CHAPTER FIVE

Regulation of GATA4 Transcriptional Activity in Cardiovascular Development and Disease

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Contents

Abstract

Transcription factors regulate formation and function of the heart, and perturbation of transcription factor expression and regulation disrupts normal heart structure and function. Multiple mechanisms regulate the level and locus-specific activity of transcription factors, including transcription, translation, subcellular localization, posttranslational modifications, and context-dependent interactions with other transcription factors, chromatin remodeling enzymes, and epigenetic regulators. The zinc finger transcription factor GATA4 is among the best-studied cardiac transcriptional factors. This review focuses

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on molecular mechanisms that regulate GATA4 transcriptional activity in the cardiovascular system, providing a framework to investigate and understand the molecular regulation of cardiac gene transcription by other transcription factors.

1. Introduction: GATA Factors in Cardiac Specification and Development

Development of the mammalian heart is an intricate dance involving multiple cell types that arise from several sources. The initial heart tube, composed of a layer of cardiomyocytes overlying a layer of endocardial cells, grows through the addition of cardiomyocytes and endothelial cells at both poles. The elongating heart tube forms a rightward loop, and the looped heart tube is then divided into four chambers by growth of the muscular interatrial and interventricular septae and by expansion of the endocardial cushions, which form central portions of these septae as well as the developing heart valves. Division of the outflow tract into systemic and pulmonary arterial circuits requires the coordination of cells arising from neural crest, second heart field, and endocardial cushions. Cells arising from the proepicardium migrate onto the surface of the heart, forming an epithelial sheet known as the epicardium. This sheet of cells undergoes epithelial-to-mesenchymal transition, thereby generating mesenchymal cells that contribute to most of the stromal cells of the heart.

A network of transcription factors precisely choreographs this process. Not surprisingly, the transcription factor network is regulated by multiple mechanisms, and disruption of the structure or activity of transcription factors underlies a significant portion of congenital heart disease, the most common type of major congenital malformation (Bruneau, 2008; Srivastava, 2006). The transcription factor GATA4 is positioned high in the cardiac transcriptional network. Proper regulation of GATA4 levels and activity are crucial for normal heart specification and development, and GATA4 regulatory mechanisms have been intensively studied. Here, we review these mechanisms, providing a framework to understand regulation of transcription factor activity in the developing heart.

1.1. Structure and function of GATA factors

The mammalian genome encodes six GATA factors, GATA1–6, which share a highly conserved region spanning 109 amino acid residues, of which 82 residues (75%) are identical across the six proteins (Fig. 5.1). This region encompasses the DNA-binding domain and contains two C-X_2-C-X_{17}-C-X_2-C zinc fingers and adjacent residues (Evans and Felsenfeld, 1989;

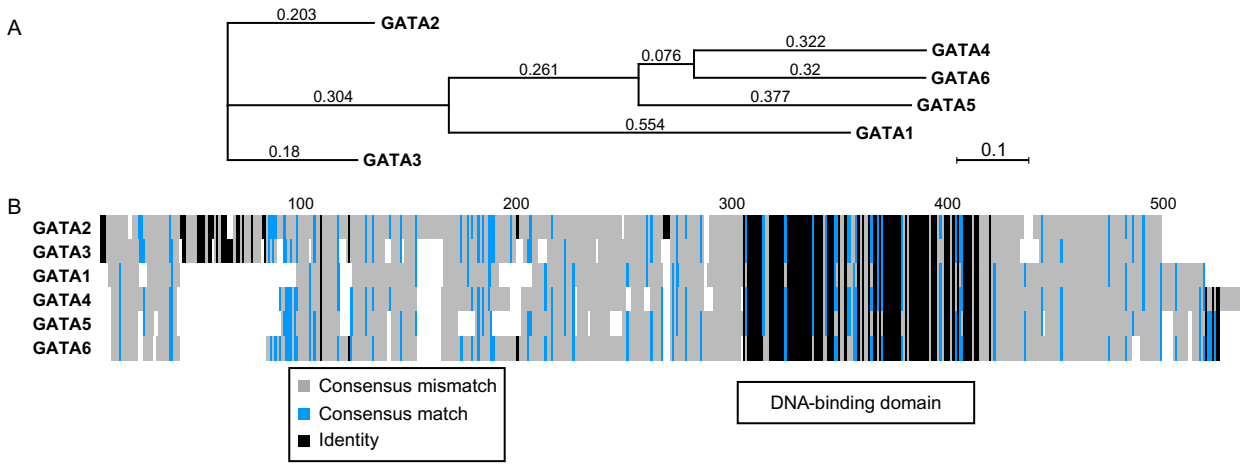

Figure 5.1 Sequence conservation of GATA proteins. (A) Phylogenetic tree shows close relationship of GATA4/5/6. The GATA4/5/6 cluster is more closely related to GATA1 than GATA2/3. (B) Conservation of primary sequence. GATA factors share a highly conserved DNA binding domain. Flanking regions show less sequence conservation.

Molkentin, 2000; Tsai *et al.*, 1989). The C-terminal zinc finger and adjacent basic residues are sufficient for specific DNA binding (Martin and Orkin, 1990; Yang and Evans, 1992). The structure of the C-terminal finger bound to DNA, determined by X-ray crystallography and NMR spectroscopy for GATA3 and GATA1, respectively (Bates *et al.*, 2008; Omichinski *et al.*, 1993), reveals direct base-specific protein-DNA contacts. The N-terminal zinc finger stabilizes DNA–protein interactions and also participates in specific protein–protein interactions. Using protein binding microarrays and other binding site selection approaches (Berger *et al.*, 2006; Merika and Orkin, 1993; Newburger and Bulyk, 2009; Sakai *et al.*, 1998), the DNA sequence recognized by GATA proteins was defined as (a/t) GATAA(g), and the *in vivo* preference of GATA1, GATA2, and GATA4 for this sequence was verified by chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) (Fujiwara *et al.*, 2009; He *et al.*, 2011; Yu *et al.*, 2009). Outside of the DNA-binding domain, the GATA4/5/6 proteins retain the modest similarity to one another but show more distant relationships to GATA1/2/3 (Fig. 5.1).

GATA4/5/6 are predominantly expressed in the cardiovascular system, the gonads, and endodermal derivatives, while GATA1/2/3 are predominantly expressed in the hematopoietic system. However, there are exceptions; for instance, GATA2 is an important transcriptional regulator in endothelial cells (Linnemann *et al.*, 2011). The GATA4/5/6 proteins are closely related, while GATA1 is more distantly related to GATA2 (Fig. 5.1B). Perhaps consistent with this sequence divergence, GATA1 is often functionally dissimilar from GATA2/3, and changes in chromatin occupancy between GATA1 and GATA2 are important for normal progression of hematopoiesis (Bresnick *et al.*, 2010). In contrast, the functional importance of such a "GATA switch" has not been established for GATA4/5/6.

Structure–function relationships for GATA4 have been analyzed by systematic mutagenesis, with the major readouts being activity in DNA-binding and luciferase reporter assays (Fig. 5.2) (Durocher *et al.*, 1997; Lee *et al.*, 1998; Morin *et al.*, 2000; Morrisey *et al.*, 1997). The N-terminal region of GATA4 was necessary and sufficient for transcriptional activation, containing two independent transcriptional activation domains that were moderately conserved in GATA5/6 (Morrisey *et al.*, 1997). While the C-terminal region was not sufficient to activate reporter transcription when fused to a heterologous DNA-binding domain, it was necessary for GATA4 transcriptional activity (but not DNA-binding). Subsequent analysis has revealed that the C-terminus is the target of acetylation and sumoylation (Takaya *et al.*, 2008; Wang *et al.*, 2004) (Fig. 5.2), which increase GATA4 transcriptional activity (see below). In addition, the C-terminal domain was essential for GATA4 transcriptional synergy with a subset of interacting transcription factors, but not their binding (Fig. 5.2). Thus, the C-terminal domain acts through unclear mechanisms to regulate GATA4 transcriptional activity.

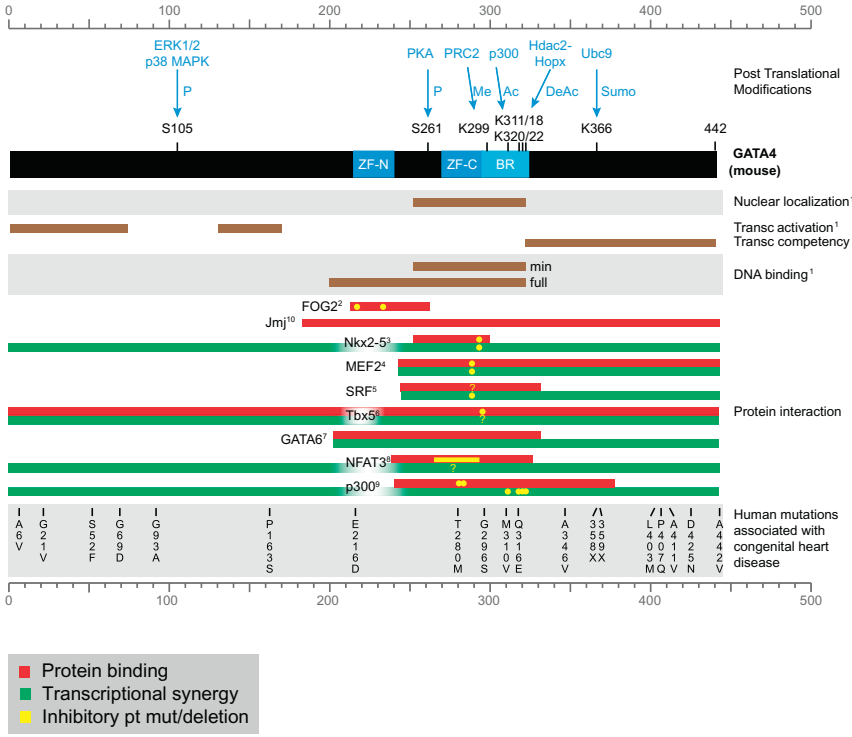

Figure 5.2 GATA4 primary sequence annotated with functional domains, posttranslational modifications, protein–protein interaction domains, and identified pathogenic human mutations. Numbers indicate amino acid residue. The GATA4 schematic shows the N- and C-terminal zinc finger domains (ZF-N and ZF-C) and the basic region (BR). Posttranslational modifications are shown above the GATA4 schematic. Ac, acetylation; DeAc, deacetylation; P, phosphorylation; Sumo, Sumoylation. Domains of biological activity are shown below the GATA4 schematic, with bars representing the minimum region sufficient for the indicated activity. Red and green bars indicate protein binding and transcriptional synergy, respectively. Yellow indicates point mutations or internal deletions that impair the indicated biological activity. Only proteins where mutational analysis of GATA4 interaction has been performed are shown. 1. Morrisey *et al.* (1997). 2. Lu *et al.* (1999), Crispino *et al.* (2001), Svensson *et al.* (2000). 3. Lee *et al.* (1998), Durocher *et al.* (1997). 4. Morin *et al.* (2000). 5. Belaguli *et al.* (2000). 6. Garg *et al.* (2003). 7. Charron *et al.* (1999). 8. Molkentin *et al.* (1998). 9. Dai and Markham (2001), Takaya *et al.* (2008). 10. Kim *et al.* (2004). Human mutations found in patients with congenital heart disease but not controls are indicated. Corresponding references are provided in the text.

1.2. GATA4 function in cardiac specification, development, and function

GATA4 is expressed in cells of the cardiac lineage from the time of their specification through adulthood, and it is a key regulator of gene expression and cellular activity at each of these stages. GATA4 promotes cardiogenesis, probably through both cell autonomous and cell nonautonomous mechanisms. During embryoid body differentiation of embryonic stem (ES) cells, GATA4 overexpression increased formation of cardiomyocytes (Grepin *et al.*, 1997; Holtzinger *et al.*, 2010). However, this effect was through non-cell autonomous mechanisms, as GATA4-overexpressing cells differentiated into $Sox17^{+}$ endoderm, which then stimulated cardiac differentiation of neighboring cells through secretion of cardiac-inducing paracrine factors such as BMPs and Wnt inhibitors. The cardiogenic effect of GATA4-induced endoderm is reminiscent of similar activity of endoderm observed in developing embryos (Foley *et al.*, 2006).

GATA4 stimulates cardiogenesis through cell autonomous mechanisms as well. Uncommitted mesodermal cells forced to express ectopically GATA4 and the chromatin remodeling factor BAF60C expressed cardiomyocyte markers (Takeuchi and Bruneau, 2009). Similarly, in combination with additional cardiac transcription factors TBX5 and MEF2C, GATA4 was also reported to reprogram fibroblasts into cardiomyocytes (Ieda *et al.*, 2010). Conversely, GATA4/6 deficient ES cells failed to form cardiomyocytes, in both EB differentiation systems and in embryos (rescued from visceral endoderm defects; Narita *et al.*, 1997; through tetraploid complementation; Zhao *et al.*, 2008). These latter experiments did not determine the cell type in which GATA4/6 are required for cardiogenesis, but collectively the data suggest that GATA4/6 act in cardiac progenitors to promote cardiomyocyte differentiation. Consistent with this conclusion, GATA4 participates in the transcriptional regulatory network that regulates second heart field differentiation (Black, 2007). Both *Mef2c* and *Hand2*, important cardiac transcription factors required for development of second heart field derivatives, have been identified as direct downstream targets of GATA4 in this population of heart progenitors (Dodou *et al.*, 2004; McFadden *et al.*, 2000; Zeisberg *et al.*, 2005).

In the developing heart, GATA4 is expressed in proepicardium, epicardium (Watt *et al.*, 2004), myocardium (Arceci *et al.*, 1993), endocardium, and endocardial cushions (Rivera-Feliciano *et al.*, 2006), and its activity is required in each of these compartments. GATA4 deficient embryos, rescued from visceral endoderm defects through tetraploid complementation, showed myocardial abnormalities and absence of the proepicardium (Watt *et al.*, 2004). Selective ablation of GATA4 in endothelial cells caused these cells to fail to undergo EMT, leading to a paucity of valvular mesenchymal cells (Rivera-Feliciano *et al.*, 2006). A point mutation of GATA4 that

blocks its interaction with FOG cofactors rescued this defect, but later valve remodeling remained abnormal, indicating continuing requirement for GATA4 in later stages of valve formation (Rivera-Feliciano *et al.*, 2006). GATA4 inactivation in myocardium caused marked myocardial hypoplasia due to decreased cardiomyocyte proliferation (Zeisberg *et al.*, 2005). Broad abnormalities of cell cycle gene expression were identified in GATA4 deficient cardiomyocytes, and Cyclin D2 and Cdk4 were identified as likely direct downstream targets (Rojas *et al.*, 2008). Strikingly, GATA4 inactivation early in heart morphogenesis caused severe, selective defects in right ventricular morphogenesis, due in part to downregulation of *Hand2* (Zeisberg *et al.*, 2005).

GATA4 inactivation in later fetal life or postnatally caused progressive, severe defects in myocardial function, and rapid decompensation with pressure overload stress (Oka *et al.*, 2006). Following pressure overload, GATA4 knockout cardiomyocytes were unable to hypertrophy and underwent increased apoptosis. GATA6 inactivation in adult heart caused similar phenotypes, suggesting that GATA4 and GATA6 function additively in regulating cardiomyocyte function and stress responses (van Berlo *et al.*, 2010). Cardiomyocyte GATA4 was also found to support normal and pressure-overload stimulated increases in myocardial capillary density by upregulating proangiogenic factors including VEGFA and inhibiting anti-angiogenic gene expression (Heineke *et al.*, 2007; Zhou *et al.*, 2009).

The level of GATA4 is critical for normal embryo development and survival, as revealed by a series of alleles that express different levels of GATA4 (Pu *et al.*, 2004). Moreover, *Gata4* heterozygous embryos suffered from a high incidence of cardiac (myocardial hypoplasia and endocardial cushion defects) and extracardiac (diaphragmatic hernia) congenital defects in the pure C57BL6/J strain background (Jay *et al.*, 2007; Rajagopal *et al.*, 2007). These defects were milder forms of phenotypes observed in null mice. In a mixed strain background, *Gata4* heterozygous mice survived to adulthood but were susceptible to pressure-overload induced heart failure due, at least in part, to increased cardiomyocyte apoptosis (Bisping *et al.*, 2006). On the other hand, cardiomyocyte overexpression of GATA4 caused cardiac hypertrophy and progressive heart failure (Liang *et al.*, 2001a). These data underscore the importance of precisely regulating GATA4 level and activity.

Consistent with key dosage sensitive roles of *Gata4* in regulating organ development and function, heterozygous mutation of *GATA4* has been linked to abnormalities of heart, diaphragm, and gonad development or function in humans (Bielinska *et al.*, 2007; Butler *et al.*, 2010; Chen *et al.*, 2010a,b; Garg *et al.*, 2003; Lourenco *et al.*, 2011; Nemer *et al.*, 2006; Peng *et al.*, 2010; Rajagopal *et al.*, 2007; Tomita-Mitchell *et al.*, 2007; Zhang *et al.*, 2008, 2009). Missense and nonsense mutations of *GATA4* cause atrial and ventricular septal defects, often in association with pulmonary stenosis,

and endocardial cushion defects (Garg *et al.*, 2003; Rajagopal *et al.*, 2007). GATA4 mutation has also been linked to complex cardiovascular defects involving right ventricular hypoplasia (Rajagopal *et al.*, 2007). Interestingly, human *GATA4* mutation has not been reported to cause ventricular dysfunction, a highly penetrate phenotype in heterozygous mice. Microdeletions involving *GATA4* are also associated with congenital diaphragmatic hernia (Bielinska *et al.*, 2007), and with disorders of sexual development (Lourenco *et al.*, 2011).

2. Regulation of GATA4 Expression Level and Activity

Given the importance of maintaining appropriate levels of GATA4 activity for normal organ development and function, a panoply of mechanisms have evolved to regulate GATA4 level and activity (Fig. 5.3).

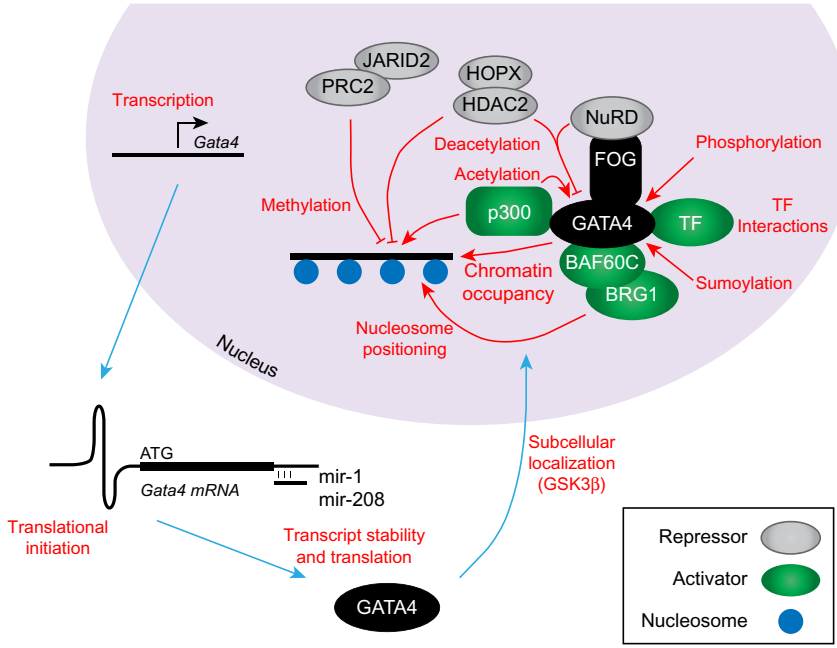

Figure 5.3 Regulation of GATA4 expression and activity. GATA4 is regulated by transcriptional, translational, and posttranslational mechanisms. Locus-specific transcriptional activity is regulated by interactions with other TFs, coactivators, corepressors, covalent modifications, and interactions with chromatin remodeling and modifying enzymes.

The remainder of this review focus on these regulatory mechanisms. These mechanisms undoubtedly pertain to other transcription factors, and thus GATA4 serves as a case study of mechanisms used to regulate transcription factor activity in the developing and adult heart.

2.1. Regulation of GATA4 expression

GATA4 expression level varies between tissues, between developmental stages, and in disease states. Although often used as a marker of cardiomyocytes, in fetal heart GATA4 expression is the highest in the proepicardium followed by the endocardial cushions and then cardiomyocytes. GATA4 expression in the adult heart has been reported to increase by approximately twofold in heart disease (Diedrichs *et al.*, 2004; Hall *et al.*, 2004). However, little is known about the mechanisms that govern GATA4 expression.

The Black lab has identified several *Gata4* enhancers with expression restricted to endoderm or portions of lateral mesoderm including the septum transversum, but not the heart itself (Rojas *et al.*, 2005, 2009, 2010). These enhancers were regulated by GATA factors themselves in combination with Forkhead and homeodomain family transcription factors. We identified two *Gata4* regulatory sequences that drove cardiomyocyte expression in transient transgenic embryos, located immediately upstream and −93 kbp upstream of the GATA4 transcriptional start site (He *et al.*, 2011). However, neither of these sequences exhibited strong cardiac expression compared to the endogenous gene, and the −93 kb site appears to have predominant activity in endocardium and endocardial cushion mesenchyme. Thus, the sequences and mechanisms that regulate GATA4 in heart development and disease remain poorly described.

GATA4 protein levels are also regulated posttranscriptionally. The cardiac-specific microRNA miR-208 appears to regulate GATA4 translation, as GATA4 protein was upregulated in miR-208 knockout mice (Callis *et al.*, 2009). The regulation likely involves direct miR-208 interaction with the *Gata4* 3′untranslated region (UTR), as it contains a putative miR-208 binding site and is sufficient to decrease expression of a linked luciferase reporter in response to miR-208. GATA4 protein translation was also regulated by the cardiac-enriched microRNA miR-1, as GATA4 levels were regulated antithetically to miR-1 in neonatal rat ventricular cardiomyocytes (Ikeda *et al.*, 2009). However, the *Gata4* 3′UTR does not contain a predicted miR-1 binding site, suggesting that miR-1 may regulate GATA4 expression indirectly.

GATA4 translation is also regulated through sequences in the *Gata4* 5′ UTR (Sharma *et al.*, 2007). At 518 nt, this region is longer than the typical 5′UTR. Moreover, it contains 18 upstream ATG sequences and a highly stable predicted secondary structure. The 5′UTR possessed activity of an internal ribosome reentry site (IRES), supporting cap-independent

translation and remaining active when cap-dependent translation was inhibited. This sequence stimulated GATA4 translation in a rat embryonic heart-derived cell line (H9c2) treated with the hypertrophic agonist vasopressin. Ca^{2+}- and protein kinase C mediated this effect. Further work is needed to delineate the impact of regulated GATA4 translation on fetal development and adult heart function.

2.2. Regulation of GATA4 activity by posttranslational modifications

A host of posttranscriptional modifications modulate GATA4 transcriptional activity. GATA4 lysine 366 has been reported to be covalently linked to the small ubiquitin-like modifier SUMO-1 (Wang *et al.*, 2004). The reaction was catalyzed by the conjugating enzyme Ubc9, and expedited by the E3 ligase PIAS1, which favored attachment of poly SUMO chains. Sumoylated GATA4 exhibited increased transcriptional activity on GATA4-regulated luciferase reporters. Interestingly, sumoylation of GATA1 and GATA2 had the opposite effect, repressing their transcriptional activity. The combination of GATA4, SUMO-1, and PIAS1 activated transcription of selected cardiac genes in 10T1/2 fibroblasts, suggesting that GATA4 sumoylation may be required for the GATA4 cardiogenic activity.

GATA4 activity is also regulated through phosphorylation. The best studied phosphorylation site is serine 105, which was reported to be phosphorylated by both Erk and p38 MAPK kinases (Charron *et al.*, 2001; Liang *et al.*, 2001b). GATA4 phosphorylation at this site within the N-terminal activation domain increased its DNA-binding affinity and transcriptional activity. Recently, the importance of this posttranslational modification was tested *in vivo* by mutating GATA4 S105 to alanine (van Berlo *et al.*, 2011). This point mutation was compatible with survival to adulthood and normal heart development. However, mutant hearts showed blunted hypertrophy to biomechanical stresses such as phenylephrine infusion and were more susceptible to heart failure and cardiac dilation after pressure overload. Moreover, these mutant hearts were resistant to hypertrophy driven by a MEK1 transgene, indicating that GATA4 is an essential mediator of cardiac hypertrophy downstream of MEK1.

GATA4 phosphorylation has been implicated in its regulation of gene transcription downstream of cyclic adenosine monophosphate (cAMP) signaling. In response to cAMP elevation in MA-10 Leydig tumor cells, GATA4 was phosphorylated by protein kinase A on serine 261, located between the two zinc finger domains (Tremblay and Viger, 2003; Tremblay *et al.*, 2002). S261 phosphorylation enhanced GATA4 activation of cAMP-responsive Leydig cell promoter transcription, at least in part by increasing GATA4 physical interaction with the transcriptional coactivator CBP (CREB (cAMP-response element-binding) binding protein). Interestingly, transcriptional activity of

CBP itself is increased by PKA phosphorylation, suggesting that the GATA4/CBP complex may coordinate regulation of Leydig cell gene transcription downstream of cAMP. cAMP signaling is also important in regulating cardiomyocyte function; however, GATA4 or S261 phosphorylation has yet to be studied in regulation of heart development or function.

GATA4 is an important regulator of cardiac gene expression changes triggered by hypertrophic agonists. The β-adrenergic agonist isoproterenol was reported to increase GATA4 transcriptional activity by enhancing its nuclear accumulation. In unstimulated cells, GATA4 interacted with and was directly phosphorylated by GSK3β at undetermined residue(s), leading to active GATA4 export from the nucleus (Morisco *et al.*, 2001). β-Agonist stimulation inhibited GSK3β, reduced nuclear export, and thereby increased nuclear accumulation. The *in vivo* significance of GSK3β regulation of GATA4 activity and subcellular localization has not been verified *in vivo*.

Protein acetylation is another important form of posttranslational modification. GATA4 physically interacts with p300 and CBP, both acetyltransferases. GATA1 was initially reported to be acetylated by p300, resulting in increased GATA1 DNA binding and transcriptional activity (Boyes *et al.*, 1998). Subsequently, p300 was also found to acetylate GATA4 (Yanazume *et al.*, 2003). As with GATA1, GATA4 acetylation increased its DNA binding affinity and its *in vitro* transcriptional activity, and GATA4 acetylation was implicated as an important mechanism that regulates cardiac hypertrophy (Yanazume *et al.*, 2003). Several residues (K311, K318, K320, K322) were found to be acetylated by p300 and required for p300 stimulation of GATA4 transcriptional activity in luciferase reporter assays (Takaya *et al.*, 2008). Mutation of all four of these residues blocked GATA4 acetylation by p300 and blunted cardiac hypertrophy induced by GATA4 overexpression. Thus, GATA4 acetylation may be an important mechanism to regulate GATA4 transcriptional activity *in vivo*.

Protein acetylation is counterbalanced by histone deacetylases (HDACs). Although initially identified by their ability to deacetylate histones, these enzymes are active on a broad range of proteins, including transcription factors (Glozak *et al.*, 2005). HDACs are key regulators of cardiac development and hypertrophy (Kook *et al.*, 2003; Zhang *et al.*, 2002). The cardiac homeodomain only protein HOPX is selectively expressed in heart and essential for normal cardiac growth and differentiation (Ismat *et al.*, 2005; Shin *et al.*, 2002). This protein, which lacks intrinsic DNA-binding capacity, modulates cardiac gene transcription by recruiting HDAC2 (Kook *et al.*, 2003). Ablation of *Hopx* and *Hdac2* caused severe heart malformation and excessive cardiomyocyte proliferation that was linked to GATA4 hyperacetylation (Trivedi *et al.*, 2010). These data suggest that HOPX serves as an adapter to facilitate HDAC2 recruitment to GATA4, thereby regulating its activity by modulating the balance of GATA4 acetylation/deacetylation.

Recently we reported that GATA4 is also regulated by protein methylation (He *et al.*, 2012). The histone methyltransferase complex PRC2 (polycomb repressive complex 2) specifically binds to and methylates GATA4 at lysine 299. PRC2 methylation of GATA4 inhibited its transcriptional activity. This inhibition of transcriptional potency was due to reduced GATA4 binding to and acetylation by p300.

2.3. Regulation of GATA4 activity by interaction with other transcription factors and cofactors

GATA4 forms protein complexes with several other transcription factors expressed in the heart. Their synergistic interactions have been proposed to regulate cardiac gene transcription, and disruption of these interactions has been reported to underlie some cases of congenital heart disease.

2.3.1. FOG1/2

The N-terminal zinc finger of GATA proteins strongly interacts with Friend of GATA (FOG) cofactors. This protein family contains two members, FOG1 and FOG2, with FOG1 predominantly coexpressed with GATA1/2/3 in hematopoietic cells, and FOG2 predominantly coexpressed in with GATA4/5/6 endodermal and mesodermal derivatives. FOG proteins do not detectably bind DNA directly, but rather associate with other transcription factors, principally GATA factors. FOG2 has been reported to largely repress GATA4/5/6 transcriptional activation. However, FOG1 both facilitates and represses GATA1/2/3-mediated transcription in a context dependent manner, suggesting that FOG2 likely also increases GATA4 transcriptional activity at a subset of targets. Consistent with this idea, Tevosian found that FOG2 stimulates GATA4 activation of *Tnnt1* transcription (Manuylov and Tevosian, 2009).

The function of GATA4–FOG2 interaction has been probed by inactivation of *Fog2* (Svensson *et al.*, 2000; Tevosian *et al.*, 2000; Zhou *et al.*, 2009), and by point mutation of *Gata4* to substitute glycine for valine 217. This substitution selectively ablates GATA4–FOG2 interaction (Crispino *et al.*, 2001; Rivera-Feliciano *et al.*, 2006; Zhou *et al.*, 2009). FOG2 deficient mice died at midgestation with cardiac defects, most notably a marked deficiency of the coronary endothelial plexus (Tevosian *et al.*, 2000). Although FOG2 is expressed in endocardial, myocardial, and epicardial lineages, targeted deletion of FOG2 in cardiomyocytes by Nkx2-5 recapitulated the $Fog2^{-/-}$ phenotype. This result indicates that cardiomyocyte FOG2 promotes coronary plexus formation through cell nonautonomous mechanisms (Zhou *et al.*, 2009). The proangiogenic activity of cardiomyocyte FOG2 continues into adulthood, as adult cardiomyocyte-restricted *Fog2* ablation caused heart failure, decreased myocardial perfusion, and reduced microvascular density (Zhou *et al.*, 2009). The GATA4-V217G

mutant largely recapitulated the FOG2 phenotype, suggesting that these activities of FOG2 are mediated through GATA4 interaction (Crispino *et al.*, 2001; Zhou *et al.*, 2009). In keeping with this result, GATA4 and FOG2 ChIP-seq in HL1 cardiomyocyte-like cells showed that nearly all FOG2 binding sites coincide with GATA4 binding sites (P. Zhou and W.T. Pu, unpublished).

Use of the GATA4-V217G mutant also identified an instance of functional GATA4–FOG1 interaction. Embryos with loss of GATA4–FOG interaction restricted to endocardium and endocardial cushion mesenchyme developed atrioventricular canal defects (Rivera-Feliciano *et al.*, 2006). However, FOG1 but not FOG2 is required for atrioventricular valve formation (Katz *et al.*, 2003). Thus, GATA4–FOG1 interaction is essential for atrioventricular valve remodeling.

2.3.2. GATA6

GATA4 and GATA6 proteins physically interact and synergistically activate target gene transcription (Charron *et al.*, 1999). The heterodimeric interaction occurs through the GATA4 DNA-binding domain, but GATA4 DNA-binding activity was dispensible for both GATA6 interaction and GATA6 transcriptional synergy. The crystal structure of GATA3 bound to DNA illustrates how the DNA binding domain mediates dimer formation, and suggests that GATA factors exist as homotypic and heterotypic dimers on DNA (Bates *et al.*, 2008).

GATA4 and GATA6 show similar expression patterns during early murine development, with both expressed in the precardiac mesoderm, the embryonic heart tube, and the developing endoderm (Morrisey *et al.*, 1996). As described in Section 1.2, GATA4 and GATA6 are redundantly required for cardiomyocyte specification. In later stages of heart development, examination of GATA4 and GATA6 doubly heterozygous embryos revealed a genetic interaction between these factors. Doubly heterozygous embryos developed myocardial hypoplasia and defects in ventricular and aortopulmonary septation (Xin *et al.*, 2006). In adult heart, inactivation of either GATA4 or GATA6 caused systolic dysfunction and impaired cardiomyocyte hypertrophy. Inactivation of both GATA4 and GATA6 caused more severe cardiac dysfunction than either alone. Thus, GATA4 and GATA6 function redundantly to maintain adult heart function (van Berlo *et al.*, 2010), consistent with GATA4/6 physical interaction.

Given the similarity of GATA4 and GATA6 loss-of-function phenotypes, it has been difficult to determine whether these proteins function redundantly in a cumulative dosage-dependent manner, or whether there are distinct functions attributable to either protein alone or to interactions between these proteins. Ablation of FOG interaction has revealed at least one qualitative distinction between GATA4 and GATA6. Unlike the dramatic embryonic lethal phenotype of GATA4-V217G mutants, the comparable GATA6

mutants have no discernable cardiovascular phenotype (J. Wang and S. H. Orkin, unpublished). Thus, cardiovascular FOG-dependent functions appear to be predominantly mediated through GATA4 rather than GATA6.

2.3.3. Nkx2-5

The C-terminal zinc finger of GATA4 binds to the cardiac homeodomain protein Nkx2–5, and this interaction leads to synergistic transcriptional activation of reporter genes *in vitro* (Durocher *et al.*, 1997; Lee *et al.*, 1998; Sepulveda *et al.*, 1998). Nkx2-5 DNA-binding activity was required for physical interaction, but GATA4 DNA-binding activity was not (Garg *et al.*, 2003). Consistent with physical interaction between Nkx2-5 and GATA4, the Nkx2-5 consensus binding site was significantly enriched in chromatin regions occupied by GATA4 in HL1 cardiomyocyte-like cells (He *et al.*, 2011). However, *in vivo* evidence of the biological significance of the Nkx2-5 and GATA4 interaction is lacking. Double heterozygosity for GATA4 and Nkx2-5 did not appear to affect fetal survival beyond the effect of heterozygosity for each factor alone (W.T. Pu, unpublished).

2.3.4. TBX5

GATA4 and TBX5 physically interact and synergistically activate reporter gene transcription (Garg *et al.*, 2003), although deletion analysis to identify the interacting domains has not been reported. The human GATA4 G296S mutation ablated both GATA4 binding to both DNA and TBX5 and blocked transcriptional synergy with TBX5 (Garg *et al.*, 2003). Although disruption of TBX5 interaction by this mutation may contribute to malformation of the heart, its abrogation of DNA binding is also likely significant. Further studies in mice showed that double heterozygosity for GATA4 and TBX5 caused more severe cardiovascular defects than single heterozygosity for each factor, thus establishing a genetic interaction between GATA4 and TBX5 (Maitra *et al.*, 2009). GATA4 and TBX5 interact in endocardial cells to promote atrial septation, and this genetic interaction was linked to synergistic activation of endocardial NOS3 expression (Nadeau *et al.*, 2010). Further work is required to define whether this interaction occurs in cardiomyocytes as well, and whether or not the genetic interaction reflects functionally required physical interaction between these factors.

2.3.5. SRF and MEF2

The MEF2 transcription factor family (MEF2A/B/C/D) and SRF contain MADS DNA binding domains. Both muscle and nonmuscle cells express these factors, yet in muscle cells they are key drivers of muscle gene expression (Niu *et al.*, 2007; Potthoff and Olson, 2007). SRF, MEF2A, MEF2C, and MEF2D are each individually required for cardiac development and/or function (Kim *et al.*, 2008; Lin *et al.*, 1997; Naya *et al.*, 2002; Niu *et al.*, 2005). The C-terminal finger of GATA4 and the MADS domain of SRF/MEF2 factors are sufficient for protein–protein interaction

(Belaguli *et al.*, 2000; Morin *et al.*, 2000, 2001). GATA4 and SRF/MEF2 synergistically activated reporter gene transcription *in vitro*, and in both cases, the GATA4 C-terminal region was required in addition to the DNA binding domain for transcriptional synergy. However, *in vivo* evidence that GATA4–SRF or GATA4–MEF2 interaction is functionally significant is lacking currently.

2.4. Chromatin occupancy

The human or mouse genomes contains approximately 7×10^6 GATA motifs, but ChIP-seq experiments show that GATA4 occupies only a small fraction ($10–50\times10^3$) of these potential sites (He *et al.*, 2011). In the case of GATA2, where genome-wide chromatin occupancy has been analyzed in different cell types, the majority of GATA2 occupied sites differed between endothelial and leukocyte cell lines, indicating that sites of chromatin occupancy are regulated in a cell type specific manner that is related to tissue-restricted transcription factor activity (Linnemann *et al.*, 2011). The mechanisms that underlie tissue-restricted transcription factor chromatin occupancy are not well understood but are pivotal to understand the activity of transcription factors like GATA4, which drive tissue-restricted gene expression programs in diverse tissues such as heart, gut, and gonads. Likely, mechanisms involve combinatorial interactions with other tissue-restricted transcription factors, and chromatin structure and accessibility.

Combinatorial interactions between transcription factors have been long proposed to regulate GATA4 and contribute to its regulation of cardiac-specific gene expression. Like GATA4, other major cardiac transcription factors such as SRF, MEF2, TBX5, and NKX2-5 are expressed in multiple tissues. However, combinatorial synergy may enhance specificity for the cardiac gene program. We used ChIP-seq to define genome-wide the chromatin occupancy of GATA4, NKX2-5, TBX5, SRF, and MEF2A in HL1 cardiomyocyte-like cells (He *et al.*, 2011). Our data supported substantial protein–protein interaction between these factors, as over 20% of chromatin regions were bound by two or more factors ($P<10^{-16}$). Four or more of these factors co-occurred within 500bp of one another at 1715 sites, and these sites were highly overrepresented for genes with enriched cardiac expression. In fact, multiple transcription factor binding was an effective criteria to identify enhancers with cardiac activity. The large majority of these multiple transcription factor bound loci were occupied by GATA4, suggesting that it plays an important role in determining cardiac enhancer chromatin occupancy.

Chromatin structure and accessibility are similarly important determinants of transcription factor chromatin occupancy. Chromatin structure is governed by the interaction of epigenetic regulatory machinery with sequence-specific transcription factors. The interaction is likely bidirectional and mutually reinforcing, as chromatin modifying enzymes may

induce a more accessible chromatin conformation and thereby recruit transcription factor occupancy. At the same time, sequence specific transcription factors may recruit epigenetic regulators that modify local chromatin structure and accessibility, thereby strengthening transcription factor occupancy and enhancing binding of interacting transcription factors.

The interaction between the histone acetyltransferase p300 and GATA4 is an example of cross talk between transcription factors and epigenetic regulators. p300 is an important transcriptional coactivator in multiple organs including the heart. Embryos lacking p300 or p300 acetyltransferase enzymatic activity die from heart defects (Shikama *et al.*, 2003; Yao *et al.*, 1998), and cardiac hypertrophy in response to pressure overload was quantitatively linked to p300 expression levels (Miyamoto *et al.*, 2006; Wei *et al.*, 2008). p300 is selectively recruited to active transcriptional enhancers, where it acetylates histones and stimulates transcription. Indeed, p300 chromatin occupancy has been shown to identify tissue-restricted transcriptional enhancers, including heart-specific enhancers (Blow *et al.*, 2010; Visel *et al.*, 2009). p300 interacts with a large number of transcriptional regulators including GATA4 (Yanazume *et al.*, 2003), and presumably these interactions govern p300 recruitment to transcriptional enhancers. We measured p300 chromatin occupancy in HL1 cardiomyocyte-like cells by ChIP-seq, and found that nearly 80% of chromatin sites bound by p300 were also bound by GATA4 (He *et al.*, 2011). Thus, GATA4 recruitment appears to be a major mechanism underlying p300 chromatin occupancy in cardiomyocytes. Since p300 also acetylates GATA4 and this increases GATA4 DNA binding affinity (Yanazume *et al.*, 2003), GATA4–p300 interaction may bidirectionally influence both p300 and GATA4 chromatin occupancy.

Specific properties of GATA4 may facilitate its occupancy of compact chromatin inaccessible to many other transcription factors. Zaret and colleagues studied FoxA2 and GATA4 in specification of hepatocytes from early endoderm progenitors (Cirillo *et al.*, 2002; Zaret *et al.*, 2008). FoxA2 and GATA4 occupied regulatory elements of the hepatocyte-specific albumin promoter, even prior to active albumin (*Alb1*) transcription. Unlike other transcription factors, FoxA2 and GATA4 were found to bind efficiently to these regulatory sequences even when compacted in nucleosome arrays. Thus, these factors were proposed to act as "pioneer" factors that could bind chromatinized regulatory regions and facilitate subsequent binding by other factors. In fact, GATA4 and a different FoxA factor (FoxA3) were central components of a set of defined factors that reprogram fibroblasts to hepatocytes (Huang *et al.*, 2011). FoxA2 and GATA4 occupancy of these regulatory elements prior to gene activation may reflect a "bookmarking" or "competence" function by establishing a chromatin state that is compatible with later expression of the gene (Zaret *et al.*, 2008). In fact, in ES cells, the Fox-factor binding site is occupied by the related FoxD3, and

this is associated with selective *Alb1* demethylation at the Fox-factor binding site. Thus, occupancy of the *Alb1* site by a series of Fox-factors may preserve competence of this regulatory region for later activity in hepatocytes, in part by promoting focal *Alb1* demethylation at the Fox-factor binding site. GATA factors (GATA4 and GATA6) are redundantly required for cardiomyocyte specification and thus GATA4 may play a pioneer and competence role in cardiac gene expression analogous to its role in hepatocyte specification.

GATA4 chromatin occupancy may be modulated by its interaction with FOG cofactors. Although FOG proteins neither bind DNA intrinsically nor modify GATA factor binding affinity or specificity for naked DNA, GATA1–FOG1 interaction was required for occupancy of specific chromatin loci (Pal *et al.*, 2004). This "chromatin occupancy facilitator" function of FOG1 was hypothesized to account in part for FOG1 modulation of GATA1 transcriptional activity. The mechanisms underlying this chromatin occupancy facilitator activity are poorly understood. The applicability of this paradigm to GATA4–FOG2 interactions has yet to be evaluated by unbiased genome-wide approaches.

Indirect GATA4 binding appears to contribute substantially to GATA4 chromatin occupancy, as a large fraction of genomic regions associated with GATA4 in ChIP-seq experiments lacked recognizable GATA motifs (He *et al.*, 2011). GATA4 was likely cross-linked to these regions as a result of its interaction with other transcriptional complexes rather than through direct DNA binding to cryptic GATA binding sites. Consistent with this, *in vitro* studies show that GATA4 interaction with some transcription factors does not require GATA4 DNA binding activity (Fig. 5.2).

2.5. GATA4 interaction with chromatin remodeling complexes

Chromatin remodeling complexes use the energy of ATP hydrolysis to displace and reposition nucleosomes, thereby altering chromatin accessibility (Ho and Crabtree, 2010). These complexes can be broadly grouped by the ATPase subunit, with the SWI/SNF family being the best studied in the cardiovascular system (Han *et al.*, 2011). SWI/SNF ATPases are large, multisubunit machines that use either BRG1 or BRM1 as the ATPase subunit. The composition of the SWI/SNF complex is under tight spatiotemporal control, and the precise complement of subunits determines the biological activity of the complex (Ho and Crabtree, 2010). The BAF60 subunit of the complex can be one of three distinct proteins, BAF60A–C (encoded by genes *Smarcd1–3*, respectively). Interestingly, BAF60C is specifically expressed in the developing heart from E7.5 to E9.5, and shRNA-mediated BAF60C knockdown caused severe defects in cardiac morphogenesis (Lickert *et al.*, 2004). BAF60C coprecipitated with GATA4 and was required for GATA4 to coprecipitate BRG1, suggesting that BAF60C functions as a cardiac-specific

adaptor that facilitates GATA4 interaction with the BRG1 chromatin remodeling complex (Lickert *et al.*, 2004). This interaction was associated with synergistic activation of cardiac reporter genes by BRG1 and GATA4 in the presence of BAF60C.

The importance of BAF60C-mediated interaction of GATA4 with the BRG1 chromatin remodeling complex was reinforced by the discovery that GATA4 and BAF60C are together sufficient to induce ectopic cardiac gene expression in mouse mesoderm (Takeuchi and Bruneau, 2009). Addition of the cardiac transcription factor TBX5 stimulated further cardiac differentiation, inducing mesoderm to form ectopic foci with spontaneously beating, a complex phenotype indicative of maturation of multiple cardiomyocyte-specific functions. At a mechanistic level, coexpression of GATA4 and BAF60C in mesoderm resulted in recruitment of GATA4 and BRG1 to regulatory elements of the cardiac genes *Tnnt2* and *Nppa*. This suggested that the BAF60C–BRG1 complex remodeled chromatin and thereby facilitated GATA4 chromatin occupancy (Takeuchi and Bruneau, 2009). Alternatively, pioneer activity of GATA4 facilitated chromatin occupancy of these regulatory elements, which was subsequently stabilized by the BAF60C/BRG1-containing remodeling complex. BAF60C continues to be expressed in the adult heart, where both BRG1 and GATA4 are essential for normal cardiac function and responses to biomechanical stress (Bisping *et al.*, 2006; Hang *et al.*, 2010; Oka *et al.*, 2006; Takeuchi *et al.*, 2011). This suggests that GATA4–BAF60C–BRG1 continue to regulate adult heart chromatin remodeling, although additional experiments are required to test this hypothesis.

2.6. GATA4 interaction with histone modifying complexes

GATA4 has also been reported to interact with three distinct complexes containing enzymes that covalently modify histones. First, as mentioned above GATA4 coprecipitates with HOPX–HDAC2. While this interaction was studied in the context of GATA4 deacetylation, a GATA4–HOPX–HDAC2 complex may also repress GATA4-bound genes by HDAC2-mediated histone deacetylation. Although GATA4 has primarily been studied as a transcriptional activator, GATA1–3 are active as both transcriptional activators and repressors. The extent to which GATA4 represses transcription via HDAC recruitment requires further study.

GATA4 also forms a complex with the nucleosome remodeling and histone deacetylase (NuRD) complex through its cofactor FOG2. This unique multisubunit complex contains both ATP-dependent chromatin remodeling and histone deacetylase activities, conferred by Mi-2α/β (Denslow and Wade, 2007). The distinct functions of these HDACs in the NuRD complex, compared to HDAC activities in complexes with HOPX or in other corepressor complexes such as mSIN3a, remain to be determined.

The NuRD complex appears to function as a transcriptional repressor, both by decreasing chromatin accessibility through nucleosome repositioning and by removing activating histone acetylation marks. FOG proteins interact with NuRD through the N-terminal 12 amino acids of FOG2 (Roche *et al.*, 2008), which are necessary and sufficient for FOG2-mediated transcriptional repression (Lin *et al.*, 2004). This N-terminal repressor recruitment domain binds the MTA (metastasis associated) protein family members MTA1/2/3, components of the NuRD complex. Interestingly, HDAC inhibitors did not block FOG2-mediated repression *in vitro* (Lin *et al.*, 2004), suggesting that other mechanisms such as nucleosome repositioning mediate gene repression by FOG.

GATA4 also occurs in protein complexes with Jumonji (*Jarid2*), a gene initially identified in a gene trap screen for mutants in heart development. *Jarid2* null mutants developed ventricular septal defect (VSD), ventricular noncompaction, and double-outlet right ventricle. JARID2 and GATA4 proteins interacted at the *Nppa* promoter, where JARID2 repressed GATA4 transcriptional activity (Kim *et al.*, 2004). JARID2 was the founding member of a family of histone demethylases, but unlike other members of this family, JARID2 lacks histone demethylase activity due to substitution of key residues within the conserved catalytic domain (Takeuchi *et al.*, 2006). Recently, JARID2 was found to be an important component of Polycomb Repressive Complex 2 (PRC2), a key repressive complex that silences gene expression by establishing repressive H3K27me3 epigenetic marks (Li *et al.*, 2010; Pasini *et al.*, 2010; Shen *et al.*, 2009). In ES cells, JARID2 colocalized with PRC2 and was required for efficient PRC2 chromatin occupancy. These data are of particular interest because GATA4 interacts directly with the PRC2 complex (He *et al.*, 2012), suggesting that GATA4-JARID2-PRC2 interaction may modulate chromatin occupancy and activity of both GATA4 and PRC2 in cardiac cells.

3. Summary

Achieving normal heart development and function requires precise regulation of transcription factor dose and activity. Every step including transcription factor gene transcription, translation, subcellular localization, chromatin occupancy, and interaction with the transcriptional machinery is regulated, permitting the cell to precisely tune transcription factor activity at each target gene locus. While the work summarized in this review has moved our understanding of transcription factor regulation forward considerably, a number of fundamental questions remain. What determines tissue- and stage-specific transcription factor chromatin occupancy and tissue-specific transcriptional activation? What is the role of

three-dimensional chromatin structure in gene expression regulation, and how is the structure initiated, maintained, and regulated? What are the transcription factor-epigenetic factor/chromatin remodeling enzyme interactions that sculpt the chromatin landscape? How do transcription factor occupancy and the chromatin landscape change during development, and in abnormal development and disease? How can the chromatin landscape be manipulated to promote cardiomyocyte expansion and myocardial regeneration? The expanding arsenal of tools to study chromatin structure and occupancy at a genome scale promise to shed light on these fundamental questions in the coming years.

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