiomyocytes using electron microscopy. In dilated cardiomyopathy hearts, autophagy appeared to be associated not only with clearance of damaged intracellular organelles but also with progressive destruction of cardiomyocytes (Shimomura et al. 2001). In one case, it was inferred that cardiomyocyte "autophagic death" was taking place (Kostin et al. 2003).

These findings suggested that autophagic activity may be a significant contributor to the pathogenesis of heart failure (Kostin et al. 2003; Saijo et al. 2004). For example, in patients with severe aortic valvular stenosis, the progression of LV systolic dysfunction correlates with the degree of cell loss (Hein et al. 2003). Although excessive autophagy in end-stage heart failure can be lethal to cardiomyocytes, basal levels of autophagy are fundamentally required for cell viability. In biopsy samples from patients with idiopathic dilated cardiomyopathy obtained at the time of implantation and explantation of an LVAD, it was noted that mechanical unloading of the ventricle was associated with decreased markers of autophagy (Kassiotis et al. 2009). This suggests that increased levels of autophagy may be an adaptive mechanism in the failing heart and that this phenomenon is attenuated by LVAD support (Kassiotis et al. 2009).

10.5.1.2 Animal Models of Hypertrophy and Heart Failure

The initial response of the heart to increases in afterload or postinfarct stress is hypertrophic growth (Hill and Olson 2008). If the afterload stress persists, the ventricle will eventually dilate, systolic function will decline, and a clinical syndrome of heart failure ensues. This progressive course of disease occurs commonly in patients with hypertension, moderate to severe aortic stenosis, or ischemic heart disease (St John Sutton 2009). In an animal model of pressure overload imposed surgically by transverse aortic constriction (TAC), autophagic flux in cardiomyocytes is activated. For instance, we have reported that autophagic activity increases rapidly after TAC, peaks at 72 h, and is maintained at elevated levels for at least 3–4 weeks (Zhu et al. 2007). The degree of autophagic activity correlates with the magnitude of hypertrophic growth and with the rate of transition to heart failure (Zhu et al. 2007); further, levels of autophagic flux correlate with the degree of hypertrophy (Cao et al. 2011). Consistent with these findings, cardiomyocyte-specific overexpression of a rate-limiting protein in the autophagic cascade, Beclin 1, provokes increased autophagic activity in the setting of pressure stress. These mice have exaggerated hypertrophic growth and early transition to ventricular dilation, systolic dysfunction, and mortality (Cao et al. 2011; Zhu et al. 2007). Conversely, suppression of autophagy in the context of Beclin 1 haploinsufficiency halved the stress-induced autophagic response and partially rescued the hypertrophic phenotype (Zhu et al. 2007).

10.5.1.3 HDACs in Cardiac Hypertrophy and Failure

Collectively, data such as these suggest that excessive autophagy can be maladaptive under conditions of pressure overload, a common clinical scenario in hypertension and aortic stenosis. One potential mechanism for this disease-promoting behavior is that autophagy may facilitate hypertrophic growth and allow for the sustenance of greater degrees of hypertrophy. Later, cell death induced by excessive autophagy may facilitate the onset of systolic dysfunction and heart failure. In this context, afterload-induced excessive cardiomyocyte autophagy is a potential target for therapeutic intervention. As the broad-spectrum HDAC inhibitor, trichostatin A (TSA), inhibits hypertrophic growth in mice subjected to TAC (Kong et al. 2006), we tested whether HDAC inhibition reduces autophagy in this scenario. We hypothesized that HDAC-dependent pathological autophagy may contribute to the disease process and that suppression of pathological autophagy by HDAC inhibition may underlie their beneficial effects. Consistent with this model, we found that HDAC inhibition suppresses load-induced cardiomyocyte autophagy, and this autophagic response is required for much of the pathological growth response (Cao et al. 2011) (Fig. 10.8).

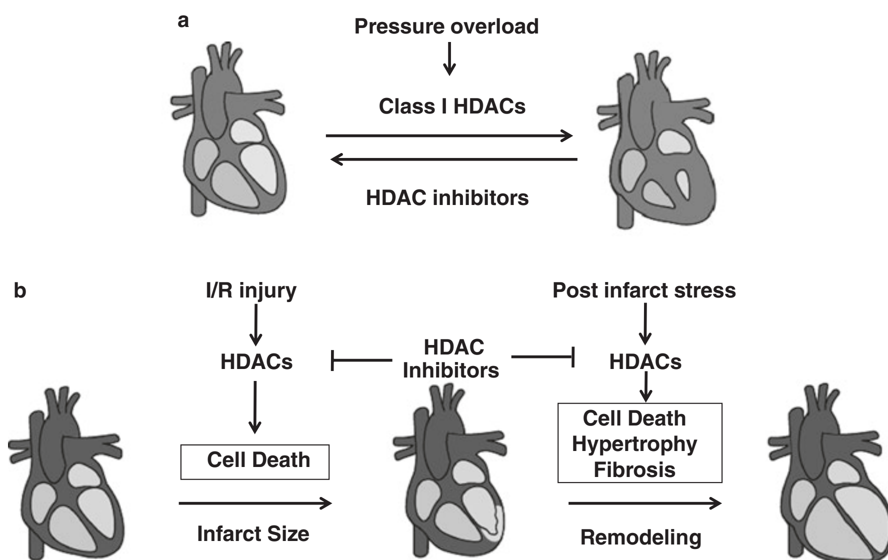

Fig. 10.8 HDAC inhibition in hypertrophic and ischemic heart disease. (**a**) Class I HDACs participate in pressure-overload induced cardiac hypertrophy. HDAC inhibitors are capable of reversing established hypertrophy through inhibiting the catalytic activity of class I HDACs. (**b**) Ischemic or ischemia/reperfusion stresses provoke cell death through HDACs. Blocking HDAC activity reduces infarct size possibly through normalizing autophagic flux. Postinfarct stresses on the myocardium induce cardiomyocyte hypertrophy, cell death (apoptosis) and fibrosis. HDAC inhibitors block these detrimental remodeling processes in part by manipulating cardiomyocyte autophagy (Adapted from reference Xie and Hill (2013))

TSA inhibits both class I and II HDACs. Therefore, it is of interest to determine which HDAC isoforms(s) are involved in the hypertrophic growth of the heart induced by afterload stress. Recent studies suggest that class I HDACs are responsible for pathological hypertrophy. Genetic models of HDAC1 and HDAC2 gene silencing demonstrate that these isoforms can compensate for one another in their ability to promote cardiomyocyte growth (Montgomery et al. 2007). However, double knockout of HDAC1 and HDAC2 is lethal at P14 and, as a result, characterization of these enzymes in adult heart has been lacking (Montgomery et al. 2007). An additional model, one in which HDAC2 is silenced specifically in the heart manifests inhibition of hypertrophy (Trivedi et al. 2007).

This question has also been addressed using inhibitors specific for class I HDACs, which demonstrated significant suppression of hypertrophic growth in heart exposed to pressure overload (Gallo et al. 2008). Overall, additional characterization of the role of class I HDACs in cardiac hypertrophy is warranted.

Data from our lab have shown that repression of class I HDACs suppresses mTOR activity in several forms of cardiac hypertrophy. Inhibition of class I HDACs induces *tuberous sclerosis complex 2* (*TSC2*) mRNA levels, which is necessary for the inhibition of mTOR (Morales et al. 2016). Overall, we propose a model in which TSC2-dependent repression of mTOR is relieved in the setting of hypertrophic growth triggers. However, when class I HDACs are inhibited, transcription of *TSC2* is induced, resulting in the formation of a TSC1/TSC2 complex, leading to reduction of mTOR activity, and repression of pathological hypertrophic growth. The mechanism by which inhibition of class I HDACs increases TSC2 levels is currently under investigation.

Whereas excessive cardiomyocyte autophagy can be detrimental, complete abrogation of the catabolic response is similarly maladaptive. For example, knockout of a rate-limiting autophagic protein, ATG5, in the heart triggers rapid-onset heart failure (Nakai et al. 2007). Consistent with this, blocking autophagy using systemic inactivation of the gene coding for the lysosomal enzyme cysteine endopeptidase cathepsin L (Ctsl) was associated with emergence of dilated cardiomyopathy (Dennemarker et al. 2010). Nutrient deprivation and mTOR suppression with rapamycin, two robust triggers of autophagy, failed to reactivate autophagic flux. Thus, impaired degradation of autolysosomal content in the absence of Ctsl can be a major mechanism underlying this cardiomyopathic phenotype (Dennemarker et al. 2010).

These observations, then, are consistent with the idea that basal levels of cardiomyocyte autophagy are critically required from cellular proteostasis. Based on this, we favor a model where titration of cardiomyocyte autophagy within an optimal, adaptive zone is an approach of therapeutic interest (Rothermel and Hill 2008). Interestingly and importantly, chronic HDAC inhibition suppresses, but does not eliminate, the autophagic response to stress and hence is an attractive strategy worthy of additional investigation (Cao et al. 2011). Similarly, HDAC inhibition restored basal autophagic flux in the setting of ischemia/reperfusion injury (see below) (Xie et al. 2014). Thus, we believe that HDAC inhibition is an attractive strategy for therapeutic manipulation of cardiomyocyte autophagy, one worthy of additional study.

10.5.2 Autophagy in Ischemic Heart Disease: Ischemia/Reperfusion Injury

10.5.2.1 Autophagy in Human Ischemic Heart Disease

Cardiomyocyte autophagy is a prominent feature in ischemic disease (Shimomura et al. 2001). That said, samples of tissue from patients with ischemic heart disease are typically confounded by co-existing heart failure (Shimomura et al. 2001). Again, due to the difficulty in obtaining human samples and lack of feasible genetic studies, most of our knowledge of autophagy in ischemic heart disease derives from preclinical, animal models. Activation of cardiomyocyte autophagy has been reported in a porcine model of chronic ischemia/reperfusion (I/R) (Yan et al. 2005), and rodent I/R models have been employed extensively in studies designed to delineate mechanism (Kong et al. 2006).

10.5.2.2 Autophagy in Animal Models of Ischemic Heart Disease

Multiple studies have demonstrated that cardiomyocyte autophagy is activated during prolonged ischemia and that suppression of that autophagic activity can be maladaptive (Matsui et al. 2007; Zhang et al. 2008). Underlying mechanisms may relate to autophagy-dependent replenishment of cellular metabolic substrates in the setting of their inadequacy coupled with elimination of dysfunctional mitochondria, which would otherwise release reactive oxygen species (ROS) and pro-apoptotic mediators (Zhang et al. 2008). However, most patients with ischemic heart disease recanalize their coronary vessels either spontaneously or by means of therapeutic intervention. As such, in most clinical scenarios, myocardial ischemia is coupled with restoration of blood flow and reperfusion injury, including robust release of ROS, calcium dysregulation, inflammation, and impaired ATP production (Yellon and Hausenloy 2007). In one study, reperfusion following coronary artery occlusion triggered robust increases in steady-state autophagy (Hariharan et al. 2011). Multiple other studies, conducted in tissue culture (Gurusamy et al. 2009), rodents (Zhu et al. 2007), and large animal models (Yan et al. 2005), have shown marked activation of steady-state autophagy during the reperfusion phase. Oxidative stress is thought to be a major underlying mechanism (Hariharan et al. 2011).

However, whether this upregulated autophagy is adaptive or maladaptive is the subject of debate. In heterozygous Beclin 1-null mice, I/R-induced autophagy was significantly attenuated compared with wild-type controls. Following experimental I/R, these mice developed smaller infarctions and less apoptosis (Matsui et al. 2007). Similar findings have been reported in cultured NRVMs exposed to simulated I/R, where chemical suppression of autophagy with 3-methyladenine (3-MA) improved cell viability (Valentim et al. 2006). In contrast, it has been reported that simulated I/R-induced autophagy in cultured cell lines can be protective (Gurusamy et al. 2009; Yitzhaki et al. 2009). Recently, it was reported that sulfaphenazole, an inhibitor of cytochrome P450-2C9, activates autophagy and is protective against I/R injury, both in tissue culture and in isolated perfused rat hearts (Kuo et al. 2010).

Based on these data, conflicting models of the role of autophagy in cardiac injury during I/R have been proposed, with some suggesting a detrimental role

and others a cardioprotective role (Hariharan et al. 2011; Ma et al. 2012a). At present, it is unclear the extent to which these discrepancies derive from differing cell types, model systems, or experimental paradigms. For example, it is important to distinguish measurements of steady-state autophagy from bona fide autophagic flux (the effective clearance and recycling of damaged organelles). A recent report using in vivo measurements of autophagic flux may have clarified these differences, suggesting that accumulation of autophagosomes after I/R damage due to impaired autophagic flux may have contributed to the confusion (Ma et al. 2012a, b).

10.5.2.3 HDACs in Ischemic Heart Disease

As mentioned above, HDAC inhibition has been shown to reduce cardiomyocyte autophagy in the context of prolonged treatment and thereby prevent pressure overload-induced hypertrophy (Kong et al. 2006). Multiple preclinical studies have demonstrated potent cardioprotective benefits of HDAC inhibitors in murine models of myocardial stress, including I/R (Kong et al. 2006; Antos et al. 2003; Zhao et al. 2007b; Granger et al. 2008). TSA, as well as another HDAC inhibitor scriptaid, reduces myocardial infarct size up to 50 % (Zhao et al. 2007b; Granger et al. 2008). Of note, HDAC inhibitor delivery as late as one hour after the onset of reperfusion still reduced infarct size to an extent similar to pretreatment (Granger et al. 2008). Furthermore, HDAC inhibition may promote myocardial repair and prevent cardiac remodeling, which seems to be dependent on c-kit signaling (Zhang et al. 2012) (Fig. 10.8).

In our lab, we tested SAHA, an HDAC inhibitor FDA-approved for cancer treatment, in a large animal (rabbit) I/R model (Xie et al. 2014). Experiments were carried out in a blinded fashion with all the rigor that is used in a human clinical trial. Rabbits were randomized into three groups: vehicle control, SAHA pretreatment (one day prior and at surgery), and SAHA treatment at the time of reperfusion only. Each arm was subjected to I/R surgery. SAHA reduced infarct size and partially rescued systolic function when administered either before surgery (pretreatment) or solely at the time of reperfusion. Additionally, cultured neonatal and adult rat ventricular cardiomyocytes were subjected to simulated I/R (sI/R) to identify mechanisms of SAHA-induced cardioprotection. Interestingly, in the infarct border zone, SAHA increased autophagy as measured by LC3-II levels and GFP-LC3 puncta formation, findings which were verified by electron microscopy. Furthermore, SAHA increased autophagic flux in the infarct border zone assayed using a tandem reporter RFP-GFP-LC3 transgenic mouse. In cultured myocytes subjected to sI/R, SAHA pretreatment reduced cell death by 40 %. This reduction in cell death correlated with increased autophagic flux in SAHA-treated cells. RNAi-mediated knockdown of ATG7 and ATG5, essential autophagy proteins, abolished SAHA's cardioprotective effects. We conclude that the cardioprotective effects of SAHA during I/R occur, at least in part, through the induction of autophagic flux. Importantly, SAHA plasma concentrations in the rabbits were similar to those achieved in cancer patients (Xie et al. 2014).

Thus, data from our lab support a model of decreased autophagic flux during reperfusion and the protective nature of restoring autophagic flux back to basal

levels in limiting reperfusion injury. Indeed, we have reported that reinduction of I/R-suppressed cardiomyocyte autophagy by short-term treatment with HDAC inhibitors at the time of reperfusion affords significant cardioprotection (Xie et al. 2014). Again, HDAC inhibition is unique in that it can both suppress excessive cardiomyocyte autophagic flux during pressure-overload (long-term treatment) and restore I/R-suppressed autophagy (short-term treatment). In light of this, HDAC inhibition might be an effective therapeutic measure in reperfusion injury, which lacks efficacious therapy in the clinical setting (Xie et al. 2014; Yellon and Hausenloy 2007; Turer and Hill 2010).

10.5.3 Autophagy in Anticancer Drug-Induced Cardiomyopathy

Cancer chemotherapy, particularly with anthracyclines, has long been associated with significant cardiotoxicity, cardiomyopathy, and heart failure. However, the fact that cancer patients are typically treated with multiple drugs in combination has made it difficult to pinpoint a unique culprit. Again, availability of human tissues in this context is rare. However, in a rat model of doxorubicin-induced cardiomyopathy, cardiomyocyte autophagy was implicated as a catabolic pathway active in the development of heart failure (Lu et al. 2009; Li and Hill 2014). Furthermore, in cancer patients treated with the reversible proteasome inhibitor bortezomib, drug-related cardiotoxicity has been observed (Nowis et al. 2010). Rats exposed to bortezomib developed heart failure, and ER stress and upregulated autophagy have been described (Nowis et al. 2010).

Doxorubicin-induced cardiomyopathy has been studied extensively (Lu et al. 2009; Kobayashi et al. 2010). Current management of doxorubicin-induced cardiotoxicity includes regular monitoring of cardiac function and limiting the maximum dose of exposure. Given these inadequate tools, it is important to identify precise underlying mechanisms. Cardiomyocyte death by apoptosis and necrosis is thought to be the primary mechanism of doxorubicin-induced cardiomyopathy. In a rat model of doxorubicin-induced cardiomyopathy, pharmacological suppression of autophagy was associated with significant rescue of cardiac function (Lu et al. 2009). Furthermore, doxorubicin-induced autophagic vacuoles were blocked by 3-MA in failing rat heart (Lu et al. 2009). A recent report showed that the transcription factor GATA4 inhibits doxorubicin-induced autophagy and cardiomyocyte death by upregulating the survival factor BCL2 and downregulating autophagy-related genes (Kobayashi et al. 2010). Given the favorable effects of HDAC inhibition on cardiomyocyte autophagy (Xie et al. 2014; Cao et al. 2011). HDAC inhibitor therapy could be considered for further characterization.

Recent work from our group has shown that doxorubicin blocks autophagic flux in cardiomyocytes by impairing lysosome acidification and lysosomal function (Li et al. 2016). We went on to demonstrate that reducing autophagy initiation protects against doxorubicin cardiotoxicity, possibly by decreasing demand on a dysfunctional system, allowing for the proper processing of lysosomal content (Li et al. 2016).

10.5.4 Autophagy in Glycogen Storage Disease-Related Cardiomyopathy

Glycogen storage disease can present as hypertrophic cardiomyopathy (Arad et al. 2005; Tanaka et al. 2000; Nishino et al. 2000). This is particularly the case for Danon disease, a condition characterized by defective autophagosome-lysosome fusion owing to a mutation in the gene coding for the lysosomal membrane protein LAMP2. Consequent accretion of unprocessed autophagosomes provokes cardiomyopathy (Tanaka et al. 2000). In a mouse model of Pompe disease, a disorder marked by defective metabolism of glycogen due to insufficiency of lysosomal acid alpha-glucosidase, suppression of the initiation steps of autophagy by inactivating ATG7 facilitates successful enzyme replacement therapy (Raben et al. 2010). A novel LAMP-2-positive dilated cardiomyopathy has also been reported (Sugimoto et al. 2007). This late onset cardiomyopathy is characterized by increased autophagic vacuoles, but its clinical features are similar to Danon disease (Sugimoto et al. 2007). As short-term HDAC inhibition can induce basal levels of cardiomyocyte autophagy (Xie et al. 2014; Cao et al. 2011), HDAC inhibition could be considered for further study in glycogen storage disease-related cardiomyopathy.

Conclusions

Autophagy, a highly conserved physiological process, is regulated at multiple levels by reversible protein acetylation. Further, autophagy is involved in the pathogenesis of numerous cardiovascular diseases, and HDAC inhibition is effective in preventing hypertrophy and the onset of heart failure. Equally important, HDAC inhibition reduces infarct size during I/R and blunts the onset of heart failure. Thus, convergence exists around these biological processes which warrants further characterization.

In animal studies, pressure overload can induce excessive autophagy and I/R reduces basal autophagy, events which can be maladaptive and promote cell death. Long-term HDAC inhibition can prevent hypertrophy and heart failure by blunting excessive autophagy. Interestingly, short-term HDAC inhibition also restores I/R-induced suppression of autophagy back to basal levels, reducing infarct size. This surprising, bidirectional regulation of autophagy by HDAC inhibition is, in fact, a favorable characteristic for therapeutic exploitation.

10.6 Perspectives

Use of HDAC inhibitors in the treatment of hypertrophy or reducing infarct size in patients may be on the horizon. However, formulating the timing and dosing of HDAC inhibitor therapy requires further understanding of HDAC-dependent control of autophagy. Regulation of autophagy by HDACs is most widely studied in cancer biology, and much work remains to delineate the precise role of HDACs in cardiomyocyte autophagy. However, prospects for translation to the clinical context are promising.

Conflicts of Interest None

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Sirtuins as Regulators of Cardiac Hypertrophy and Heart Failure

11

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Abstract

Sirtuins are emerging as key regulators of a number of biological functions ranging from cell growth, differentiation to longevity. Mammalian genome encodes seven sirtuins isoform (SIRT1–SIRT7), which are distributed in different compartment of the cells. Sirtuins need NAD^+ for their enzymatic reaction. Because of their dependency on NAD, they can respond to energy need of the cells and modulate key enzymatic pathways relevant to the maintenance of cellular health. Throughout the evolution, from prokaryotes to mammals, sirtuins are mainly valued for their ability to bestow cells with efficient adaptation to stress conditions. Sirtuins target histone and nonhistone proteins to ameliorate varied pathophysiologic conditions by controlling gene transcription and fitness of mitochondria. Many sirtuin isoforms have been also studied for their roles in regulating cardiac development and protecting the heart from pathological stress. In this review, we will discuss impact of sirtuins in overall health of the cardiovascular system.

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11.1 Introduction

Heart failure (HF) remains an important clinical and societal problem. In the United States alone, about 600,000 people die of heart disease, and approximately 550,000 new cases are recognized annually. It is estimated that there are nearly five million Americans currently living with congestive heart failure (CHF) and it is the first listed diagnosis in around 900,000 hospitalizations each year (Go et al. 2013). Although there have been important advances in our understanding of the mechanisms, etiologies, and treatments of HF over the past two decades, the morbidity and mortality attributed to HF continues to rise (Massie and Shah 1997). In general terms, HF is recognized as a pathophysiological state in which the heart is unable to pump blood at a rate commensurate with the requirements of the metabolizing tissues. HF is usually caused by a defect in myocardial contraction. It can arise from multiple conditions such as coronary heart diseases (CHD), high blood pressure, metabolic disorders, excessive alcoholism, and also as a consequence of aging. In addition, genetic factors could also contribute to the evolution of HF. Defect in myocardial contractile function causing HF may result from decrease in number of viable fully functional myocytes, decrement in the function of viable myocytes, or alterations to the intrinsic contractile activity of individual myocytes (Anversa et al. 1990). Several functional abnormalities involving calcium homeostasis, contractile protein function, mitochondrial bioenergetics, and cytoskeleton rearrangement have been identified in the hypertrophied and failing myocardium (Colucci 1997; Frey and Olson 2003; Katz 1990). At the molecular level, alterations have been observed in the expression of numerous genes that are central to the normal structure and function of the myocardium; however, the basic mechanism of gene dys-regulation that contributes to the evolution of HF is not yet fully understood.

There are multiple layers of controls involved in gene expression, which include location of regulatory sequences such as promoters and enhancers in the gene, various histone modifications, and multiprotein complexes that remodel chromatin (Bernstein et al. 2007). Common epigenetic modifications, such as methylation and acetylation of histones (Allfrey et al. 1964) or chromatin-associated nonhistone proteins, also play critical roles in gene expression (Thiagalingam et al. 2003; Wu et al. 2009). One such epigenetic modification is reversible acetylation of specific lysine residues in the histones and nonhistone proteins (Glozak et al. 2005). This type of modification has gained significant interest in recent years because of its versatile role in the regulation of diverse cellular pathways (Norris et al. 2009; Spange et al. 2009). Defects in protein lysine acetylation have been attributed to several human diseases including heart failure (Batta et al. 2007; Morris 2013).

11.2 Role of Lysine Acetylation as an Epigenetic Modifier

Protein acetylation is carried out by the addition of an acetyl moiety to the epsilon-amino group (ε) of lysine residues within a protein. In this process, acetyl group from acetyl-coenzyme A (acetyl-CoA) is added to the lysine residue by either an

enzymatic or a nonenzymatic mechanism (Wagner and Hirschey 2014). Enzymatic addition is facilitated by a class of enzymes called histone acetyl-transferases (HATs), while the nonenzymatic addition depends on various factors, such as concentration of acetyl-CoA and pH of the milieu interior of the cell. It is proposed that acetyl-CoA concentrations can be a determining factor for the metabolic status of the cell (Cai and Tu 2011). Since acetyl-CoA is necessary for acetylation reaction, it can influence activation of gene expression via acetylation of regulatory factors and/or histones. Histones that comprise the core of nucleosomes when acetylated get repelled from the negatively charged DNA, thereby facilitating access of transcription factors to promoters and enhancers of the gene. The reverse direction of this reaction is removal of acetyl group from the lysine residue of the histones, a process called deacetylation. Histone deacetylation results in compaction of the chromatin, and thus, it shuts off the gene transcription (Wolffe 1996). Deacetylation of histone or nonhistone proteins is carried out by a group of enzymes termed as lysine deacetylases (HDACs or KDACs).

Recent studies have brought HDACs into limelight because of the wide spectrum of involvement they demonstrate in different facets of cellular processes. HDACs are classified into four classes and comprise of 18 members in eukaryotes (Ekwall 2005). Class I HDACs that include HDAC 1, 2, 3, and 8 are largely known to associate with multiprotein chromatin-remodeling complexes in the nucleus. However, HDAC3 was also found in cytoplasm where it regulates contractility of myofilaments (Samant et al. 2011). Class II HDACs include HDAC 4, 5, 6, 7, 9, and 10 that can shuttle between nucleus and cytoplasm based on the cellular cues. HDAC11 is separately classified into Class IV. These three classes of HDACs are zinc-dependent enzymes. In the heart, majority of Class I HDACs seems to act as pro-hypertrophic molecules (Backs and Olson 2006). HDAC2 overexpression in mouse heart has been shown to induce hypertrophy through activation of PI3K–Akt–GSK3β-signaling pathway (Trivedi et al. 2007). On the other hand, members of Class II HDACs repress activity of several pro-hypertrophic transcription factors including GATA4, NFAT, MEF2, SRF and thus act as antihypertrophic agents (Backs and Olson 2006; Chang et al. 2004; Zhang et al. 2002). Class III HDACs, more commonly known as "Sirtuins," are structurally distinct from the other classes of deacetylases. These are NAD^+-dependent lysine deacetylases that are highly conserved during evolution.

11.3 Sirtuin Family of Lysine Deacetylases

Members of sirtuin family are found in as primitive form of life as eubacteria and archaea. Their expression precedes histones and even the existence of chromatin-like entity (Frye 2000). It is suggested that fluctuations in the intracellular acetate and NAD^+ levels in response to environmental stress could regulate cellular growth and metabolic processes via dynamic interplay between lysine acetylation–deacetylation of key proteins. A good example of such reversible regulation goes back in evolution to the single-cell prokaryotes called archaea, which were first found in

extreme environments such as volcanic hot springs (Brock and Freeze 1969). Archaea are considered ancestors of eukaryotes, which were first to possess chromatin-like structures (Pereira et al. 1997). Archaeal sirtuin, Sir2 was found to deacetylate another conserved chromatin-binding protein *Alba* to modulate its affinity toward DNA, and thereby regulating genome organization (Bell et al. 2002). It appears that as the complexity of systems increased diversification of sirtuins became a necessity for the multicellular organism to fulfill the unique, but intimately coordinated signaling networks of the cell, to maintain unanimity with the environmental stress.

The mammalian genome encodes seven highly conserved sirtuin isoforms, SIRT1 to SIRT7 (Frye 2000). These seven isoforms are localized to different subcellular compartments and regulate variety of cellular functions ranging from cell growth, metabolism to lifespan. Because of their dependency on NAD^+ as a cofactor, these deacetylases are considered to be sensors of energy crisis in the cell. They are prominently involved as regulators of key members of the critical signaling pathways (Nakagawa and Guarente 2011; Vassilopoulos et al. 2011). During deacetylation reaction, sirtuins remove the acetyl group from the ε-acetyl-lysine substrates and transfer it to ADP-ribose moiety of NAD^+, leaving nicotinamide (NAM) and O-acetyl-ADP-ribose (OAADPR) as the by-product of the reaction (Tanner et al. 2000). Some of the sirtuin isoforms also harbor other enzymatic activities, such as ADP ribosyl-transferase (ART), demalonylation, and desuccinylation, though much less has been understood for their roles in these modifications, compared to deacetylation (Du et al. 2011; Nakagawa and Guarente 2011; Peng et al. 2011).

Of the seven sirtuins, SIRT1, SIRT6, and SIRT7 mainly reside in the nucleus (Michishita et al. 2005). SIRT1 is also shown to migrate to cytoplasm and to plasma membrane during development and growth conditions (Sundaresan et al. 2011a; Tanno et al. 2007), while SIRT7 is the resident of nucleolus (Michishita et al. 2005). SIRT2 is primarily localized to the cytoplasm but can shuttle to the nucleus to regulate cell cycle (Inoue et al. 2007). SIRT3, SIRT4, and SIRT5 are predominantly mitochondrial (Nakagawa and Guarente 2011). A lesser amount of SIRT3 is also present in the nuclear and cytoplasmic compartments (Sundaresan et al. 2008). Research in the past decade has highlighted beneficial effects of sirtuins in maintaining cellular growth, metabolic homeostasis, and longevity (Haigis and Guarente 2006; Haigis and Sinclair 2010; Michan and Sinclair 2007). As these processes are dependent upon the proper balance of gene regulation, a role of sirtuins in gene transcription is obvious. Regulation of gene transcription by sirtuins could be directly mediated through their interactions with relevant transcription factors or indirectly as a member of different multiprotein chromatin-remodeling complexes. In addition to deacetylation activity, sirtuins are now being implicated in the process of protein–deacylation where lysine residues are stripped off modifications by succinyl-CoA, malonyl-CoA, glutaryl-CoA, etc. SIRT5 possesses lysine desuccinylase, demalonylase, and deglutarylase activity (Du et al. 2011; Peng et al. 2011), while SIRT6 displays lysine demyristoylase and depalmitoylase activity (Jiang et al. 2013). This implies that these NAD^+-dependent enzymes can multitask to regulate several signaling pathways in different subcellular compartments to maintain cellular homeostasis and combat environmental stresses.

11.4 Sirtuins as Histone Modifiers

The basic unit of DNA packaging in eukaryotic chromatin, called as nucleosome, is made up of 147 bp of DNA strand wound around a histone octamer, consisting of two copies of each of the four histones (H2A, H2B, H3, and H4) (Kornberg and Lorch 1999). When sirtuins get incorporated in the multiprotein regulatory complexes residing at the gene promoters, they modulate distribution of nucleosomes at these sites. Covalent modifications of specific amino-acid residues in the N-terminal tail of core histones alter interaction of negatively charged DNA with its binding proteins, including histone and other nonhistone proteins. So far, in addition to acetylation, a wide array of post-translational modifications of histones is reported. These include methylation, phosphorylation, ubiquitination, ADP-ribosylation, sumoylation, etc. (Jenuwein and Allis 2001). All these reversible modifications are intricately regulated via balanced coordination between counteracting enzymes. Based on the position of the modified amino acid residue in histones or the type of modification, higher order structure of the chromatin gets altered, and thereby a specific gene or different set of genes are repressed or activated (Batta et al. 2007).

In mammals, more than two thousand proteins are reported to be acetylated making lysine acetylation/deacetylation a major player in the field of cellular regulatory mechanisms (Guan and Xiong 2011; Lundby et al. 2012). Sirtuins, not only on their own are significant contributors to epigenetic modifications of histones, but also work in conjunction with methylation, the other well-studied histone modification (Papait et al. 2013). Together, they create an "epigenetic code" for chromatin that can result into transcriptionally active or silenced domains. Coordination between histone acetylation and methylation is observed in important cellular processes, such as DNA replication, repair, and gene transcription (Lorenzen et al. 2012).

Of the four core histones, the major two which are reported to be common targets of multiple sirtuins are histone H3 and H4. Lysine 9 in H3 (H3K9) and lysine 16 in H4 (H4K16) are highly conserved and commonly acetylated residues throughout the evolution (Bosch-Presegue and Vaquero 2014). H3K9 acetylation is reported in many actively transcribed gene promoters. However, H4K16 acetylation is unique in the sense it is linked to both transcriptional activation and repression (Martinez-Redondo and Vaquero 2013). Table 11.1 summarizes the different lysine residues of histones targeted by sirtuins.

11.5 Sirtuins as Regulators of Gene Expression in Cardiovascular Diseases

Most common causes of heart failure are coronary artery disease, which leads to myocardial infarction. These cardiac pathologies ultimately result in loss of functioning myocytes, development of fibrosis, and remodeling of the left ventricle. Of the seven sirtuins expressed in mammals, so far the roles for SIRT1, SIRT3, SIRT6, and SIRT7 have been studied in some detail for their ability to regulate the development of cardiac remodeling and heart failure (Fig. 11.1). However, relatively less is

Table 11.1 Lysine residues in histones targeted by different sirtuin isoforms

Histone substrate	Sirtuin	References
H1K26	SIRT1	(Vaquero et al. 2004)
H3K9	SIRT1	(Imai et al. 2000; Vaquero et al. 2007)
	SIRT3	(Scher et al. 2007)
	SIRT6	(Kawahara et al. 2009; McCord et al. 2009; Michishita et al. 2008; Sundaresan et al. 2012; Zhong et al. 2010)
H3K14	SIRT1	(Imai et al. 2000)
H3K18	SIRT7	(Barber et al. 2012)
H3K56	SIRT1	(Yuan et al. 2009)
	SIRT2	(Vempati et al. 2010)
	SIRT6	(Michishita et al. 2009; Yang et al. 2009)
H4K16	SIRT1	(Imai et al. 2000)
	SIRT2	(Vaquero et al. 2006)
	SIRT3	(Iwahara et al. 2012; Scher et al. 2007)

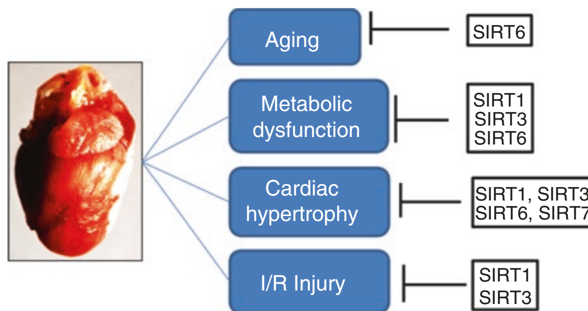

Fig. 11.1 Sirtuins in cardiovascular diseases
Sirtuin 1, 3, 6, and 7 contribute positively to improve cardiovascular health in spite of their different subcellular locations. In addition to their role as epigenetic modulators of histones, these NAD^+-dependent class III deacetylases regulate multitude of key pathways associated with oxidative stress, calorie restriction, aging, metabolic disorders, pressure overload, etc

known for their effects in maintaining fitness of the vascular system. Here, we will discuss these sirtuin isoforms individually for their involvement in protecting the heart from going into failure.

11.5.1 SIRT1

SIRT1 is a prototype member of the sirtuin family, and it has been studied most for its role in regulating growth and functioning of the heart. We and others have previously reviewed the role of SIRT1 in the heart and its critical participation in signaling pathways contributing to development of cardiac hypertrophy. Interested

readers can consult these reviews for details (Giblin et al. 2014; Sundaresan et al. 2011a; Zhang and Kraus 2010). SIRT1 has been shown to play a critical role in the development of the embryonic heart (Cheng et al. 2003; McBurney et al. 2003). SIRT1 whole-body knock-out mice on an inbred genetic background show cardiac malformations and succumb to death soon after birth (Cheng et al. 2003; McBurney et al. 2003). Occasionally, few pups with complete SIRT1 deficiency survive, and they display retarded body size and defects in the development of ventricular septum, valves, and endocardial cushion (Cheng et al. 2003). Studies conducted to determine subcellular localization of SIRT1 during development have revealed that it is exclusively nuclear in embryonic heart but become expressed both in the nucleus and in the cytoplasm in the adult heart (Tanno et al. 2007), thus suggesting a shifting role for SIRT1 to accommodate needs of the adult heart. Based on biochemical studies, it is suggested that in the absence of SIRT1, hyperacetylation of p53 and dysregulation of the apoptotic process, which is necessary for cardiac development, may contribute to cardiac developmental defects (Cheng et al. 2003). Also, acetylation of myocyte enhancer factor 2 (Mef2) was reported in SIRT1KO hearts. Since MEF2 is a critical transcription factor necessary for the normal growth and development of the heart, the acetylation of this factor is also proposed to be root cause of cardiomyopathy seen in SIRT1KO hearts. At the cellular level, these SIRT1KO hearts show reduced size of cardiomyocytes, lack of extracellular matrix and defects in mitochondrial structure and function (Planavila et al. 2012).

SIRT1 has been also shown to play an essential role in regulating activity of the Akt-signaling pathway (Pillai et al. 2014; Sundaresan et al. 2011b). Same as for Akt, which plays contrasting roles in regulating growth of the heart depending on the degree of activation (Catalucci and Condorelli 2006), SIRT1 has been also shown to exert a dose-dependent effect for the development of cardiac hypertrophy (Alcendor et al. 2007). In primary cultures of cardiomyocytes, SIRT1 expression seems to be necessary for the induction of cellular hypertrophy (Alcendor et al. 2004). Ischemia reperfusion causes more injury to SIRT1-deficient, but attenuation in SIRT1 over-expressing heart (Hsu et al. 2010). In the *in vivo* models of cardiac I/R injury, long-term calorie restriction (CR) was shown to restrain infarct size with concomitant increase in nuclear localization of SIRT1 (Shinmura et al. 2008). However, studies conducted with transgenic mice having cardiac-specific overexpression of SIRT1 showed induction of cardiac hypertrophy with impaired diastolic function in a dose-dependent manner. Only moderate overexpression (2.5-fold) of SIRT1 in transgenic hearts was reported to be beneficial to protect the heart from oxidative stress, age-associated cardiac hypertrophy, fibrosis, and apoptosis (Alcendor et al. 2007). Mechanistic studies have demonstrated that during oxidative stress, SIRT1 inhibits the activity of pro-apoptotic transcription factors, p53 and FOXO3a by deacetylation, while it activates FOXO1-dependent expression of the antioxidant enzymes such as MnSOD and catalase, thereby suppressing cardiomyocyte death (Alcendor et al. 2004). On the other hand, more than ninefold overexpression of SIRT1 leads to cardiac hypertrophy due to overactivation of the Akt-signaling pathway (Alcendor et al. 2007). Hyperexpression of SIRT1 in

spontaneously hypertensive rat hearts (Li et al. 2009) and in diabetic rats (Vahtola et al. 2008) is also reported to be associated with the evolution of cardiac hypertrophy.

In addition to dose dependency, beneficial effects of SIRT1 are also reliant on availability of NAD^+, the essential cofactor needed for its enzymatic activity. Evidence for NAD^+'s role in conferring favorable effect of SIRT1 comes from the hearts of fructose fed (Pillai et al. 2008) or exercise-trained mice (Ferrara et al. 2008). These hearts show mild induction of SIRT1 with abundant supply of NAD^+ and therefore suppression of stress-induced cardiac hypertrophy. Also, in the rat model of cardiac hypertrophy where poly-ADP ribose polymerase 1 (PARP1), another NAD^+-consuming enzyme was activated; concomitant decrease in SIRT1 activity was reported (Pillai et al. 2005). Replenishing NAD^+ or activating enzymes necessary for NAD^+ biosynthesis (NAMPT or NMNAT) prevented loss of SIRT1 activity (Zhang et al. 2009) and helped to improve cardiomyocyte survival (Pillai et al. 2005). In another study, PARP1-mediated cardiomyocyte death was shown to be averted by SIRT1-mediated deacetylation of PARP1, as well as by reducing its expression through the inhibition of PARP1 promoter (Rajamohan et al. 2009).

Defects in mitochondrial structure and function are also hallmarks of the failing heart (Nisoli et al. 2007). Expression of genes involved in nuclear-encoded mitochondrial genes is downregulated in human hearts undergoing failure. Many of these genes are targets of estrogen-related receptors (ERRs), a nuclear receptor family. In the failing heart, peroxisome proliferator-activated receptor-α (PPAR-α) makes a complex with SIRT1 and directly interacts with ERR response elements (ERRE) in the target promoters to repress their transcription (Oka et al. 2012). Pressure overload that downregulates many ERR target genes can be rescued partially by knocking down PPAR-α and SIRT1 together (Oka et al. 2012). SIRT1 appears to be an essential functional partner for the suppressive transcriptional action of PPAR-α (Oka et al. 2012). Additionally, liver-specific SIRT1 knock-out mice show impaired PPAR-α target gene expression (Purushotham et al. 2009). However, another study reports that activation of PPARα pathway through the upregulation of SIRT1 protects the heart from the development of hypertrophy. In this study, activation of SIRT1 by resveratrol (RSV) prevented agonist-induced cardiac hypertrophy, metabolic dysregulation, and inflammation in a PPARα-dependent manner (Planavila et al. 2011). These opposing effects of SIRT1 involving the same pathway, emphasize how subtle changes in sirtuin levels can impact delicate regulation of transcription factors. In the first study, where SIRT1 was found to be detrimental, cardiac hypertrophy was induced via pressure overload, while in the second study where its activation was found to be protective, hypertrophy was induced by isoproterenol infusion. It is possible that the opposite effects of SIRT1 could be dose-dependent as was the case with SIRT1 overexpressing transgenic mice, or due to duration of activation. Higher doses of SIRT1 may impose detrimental effects due to indiscriminate deacetylation of proteins that may not occur in conditions where SIRT1 activation is mild. RSV-induced SIRT1 activity was also reported to improve cardiac contractility in rat hearts subjected to trauma-hemorrhage injury (Jian et al. 2012). Elevated expression of SIRT1 upon RSV treatment in this model

was found to be associated with decreased levels of c-Myc, cytosolic cytochrome C, and plasma TNF-α, while an increased cardiac ATP contents and augmented PGC-1α expression. This positive effect of RSV was abolished upon treatment with SIRT1 inhibitor sirtinol-reiterating SIRT1 dependency.

One of the factors that play a pivotal role in regulating mitochondrial biogenesis and fatty acid metabolism is PGC1α, and its optimal activity is necessary for maintaining energy demand of the heart. In human failing heart, PGC-1α levels are approximately 30 % of that in the nonfailing heart. PGC-1α levels were also found to be reduced in aging mouse hearts concomitant with decreased levels of SIRT1 (Garnier et al. 2009). PGC1α is a direct target of SIRT1. SIRT1 physically interacts with and deacetylates PGC1α at multiple lysine residues leading to an increased activity of PGC1α (Rodgers et al. 2005). Therefore, observed activation of PGC-1α through SIRT1-mediated deacetylation is expected to improve energy metabolism of the failing heart. In accordance with this presumption, it was reported that SIRT1 activator resveratrol ameliorates cardiac dysfunctions by preserving mitochondrial biogenesis in a rodent model of heart failure (Lagouge et al. 2006). Additionally, in mice fed with high-calorie diet, impaired insulin sensitivity was correlated with pathological histology of hearts. This pathology was attenuated by treatment with resveratrol in the SIRT1-dependent manner. Also, in high-fat-fed mice, SIRT1 was implicated in improving insulin sensitivity by repressing protein tyrosine phosphatase (PTP) 1B transcription at the level of chromatin. PTP1B is a key insulin-receptor phosphatase, which reverses tyrosine kinase effect to negatively regulate insulin-signaling transduction cascade (Moller 2001). SIRT1 was shown to improve contractile function of failing myocardium through the regulation of insulin sensitivity (Sun et al. 2007).

Cardiac hypertrophy is dependent on coronary angiogenesis. Loss of coordination between angiogenesis and cardiac muscle growth culminates into development and progression of pathologic hypertrophy (Shiojima et al. 2005). In aging-associated cardiovascular diseases such as hypertension, diabetic cardiomyopathy, and atherosclerosis, endothelial dysfunction arising from oxidative stress and premature senescence of endothelial cells is very common (Taddei et al. 2006; Voghel et al. 2007). SIRT1 plays a dual role in the activation of endothelial nitric oxide synthase (eNOS) by increasing its expression and by deacetylation to stimulate its activity, and thereby promoting endothelium-dependent vasodilation (Mattagajasingh et al. 2007; Napoli et al. 2008). SIRT1 has also been shown to interfere with the NF-kB signaling pathway and thereby down-regulating expression of pro-inflammatory cytokines in the endothelial cells and macrophages (Stein and Matter 2011). This effect of SIRT1 has been implicated for its ability to prevent chronic inflammatory diseases such as atherosclerosis and atherothrombosis (Stein and Matter 2011). It has also been demonstrated that sustained pressure overload results in the accumulation of p53, which inhibits HIF-1α activity leading to impaired cardiac angiogenesis, and thus promoting progression of hypertrophy to failure (Sano et al. 2007). As mentioned above SIRT1 deacetylates and reduces transcriptional activity of p53 in cardiomyocytes, and this can help in inhibiting antiangiogenic properties of p53 by stabilizing HIF1α. Additionally, FOXO1 transcription factor

that is a negative regulator of blood vessel formation is deacetylated and deactivated by SIRT1, leading to promotion of angiogenesis (Potente et al. 2007). Resveratrol was also shown to ameliorate cardiac damage caused by myocardial infarction (MI) by upregulating expression of vascular endothelial growth factor (VEGF), and its downstream-signaling pathway leading to angiogenesis (Fukuda et al. 2006).

Other modes controlled by sirtuins to keep heart healthy are by inhibiting cardiomyocyte cell death and preventing cardiac fibrosis. In the mitochondria-mediated cell death pathway, stress-induced acetylation of Ku70 leads to its dissociation from pro-apoptotic protein, Bax. Bax released from Ku70 enters into mitochondria to induce apoptosis (Cohen et al. 2004a). SIRT1 maintains Ku70 in the deacetylated state and thus keeps it in the complex with Bax to suppress the stress-induced apoptosis (Cohen et al. 2004b). SIRT1 also deacetylates p53 to inhibit its pro-apoptotic activity at the p53-target genes. A recent study in a mouse model of accelerated aging, demonstrated that long-term (8 months) treatment with resveratrol-restored SIRT1 activity, and this in turn mitigated FOXO1- mediated pro-apoptotic signaling in the senescent heart (Sin et al. 2014). SIRT1-mediated deacetylation of FOXO1 was also shown to be required for the upregulation of starvation-induced autophagy in the mouse heart, and this was implicated in maintaining left ventricular function during starvation (Hariharan et al. 2010). Deacetylated FOXO1 elevates expression of a small GTP-binding protein (Rab7), which is needed for autophagosome-lysosome fusion to increase autophagic flux (Hariharan et al. 2010). Another pathway through which SIRT1 may influence autophagy in response to DNA damage is by the regulating activity of E2F1, a cell cycle and apoptosis-regulating transcription factor. Mutual regulation between SIRT1 and E2F1 is demonstrated to impact cellular response to DNA damage. While SIRT1 deacetylates E2F1 to inhibit its transactivation activity, DNA damage-induced E2F1 upregulates SIRT1 at transcriptional level (Wang et al. 2006). E2F1 has also been shown to upregulate the expression of several autophagy-related genes (ATGs) and also of the damage-regulated autophagy modulator (DRAM) (Polager et al. 2008).

SIRT1 is also shown to modify cardiac hypertrophic response by deacetylating histones. The histone H2A variant H2A.z, which is essential for development in mammals, is found to be upregulated during cardiac hypertrophy. Knock-down of H2A.z is shown to inhibit the expression of hypertrophic growth-related genes, including cyclin-dependent kinase (Cdk)-7 and ribosomal protein S6. H2A.z is deacetylated on K15 by Sir2α, the mouse homolog of yeast Sir2, which leads to the ubiquitination of distant lysine residues (K115 and K121) and degradation of the histone (Chen et al. 2006). Thus, it appears that SIRT1 exerts its gene silencing effect in the cells via different routes either by remodeling chromatin through epigenetic modifications of histones or by regulating the activity of other transcription factors.

SIRT1 has been also shown to promote cardiac contraction by regulating intracellular calcium (Ca^{2+}) handling. In the heart, SERCA2a plays a major role in controlling Ca^{2+} uptake into sarcoplasmic reticulum. Impaired SERCA2a function is associated with human dilated or ischemic cardiomyopathy and heart failure (Arai et al. 1993; Limas et al. 1987). We have reported earlier that resveratrol could

enhance SERCA2a promoter activity in cultured cardiomyocytes in SIRT1-dependent manner (Sulaiman et al. 2010). In another study utilizing the rat model of myocardial infarction, resveratrol treatment improved cardiac function and reduced mortality via activation of SIRT1-AMPK axis of signaling (Gu et al. 2014).

11.5.2 SIRT3

SIRT3 is mainly localized to mitochondria where most of its targets have been identified. We have recently reported that cardiomyocyte death induced by doxorubicin, a cardiotoxic anticancer drug, is prevented via deacetylation and activation of OPA1, a quintessential mitochondrial inner membrane fusion protein (Samant et al. 2014). SIRT3-mediated deacetylation primes cells to successfully combat cellular stress by improving mitochondrial health, facilitating ATP production and reducing excessive ROS (reactive oxygen species) synthesis. SIRT3 has also been shown to deacetylate and activate the antioxidant MnSOD, thereby increasing ROS-detoxifying capacity of cells. SIRT3 is considered a guardian of mitochondria because of its wide range of effects to maintain mitochondrial fitness, reviewed in references (Giralt and Villarroya 2012; Pillai et al. 2010a; Sack 2012). SIRT3 is expressed in two isoforms, a short form of processed SIRT3 that is present exclusively in mitochondria, and a full-length long form of SIRT3, which is present in the nucleus and in cytoplasm (Iwahara et al. 2012; Sundaresan et al. 2008). Nuclear full-length (FL)-SIRT3 is reported to repress gene transcription through gene-specific deacetylation of histones H3K9 and H4K16 under physiological conditions (Iwahara et al. 2012; Scher et al. 2007). It is demonstrated that, when cells are stressed, for example by treating them with DNA-damaging agents, nuclear FL-SIRT3, but not the mitochondrial short form, gets ubiquitinated and rapidly degraded. Upon degradation, the repressive effect of FL-SIRT3 is eliminated and the target genes are rapidly activated to combat cellular insult (Iwahara et al. 2012). Similar to SIRT1, FL-SIRT3 deacetylates Ku70 to prevent stress-mediated death of cardiomyocytes by sequestering the proapoptotic BAX to preclude it from entering into mitochondria (Sundaresan et al. 2008). We and others have reported that SIRT3KO mice show no obvious developmental defect at birth, but they spontaneously develop cardiac hypertrophy as they reach to adulthood. SIRT3KO mice are also highly sensitive to hypertrophic agonists (Hafner et al. 2010; Sundaresan et al. 2009) and to IR-injury (Porter et al. 2014). The transgenic mice with cardiac-specific overexpression of SIRT3 are protected from developing pathologic cardiac hypertrophy (Sundaresan et al. 2009). The antihypertrophic role of SIRT3 is linked to its ability to retain FOXO3A in the nucleus, and thereby promoting expression of antioxidant enzymes, MnSOD and catalase. SIRT3-induced activity of antioxidants was also shown to help in preventing ROS-mediated RAS activation in cardiomyocytes (Sundaresan et al. 2009). Interestingly, we also observed that the pro-hypertrophic Akt signaling was suppressed by SIRT3, through the deacetylation of LKB1 and thereby activating antihypertrophic LKB1-AMPK signaling in the heart (Pillai et al. 2010b). All these study demonstrated a protective role of SIRT3 to maintain growth and function of the heart.

11.5.3 SIRT6

Another sirtuin that has been studied for its role in regulating cardiac hypertrophy is SIRT6. This is a nuclear, chromatin-associated sirtuin that controls diverse biological pathways related to genomic stability and metabolic homeostasis. SIRT6 possesses dual enzymatic activities, lysine deacetylation, and mono-ADP ribosylation. Through deacetylation of H3K9 and H3K56, it regulates the maintenance of telomeric chromatin, accessibility of DNA repair factors to chromatin, gene expression pertaining to inflammation and glucose/lipid metabolism (Kugel and Mostoslavsky 2014; Michishita et al. 2008; Michishita et al. 2009; Yang et al. 2009). As a histone H3K9 deacetylase, SIRT6 is recruited to the promoters of NF-kB target genes involved in apoptosis and cell senescence, via physical interaction with RELA subunit of NF-kB to attenuate aging-related inflammation (Kawahara et al. 2009). The involvement of SIRT6 in maintaining genome stability through H3K9 and H4K56 deacetylation is linked to its ability to regulate lifespan of the organism. SIRT6 as a H3K9 deacetylase recruits Werner helicase (Wrn) to telomeres and preserves telomere integrity. SIRT6KO mice exhibit pleiotropic effects resembling a human degenerative syndrome of premature aging called progeria, Werner syndrome (Michishita et al. 2008; Michishita et al. 2009). McCord et al., demonstrate that SIRT6 dynamically associates with chromatin at the site of double-stranded break (DSB) in the DNA to deacetylate H3K9Ac and mobilizes catalytic subunit of DNA-dependent protein kinase to the repair site (McCord et al. 2009). SIRT6-mediated mono-ADP-ribosylation and activation of PARP1 is reported to be necessary for DSB repair in oxidative stress-induced DNA damage (Mao et al. 2011). Another study demonstrated that SIRT6 increases accessibility of chromatin to DNA repair factors for efficient DSB repair by deacetylating H3K56 and recruiting SNF2H, an ATP-dependent chromatin remodeler to the break site (Toiber et al. 2013). Human SIRT6 is shown to regulate global and telomere-specific levels of H3K56Ac correlating with cell-cycle phases, being highest at S-phase and substantially reduced by G2/M phase (Michishita et al. 2009). We have demonstrated that SIRT6 through its interaction with c-Jun is recruited to target promoters of genes involved in IGF/Akt signaling. SIRT6 represses the expression of different components of IGF/Akt signaling by the deacetylation of histone H3K9 and thus blocks the development of pathologic cardiac hypertrophy (Sundaresan et al. 2012).

Senescence of endothelial cells is also a major contributory factor in the development of cardiovascular maladies. It is observed that SIRT6 prevents onset of premature senescence and a decrease in replicative capacity of human endothelial cells (Cardus et al. 2013). Recently, a study has reported that NMNAT2 (nicotinamide mononucleotide adenylyltransferase), a central enzyme in NAD^+ synthesis, protected neonatal rat cardiomyocytes from angiotensin II-induced hypertrophy through the activation of SIRT6, but not SIRT1 (Cai et al. 2012). Another study reports that cardiomyocytes isolated from transgenic mice overexpressing SIRT6 show resistance to hypoxic damage. Authors of this study demonstrate that, SIRT6-mediated protection from hypoxia-impeded apoptosis-necrotic pathways involving the activation of pAMPKα pathway, increased expression of Bcl-2, inhibition of NF-kB, reduction in

ROS levels and decreased levels of pAkt (Maksin-Matveev et al. 2014). The contribution of SIRT6 in various cellular processes and impact of its deregulation on human diseases is reviewed in reference (Kugel and Mostoslavsky 2014).

11.5.4 SIRT7

Similar to SIRT1, mice deficient in SIRT7 display hyperacetylation of p53 develop progressive cardiac hypertrophy, extensive fibrosis, and inflammatory cardiomyopathy. Unlike the postnatal lethality observed for SIRT1 and SIRT6 knock-out mice, deficiency for SIRT7 shortens the lifespan of SIRT7-KO mice to 11 months of age, emphasizing unique and diverse roles for these sirtuins at different stages of heart development (Vakhrusheva et al. 2008). The role SIRT7 in different models of cardiac diseases has not been studied yet.

11.6 Concluding Remarks

Sirtuins have multitask action in the cardiovascular system at several levels; from regulating cardiac development, angiogenesis, metabolism, cell survival to managing myocardial stress. Given a wide range of sirtuin targets in different compartments of cells, it seems that majority (if not all) of cellular pathways are directly or indirectly regulated by this class of NAD^+-dependent deacetylases. Though our understanding of sirtuin biology has expanded remarkably over the past decade, their role as epigenomic modulators in cardiovascular field is not yet completely understood. Because of their NAD^+ dependency, sirtuins are many times uniquely positioned as the "first responders" to combat metabolic stress on the cell, either in physiologic or in pathologic scenario. Sirtuins in terminally differentiated cells, such as cardiomyocytes, may play a very different role than the one in dividing cells such as cardiac fibroblasts and cancer cells. However, with "epigenetic nutriceuticals" already being on the market, a word of caution is necessary when considering sirtuins as potential targets for drug development because of their apparently conflicting functions in different cell-specific contexts.

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Conflict of Interest The authors declare that they have no conflict of interest.

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BET Bromodomains and P-TEFb in Cardiac Transcription and Heart Failure Pathogenesis

12

Priti Anand, Amir Munir, and Saptarsi M. Haldar

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Abstract

Stress-activated cardiac signaling cascades ultimately converge on defined transcriptional pathways that drive pathologic gene expression programs. Excessive or prolonged activation of these pathways culminates in hypertrophy, fibrosis, and contractile dysfunction. As the gene-regulatory machinery functions as a distal signal integrator in this disease process, defining mechanisms by which upstream pathways couple to chromatin-dependent signal transduction in cardiomyocytes (CMs) has been an area of intense scientific and therapeutic interest. It has long been recognized that dynamic positioning of acetyl-lysine on nucleosomal histone tails, regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes, plays a central role in cardiac plasticity and HF pathogenesis. In this chapter, we will discuss signaling events downstream of local chromatin acetylation in the heart and their role in pathologic cardiac plasticity and HF pathogenesis. We will highlight recently published studies that implicate BET family bromodomain-containing coactivator proteins as a critical link between

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activated cardiac enhancers, P-TEFb (positive transcription elongation factor b), and RNA polymerase II (Pol II) dynamics in the stressed myocardium.

12.1 Introduction

Stress-activated cardiac signaling cascades ultimately converge on defined transcriptional pathways that drive pathologic gene expression programs. Excessive or prolonged activation of these pathways culminates in hypertrophy, fibrosis, and contractile dysfunction (Hill and Olson 2008; van Berlo et al. 2013). As the gene-regulatory machinery functions as a distal signal integrator in this disease process, defining mechanisms by which upstream pathways couple to chromatin-dependent signal transduction in cardiomyocytes (CMs) has been an area of intense scientific and therapeutic interest. It has long been recognized that dynamic positioning of acetyl-lysine on nucleosomal histone tails, regulated by histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes, plays a central role in cardiac plasticity and HF pathogenesis (Di Salvo and Haldar 2014; McKinsey 2012). In this chapter, we will discuss signaling events downstream of local chromatin acetylation in the heart and their role in pathologic cardiac plasticity and HF pathogenesis. We will highlight recently published studies that implicate BET family bromodomain-containing coactivator proteins as a critical link between activated cardiac enhancers, P-TEFb (positive transcription elongation factor b), and RNA polymerase II (Pol II) dynamics in the stressed myocardium.

12.2 P-TEFb

P-TEFb is a protein complex with cyclin-dependent kinase (CDK) activity that plays a central role in the regulation of transcription by Pol II. Productive transcription by Pol II is stalled at many genes as it gets trapped near the promoter by negative elongation factors, which inhibit Pol II transcriptional activity, thereby resulting in premature transcription termination. Productive transcription requires the recruitment of P-TEFb activity, which phosphorylates negative transcription factors and the CTD (carboxy-terminal domain) of Pol II. Activation by P-TEFb increases the processivity of paused Pol II, facilitating the stimulus-coupled production of full-length mRNA transcripts (Zhou and et al. 2012). Here, we will review some basic features of P-TEFb-dependent signaling and discuss the role of this critical transcriptional regulatory complex in cardiac plasticity.

12.2.1 Identification of P-TEFb

In Drosophila, P-TEFb is made up of two subunits: a larger catalytic unit (CDK9) and a smaller regulatory unit (cyclin T). The human P-TEFb complex has one CDK9 that can interact with one of the smaller cyclin subunits: T1 (and splice variants T2a

and T2b) (Peng et al. 1998), and cyclin K (Fu et al. 1999). P-TEFb and its role in transcription regulation was originally identified in an *in vitro* system using ATP analogs such as 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). Treatment of mammalian cells with DRB causes inhibition of heterogeneous nuclear RNA synthesis and a substantial reduction in mRNA production (Sehgal et al. 1976). Initially, DRB was considered to participate in the inhibition of transcription initiation (Sehgal et al. 1976), but subsequent studies showed that the principal function of DRB was to inhibit the elongation phase of transcription (Chodosh et al. 1989; Marshall and Price 1992). DRB-sensitive kinase activity was postulated to be required for productive elongation (Zandomeni et al. 1986). Studies using isolated transcription machinery reported premature termination of transcription due the lack of such "positive elongation activity" and the restoration of productive transcription upon addition of this activity (Marshall and Price 1992). These studies were followed by the purification of the P-TEFb complex from fractionated nuclear extracts of Drosophila cells and its characterization as a Pol II CTD kinase by the laboratory of David Price, confirming its essential role in productive elongation following transcription initiation (Marshall et al. 1996; Marshall and Price 1995).

12.2.2 P-TEFb Function and Regulation

Important substrates of P-TEFb include the CTD of Pol II and the elongation repressor proteins DSIF (DRB sensitivity-inducing factor) (Wada et al. 1998) and NELF (negative elongation factor) (Yamaguchi et al. 1999). The kinase component of P-TEFb phosphorylates serine residues in the C-terminal heptad-repeat domain ($Y_1S_2P_3T_4S_5P_6S_7$) of the largest subunit of Pol II (Eick and Geyer 2013), an event that facilitates release of paused Pol II (Marshall et al. 1996). P-TEFb accumulates in sites of active transcription (Ni et al. 2004) and preferentially phosphorylates Ser2 of the Pol II CTD (Shim et al. 2002). P-TEFb also participates in transcription elongation by phosphorylating the C-terminal region (CTR) of the larger subunit (Spt5) of DSIF that promotes the transition of DSIF from its role as a repressor to being an activator of elongation (H. Chen et al. 2009). In addition, P-TEFb-mediated phosphorylation of NELF (on the RD subunit) is also required for triggering pause release (Fujinaga et al. 2004). Although it was believed that transcription termination upon treatment of cells with flavopiridol (a highly potent P-TEFb inhibitor) was predominantly attributed to the loss of Ser2 phosphorylation at CTD (Ni et al. 2004), the discovery of other CDKs (and their role in CTD phosphorylation) suggests that P-TEFb may not be the only regulatory complex governing Pol II dynamics (Zhou et al. 2012).

The majority of P-TEFb exists in an inactive pool (Yang et al. 2005), bound to the 7SK small nuclear RNA (snRNA), a 332-nucleotide RNAP III-synthesized transcript. This ribonucleoprotein complex is reversibly assembled with the double-stranded RNA-binding protein HEXIM1 or HEXIM2 (hexamethylene bisacetamide-inducible protein), which represses P-TEFb by inhibiting its CDK9 kinase activity (Michels et al. 2004). This inactive complex also includes LARP7

(lupus antigen-related protein 7) that binds to 3′end of 7SK and confers protection from 3′ exonucleases (He et al. 2008; Krueger et al. 2008) and MePCE (methyl phosphate capping enzyme), which transfers a methyl group to the gamma phosphate of the first nucleotide of the 5′ end of 7SK (Jeronimo et al. 2007). Both LARP7 and MePCE are crucial for maintaining the stability of the complex (Zhou et al. 2012) and regulating the kinase activity of CDK9. It is important to note that P-TEFb can bind with 7SK in the absence of HEXIM1, but the binding of the latter is required for the suppression of P-TEFb activity (Yik et al. 2003).

Accumulating evidence suggests that P-TEFb can be released from this inhibitory complex in a signal-dependent manner to activate productive elongation (Biglione et al. 2007; Peterlin et al. 2012). Extraction of P-TEFb from this complex leads to a conformational change in the 7SK complex, the removal of HEXIM (Krueger et al. 2010) and replacement of the removed proteins with hnRNAs that stabilize the complex (Barrandon et al. 2007). The stress-induced release of P-TEFb from 7SK requires dephosphorylation of Thr 186 in the catalytic site of CDK9 (R. Chen et al. 2004). For cellular homeostasis, P-TEFb equilibrium is maintained by the induction of HEXIM1 expression and by the immediate reassembly of 7SK upon detection of an excess of the newly released P-TEFb activity (Liu et al. 2014). Following the completion of transcription, P-TEFb is recaptured by the reassembled 7SK (Zhou et al. 2012). Of note, in the setting of certain viral infections, such as HIV, host P-TEFb plays an important role in viral transcription. The HIV-encoded transactivator protein TAT seizes host P-TEFb by forming contacts with CyclinT1 and CDK9 (Tahirov et al. 2010) and thus recruits P-TEFb to the transactivation region (TAR) in the viral genome. This leads to the formation of full-length viral transcripts by recruiting host transcription elongation machinery (Zhu et al. 1997). In general, the extraction of P-TEFb from the inactive complex is mediated by three major mechanisms: (1) direct recruitment by transcriptional activators (e.g., c-MYC, p65 NFkB, or the HIV Tat protein) (Barboric et al. 2001; Eberhardy and Farnham 2002; Kanazawa et al. 2003; Zhu et al. 1997), (2) recruitment by the coactivator protein BRD4, which in enriched at acetylated enhancers (Jang et al. 2005; Yang et al. 2005), and (3) recruitment via the mediator complex (Takahashi et al. 2011).

12.3 P-TEFb and Pol II Activation in Hypertrophy and HF

In response to excessive or sustained stress, CMs undergo pathologic transformation that is characterized by hypertrophy, inflammatory activation, and metabolic changes that ultimately culminate in contractile dysfunction. Pathologic hypertrophy of CMs is associated with the induction of a large number of genes and a general state of transcriptional anabolism. In an elegant series of experiments, the laboratory of Michael Schneider has demonstrated that PTEF-b activity is a critical driver of transcriptional anabolism in CMs during stress that contributes to heart failure pathogenesis (Sano et al. 2002). Using antibodies that can discriminate hyperphosphorylated Pol II, they showed that cardiac Pol II is dynamically hyperphosphorylated in response to pathologic stress. In cultured neonatal rat ventricular

myocytes (NRVM), the hypertrophic agonist endothelin-1 (ET-1) activates CDK9 and inhibition of CDK9 activity (using adenoviral delivery of a dominant negative CDK9 construct) potently attenuates ET-1-mediated hypertrophy. Mechanistic studies demonstrated that hypertrophic signals rapidly lead to dissociation of PTEF-b from the 7SK RNA inhibitory complex. Finally, CM-specific overexpression of Cyclin-T1, the regulatory component of PTEF-b that activates CDK9, was sufficient to drive pathologic cardiac hypertrophy in adult mice *in vivo* (Sano et al. 2002). Furthermore, excessive PTEF-b activation sensitized mice to cardiac decompensation and heart failure following pressure overload, a phenotype that was associated with substantial suppression of genes encoding mitochondrial proteins and perturbed mitochondrial function (Sano et al. 2004). Intriguingly, miR-1, a muscle-specific micro-RNA that functions as a negative regulator of cardiomyocyte hypertrophy can suppress the expression of several growth-promoting genes including *Cdk9* (Sayed et al. 2007), although the specific function of the miR-1-CDK9 axis is not known.

12.4 P-TEFb Regulation by HEXIM1 in the Heart

HEXIM1 (hexameththylene bis-acetamide inducible 1), also known as CLP-1 (cardiac lineage protein 1), is an important regulator of P-TEFb function (Kwak and Lis 2013) (Espinoza-Derout et al. 2007). A significant fraction of cellular P-TEFb (i.e., Cyclin T1/CDK9) is maintained in an inactive pool complexed with HEXIM1 and the 7SK RNA scaffold. These protein–protein and protein–RNA interactions maintain the poised CyclinT1/CDK9 module in a catalytically inactive state. As mentioned earlier, P-TEFb can be extracted from the inactive pool to transcriptionally active genomic loci via several mechanisms, including interactions with Mediator, BRD4, and a number of sequence-specific TFs (Kwak and Lis 2013).

Consistent with the role of HEXIM1 as an endogenous negative regulator of cardiac PTEF-b activity, several studies have implicated HEXIM1 in cardiac hypertrophy. In cultured cardiomyocytes subjected to mechanical stretch or ET-1 treatment, Cyclin T1 dynamically dissociates from HEXIM1 (Espinoza-Derout et al. 2007), consistent with the known activation of P-TEFb during hypertrophic stress (Sano et al. 2002). Experiments using the JAK2 kinase inhibitor AG490 show that the dissociation of P-TEFb from HEXIM1 during mechanical stretch is, in part, dependent on JAK-STAT signaling (Espinoza-Derout et al. 2007). Mice with global deletion of *Hexim1* have embryonic lethality in late fetal stages [E16.5], a phenotype associated with a dysmorphic left ventricular (LV) architecture, thickened LV walls, and heightened expression of several fetal genes known to be reactivated in stressed adult hearts (Hang et al. 2010). Mice harboring an insertional mutation that results in the disruption of the C-terminus of HEXIM1 also exhibited defects in cardiac development that were associated with decreased myocardial vascularity and vascular endothelial growth factor (VEGF) expression (Montano et al. 2008). Studies by Siddiqui et al., using mice with systemic haploinsufficiency of HEXIM1 strongly corroborate a role for this protein as a negative regulator of cardiac P-TEFb function and cardiac

hypertrophy *in vivo* (Espinoza-Derout et al. 2009). In a model of pathologic cardiac hypertrophy caused by cardiomyocyte-specific overexpression of active Calcineurin-A, it was shown that Cyclin T1 dynamically dissociated from HEXIM1, consistent with the activation of myocardial PTEFb *in vivo*. Furthermore, crossing Cyclin T1-overexpressing mice with HEXIM1 haploinsufficient mice resulted in exaggerated cardiac hypertrophy and Pol II Ser2P abundance *in vivo* (Espinoza-Derout et al. 2009). It will be interesting to assess whether HEXIM1 deficiency also renders mice susceptible to pathologic cardiac hypertrophy in other settings (e.g., pressure overload, myocardial infarction, neurohormonal stimulation).

Effects of ectopically expressing HEXIM1 in the heart appear to differ depending on the transgenic approach that is employed. Transgenic overexpression of HEXIM1 in CMs using a constitutive α-MHC promoter led to the attenuation of pulmonary hypertension-induced right ventricular hypertrophy (RVH) (Yoshikawa et al. 2012). Interestingly, inducible cardiac-specific expression of ectopic HEXIM1 in adult mice (using a tetracycline-responsive system) led to a reversible growth response resembling some features of physiological hypertrophy, with increased VEGF expression, cardiac vascularization, and ventricular performance (Montano et al. 2013). In this inducible model, myocardial expression of *c-myc* and several metabolic genes (e.g., *Ppara, Acadm, CytC*) was observed without any induction of natriuretic peptide genes. In addition, cardiac HEXIM1-overexpressing mice had increased endurance exercise capacity on a treadmill (Montano et al. 2013). As these HEXIM1-overexpressing mice also had sinus bradycardia and electrocardiographic evidence of QT interval prolongation (Montano et al. 2013), it will be important to determine whether longer-term activation of this protein is arrhythmogenic or cardiotoxic. In addition, it will be interesting to see whether these HEXIM1-overexpressing mice are resistant to pathologic stress.

12.5 P-TEFb Regulation by CTIP2 in the Heart

COUP-TF-interacting protein 2 (CTIP2/Bcl11b) has recently been implicated as an important negative regulator of P-TEFb-dependent transcription (Cherrier et al. 2013). CTIP2 is known to bind DNA and associate with transcriptional repressor complexes (e.g., HDAC2, SUV39H1). CTIP2 has been implicated in thymocyte development, neurogenesis, cancer pathogenesis, and silencing of HIV transcription (Arlotta et al. 2005; Ganguli-Indra et al. 2009; Ikawa et al. 2010; Li et al. 2010; Marban et al. 2007). In a recent study, CTIP2 was found to have a novel association with the inactive PTEFb complex, forming direct interactions with defined regions of the 7SK RNA and HEXIM1 (Cherrier et al. 2013). Overexpression and knockdown of CTIP2 in HEK293T cells demonstrated that this protein functions as an endogenous inhibitor of CDK9 activity, Pol II CTD (Ser 2) phosphorylation, and P-TEFb-dependent gene expression. Cherrier et al. compared the set of CTIP2-regulated genes in HEK293 cells with gene expression profiles from adult mice with cardiac hypertrophy (mediated by phenylephrine administration via subcutaneous osmotic minipump for 15 days). Bioinformatic analyses revealed significant

correlation between genes regulated by CTIP2 knockdown (293 cells), CDK9 overexpression (293 cells) and hypertrophied mouse myocardium, suggesting that CTIP2 might function as a negative regulator of P-TEFb during cardiac hypertrophy. Consistent with this correlative data, ChIP-qPCR analyses showed that CTIP2, HEXIM1, CDK9, and Cyclin-T1 were all enriched at the proximal promoter of the *Myh7* gene. Together, these studies define CTIP2 as a novel endogenous repressor of P-TEFb and suggest that CTIP2 might function as a negative regulator of cardiac hypertrophy (Cherrier et al. 2013). Given the established role for P-TEFb activation in cardiac pathology (Sano et al. 2002), further gain- and loss-of-function studies of CTIP2 in cultured cardiomyocytes and in mouse models of heart failure will be particularly informative.

12.6 BET Bromodomains in the Heart

Over the past decade, studies in cultured CMs and mutant mouse strains have implicated a defined set of sequence-specific DNA-binding TFs that function as drivers of cardiac hypertrophy when excessively activated (e.g., NFAT, GATA4, NFkB, MEF2, c-Myc) (Hill and Olson 2008; van Berlo et al. 2013; Zhong et al. 2006). In response to pathological stress, coordinate activation of several such TFs lead to their increased recruitment to regulatory regions of the genome (enhancers), where they trigger aberrant myocardial gene expression (Hill and Olson 2008; van Berlo et al. 2013). Transcriptionally active enhancers are associated with local N-ε-acetylation of lysine sidechains (Kac) on nucleosomal histone tails (Lee and Young 2013; Schreiber and Bernstein 2002). Dynamic positioning of Kac, which arises from the interplay of so-called epigenetic "writers" (HATs) and "erasers" (HDACs) (Dawson et al. 2012; Lee and Young 2013), has been shown to play a critical role in HF pathogenesis (Di Salvo and Haldar 2014; McKinsey 2012; Trivedi et al. 2007; Wei et al. 2008; Xie and Hill 2013; Zhang et al. 2002). However, the precise signaling events by which hyperacetylated cardiac enhancers signal downstream to Pol II at transcriptional start sites (TSS) in the heart was previously unknown.

Context-specific recognition of Kac at regions of actively transcribed euchromatin is mediated by epigenetic "reader" proteins possessing a Kac-recognition module, or bromodomain (Filippakopoulos et al. 2010). While both epigenetic "writers" (e.g., EP300) and "erasers" (e.g., HDACs) had previously been implicated in HF pathogenesis (McKinsey 2012; Trivedi et al. 2007; Wei et al. 2008; Xie and Hill 2013; Zhang et al. 2002), little was known about epigenetic readers in the heart. In 2013, two contemporaneous papers reported that BRD4, a member of a conserved family of epigenetic reader proteins termed BETs (Bromodomain and Extra-Terminal), was a positive regulator of cardiac hypertrophy and HF pathogenesis (Anand et al. 2013; Di Salvo and Haldar 2014; Spiltoir et al. 2013). There are four mammalian BET proteins (BRD2, BRD3, BRD4, and testis-specific BRDT) that contain two tandem N-terminal bromodomains. Of these, BRD4 is selectively induced with pathological stress (Spiltoir et al. 2013) and can directly interact with CDK9 (Bisgrove et al. 2007). Systemic *Brd4* or *Brd2* deletion in mice results in

early zygotic implantation defects and haploinsufficiency of either allele results in severe multisystem developmental/growth abnormalities (Houzelstein et al. 2002), limiting their usefulness in the study of postnatal cardiac plasticity. Mice harboring conditionally targeted alleles for these loci are in active development.

Our ability to probe BET function in mammalian biology was rapidly accelerated by the creation of the small molecule JQ1 by the laboratory of James Bradner (Filippakopoulos et al. 2010). JQ1 is a first-in-class, potent, and specific inhibitor of BET bromodomains that functions by competitively displacing BETs from acetylated histone, thereby disrupting downstream chromatin-based signaling events (Filippakopoulos et al. 2010). JQ1 is extremely potent with highest affinity for the BRD4 bromodomain (low nanomolar EC50) (Filippakopoulos et al. 2010). JQ1 does not interact with any of the 38 non-BET mammalian bromodomain-containing proteins and is therefore highly specific (Filippakopoulos et al. 2010). It has rapid onset of action (hours), is reversible, and is well tolerated in experimental animals (Filippakopoulos et al. 2010). Using this novel chemical biologic tool, our group and others have defined critical roles for BRD4 in several developmental/disease contexts (Delmore et al. 2011; Filippakopoulos et al. 2010; Matzuk et al. 2012), including HF (Anand et al. 2013; Di Salvo and Haldar 2014; Spiltoir et al. 2013).

Studies by our group and a group led by Timothy McKinsey contemporaneously reported that BET bromodomains function as critical transcriptional coactivators of specific, stress-induced pathologic gene expression programs in the rodent myocardium (Anand et al. 2013; Haldar and McKinsey 2014; Spiltoir et al. 2013). Nanomolar concentrations of JQ1 block agonist induced hypertrophy in NRVM. Similarly, specific siRNA-mediated knockdown of BRD4 in NRVM also blocks agonist induced hypertrophy, corroborating an important cell autonomous role for BRD4 in this process. JQ1 administration to adult mice potently attenuated pathologic cardiac hypertrophy and HF pathogenesis in models of pressure overload (transverse aortic constriction) and chronic phenylephrine infusion. Integrated transcriptomic and epigenomic analyses in NRVM and mouse heart tissue revealed that (a) BRD4 is enriched at active cardiac enhancers genome wide, (b) BETs coactivate specific stress-inducible transcriptional networks in the myocardium (including gene expression programs driven by hyperactivation of NFAT, GATA4, NFKB, IRF, and ETS transcription factors), (c) an important mechanism by which BETs coactivate stress-induced myocardial gene expression is via facilitating pause release of Pol II at transcriptionally active genes, via recruitment of P-TEFb activity. Collectively, this work demonstrates that BRD4 functions as a nodal signal integrator that relays activity from acetylated cardiac enhancers downstream to transcriptional start sites, where it triggers Pol II elongation and drives pathologic gene expression in the heart (Fig. 12.1). Ongoing studies are aimed at understanding more detailed molecular mechanisms by which BET activation is coupled to upstream stressors and how BETs achieve target specificity in the myocardium. Furthermore, cell- and gene-specific roles for individual BET family members *in vivo* will be facilitated by the development of conditionally targeted mouse strains.

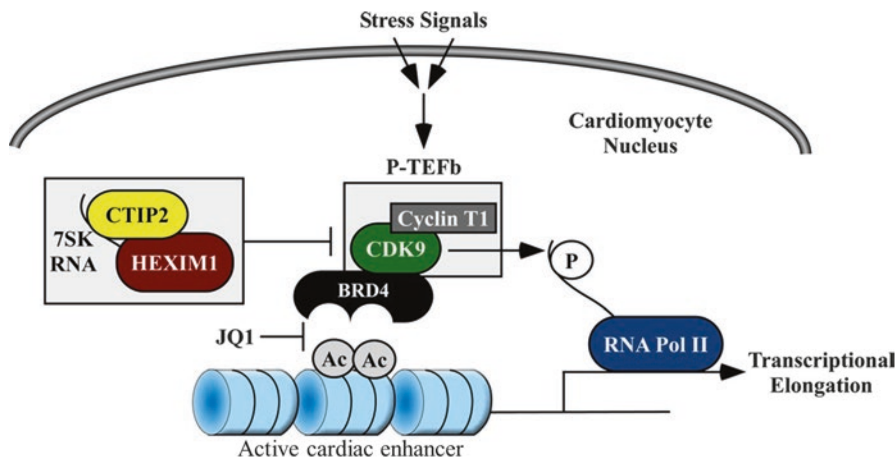

Fig. 12.1 General framework for BDR4- and P-TEFb-dependent signaling during cardiac hypertrophy (reproduced with permission from Haldar and McKinsey 2014)

A recent study from the laboratory of Scott Lowe reported the development of a mouse strain-harboring global expression of an inducible and reversible transgenic shRNA that targets Brd4 (Bolden et al. 2014). This model produces potent and sustained suppression of total BRD4 protein abundance in multiple tissues and does so in a temporally restricted manner. This study revealed that potent and sustained BRD4 suppression caused abnormalities in certain proliferating cellular compartments *in vivo*, such as the epidermis, hair follicles, and intestinal crypts. Importantly, these phenotypic abnormalities were reversible when the transgenic RNAi was turned off. These data suggest that sustained suppression of total BRD4 protein can have potential toxicity in normal tissues. Interestingly, this degree of toxicity has not been observed with small molecule BET bromodomain inhibitors, such as JQ1, which are well tolerated in experimental animals (Shi and Vakoc 2014). These observations may reflect the different consequences of sustained depletion of total BRD4 protein (as occurs with genome targeting or RNAi-mediated silencing) versus phasic, competitive and reversible displacement of BETs from acetyl-lysine (as occurs with small molecules such as JQ1) (Shi and Vakoc 2014).

The use of the small-molecule BET bromodomain inhibitor JQ1 provides proof of concept that these chromatin-dependent signaling events can be pharmacologically targeted in HF. Further delineation of the efficacy and therapeutic index of pharmacologic BET bromodomain inhibitors in HF and other cardiovascular disease are important areas of active investigation. A number of BET bromodomain inhibitor drugs have been developed (including drug derivatives of JQ1), a number of which are in early-phase human trials for cancer (Bradner 2013; Rohn 2012). Safety and efficacy data from these cancer trials will be important benchmarks for the development of near-term cardiovascular applications. We note that the BET inhibitor RVX-208, which has a pharmacologically different mechanism of action than JQ1 (Picaud et al. 2013), is currently being evaluated for its ability to raise

HDL and promote atheroprotective effects (Nicholls et al. 2011). While the overall efficacy of RVX-208 in humans has yet to be fully determined, these trials suggest that the therapeutic index of BET inhibition in humans may be tractable for cardiovascular disease and other noncancer applications.

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Conflict of Interest The authors declare that they have no conflict of interest.

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Long Noncoding RNAs in Heart Disease

13

Constantin Kühl and Norbert Frey

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Abstract

Advances in research technology with systematic and unbiased measurements of transcriptional activity revealed the surprising fact of pervasive transcription of mammalian genomes. However, most of these transcripts are not obviously protein coding nor do they reveal easily inferable biological relevance and thus have been termed “noncoding”. This universe of noncoding RNAs with diverse and versatile families such as transfer RNAs (tRNA), ribosomal RNAs (rRNA), micro-RNAs (miRNA) small nucleolar RNAs (snoRNA) has fuelled an entire

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new branch of research and already challenged major dogmas in molecular biology. Among the diverse classes of noncoding RNAs, long noncoding RNAs (lncRNA) have emerged as major regulators of transcription, nucleolar organization, and chromatin-modifying complexes. The goal of this chapter is to present the state of research of lncRNAs in the context of heart disease and heart failure.

Abbreviations

ChIP-Seq	Chromatin immunoprecipitation sequencing
eRNA	Enhancer-associated RNA
lincRNA	Long intergenic/intervening noncoding RNA
lncRNA	Long noncoding RNA
ncRNA	Noncoding RNA
PARP	Poly ADP ribose polymerase
RPKM	Reads per kilobase per million mapped reads
TAC	Transverse aortic constriction
UTR	Untranslated region

13.1 Introduction

Heart failure is among the most prevalent causes for morbidity and mortality in the Western world with as many as 1–2 % of the adult population afflicted (McMurray et al. 2012). Five-year survival is as a low as 50 %, numbers that underscore the severity of this condition (Go et al. 2014; Wong et al. 2014). Heart failure develops if the cardiac muscle is unable to comply with the oxygen demands of the organism due to either a decrease in systolic function (heart failure with reduced ejection fraction) and/or an increase in ventricular-filling pressures (heart failure with preserved ejection fraction), both leading to a decrease in cardiac output and to clinical symptoms such as dyspnea, edema, and sudden cardiac death (Hill and Olson 2008).

Heart failure is a dynamic process and the consequence of an intricate network of cardiac remodeling processes, including cardiac physiological and pathological hypertrophy, atrophy, and dilatation (Frey et al. 2004; Burchfield et al. 2013; Lyon et al. 2015; van Berlo et al. 2013). A mechanistic understanding of cardiac signaling cascades and structural cellular components, including receptors and kinases, sarcomeric “hubs,” such as the z-disk, transcription factors, and miRNAs, has helped to advance current and future medical therapy, for example, medications modulating the renin–angiotensin–aldosterone axis (Frank et al. 2006; Packer et al. 2015; Olson 2014).

Elucidating the genomic sequence of humans has promised to deliver the blueprint for combatting disease in a causal fashion, directly aimed at its molecular roots. Nevertheless, the promises of genomic medicine to develop novel therapies

have not been fulfilled yet, but helped to elucidate further levels of complexity in the regulation of cellular processes. Research efforts have now turned from individual genomic sequences toward sequencing thousands of individuals to unravel genomic variation (Lander et al. 2001; 1000 Genomes Project Consortium et al. 2012).

Furthermore, the efforts of research consortiums, such as ENCODE, FANTOM, or NIH Roadmap Epigenomics to catalog and analyze regulatory and functional elements of different genomes and their epigenetic states are thus only the beginning and leave the research community with vast amounts of data of a complex regulatory landscape that now need individual characterization and further refinement (ENCODE Project Consortium 2012; Forrest et al. 2014; Kundaje et al. 2015).

Epigenetics is classically defined as the field of genetic research that analyses the phenotypic changes imposed by mechanisms, which do not depend on changes of the initial genomic sequence (Waddington 1942; Berger et al. 2009).

Epigenetics has recently contributed substantially to the deciphering of many key-regulating factors of transcriptional and translational activity and to our understanding of a variety of pathologic conditions, especially in cardiovascular and cancer research (Backs and Olson 2006; Chang and Bruneau 2012; Weichenhan and Plass 2013).

Advances in research technology with systematic and unbiased measurements of transcriptional activity revealed the surprising fact of pervasive transcription of mammalian genomes (Carninci et al. 2005). However, most of these transcripts are not obviously protein coding nor do they reveal easily inferable biological relevance and thus have been termed "noncoding". This universe of noncoding RNAs with diverse and versatile families such as transfer RNAs (tRNA), ribosomal RNAs (rRNA), micro-RNAs (miRNA) small nucleolar RNAs (snoRNA) has fuelled an entire new branch of research and already challenged major dogmas in molecular biology (Cech and Steitz 2014). Among the diverse classes of noncoding RNAs, long noncoding RNAs (lncRNA) have emerged as major regulators of transcription, nucleolar organization, and chromatin-modifying complexes. The main goal of this chapter is to present the state of research of lncRNAs in the context of heart disease and heart failure.

13.2 Pervasive Transcription and the Characterization of lncRNAs

The history of research in noncoding RNAs can be traced back to the 1950s with the discovery of rRNAs and tRNAs, followed by snRNAs and antisense transcription in the 1980s and 1990s. Apart from individual lncRNAs such as H19 and Xist, it was not before the technological advancements of the years after 2000, especially tiling microarrays and next-generation sequencing, together with sophisticated immunoprecipitation approaches, that a systematic understanding of the RNA landscape emerged (Willingham and Gingeras 2006).

Pioneering research was conducted using tiling microarrays of human chromosomal transcription that allowed analysis of transcription at windows of only a

couple of nucleotides. Thereby it became evident that large parts of the human genome are pervasively transcribed and a high number of these new transcripts have very low or no potential at all to encode for proteins. Sequence analysis of mouse-noncoding transcription revealed a lesser degree of conservation on average compared to 5′- or 3′-UTRs. Conversely, the putative promoter regions of ncRNAs were more conserved than those of protein-coding genes. Noncoding RNAs were arbitrarily divided into short- and long-noncoding RNAs at a length of 200 nucleotides. Long-noncoding transcripts show specific expression patterns in cellular compartments with partly restricted expression to the nucleus or cytosol and high degrees of tissue-specific expression. They tend to be lower expressed, compared to protein-coding transcripts (Kapranov et al. 2002; Rinn et al. 2003; Bertone et al. 2004; Cawley et al. 2004; Kampa et al. 2004; Cheng et al. 2005; Carninci et al. 2005; Kapranov et al. 2007).

The research conducted by the ENCODE-consortium added further understanding to the class of long noncoding RNAs. The combination of RNA-sequencing maps of chromatin regulatory marks delineated that in fact 62.1 % and 74.7 % of the human genome give rise to processed transcripts (Djebali et al. 2012).

An in-depth analysis of all human coding and noncoding sequences exemplified a variety of important characteristics of long noncoding RNAs: The GENCODE analysis identified 14,880 human lncRNA transcripts, 9,518 of them from intergenic and 5,362 from intragenic transcriptional origin. Transcriptional start sites of long noncoding RNAs are comparable to protein-coding genes with respect to active histone marks (H3K4me2, H3K4me3, H3K9ac, H3K27ac), but show a higher level of marks associated with both silencing (H3K27me3) and activity (H3K36me3). LncRNAs are transcribed via polymerase II-dependent mechanisms and most of them are post-transcriptionally modified via 5′-capping and 3′-polyadenylation, although other modifications of lncRNAs, such as methylation at N(6)-methyladenosine, have been described (Ponting et al. 2009; Zhang et al. 2014; Fu et al. 2014).

There is a high degree of correlation between expression of long noncoding RNA transcripts and neighboring protein-coding genes, both in *cis* and in *trans*, but generally more pronounced in *cis*. This observation of expression correlation holds also true for long noncoding RNAs that are intertwined with protein-coding genes and is partly attributed to transcriptional coregulation mechanisms (Derrien et al. 2012; Orom et al. 2010; Kim et al. 2010).

Classification of long noncoding RNAs correspond to genomic organization and association with DNA-regulatory elements, this is summarized in Table 13.1 and exemplified in Fig. 13.1.

Following these initial results, several groups reported catalogs of lncRNAs and refinements of systematic analyses, confirming this initially reported characteristics of lncRNAs. Complementary approaches using correlation of expression data with histone marks of activated protein and ribosome profiling further advanced our understanding, especially considering biological relevance and coding potential (Guttman et al. 2009; Mikkelsen et al. 2007; Guttman et al. 2013).

Table 13.1 Classification of long noncoding RNAs

Genomic localization	Intergenic	
	Genic	Exonic
		Intronic
		Overlapping
Genomic strand	Sense	
	Antisense	
Direction of transcription	Sense	
	Antisense	
	Bidirectional	
Association with DNA regulatory elements	Promoter associated	
	Enhancer associated	

(Modified after Derrien et al., Genome Res 2012)

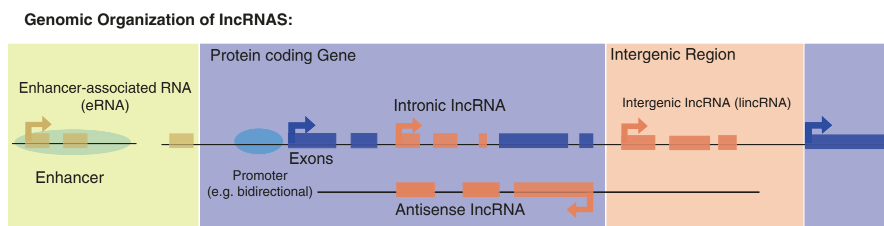

Fig. 13.1 Exemplifies the genomic organization of long noncoding RNAs, together with different regulatory elements e.g. promoter and enhancer regions and protein coding genes

13.3 Evolutionary Conservation of Long Noncoding RNAs

Strong evolutionary conservation is generally viewed as an indicator of biological significance, as evolution can be seen as nature's experiment of function on the scale of millions of years. Thereby, understanding evolutionary conservation may aid understanding relevance. In the case of long noncoding RNAs, this has become increasingly more difficult. The combination of comparably lower transcriptional activity and interspecies sequence variation in noncoding regions led to the initial notion that transcription at noncoding loci is genomic "junk", leading to "transcriptional noise" without any biological significance (Ponjavic et al. 2007).

Two systematic analyses of lncRNA ancestry in 6 different mammals and 11 tetrapod species identified families of homologous lncRNAs at different evolutionary stages. The highest degree of conservation and sequence constraints was found in older lncRNA families. However, newer families showed evidence of purification selection, especially at the promotor level. As a class, lncRNA are considered to be able to evolve fast and to show strong tissue specificity (Necsulea et al. 2014; Washietl et al. 2014). Hezroni et al., directly compared transcriptomes from 17 different species using a novel computational pipeline to identify lncRNAs, sequence

homology, orthologous and syntenic sequences. Their results confirmed several previous findings about long noncoding RNAs, but additionally led to the discovery that conserved lncRNAs are biased towards sequence conservation at their 5′ regions. Furthermore, exoneration of transposable elements plays a pivotal role in the evolutionary development of lncRNA sequences (Hezroni et al. 2015).

Diederichs summarizes the current research findings and distinguishes four different entities of long noncoding RNA conservation: sequence, structure, function, and expression from syntenic loci (Diederichs 2014).

These results led to interesting conclusions: First, lack of sequence similarity does not necessarily exclude evolutionary conservation and thus high biological relevance. Second, understanding of a long noncoding RNA can only be achieved in its genomic context and with understanding of the associated regulatory elements. Evolutionary conservation will be especially relevant to translate findings from research in cardiovascular model systems to humans, as morphology and physiology of the heart as an organ changed profoundly over the course of evolution, with obvious significance for human disease, that is, the low regenerative potential of the mammalian heart.

13.4 Long Noncoding RNAs in Cardiovascular Research: Bench

Research in mouse models of heart embryonic development and models mimicking human pathology have led to important discoveries and characterized lncRNAs as potent epigenetic regulators of cardiac gene expression and chromatin modification:

13.4.1 Polycomb, Trithorax, and Noncoding RNAs: Fendrr and Braveheart

Polycomb and Trithorax Group proteins are central modulators of epigenetic gene regulation, development and differentiation and are associated with cardiac development and disease (Wang 2012). Proteins of the TrxG/MLL complex catalyze H3K4 methylation and act toward the activation of transcription (Schuettengruber et al. 2011). Proteins of the PRC2 complex act as a methyltransferases and catalyze methylation of H3K27, thereby repressing gene expression (Di Croce and Helin 2013). It has been shown that components of both the PRC2 and the TrxG/MLL complexes are able to interact with long noncoding RNAs (lncRNAs) and this interaction might constitute an important regulatory mechanism (Khalil et al. 2009; Wang and Chang 2011; Brockdorff 2013). RNA-immunoprecipitation of PRC2 components combined with RNA-sequencing (RIP-Seq) in mouse embryonic stem cells led to discovery of more than 9000 different RNAs that bind to PRC2 (Zhao et al. 2010). This is mediated through specific binding mechanisms, as well as promiscuous binding of RNAs. This led to ongoing controversy regarding the

prevailing mechanism and possible experimental artifacts (Davidovich et al. 2013). However, new data confirmed both specific and promiscuous RNA binding *in vitro* (Davidovich et al. 2015). Polycomb and Trithorax proteins act antagonistically through DNA regulatory responsive elements. Interestingly, also Polycomb/Trithorax response elements show transcriptional activity and can switch their respective function.

Recently, important roles for long noncoding RNAs in mediating regulatory effects via PRC2/TrxG in the heart have been elucidated: Long noncoding RNA "Fetal-lethal noncoding developmental regulatory RNA" (Fendrr) was identified from RNA-Seq and ChIP-Seq analysis from early somite stage mouse embryos. Fendrr is divergently transcribed upstream of the Foxf1-locus and predominantly expressed in lateral plate mesoderm. Knockdown of Fendrr leads to embryonic lethality around E13.75, accompanied by cardiac hypoplasia and omphalocoele. Fendrr is predominantly expressed in EOMES-positive cells of the early cardiac mesoderm lineage. Knockdown of Fendrr leads to increase of H3K4-promoter trimethylation of key cardiac developmental transcription factors Gata6 and Nkx2-5 and increased their expression at E8.5. At later stages, Gata6, Foxf1, together with Irx3 and Pitx2 show higher expression in mutant embryos. Fendrr mutant embryonic stem cells show reduced promoter occupancy of Ezh2 and Suz12 at several potential target genes of Fendrr. Fendrr directly binds PRC components Ezh2 and Suz12 and TrxG/MLL component Wdr5. No binding could be observed for the parts of PRC1, suggesting specificity of discrimination between different histone-modifying complexes. Furthermore, a sequence domain of Fendrr is able to bind double-stranded Foxf1 and Pitx2 promoter regions. Taken together, Fendrr links PRC2 to its target promotors, thereby increasing PRC2 occupancy and methylation of H3K27, which subsequently leads to the modulation of cardiac developmental gene expression (Grote and Herrmann 2013; Grote et al. 2013).

Expression analysis of different mouse embryonic stem cells and the adult heart led to identification of the long noncoding RNA "Braveheart" (Bvht). Although knockdown of Bvht did not impede differentiation of embryonic stem cells toward all three lineages, differentiation of embryoid bodies toward cardiac myocytes was severely impaired. Transcriptome analysis of such depleted cells revealed failed activation of key cardiac transcription factors (among them MesP1, Hand1/2, Nkx2-5, Tbx20) and transcripts for epithelial–mesenchymal transition (Snai, Twist). A significant overlap between Bvht-depletion and MesP1-regulated genes was observed. Forced expression of MesP1 in Bvht-depleted embryonic stem cells was able to rescue this phenotype, that is, permitting differentiation to the cardiac lineage. Therefore, the authors concluded Bvht to function upstream of MesP1. Mechanistically, Bvht interacts with Suz12 as part of PRC2. Furthermore, knockdown of Bvht in neonatal cardiac myocytes leads to disruption of myofibrillar ultrastructure and reduced expression of cardiac structural proteins. Taken together, the authors reveal Bvht as a novel key regulator of cardiac development (Klattenhoff et al. 2013).

13.4.2 Antisense Transcription and Chromatin Remodeling at the Myosin Locus

The switch of cardiac myosin isoform expression between adult Myh6 (α-myosin) and fetal Myh7 (β-myosin) is a hallmark of cardiac hypertrophy and heart failure (Miyata et al. 2000). A role for antisense transcription in regulating myosin expression was described earlier; nevertheless, an understanding of downstream mechanism has been lacking (Haddad et al. 2003). Antisense transcription and alternative splicing from exonic and intergenic regions of the Myh7-locus give rise to a cluster of cardiac specific long noncoding RNAs, named “Myhrt”. The most abundant isoform, Mhrt770, was characterized as a potent inhibitor of pathologic cardiac hypertrophy. Cardiac-specific overexpression of Mhrt is able to alleviate early and late cardiac hypertrophy in mice after TAC surgery. Mechanistically, Mhrt770 binds Brg1, a member of the SWI/SNF ATP-dependent chromatin-remodeling complex to form a feedback circuit. Brg1 interacts with several HDACs and PARPs to form a complex on Myh6 and Myh7 promoters for antithetic regulation (Hang et al. 2010). Mhrt770 prevents Brg1 to bind to its target genomic region at the promoters of Myh6, Myh7, and Opn. These findings could be translated to human cardiomyopathies: Expression of MHRT is reduced in hearts from patients with cardiomyopathies (Han et al. 2014) (Fig. 13.2).

13.4.3 Fetal Cardiac Enhancer-Associated Long Noncoding RNAs

Enhancers are defined as “intergenic or intragenic regulatory sequences that can activate gene expression […]” (Orom and Shiekhattar 2013). Enhancers harbor transcriptional activity and can even give rise to transcription of lncRNAs (Kim et al. 2010). These enhancer-associated long noncoding RNAs themselves can provide an enhancer-like function in regulating transcriptional activity (Orom et al. 2010). Furthermore, they can be distinguished as a class from promoter-associated lncRNAs with distinct characteristics concerning tissue specificity and expression levels, as well as evolutionary conservation. (Marques et al. 2013) Recently, analyses of differential lncRNA expression at fetal cardiac enhancers during cardiac progenitor cell differentiation, after TAC surgery and LAD ligation were able to identify many dynamically transcribed lncRNAs. These were correlated with transcriptional activity at neighboring genes. Knockdown of two selected cardiac-enhancer-associated lncRNAs led to the suppression of gene expression of the corresponding genomic locus (Ounzain et al. 2014b).

13.4.4 Interactions Between miRNAs and lncRNAs

Long noncoding RNAs have also been characterized for their interactions with microRNAs via miRNA-binding sequence domains. Thereby, they serve as competing endogenous RNAs (ceRNAs) and bind miRNAs (as “sponges”), which reduces

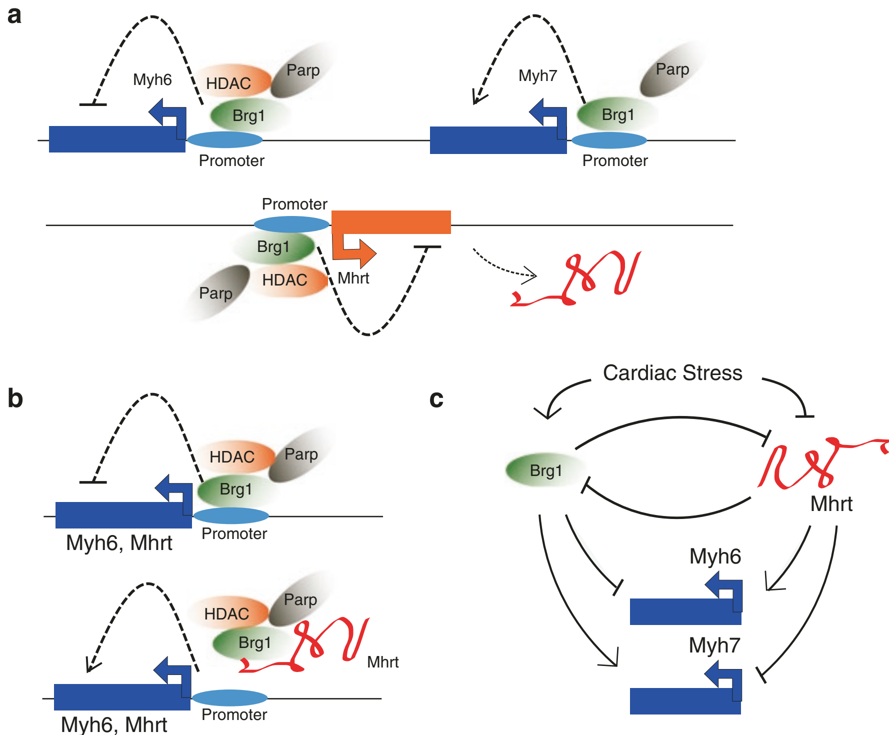

Fig. 13.2 LncRNA Mhrt modulates DNA binding of Brg1–chromatin repressor complex in cardiac hypertrophy: **(a)** During conditions of cardiac stress, chromatin repressor complex Brg1-Parp-Hdac suppresses Myh6 and activates Myh7 transcription, promoting the myosin isoform switch. LncRNA Myhrt is an antisense-lncRNA, overlapping parts of the Myh7 locus and also transcriptionally repressed by the Brg1 complex, **(b)** The Brg1–Parp–Hdac complex interacts with the Myh6 promoter to repress the transcription of Myh6 and Mhrt. Mhrt binds the Brg1-helicase domain and thereby impedes Brg1 to recognize its chromatin targets, **(c)** Pathological stress activates the Brg1–Hdac–Parp chromatin repressor complex to inhibit Mhrt transcription in the heart. Brg1 and Mhrt form a negative feedback loop. Brg1 is a mediator of cardiac hypertrophy and functions as a repressor of Myh6 and activator of Myh7. Mhrt inhibits Brg chromatin repressor complexes to recognize their targets and thereby leads to a normalization of Myh6/Myh7-ratio, *Parp* poly (ADP ribose) polymerase, *HDAC* histone deacetylase, *Myh6* alpha-myosin heavy-chain, *Myh7* beta-myosin heavy-chain, *Mhrt* myosin heavy-chain-associated RNA transcripts

target miRNA levels and alleviates its downstream effect (Salmena et al. 2011; Tay et al. 2014). The relevance of this interaction has been established in several examples for skeletal muscle transcriptional regulation and recently also in the heart (Cesana et al. 2011; Dey et al. 2014).

Long noncoding RNA AK048451, named “cardiac hypertrophy-related factor” (CHRF), is significantly induced after the treatment of cardiac myocytes with angiotensin-II and harbors a binding site for miR-489, with high interspecies sequence conservation. Knockdown and overexpression of CHFR modulates

expression levels of miR-489, followed by impaired or exaggerated hypertrophic response via change of sequestration of miR-489 and changed expression of downstream target Myd88 (Wang et al. 2014a).

Prohibitin subunit 2 (Phb2) is downregulated in cardiac myocytes after exposure to anoxia. Overexpression of Phb2 reduces mitochondrial fission, apoptosis, and size of myocardial infarction in mice. Vice versa, the knockdown increases mitochondrial fission and infarct size. Phb2 is specifically regulated though microRNA miR-539 via binding at the 3′ UTR. Downregulation of miR-539 inhibited both mitochondrial fission and apoptosis induced by anoxia. These effects were abrogated by the knockdown of Phb2, therefore indicating a functional link of miR-539 and downstream Phb2. Using bioinformatics, lncRNA AK017121 (named "CARL") was identified, that is able to specifically bind miR-539 and, by modulating its expression, CARL exert functional effects on apoptosis and mitochondrial fission in cardiac myocytes (Wang et al. 2014b).

Autophagy is a key stress response in cardiac myocytes, aimed at preventing cell death at the expense of digesting short-term dispensable proteins and/or organelles. Atg7 encodes the E1 enzyme in the autophagosome and is involved in the regulation of autophagy and cell death. MicroRNA miR-188-3p is able to specifically bind to the 3′-UTR of Atg7 and thereby exerts an inhibitory effect on autophagic response of cardiac myocytes. LncRNA AK079427 (named "AFP") is able to specifically bind miR-188-3p and thereby interferes with this signaling cascade. AFP participates in the autophagic regulation both in cardiac myocytes and in an in vivo model of ischemia-reperfusion injury (Wang et al. 2015).

13.4.5 LncRNAs, Short Open Reading Frames and Small Peptides

Attributing the coding potential of a transcript is usually achieved using a combination of several bioinformatics approaches, combining measures of evolutionary conservation of amino acid sequences known protein domains and other data (e.g., from ribosome sequencing, mass spectroscopy). This is especially difficult for short transcripts and is prone to error. Short open reading frames can be defined by their size ranging from 2 to 100 codons. Several transcripts originally attributed as noncoding were found to be coding after experimental validation, some of them being translated to micropeptides with important regulatory functions (Andrews and Rothnagel 2014): ELABELA is a 32 amino acid protein that is highly conserved between species. Experimental deletion in zebrafish leads to cardiac dysplasia of varying severity and even aplasia of the heart, due to impaired endodermal differentiation and possibly signaling as a novel apelin-receptor ligand (Chng et al. 2013).

LncRNA gene RNA 003 in 2 L (pncr003:2 L) in fruit flies was detected to harbor two evolutionary conserved small open reading frames of 28 and 29 amino acids length. Both transcripts are strongly expressed in somatic muscles and the postembryonic heart. Overexpression and knockdown modulate calcium transients and induce cardiac arrhythmia. Both peptides show homology to sarcolipin and

phospholamban and were therefore named sarcolamban, as strong phylogenetic evidence points toward a shared ancestral peptide (Magny et al. 2013).

Myoregulin is a conserved micropeptide encoded by a skeletal muscle-specific transcript, also originally attributed as a lncRNA. Myoregulin is a direct interaction partner of the calcium pump SERCA and inhibits calcium transport activity into the sarcoplasmatic reticulum. Genetic knockdown improves skeletal muscle exercise performance in mice (Anderson et al. 2015).

13.4.6 Signatures of Long Noncoding RNAs in Mouse Models of Cardiac Development and Disease

Several research groups reported differential ncRNA expression data from high-throughput assays from different models of cardiac development and disease, such as pressure overload due to transverse aortic constriction or coronary artery ligation to induce myocardial infarction (Tarnavski 2009). We summarized the most significant findings in Table 13.2 and Fig. 13.3.

13.5 Long Noncoding RNAs in Cardiovascular Research: Bedside

Low evolutionary conservation of lncRNAs makes translation of research results from experimental models difficult. Nevertheless, some groups already reported a role for lncRNAs in human myocardial infarction, heart failure and of course, sequence variation in noncoding regions.

13.5.1 Heart Failure

Mitochondrial lncRNA LIPCAR was identified in serum of patients post myocardial infarction and characterized as a biomarker for heart failure. Initially, LIPCAR-plasma levels decrease after myocardial infarction but show strong induction in patients who develop pathologic left ventricular (LV) remodeling in the future. Furthermore, it is also induced in patients suffering of heart failure from nonischemic etiology and expression levels strongly and independently correlate with mortality, with incremental diagnostic value compared to a model comprised of classic risk factors (Kumarswamy et al. 2014).

Analyses of human LV samples from ischemic and nonischemic cardiomyopathies before and after implantation of a mechanical assist device (LVAD) compared to control hearts using RNA-sequencing identified signatures of lncRNAs that are able to distinguish between etiologies of heart failure and discriminate patients between pre- and post-LVAD state. Furthermore, correlation analysis of lncRNA and neighboring mRNA expression revealed a significant prevalence of cis-regulation of lncRNAs and neighboring protein-coding genomic loci (Yang et al. 2014).

Table 13.2 High-throughput screening experiments in cardiac disease models

Authors	Species	Model	Assay	Key findings
Lee et al. (2011)	Mouse	TAC	RNA-Seq	15 and 135 differentially regulated lncRNAs between hypertrophy and control and failing and control, respectively. Strong induction of lncRNA H19. LncRNAs show a higher degree of tissue specific expression compared to protein coding genes
Li et al. (2013)	Mouse	Isoproteronol	Microarray	Identification of 32 lncRNAs with the patterns of regulation in plasma, heart tissue, and peripheral blood cells that might be used as potential biomarkers for heart failure
Liu et al. (2014)	Mouse	Ischemia reperfusion (LAD ligation)	Microarray	Identification of 151 differentially regulated lncRNAs
Matkovich et al. (2014)	Mouse	Embryonic heart E14.5, adult heart, TAC	RNA-Seq	Cardiac hypertrophy does not lead to the activation of embryonic expressed lncRNAs. Larger extend of differentially regulated lncRNAs between embryonic and adult conditions than between adult and hypertrophic/failing state
Ounzain et al. (2014a)	Mouse	LAD ligation	RNA-Seq	Identification of differentially regulated lncRNAs in a LAD-ligation mouse model, correlation with chromatin-state maps (strongest correlation of lncRNAs with chromatin marks of enhancer activity) and validation of evolutionary conserved lncRNAs in human-diseased tissues
Ounzain et al. (2014b)	Mouse	Embryonic stem cell differentiation, LAD ligation, TAC	RNA-Seq	Identification and characterization of foetal cardiac enhancer-associated lncRNAs. These are differentially regulated in the models of ischemia and pressure overload, knockdown of eRNAs regulates the transcription of associated target genomic locus
Zangrando et al. (2014)	Mouse	LAD ligation	Microarray	30 differentially regulated lncRNAs, most robustly induced are Miat1 and Miat2
Zhu et al. (2013)	Mouse	Embryonic hearts E11.5, E14.5, E18.5	Microarray	1237 differentially regulated long noncoding RNAs

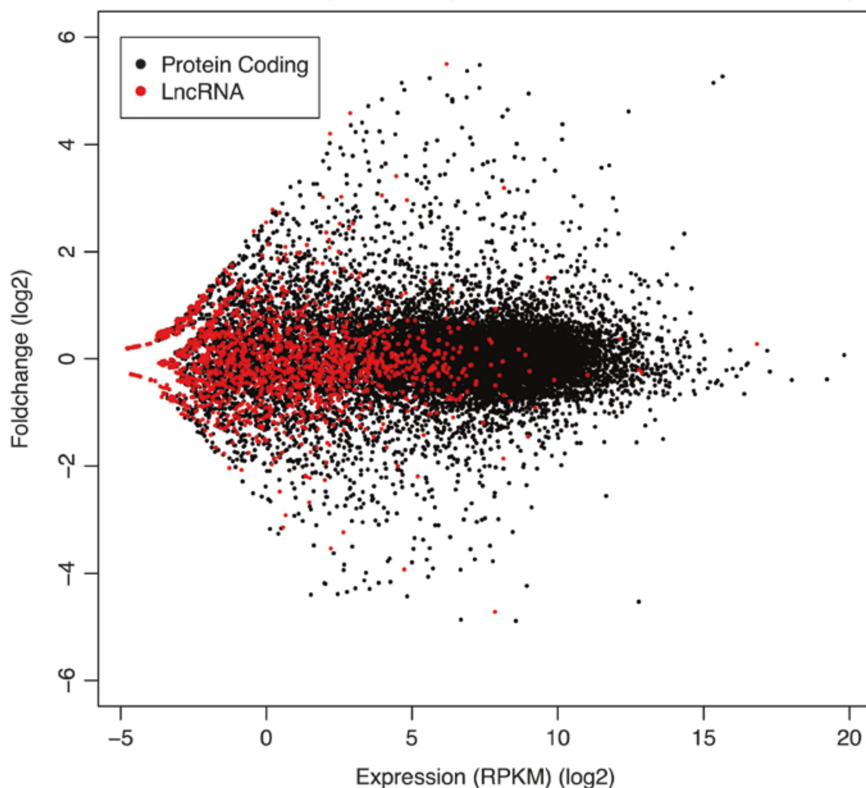

Fig. 13.3 LncRNA expression levels are lower compared to protein coding transcripts and show a lower degree of differential regulation. RNA-Seq data from Calcineurin-transgenic mouse hearts and controls (unpublished data Kühl/Frey)

13.5.2 Myocardial Infarction

Vausort et al. measured expression levels of five long noncoding RNAs with known relevance in the cardiovascular system in peripheral blood lymphocytes of 414 patients with acute coronary syndromes and 86 control patients. They selected antisense hypoxia-inducible factor 1α (aHIF), ANRIL within the 9p21-risk locus for coronary heart disease, KCNQ1-overlapping transcript 1 (KCNQ1OT1) that regulates the imprinting of KCNQ1 sodium channel, myocardial infarction-associated transcript (MIAT), and metastasis-associated lung adenocarcinoma transcript 1 (MALAT), one of the most abundantly expressed lncRNAs. In ST-elevation-myocardial infarction, the expression of ANRIL, KCNQ1OT1, MIAT, and MALAT was reduced, compared to expression levels in non-ST-elevation-myocardial infarction. The authors later used univariate and multivariate analysis to identify several associations between ejection fraction, age, diabetes mellitus, and hypertension and

expression values of lncRNAs. The measurement of ANRIL and KCNQ10T1 added significant discriminatory value in identifying patients at risk of developing LV dysfunction using multivariate analysis (Vausort et al. 2014).

13.6 Human Noncoding Variation and Heart Disease

The understanding of human genomic variation and mutations in noncoding regions and the effect on pathogenesis of human disease is incomplete. Data from the ENCODE and NIH Roadmap Epigenomics Project provide a resource to link genomic sequence, transcriptional activity, and chromatin state (Bernstein et al. 2010).

An analysis of data from the "1000 genomes project" aimed to characterize variations in noncoding regions of the human genome. Transcription factor-binding sites and noncoding RNAs were less constrained for SNPs compared to protein-coding sequences. Per contra, noncoding RNAs showed a higher degree of evolutionary constraint for insertions and deletions. A higher degree of purifying selection in a lncRNA correlated with higher levels of transcriptional expression (Mu et al. 2011).

The human genomic region 9p21.3 is among the most thoroughly studied susceptibility loci for several entities of cardiovascular disease and has been characterized in different populations (Roberts 2014; Johnson et al. 2013). Of note, this region contains the protein-coding genes CDKN2A and CDKN2B and the long noncoding RNA ANRIL ("antisense non-coding RNA in the INK4 locus"), which is strongly associated with several major SNPs of cardiac disease (Pasmant et al. 2011).

Ishii et al. conducted a case–control genetic association study and identified a new susceptibility locus for myocardial infarction located on chromosome 22q12.1 and a novel long noncoding RNA, named MIAT ("myocardial infarction-associated transcript") and characterized the impact of several SNPs that changed MIAT expression and binding to nuclear proteins (Ishii et al. 2006).

A large genomewide association trial of dilated cardiomyopathy in more than 4,100 cases and 7,600 controls reported several single nucleotide polymorphisms (SNPs) on chromosome 6p21. Highest significance after replication was attributed to a SNP that mapped to a noncoding gene, HCG22 (Meder et al. 2014).

A recent systematic analysis of published genomewide association studies found that 93% of disease and trait-associated variants lie within noncoding sequences, which comprises noncoding DNA-regulatory regions such as enhancers, promoters, DNAse hypersensitivity sites but also lncRNAs (Maurano et al. 2012).

13.7 Summary

Research into long noncoding RNAs has led to important discoveries in cardiac physiology and pathophysiology. Characterizing lncRNAs sparked the discovery of intricate links between transcription, chromatin modification, and organization that will be translated to an improved understanding of cardiac pathophysiology and disease. Hopefully, this also will lead to new targets and novel therapies. For

example, targeting antisense transcripts instead of the coding transcript allows modifying transcriptional activity at a genomic locus without changing the locus itself. As expression of many lncRNA is restricted to a specific tissue, targeting via systemically delivered gene therapy potentially causes fewer systemic off-target effects.

Looking at the growing body of research, several gaps remain. First, the wealth of long noncoding RNA sequences so far lacks clear annotation and is divided into a variety of databases. A unifying nomenclature has recently been proposed for human long noncoding RNA but needs to be introduced into all fields of lncRNA research (St Laurent et al. 2015; Wright 2014). The quality and depth of chromatin maps from human and mouse heart that allowed a more detailed understanding of cardiac lncRNAs is still not available for rat and zebrafish, two relevant species for cardiovascular research. Also, for an understanding of lncRNAs in the heart, we need to replicate the findings from ENCODE that did not include cells from the heart. So far, most results were obtained in heterogeneous cardiac cell populations. Thus, delineating the transcriptome of individual populations, such as cardiac myocytes, fibroblasts, smooth muscle, and endothelial cells, will be another important goal. Second, we will need to readjust our knowledge about mutations in noncoding regions of the genome and will have to include them for a deeper understanding of hereditary factors and mutations in cardiac disease, such as hypertrophic cardiomyopathies. Third, the individual characterization and delineation of long noncoding RNA mechanisms will need translation to the bedside. Apart from a use as biomarkers – and in that case, lncRNAs have to prove their added value compared to classic biomarkers or miRNA signatures, we will need to develop novel approaches to target and modulate cellular lncRNA expression levels.

Conflict of Interest The authors declare that they have no conflict of interest.

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