Published in final edited form as:

Proteomics. 2014 October ; 14(19): 2115–2126. doi:10.1002/pmic.201400131.

How the proteome packages the genome for cardiovascular development

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Abstract

The devastating impact of congenital heart defects has made mechanisms of vertebrate heart and vascular development an active area of study. Because myocyte death is a common feature of acquired cardiovascular diseases and the adult heart does not regenerate, the need exists to understand whether features of the developing heart and vasculature—which are more plastic—can be exploited therapeutically in the disease setting. We know that a core network of transcription factors governs commitment to the cardiovascular lineage, and recent studies using genetic loss-of-function approaches and unbiased genomic studies have revealed the role for various chromatin modulatory events. We reason that chromatin structure itself is a causal feature that influences transcriptome complexity along a developmental continuum, and the purpose of this article is to highlight the areas in which ‘omics technologies have the potential to reveal new principles of phenotypic plasticity in development. We review the major mechanisms of chromatin structural regulation, highlighting what is known about their actions to control cardiovascular differentiation. We discuss emergent mechanisms of regulation that have been identified on the basis of genomic and proteomic studies of cardiac nuclei and identify current challenges to an integrated understanding of chromatin structure and cardiovascular phenotype.

Keywords

chromatin; epigenetics; genomics; proteomics; heart; vasculature; systems biology

Introduction

Cardiac development involves signaling by stage-specific transcription factors that define branching into cellular lineages. Cardiomyocytes arise from the mesoderm (marked by *Brachyury* and *Mesp1*), in which cardiac-determined cells of the primary heart fields fuse to form the bilateral cardiac crescent structure [1-4]. The bilateral fields of the cardiac crescent merge and differentiate into progenitor cells (marked by *Nkx2-5* and *Isl1*) of the linear heart tube that then mature into cardiomyocytes (marked by *Myh6* and *Myh7*) [1-3]. Tissue from the primary heart field will develop into the left ventricle and atria while the precursor cells

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The authors report no conflicts of interest associated with this paper.

of the second heart field will contribute to the right ventricle, atria and outflow tract [5, 6]; both heart fields have potential to also contribute to the coronary vasculature [7]. Later developmental events include cardiac looping, formation of the outflow tract that will give rise to the pulmonary artery and aorta, and atrial-ventricular septation [4]. Brachyury-expressing mesoderm cells can differentiate into hematopoietic cells and myocytes [8] while *Mesp1*-positive pre-cardiac cells are restricted to smooth muscle or cardiomyocyte fate [9]. *Mesp1* can induce expression of cardiac transcription factors, including *Gata4* and *Mef2c* [9], and is required for heart tube formation [10]. Cardiac progenitors can diverge to give rise to cardiomyocytes, cardiac fibroblasts, endothelial cells and smooth muscle [7, 11]. Although *GATA4*, *NKX2-5* and *TBX5* are necessary transcription factors for myocyte proliferation and cardiac morphogenesis [12, 13], how these signaling pathways regulate chromatin architecture to fine tune global gene expression patterns and the chromatin level factors that determine specialization of cardiomyocytes into atrial, ventricular, and conduction cells remain unknown.

Genome organization in the three-dimensional nuclear space, shaped by temporal and spatial presence of protein factors, underlies differences in cell type-specific transcriptomes. DNA is wound around core histone units (H2A, H2B, H3 and H4) to form the nucleosome, the primary structural unit of chromatin. Chromatin can be loosely packaged into euchromatin, which can allow for gene expression, or tightly packaged into heterochromatin, associated with silenced regions of the genome. There are stark differences in the packaging of the genome in different cell types and along the same lineage at different stages of development (Figure 1). The mechanisms to establish and modify chromatin structure include histone post-translational modifications (PTMs), histone variants, nucleosome positioning (e.g. by ATP-dependent chromatin remodelers), non-nucleosomal chromatin structural proteins, DNA modifications and noncoding RNAs (Figure 2). During terminal differentiation of the cardiomyocytes, there is an overall shift from euchromatin to heterochromatin [14] and decrease in transcriptional activity [15] (Figure 3). These changes determine cell identity by regulating chromatin packaging in a manner conducive to production of the desired transcriptome—that is, by making the right genes accessible to transcription factors and RNA polymerase. The chromatin structural features of cardiomyocytes remain uncharacterized—furthermore, we have only limited understanding of the mechanisms that establish chromatin structure and delineate phenotype during development. While some aspects of chromatin structure are globally altered with disease, cells do not change from one type to another, implying that a baseline skeleton of the chromatin anatomy is firmly set with development. This baseline skeleton is non-random and reproducible, as determined by global chromosome conformation capture and imaging studies [16, 17]. The role of individual chromatin remodeling proteins and histone modifiers has also been recognized in cardiovascular development, yet only recently have studies begun to emerge using genome or proteome-wide studies to characterize this process in an unbiased manner. We summarize herein what is known about the key chromatin structural regulators involved in cardiac development (please see Supplemental Table for a comprehensive list of gain/loss of function studies on chromatin regulators in cardiovascular development) and place an emphasis on emergent areas in which proteomics and systems biology can advance our understanding of the epigenetics of development.

Histone Variants and Post-Translational Modifications

Histone methylation and acetylation are the best-studied histone PTMs that, depending on the residue they target, can be indicative of gene repression or activation. Trimethylation of histone H3 at lysine 4 (H3K4me3) is a mark for euchromatin [18] while methylation at lysine 27 (H3K27me3) [19] and lysine 9 (H3K9me3) [20] label heterochromatin, facultative and constitutive respectively. Promoters containing both H3K4me3 and H3K27me3 are labeled poised—meaning they can be selectively activated or silenced in a lineage-specific manner—and are characteristic of regulators of differentiation. Reader proteins that are selective for these histone modifications can further influence accessibility and packaging of the DNA. While a number of other types of histone PTMs have been identified, principally by mass spectrometry [21], the individual functions in chromatin maintenance and regulation of packaging are less established, and how these modifications interact in a coordinate fashion to mediate chromatin architecture during developmental transitions, is yet to be determined.

Although these modifications were originally described in non-cardiovascular cells, many have been shown to be operative in the cardiovascular systems, with the most abundant evidence coming from naturally occurring or experimental disruption of the histone-modifying enzymes. In one recent and unbiased example of the former, examination by exome sequencing of human congenital heart disease patient samples revealed an increased incidence of *de novo* mutations occurring at genes involved in depositing, removing and reading H3K4me3 or H2BK120 ubiquitination marks [22]. This study is strong rationale for more 'omics investigations into how myocyte-specific gene expression is coordinated across the genome: because disruption of any one of various histone modifying enzymes alone is sufficient to induce cardiac development defect, transcriptional circuits in cardiac development can be hypothesized to involve a panoply of histone modifications at various genes—some activating and some inhibiting—in addition to transcription factors. The mutations identified in Zaidi *et al.* occurred in various enzymes in the language of histones (those depositing, reading and removing modifications)—the next step will be to determine which loci are targeted by these enzymes during development, and whether networks can be built to understand convergent targets amongst the group of altered histone modifiers (e.g. Do the enzymes modified by *de novo* mutations all target the same genes?).

The Polycomb Repressive Complex 2 (PRC2) is a multiprotein complex involved in depositing H3K27me3. Evaluation of PRC2 in the mouse heart has implicated it in development and maintenance of appropriate gene expression [23]. Inactivation of Ezh2, a protein component of PRC2, resulted in hypoplasia, septal defects and atrial dilation in embryos—mice surviving to adulthood had septal defects, hypoplasia, fibrosis and impaired systolic function [23]. Chromatin immunoprecipitation sequencing (ChIP-seq) and RNA sequencing (RNA-seq) studies demonstrated that Ezh2 is critical for ventricle-specific gene expression and silencing of non-cardiomyocyte transcription factors during the early stages of heart development [23]. At the same time, removal of H3K27me3 is also required for differentiation into cardiovascular cells. JMJD3, a histone demethylase, enables expression of mesoderm marker, *brachyury*, by removing H3K27me3 at its promoter, in differentiating embryonic stem cells [24]. Jmjd3 knockout in embryonic stem cells prevents differentiation

into mesoderm and cardiomyocytes by disallowing expression of genes necessary for differentiation (i.e. *Mesp1*, *Nkx2-5* and other cardiac structural proteins) [24].

The Smyd family of lysine methyltransferases has been implicated in gene regulation, with Smyd1 shown to be specific to cardiac and skeletal muscle [25]. Knockout of Smyd1 is embryonic lethal in mice, in part due to incomplete development of the right ventricle [25]. In zebrafish, Smyd1 is important in the maintenance of sarcomere organization [26, 27], perhaps independent of its functions through chromatin. WHSC1 is another histone methyltransferase, shown to establish H3K36me3 marks [28]. WHSC1 can also interact with cardiac transcription factor NKX2-5 to suppress expression of cardiac progenitor genes [28], and loss of Whsc1 function leads to congenital heart defects [28]. Furthermore, interactions between different histone methylating complexes are highly coordinated. For example, PHF19, a reader protein, can recognize and bind to H3K36me3 in stem cells to recruit H3K36me3 demethylase NO66, while at the same time recruiting PRC2 to shift from H3K36me3 to H3K27me3 modification [29] and thereby silence Polycomb target genes. JARID2, a transcriptional repressor, has been shown to regulate histone methylation during development via association with PRC2 to promote normal differentiation of mouse embryonic stem cells [30]. In addition, stem cell differentiation entails transitioning into a more heterochromatic state through accumulation of H3K9 methylation [14, 31]. In the developing heart, JARID2 was found to interact with H3K9 methyltransferase, SETDB1 [32], highlighting another role for JARID2. JARID2 knockout mice had global reduction in H3K9 methylation as well as cardiac developmental impairments, including septal defects and hypertrabeculation [32]. It is unclear how JARID2 regulates different histone modifications observed in stem cells and developing heart tissue, and whether its role is specific to the target gene or developmental stage, and/or dependent on other unidentified factors. Histone modification complexes can interact with cardiac transcription factors to co-regulate target genes. JMJD3 closely associates with TBX5 to target promoters and regulate cell fate [33]. Overexpression of *Isl1* can promote expression of other cardiac transcription factors (*Gata4*, *Nkx2-5* and *Mef2c*) through increased H3 acetylation at these genes [34]. These findings have provided a link between the functions of chromatin structure and tissue-specific transcription factors.

Histone acetylation is another modification required for development. p300 and CBP histone acetyltransferases show peak expression during embryonic development that is reduced in the adult heart [35]. Mice with mutated p300 exhibit septal defects and ventricular hypoplasia in addition to other systemic developmental defects [36, 37] and roles in adult heart disease [38]. Class I and II histone deacetylases (HDAC) are critical for cardiac chamber development, proliferation and function. HDAC1 and HDAC2 (Class I HDACs) are functionally redundant [39], but a cardiac-specific double knockout results in inappropriate upregulation of genes associated with stress response, calcium handling and the contractile apparatus [39]. Overexpression of HDAC3 (Class I) regulates cardiomyocyte proliferation during development by repressing cell cycle inhibition genes [40]. HDAC7 (Class IIa) mutant mice have impaired vascular remodeling by upregulation of matrix metalloproteinase that interferes with vessel integrity during development [41]. HDAC5 and HDAC9 (Class IIa) serve redundant functions—suppressing stress-induced genes—and are required for development of ventricular walls and septum formation [42]. Class III sirtuins

and class IV HDACs are less characterized in the context of chromatin and cardiac development. Some studies have suggested low sirtuin activity correlates with cardiac disease phenotypes [43, 44], whereas other studies have demonstrated their requirement for normal blood vessel formation and the angiogenic response to ischemia [45]. The different classes of HDACs with inherent functional redundancy may reflect a mechanism that has evolved for tight control of chromatin structure in a stage-specific manner during development and in a cell type-specific manner in disease.

In addition to covalent modifications of histone tails, histone variants present another level of complexity to chromatin structure by altering DNA packing in the nucleosome, interactions with other core and linker histones, and interactions with DNA repair and transcriptional machinery to regulate nucleosome stability [46]. H2A.Z, an H2A variant, is present in nucleosomes near transcription start sites of active genes [47]. While associated with nucleosome depletion and open chromatin formation, H2A.Z was also found to be enriched at bivalent promoters, marked with H3K27me3, and in close proximity with transcription start sites of genes that determine cell fate, such as transcription factors, in mouse embryonic stem cells [48]. In differentiating neuronal precursor cells, H2A.Z enrichment is associated with active genes, and it is required for differentiation *in vitro* [48]. While it is known that H2A.Z is upregulated during cardiac hypertrophy [49], the specific role it plays in regulation of cardiomyocyte differentiation and heart development has not been established. Other histone variants have not been explored in detail in cardiac development, although the available proteomic literature on the adult indicates that the mouse heart expresses a wide range of variant isoforms for each of the four core histones, as well as for linker histone H1 [50].

One limitation in the histone acetylation/methylation literature in particular has been an absence of genome-wide ChIP-seq studies in cardiovascular cells, in large part due to low endogenous expression of these enzymes and cellular heterogeneity in complex tissues like heart and vasculature. These investigations are needed to determine, in a developmental stage-specific manner, the distribution of individual histone-modifying enzymes as well as their target histone PTMs along the genome. As a result, many loss-of-function studies may reveal phenotypes that are the result of overlapping chromatin-modifying enzymes. Recent studies have made headway in this area with histone marks themselves in isolated cell models of cardiac development [3, 51], as well as in the adult heart (notwithstanding the issues of uncertain cell type contribution when working from endogenous cardiac tissue) during disease [52, 53]. *In vitro* differentiation of cardiomyocytes has shown the regulation by histone PTMs at key cardiac genes [51]. Genes encoding for cardiac transcription factors are enriched with H3K27me3 marks and poised for activation at early primitive stages [51]. During differentiation and concomitant with transcription factor activation, there is a decrease in the silencing H3K27me3 mark and parallel increases in active H3K4me3 and H3K36me3 (associated with transcriptional elongation) marks [51]. Meanwhile, cardiac structural genes, when turned on, accumulate H3K4me3 and H3K36me3 and lack the additional level of regulation by H3K27me3 [51]. These findings highlight the necessity of appropriate global chromatin remodeling by histone modifications, in addition to expression of master regulators, for the coordinated activation of developmental stage-specific genes.

Future studies using ChIP-seq for endogenous histone-modifying enzymes will be required to elucidate how these proteins coordinate with cardiac transcription factors *in vivo* and at different developmental stages.

ChIP-seq studies for histone PTMs have characterized distal regulatory elements and revealed cardiac-specific enhancers important for developmental and stage-specific regulation of cardiac genes. These enhancers are not static—rather, they serve a temporal role during both differentiation *in vitro* and development [54] with precise coordination of activity with the putative binding and expression of cardiac transcription factors [3]. Observations to date have characterized enhancer function by association with the nearest gene on the chromosome without taking endogenous three-dimensional organization into account. As such, structural enhancers—that is, permissive features of chromatin that play a role in endogenous architecture, are distal from the genes they regulate on the DNA strand but near said genes in the endogenous chromatin environment—have not been examined in cardiovascular development. Future studies integrating the current enhancer datasets with three-dimensional chromatin architecture measurements [17] will be important to understand how organization of the genome is coordinated *in vivo* to orchestrate cell fate—whether these enhancers are shared or distinct from those associated with cardiac hypertrophy [52] is another question that, when answered, will advance our understanding of the scales at which cellular programming in disease mimics that in development.

ATP-Dependent Chromatin Remodelers

ATP-dependent chromatin remodelers are critical regulators of DNA accessibility for gene expression and silencing by mediating nucleosome positioning, occupancy and histone variant composition [55, 56]. There are four classes of remodelers: SWI/SNF, ISWI, NuRD and INO80. The SWI/SNF complexes have been widely studied in the heart, in both development and disease models. The BAF and PBAF complexes are examples of the mammalian SWI/SNF complex and are composed of multiple subunits, interchanging of which can confer target loci specificity (e.g. the BAF complex has the BAF250 subunit while the PBAF complex incorporates BAF200 and BAF180 [57]). BAF180 normally co-regulates target genes with nuclear receptors and its deletion results in ventricular wall hypoplasia, whereas BAF250 deletion leads to lethality [58]. BAF250a participates in normal cardiac progenitor cell differentiation—loss of BAF250a leads to pervasive cardiac developmental defects and death, acting by inhibiting cellular proliferation. These actions appear to proceed through binding of BAF250a in the promoters of various cardiac transcription factors [59], although global targets of the proteins in the heart remain unknown. BAF250 regulation of Gata4, Mef2c, Nkx2-5 and Bmp10 expression is mediated through increasing DNA accessibility at promoters [59]. In agreement with this observation, BAF250a mutations in embryonic stem cells limit pluripotency potential and prevent differentiation into cardiomyocytes [60]. In addition to its role in cardiac hypertrophy [61], the ATPase catalytic subunit BRG1 is required for cardiac development [62], and the stoichiometry of interactions amongst BRG1 and TBX transcription factors can mediate transcription of target cardiac genes [62].

BAF60c is specific to the developing heart and, like BRG1, can mediate interactions with cardiac transcription factors to specify cardiac gene expression [63]. Specific targeting of remodeling complexes may arise with from readers of histone modifications. DPF3 is a muscle-specific protein that can interact with methylated and acetylated histones as well as members of the BAF complex [64]. DPF3 is necessary for zebrafish cardiac development and can localize BAF chromatin remodeling complexes to cardiac-specific genomic loci to regulate gene expression [64]. In addition to normal development, there is evidence that chromatin remodelers are important for cardiomyocyte differentiation from an induced pluripotent state. GATA4 requires the presence of BAF60c to act on target genes, and together with TBX5, this complex is sufficient to induce differentiation of non-cardiac mesoderm into beating cardiomyocytes [65].

The roles of the other classes of ATP-dependent remodelers have not been well defined in the heart. The CHD4 of NuRD complex is important for maintenance of vascular integrity *in vivo* by directly regulating expression of extracellular matrix proteases [66]. Recently, it was found that BAF250a can interact with NuRD complex to repress transcription in P19 carcinoma cells [67] although the implications for a role of NuRD in cardiac development are unclear. The efficiency of activity of ATP-dependent chromatin remodelers can also depend on association with histones as observed with ISWI. ISWI ATPase activity requires recognition of H4 tails [68], and this interaction and level of ISWI activity are higher in the presence of H2A.Z variants [69]. ISWI ATPase *Drosophila* mutants demonstrate requirement of ATPase activity for chromosome compaction and higher-order structure, which can be mediated through recruitment of linker histones [70]. It is anticipated that roles for these remodelers will be tested soon using loss-of-function approaches in the developing heart.

A common theme with ATP-dependent chromatin remodelers is that the combinatorial composition of their subunits can confer specificity of action. Much of the knowledge of subunit composition has been imported to the cardiac field from non-cardiac systems and then tested with loss-of-function approaches, as described in the foregoing paragraphs. A frontier for cardiac-specific chromatin remodeling is the application of interactome mapping targeted at chromatin remodeling complexes in the myocyte. Affinity isolation and proteomic mass spectrometry needs to be applied to characterize these complexes in an unbiased manner at different developmental stages, to reveal not only interactions with known cardiac transcription factors, but also with other regulators of activity and gene targeting. Methods like the INTACT approach [71], which uses genetic labeling of specific proteins in a lineage of interest, hold great promise, as they obviate concerns of cell type specificity and recovery of endogenous proteins.

DNA Methylation

DNA methylation is a direct modification of cytosine, associated with gene silencing when present in promoters, enriched at CG-rich regions (CpG islands), and expression when present within gene bodies. There are global increases in DNA methylation during cellular differentiation. *De novo* methylation by DNA methyltransferases, DNMT3a and DNMT3b, is essential for early differentiation in embryonic stem cells [72]. Dnmt3b mutations in mice

are lethal, showing incomplete ventricular septum formation in addition to other systemic defects interfering with vascular and liver development [73]. A model for how DNA methylation contributes to higher-order chromatin structure (between the scale of nucleosome and chromosome) is currently lacking, and it is commonly assumed that this epigenetic mark works in concert with histone modifications to specify chromatin states.

DNA methylation can recruit different sets of protein interacting partners. Methyl-CpG binding protein (MeCP2) is a DNA methylation binding protein that is required for mouse development [74]. MeCP2 can serve as a reader of methyl-cytosine to regulate establishment of heterochromatin by recruiting remodeling machinery to modify histones, such as deacetylation of H4 and methylation of H3K9, thereby silencing genes during development [75]. In developing cardiomyocytes, there are increases in the expression of Dnmts and methyl-cytosine binding proteins [76], and DNMT activity inhibition in this system can result in net increases in euchromatic histone marks, H3 and H4 acetylation and H3K4me3 [76]. An increase in DNA methylation can result in the binucleation and terminal differentiation of cultured fetal cardiomyocytes [77]. Changes in methylation states at promoters in genes involved in cardiac development, including EGFR, Tbx5 and Nkx2-5, have been observed in congenital heart disease cases [78]. These findings implicate DNA methylation as an important regulator of cardiovascular development worthy of further exploration.

Global methylome studies using bisulfite treatment and DNA sequencing—which would reveal the totality of modified CpGs and shed light on how methylation correlates with chromatin marks and transcription on a global scale—have not been reported for the heart. Studies from adult human tissue in the setting of heart disease indicate global changes in methylation [79], but even in this arena case-control studies are lacking to understand the basal differences in DNA methylation between cells in the cardiovascular lineage. A frontier in this field will be combining DNA methylomics with protein identification—either by looking for interacting partners of known methyl-cytosine binding proteins, or by screening for novel, cardiac-specific methyl-cytosine proteins directly, as has been proposed in other systems [80]—only with this approach will we understand how DNA methylation contributes to high-order chromatin structure, by linking nucleotide modification to protein interaction with chromatin structure as a readout.

Chromatin Structural Proteins

Chromatin structural proteins, which modulate accessibility and local packing independent of direct enzymatic activity, play a role in the establishment and/or maintenance of chromatin architecture and accessibility through direct and indirect mechanisms. Non-nucleosomal structural proteins, linker histone H1 and heterochromatin-associated HP1, have dynamic interactions in stem cell nuclei and become immobile as cells differentiate to establish high-order structure [81], suggesting the proteins participate in setting the chromatin scaffold during development. Levels of histone H1 variants are associated with differentiation of stem cells, with increases in H1.0 variant occupancy at pluripotency genes allowing for silencing of these genes and progression of stem cell differentiation [82, 83]. Both linker histones and high mobility group B (HMGB) proteins have been implicated in

global increases in transcriptional activity in heart disease [84], although their role in development remains unknown. HMGA2, on the other hand, is an important regulator of cardiomyocyte differentiation and proliferation and can bind to the promoter of Nkx2-5 to directly regulate its expression [85]. HMGA proteins are expressed abundantly during early development, with low expression in mature cells [86]. Some genomic territories, like the nucleolus, are associated with specific chromatin-binding proteins [87]. Studies from zebrafish have implicated Nucleolin in ribosomal RNA biosynthesis and proper heart formation, a role that may be recapitulated in the setting of disease in mammalian models [88].

The nuclear envelope is an important site for anchoring of repressed genes and accordingly hosts silencing machinery [89, 90]. Proteins of the nuclear envelope physically interact with the cellular cytoskeleton enabling communication between cytoplasmic functions and cellular mechanics with nuclear properties and gene expression. There is also significant evidence that specific nuclear envelope proteins silence genes during cardiac lineage commitment, with lamins being a well-characterized example. Lamin A/C has been shown to tether chromatin to the nuclear envelope and reduce the plasticity in differentiated cells [91]. Stem cell-derived cardiomyocytes have increased lamin A/C expression, which is undetectable in embryonic stem cells [92]. Compared to other mature organs, the heart expresses high levels of lamin A [93]. While not required during early mouse development [94], lamin A/C knockout mice develop dilated cardiomyopathy with reduced cardiac function and have shortened lifespan, which can be improved upon reintroduction of lamin A transgene [95]. Furthermore, low lamin A levels in dilated cardiomyopathy patients was associated with disrupted nuclear morphology and heterochromatin deposition [96]. Lamin A/C knockout results in globally altered chromatin structure to adapt an inverted conversion, such that heterochromatin accumulates in the center of the nucleus [97], demonstrating the role of lamins in genomic-scale organization of heterochromatin at the nuclear envelope.

Differentiation of human myoblasts has provided evidence for global physical restructuring of chromatin and the nucleus [98]. Upon differentiation, the nucleus undergoes a reduction in volume and flattens out with dynamic changes in spatial positioning of chromosomes [98] (Figure 3). It is understood that the cytoskeleton can influence nuclear properties, but whether and how chromatin architecture can influence nuclear plasticity and cell function has not been explored in the heart.

A major unanswered question in the field of chromatin structural proteins relates to the basic logic of their function. There have been reports of binding partners for HMG proteins and linker histones, but unlike the chromatin remodeling complexes, transcription factors and histone-modifying enzymes, there is a lack of consensus for the protein complexes in which non-nucleosomal chromatin structural proteins operate. In addition, there have been recent ChIP-seq studies (albeit not from cardiovascular cells) on HMGs [99], CTCF [100] and linker histone proteins [101] suggesting that while some isoforms have apparent consensus sequences that influence their binding, others do not [102, 103]. Unlike histones, the extent and functional consequences of PTM on non-nucleosomal proteins is not well understood. Are these proteins non-specific “bricks and mortar” for genome packaging or do they form distinct species of complexes, perhaps with regulation through PTM, and have individual

target loci? Answers to these questions will require creative combination of proteomics to map protein complexes with epigenomics to determine which members of said complexes confer specificity.

Noncoding RNAs

Constituting the majority of distinct transcripts when compared to protein-coding RNA, noncoding RNAs (ncRNA) have been implicated in chromatin structure through regulation of histone-modifying enzymes [104, 105], DNA methylation [106], promoter binding [107], as well as alternative splicing and other post-transcriptional processing of mRNA [108]. miRNAs have an increasingly recognized role in cardiac development and adult heart regulation [109, 110], acting mostly through regulation of mRNA abundance [111, 112]. The individual requirements of miRNAs have been reviewed in greater detail [113, 114]. Long noncoding RNAs (lncRNA) have been proposed to regulate interactions between DNA, RNA and proteins via their secondary structure [108]. In such a role, lncRNAs are thought to serve as a scaffold between different sets of molecules to alter chromatin structure and gene expression in a locus-specific manner. Thus, lncRNAs have the potential to solve a major riddle of chromatin biology: how are chromatin remodeling enzymes and structural proteins targeted to the correct genes in a development- and stimulus-responsive manner? lncRNAs, which are expressed at low levels (in comparison to the abundance of protein-coding mRNAs), show a high degree of tissue specificity [115] and can be regulated directly by tissue-specific transcription factors [116]. lncRNAs are important for maintaining pluripotency as well as repressing tissue specification programs in mouse embryonic stem cells [117]. A large percentage of lncRNAs associate with complexes involved in writing, erasing and reading histone modifications, with H3K27me3 and H3K4me3 being well-established targets [104, 105, 117], and have specifically been shown to positively regulate transcription through recruitment and targeting of H3K4 methyltransferases [105, 118].

lncRNAs, *Braveheart* and *Fendrr*, are specific and critical for cardiac differentiation and examples of lncRNAs that regulate gene silencing machinery. *Braveheart* expression is necessary for the activation of cardiac programming in mouse embryonic stem cells by positively regulating key cardiac transcription factors (e.g. *Mesp1*, *Hand2*, *Nkx2-5*) through interactions with SUZ12 of PRC2 [119]. *Fendrr* is expressed in the lateral mesoderm and is a key regulator of cardiac development; *Fendrr*-mutant mouse embryos had impaired cardiac function, septal defects and hypoplasia, proposed to be through altered balance of H3K27me3 and H3K4me3 at promoters of transcription factors [120]. lncRNAs are an exciting interface between the transcriptome, genome and proteome for regulating developmental (and adult) phenotypes in part because so much remains unknown about how they function. What are the proteins that guide lncRNAs to the proper histone modifications? Are the lncRNA-protein complexes heterogeneous in a single cell? Are all histone modifications of a given type bound by a given lncRNA in a terminally differentiated cell (e.g. is every nucleosome with H3K27me3 bound by *Braveheart* and/or *Fendrr*)? Are the protein binding partners for individual lncRNAs dynamic at a single locus and do the complexes change genomic residence during development? Answers to these questions will likely emerge soon as crosslinking and immunoprecipitation sequencing

(CLIP-seq) [121] and other RNA localization methods (RNA Antisense Purification and RNA *in situ* hybridization) are combined with proteomic studies in the setting of cardiac development.

Technical Advances and Future Perspectives

Systems approaches have been taken to understand the global dynamics in cellular processes, which have allowed for novel identification of developmental regulators. Mass spectrometry analyses of the mouse cardiac nuclear proteome characterized nuclear proteins associated with different sub-nuclear compartments and identified novel histone variants [122], yet these types of approaches have not been leveraged in the setting of development, primarily due to small sample sizes. To overcome issues of tissue heterogeneity, a very recent study using an *isolation of nuclei tagged in specific cell types* (INTACT) approach employed transgenic methods to selectively label *Xenopus* cardiomyocyte nuclei for proteomic analysis [71]. In this system, embryos were introduced with transgenes for a fusion nucleoporin construct and BirA, a biotin ligase specific for the construct, driven by cardiomyocyte *mlc2p* promoter [71]. Enrichment of biotinylated nuclei allowed for identification of cardiomyocyte-specific peptides, the complexity of which was lower compared to previous studies performed on whole heart tissue [71]. Nascent chromatin capture techniques have been used to identify protein complexes at replication forks to understand transmission of epigenetic memory [123]. Another innovative proteomic screening technique that has overcome the issue of cell specificity and limited sample size is the FlipTrap method, which systematically targets endogenous proteins for immunohistochemical tracking or inducible knockout [124].

A well-recognized challenge to ‘omics investigations of cardiac development is tissue heterogeneity. When studying proteins in the membrane or mitochondria, for example, it is reasonable to expect that most of the protein mass comes from myocytes in a developing or mature heart. This is not the case in the setting of nuclear proteins and chromatin modifications, because in terms of cell number, myocytes—and therefore myocyte nuclei—constitute 50% or less of the heart. Recent advances in stem cell- and iPS cell-derived cardiomyocyte models have revolutionized the study of the myocyte development, with recent examples including the role of histone modifications in cardiac lineage commitment [3, 51] and the examination of genetic cardiomyopathies in patient-derived myocytes [125]. A current frontier is application of quantitative proteomics to these cells, including at intermediate developmental stages, to dissect the stage-specific changes in chromatin bound proteins that determine differential chromatin structure (Figure 3) and ultimately control cell fate.

While differentiated cell types display similar chromatin structural features on a large scale, it is unclear how the loci-specific changes occur to give rise to different cell types (Table). In other words, what dictates the environment for orchestration and targeting of chromatin structural changes during development? Ultimately, the code for development must come from the genome primary sequence—the reading of this code, however, becomes cell type-specific through the repositioning of nucleosomes, their decoration with post-translational modifications, interaction with chromatin structural proteins (and maybe lncRNAs, although

in our opinion more evidence is needed to understand the extent to which these molecules regulate chromatin structure in development and adulthood) and by modification of DNA through methylation. We reason many of the cues for cell type specificity may reside in intergenic/non-coding genomic regions that contribute to chromatin structure and thereby gene expression in a cell type-specific and non-intuitive manner (i.e. not by encoding a transcript, but rather by serving as a substrate for unique chromatin features). For transcriptional enhancers this concept has been well defined, but for regions further away from mRNA coding genes (i.e. functional DNA elements not localized near a gene in the genome) the story is less clear. Dissecting the causal (as well as temporal) relationship amongst transcription factors, chromatin remodelers, chromatin structure and transcriptome makeup is critical to understand the fundamental logic by which one cell becomes another along the developmental continuum. Many congenital heart diseases arise from mutations in cardiac transcription factors and/or chromatin structural regulators. These mutations will allow us to better understand the functions that chromatin structure can play in the establishment of cardiac gene expression during development. While the roles of these structural regulators are becoming clearer, it remains unknown how these mechanisms work in combination to mediate the spatial organization for establishing cardiac-specific gene expression. Future studies will require using global chromosome capture and chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) assays to understand how proteins mediate endogenous three-dimensional structure of the cardiac genome at different stages of development. Since transcriptomes vary widely between differentiated cell types, it is unlikely that chromatin structural measurements in non-cardiac lineage cells will effectively inform on the structural features necessary for cardiac gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of funding: Research in the Vondriska Laboratory is supported by the American Heart Association (IRG18870056) and National Institutes of Health (HL-105699 and HL-115238). E.K. is supported by the NIH Training Grant 5T32GM065823.

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Figure 1. Cell type-specific genome organization. DAPI labeling of DNA from mouse embryonic fibroblast (A), neonatal cardiomyocyte (B) and adult cardiomyocyte (C) shows specific chromatin packaging and nuclear structure of the same mouse genome in different cell types and at different developmental stages. Scale bar: 5μm.

Figure 2. Mechanisms of chromatin modification. Levels of genome packaging can be regulated in the following ways: (1) histone variants, (2) histone post-translational modifications (active, *green*, or repressive, *red*), (3) DNA modifications (*e.g.* methylation of cytosine), (4) association with chromatin structural proteins and RNAs (*light blue*) and (5) positioning of nucleosomes along DNA.

Figure 3.

Model for chromatin and nuclear architectural changes during development and disease. The cardiac nucleus undergoes dynamic structural changes to create a cell type-specific transcriptome. During differentiation, there is a shift from euchromatin to heterochromatin [14, 126], a decrease in global transcription [15] and an overall compaction of nuclear size [93, 127, 128]. With disease, these features are reverted to resemble earlier developmental stages, including a shift towards euchromatin [84], an increase in RNA production [129] and an increase in nuclear size [130].

Table**Key Questions Regarding Chromatin Regulation in Cardiovascular Development**

Questions	Current Knowledge	Future Directions
How are epigenetic changes coordinated at developmental milestones?	ChIP-seq of individual histone PTMs during cardiomyocyte differentiation	Determine how PTMs correspond to protein localization and specify global transcriptional states
What are the genomic targets of chromatin remodelers (readers, writers & erasers) and how do they control phenotype?	Loss- and gain-of-function for various chromatin enzymes	ChIP-seq for individual histone modifiers; comparison with known modification ChIP-seq datasets; CLIP-seq to prove role in transcription and explore RNA functions
How does the 3D organization of the genome change during development and is this important to specify the cardiovascular lineages?	Chromosome conformation capture analyses of genomes from non-cardiac cells	Chromosome conformation capture and ChIA-PET at various stages of development; comparison with protein localization and transcription