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Epigenetic regulation of cardiac development and function by Polycomb Group and Trithorax Group Proteins

Q. Tian Wang
Department of Biological Sciences, University of Illinois at Chicago, 900 S Ashland Ave., Chicago, IL 60607, USA

Q. Tian Wang: qtwang@uic.edu

Abstract

Heart disease is a leading cause of death and disability in developed countries. Heart disease includes a broad range of diseases that affect the development and/or function of the cardiovascular system. Some of these diseases, such as congenital heart defects, are present at birth. Others develop over time and may be influenced by both genetic and environmental factors. Many of the known heart diseases are associated with abnormal expression of genes. Understanding the factors and mechanisms that regulate gene expression in the heart is essential for the detection, treatment and prevention of heart diseases. Polycomb Group (PcG) and Trithorax Group (TrxG) proteins are special families of chromatin factors that regulate developmental gene expression in many tissues and organs. Accumulating evidence suggests that these proteins are important regulators of development and function of the heart as well. A better understanding of their roles and functional mechanisms will translate into new opportunities for combating heart disease.

Introduction

During heart development, several cardiac progenitor pools give rise to diverse cell lineages, such as cardiomyocytes, smooth muscle cells of the blood vessels, fibroblasts that form the connective tissues, and endothelial cells of the endocardium (reviewed in Brand, 2003; Vincent and Buckingham, 2010). In order for the developing heart to take on the correct form and function, the differentiation of these cell lineages must be finely orchestrated with cardiac morphogenic events, such as looping, septation, and trabeculation. A significant part of this orchestration is at the level of transcriptional regulation, and a network of cardiac transcription factors have been shown to govern the temporal and spatial patterns of gene expression in the developing heart (reviewed in Bruneau, 2002; Clark *et al.*, 2006; Conway *et al.*, 2010; Cripps and Olson, 2002; Greulich *et al.*, 2011; Kodo and Yamagishi, 2011; McCulley and Black, 2012; Nemer, 2008; Olson, 2006; Wirrig and Yutzey, 2011). Transcription factors also play important roles postnatally in maintaining the pattern of differentiated gene expression, protecting cells from apoptosis, and regulating the heart's stress response such as hypertrophic growth (Aries *et al.*, 2004; Balza *et al.*, 2006; Oka *et al.*, 2006; Oka *et al.*, 2007; Parlakian *et al.*, 2005; Toko *et al.*, 2002; Wang *et al.*, 2005; Zhang *et al.*, 2001).

During the past two decades, the importance of epigenetic mechanisms in transcriptional regulation has been gradually recognized. Eukaryotic genomic DNA is packaged into arrays of nucleosomes, collectively known as chromatin (Fig. 1A). The chromatin in a given region can exist in different configurations that either limit or facilitate local transcription activity. Each nucleosome contains a stretch of DNA wrapped around 8 histone molecules. Each histone has an N-terminal and/or C-terminal tail that protrudes from the nucleosome and can be covalently modified by acetylation, methylation, phosphorylation and other enzymatic

reactions (reviewed in Berger *et al.*, 2002; Cheung *et al.*, 2000; Nightingale *et al.*, 2006) (Fig 1B). These modifications have profound effects on chromatin configuration and, therefore, transcription. Another way to change chromatin configuration is through ATP-dependent chromatin remodeling. Chromatin remodeling can change the position of nucleosome(s), nucleosome spacing, nucleosome-DNA affinity, as well as the integrity of nucleosome(s) (reviewed in Aalfs and Kingston, 2000; Fan *et al.*, 2004; Glatt *et al.*, 2011; Hargreaves and Crabtree, 2011) (Fig. 1A).

The heart expresses many epigenetic factors, including both histone modifying proteins and chromatin remodelers. Several recent publications have provided excellent general reviews of epigenetic regulation of heart development and disease (Han *et al.*, 2011; Ohtani and Dimmeler, 2011; Vallaster *et al.*, 2012; van Weerd *et al.*, 2011). Among epigenetic factors, Polycomb Group (PcG) and Trithorax Group (TrxG) proteins are unique in that they are able to maintain repressed and activated chromatin configurations, respectively, for extended periods of time and in the absence of the transcription factors that initiate repression or activation (reviewed in Jacobs and van Lohuizen, 2002; Pirrotta, 1995). This feature makes them particularly important in the maintenance of “cellular memory” both during lineage differentiation and in adult tissues. Studies on the development of multiple organs and tissues have corroborated such importance. Until recently, there was little information on the roles of PcG or TrxG proteins in the heart. However, emerging data strongly suggest that they are central players in the specification of cardiac lineages during heart development and in the maintenance of cellular properties in the adult heart. In this review, I will focus on these data and discuss them in conjunction with current models of PcG and TrxG functional mechanisms.

PcG and TrxG proteins: essential regulators of axial patterning and lineage development

PcG proteins and their antagonists, TrxG proteins, were first identified in *Drosophila* as regulators of homeotic genes (*Hox* genes) (the early genetic studies that identified PcG and TrxG proteins were reviewed in Kennison, 1995). *Hox* genes are highly conserved transcriptional regulators of cell fates along the anterior-posterior axis (reviewed in Hueber and Lohmann, 2008). They are expressed in defined domains along the anterior-posterior axis and in defined temporal orders: individual *Hox* genes are sequentially activated in progressively posterior domains at progressively later time points (reviewed in Mallo *et al.*, 2010). The overall result is that the boundaries of *Hox* expression domains are spatially staggered (Figure 2). *PcG* genes are required for repression of *Hox* genes outside of, especially anterior to, their normal expression domains (reviewed in Pirrotta, 1995; Gould, 1997). In *PcG* mutants, *Hox* genes become derepressed in anterior cells, causing those cells to take on a more posterior fate. On the other hand, *TrxG* genes are required for keeping *Hox* genes activated within their normal expression domains (reviewed in Gould, 1997). In *TrxG* mutants, expression of *Hox* genes is not properly maintained, causing posterior cells to take on a more anterior fate.

In addition to their roles in axial patterning, PcG and TrxG proteins are involved in the development of many organs and cell lineages (reviewed in Surface *et al.*, 2010; van Lohuizen 1998). Genome-wide studies show that PcG and TrxG proteins and the histone marks regulated by them are associated with thousands of chromatin loci in embryonic stem (ES) cells, and these loci are enriched for “developmental genes” (Mikkelsen *et al.*, 2007; Ku *et al.*, 2008; Pan *et al.*, 2007; Zhao *et al.*, 2007). ES cells that are deficient in PcG activity are defective in differentiation and in activating differentiation-specific genes such as lineage markers (Pasini *et al.*, 2007; Chamberlain *et al.*, 2008; Shen *et al.*, 2009; Landeira *et al.*, 2010).

Finally, mammalian PcG and TrxG proteins have been found to participate in the regulation of proliferation and tumorigenesis (reviewed in Bracken and Helin, 2009; Hess, 2004; Reisman *et al.*, 2009). For example, the PcG protein Bmi-1 is an oncogene that can induce telomerase activity and allow cells to bypass senescence when over-expressed in mammary epithelial cells (Dimri *et al.*, 2002). Another PcG protein, Ezh2, is over-expressed in hormone-refractory, metastatic prostate cancer. Silencing of Ezh2 by siRNA inhibits proliferation of cultured prostate cells (Varambally *et al.*, 2002).

Functional mechanisms of PcG and TrxG proteins

PcG and TrxG proteins function at the level of chromatin, and their functional mechanisms are highly conserved. PcG proteins function in multi-protein complexes (reviewed in Muller and Verrijzer, 2009; Schuettengruber and Cavalli, 2009). Four such complexes have been identified in *Drosophila*: PhoRC, PRC1, PRC2, and PR-DUB. With the exception of PhoRC, corresponding complexes have also been identified in mammals. The PhoRC complex contains sequence-specific DNA binding activity and also interacts with mono- and di-methylated lysine 27 of histone H3 (H3K27) (Klymenko *et al.*, 2006). It has been proposed that PhoRC plays the critical role of recognizing hypomethylated nucleosomes around upstream regulatory elements of PcG target genes. The PRC2 complex contains methyl-transferase activity and induces trimethylation of H3K27 (Czermin *et al.*, 2002; Muller *et al.*, 2002). H3K27me3 is a well-known mark for silenced chromatin and is associated with the promoters and regulatory elements of PcG target genes. The PRC1 complex binds trimethylated H3K27 and induces chromatin compaction, thereby maintaining target chromatin regions in the silenced state (Fischle *et al.*, 2003; Francis *et al.*, 2004) (Figure 3A). PRC1 also mono-ubiquitinates histone H2A at lysine 119, though the ubiquitinase activity of PRC1 appears to be dispensable for its silencing function (Eskeland *et al.*, 2010). The PR-DUB complex has histone deubiquitinase activity that is specific to mono-ubiquitinated H2A (uH2A) in *in vitro* assays (Scheuermann *et al.*, 2010). The exact role of H2A ubiquitination/deubiquitination in PcG-mediated repression is still unclear.

TrxG proteins also function in multi-subunit complexes (Figure 3B) (reviewed in Schuettengruber *et al.*, 2011). Three TrxG complexes, the MLL complex, the BRM/BAF complex and a supercomplex, have been purified in mammalian cells. The MLL complex contains histone methyltransferase (HMTase) activity and trimethylates lysine 4 of histone H3 (H3K4) (Yokoyama *et al.*, 2004). H3K4me3 is tightly associated with the promoter regions of transcriptionally active loci (Bernstein *et al.*, 2005; Schneider *et al.*, 2004). The BRM/BAF complex contains the SWI/SNF chromatin-remodeling ATPase Brm/Brg1 and mediates ATP-dependent nucleosome sliding (Papoulas *et al.*, 1998; Wang *et al.*, 1996). The supercomplex contains both HMTase activity and chromatin remodeling activity (Nakamura *et al.*, 2002).

Several lines of evidence show that PcG and TrxG proteins antagonize the function of each other. First of all, *PcG* and *TrxG* mutations have opposite effects on axial patterning. *PcG* mutations cause posterior transformations, while *TrxG* mutations cause anterior transformations (reviewed in Kennison, 1995). Secondly, genetic experiments show that most *PcG* and *TrxG* mutations are reciprocally suppressive (Daubresse *et al.*, 1999; Ingham, 1983; Kennison and Tamkun, 1988). Finally, the PRC1 complex blocks the chromatin remodeling ability of the BRM/BAF complex on an *in vitro* assembled nucleosomal template (Shao *et al.*, 1999). Conversely, the active histone mark H3K4me3, which is generated by SET1-like and MLL complexes, inhibits histone methylation by PRC2 (Schmitges *et al.*, 2011). Together, PcG and TrxG proteins comprise a highly conserved system that keeps the transcription state of target genes in finely controlled balance.

PcG and TrxG proteins in the maintenance of "cellular memory"

In multi-cellular organisms, different cell lineages have distinct patterns of gene expression. During differentiation, a cell's chromatin is configured to facilitate a lineage-specific gene expression pattern. Both the chromatin configuration and the gene expression pattern it ensures are inherited by the cell's progenies through many cell divisions, even when the developmental cues that initiate the chromatin configuration are no longer present (reviewed in Margueron and Reinberg, 2010; Simon, 1995). PcG and TrxG proteins are thought to be particularly important for the maintenance of such "cellular memory" (reviewed in Jacobs and van Lohuizen, 2002; Pirrotta, 1997).

Genetic experiments in *Drosophila* provided the early evidence that PcG proteins have a role in the long-term maintenance of gene silencing. For example, the expression pattern of the *Hox* gene *Ubx* is established shortly after gastrulation during *Drosophila* embryogenesis. In embryos mutant for the *PcG* gene *esc*, *Ubx* expression domain was initiated normally, but ectopic expression arose after the germ-band extension stage (Struhl and Akam, 1985). Thus, *esc* is dispensable for setting up the *Ubx* expression domain, but is needed in later stages to ensure that *Ubx* remains silenced in regions where it was not initially activated. On the other hand, TrxG proteins function to maintain gene activation. For example, in mouse embryos that lack the TrxG gene *Mll2*, the expression of *Mox1* and *Hoxb1*, two Mll2 targets, was induced normally but later became degenerate and was eventually lost completely (Glaser *et al.*, 2006).

How can PcG/TrxG proteins facilitate the transmission of chromatin configuration through cell divisions? While the exact mechanisms are far from well understood, some clues can be found in recent biochemical and structural studies of the PRC2 complex. During S phase, core components of PRC2 are recruited to sites of DNA replication (Hansen *et al.*, 2008). One of the core components, EED, binds to pre-existing H3K27me3 marks through its WD40 repeats (Margueron *et al.*, 2009). EED-H3K27me3 interaction stimulates the methyltransferase activity of PRC2. Thus, pre-existing H3K27me3 not only provides a way to recruit PRC2 complex, but also activates the complex to tri-methylate H3K27 on newly incorporated histones, providing a mechanism for efficient "self-renewal" of the H3K27me3 mark during mitosis.

Emerging roles for PcG and TrxG proteins in the regulation of heart development and function

Multiple *PcG* and *TrxG* genes are normally expressed in the mouse heart. Due to the essential function of *PcG/TrxG* genes, constitutive knockouts of key *PcG* or *TrxG* genes often result in lethality during early embryogenesis before cardiac phenotypes can be analyzed (Bultman *et al.*, 2000; O'Carroll *et al.*, 2001; Voncken *et al.*, 2003). Aided by conditional knockout models, studies in the past decade have uncovered crucial roles for two PcG complexes and one TrxG complex during cardiac development and/or in the adult heart (Fig. 4, Supplementary Table 1). The major findings of these studies are reviewed below.

Polycomb Repressor Complex 2 (PRC2) core components: Ezh2 and Eed

Ezh2 is a SET-domain histone methyltransferase and the core subunit of PRC2 (Czermin *et al.*, 2002; Muller *et al.*, 2002). Homozygous *Ezh2* knockout embryos die before completion of gastrulation (O'Carroll *et al., 20*01), suggesting that *Ezh2* is essential for early embryonic development. Ezh2 is highly expressed in the developing heart but down-regulated in the adult heart, while its homolog Ezh1 shows the reverse pattern (Sdek *et al.*, 2011). Two recent studies that inactivated Ezh2 in specific cell populations in the heart showed that

Ezh2 plays important roles both during heart development and in the adult heart (He *et al.,* 2012a; Delgado-Olguin *et al.*, 2012).

Inactivation of *Ezh2* in ventricular cardiomyocytes using *Nkx2.5::Cre* ($Ezh2^{NK}$) resulted in perinatal lethality and an array of cardiac abnormalities including hypoplasia in the compact myocardium, excessive trabeculation, septal defects and dilation in the right atrium (He *et al.*, 2012a). Perinatal lethality and thinning of the myocardium were also observed when *Eed* was deleted in differentiated cardiomyocytes by *TnT::Cre*, which is active slightly later than *Nkx2.5::Cre* (He *et al.*, 2012a). However, Eed^{TnT} embryos did not exhibit septal defects or atrial dilation. Taken together, these results suggest that PRC2 activity is required for multiple aspects of heart morphogenesis at multiple time points. What is the molecular basis for the morphological defects in $Ezh2^{NK}$ and Eed^{TnT} hearts? He *et al.* have identified more than 50 genes that are directly repressed by PRC2 in the developing heart. The list includes multiple transcription factors with known roles in various steps of heart morphogenesis, such as *Isl1*, *Tbx2*, *Tbx3*, *Hand1*, *Irx5* and *Six1* (Cai *et al.*, 2003; Costantini *et al.*, 2005; Guo *et al.*, 2011; McFadden *et al.*, 2005; Mesbah *et al.*, 2008; Risebro *et al.*, 2006; Ribeiro *et al.*, 2007; Riley *et al.*, 1998; Singh *et al.*, 2011). This suggests that PRC2 is critically involved in the developmental coordination of cardiac gene expression programs. In addition, PRC2 directly represses the cyclin-dependent kinase inhibitors *Ink4a/b*, which may explain the hypoplasia phenotype in $Ezh2^{NK}$ embryos (He *et al.*, 2012a) (Fig. 5A).

A study by Delgado-Olguin *et al.* used a *MEF2c-ANF::Cre* to inactivate *Ezh2* from E7.5 on in cardiac progenitors of the second heart field (SHF, also known as the anterior heart field or AHF) and their derivatives. Interestingly, $Ezh2^{SHF}$ mice did not exhibit overt defects in cardiac morphogenesis; instead, such animals survived to adulthood but developed cardiac hypertrophy and fibrosis in the SHF-derived right ventricle (Delgado-Olguin *et al.*, 2012) (Fig. 6). *Six1* was identified as the main effector of Ezh2 function in cardiac hypertrophy. Removing one copy of *Six1* rescued the hypertrophy and fibrosis phenotypes caused by SHF-specific deletion of *Ezh2* (Delgado-Olguin *et al.*, 2012). *Six1* is normally expressed in progenitors in the SHF at E7.5-E8.5, but is down-regulated quickly upon differentiation (Guo *et al.*, 2011). In $Ezh2^{SHF}$ hearts, *Six1* expression persists throughout cardiogenesis and in the adult myocardium. An interesting question is whether Ezh2 (and PRC2 activity) plays an initiating or maintenance role in the developmental silencing of *Six1* and other cardiac targets. In *Drosophila*, the developmental silencing of *Hox* genes can be divided into initiating and maintenance phases, which require distinct regulatory elements, and PcG activity is specifically required during the maintenance phase (Ringrose and Paro, 2007). Thus we might expect that the activity of mammalian PRC2 is continuously required, throughout adulthood, to keep *Six1* in a silent state. In other words, *Six1* repression may have been initiated in $Ezh2^{SHF}$ hearts but was not maintained. Alternatively, mammalian PRC2 may be needed transiently for initiating *Six1* silencing but becomes dispensable afterwards. Differentiating between these two scenarios will impact on the design of therapies that target the PRC2 pathway. It is worth noting that the adult heart predominantly expresses *Ezh1* instead of *Ezh2* (Sdek *et al.*, 2011). However, Ezh1 function was not sufficient to repress *Six1* in adult $Ezh2^{SHF}$ hearts (Delgado-Olguin *et al.*, 2012). This may be due to functional divergence between Ezh1 and Ezh2. Although Ezh1 has been shown to methylate H3K27 and complement Ezh2 in ES cells and skin tissue (Ezhkova *et al.*, 2011; Shen *et al.*, 2008), two studies have highlighted important differences between the functions of the two homologues, including a transcriptional activating role for Ezh1 (Margueron *et al.*, 2008; Mousavi *et al.*, 2012).

In addition to methylating H3K27, Ezh2 was also found to methylate the cardiac transcription factor GATA4 and inhibit GATA4 activity both *in vivo* and *in vitro* (He *et al.*, 2012b) (Fig. 7). This makes GATA4 the first known non-histone substrate of PRC2 and

reveals a novel mechanism by which PRC2 may regulate transcription. Future experiments are needed to elucidate the relative contribution by H3K27 methylation *versus* GATA4 methylation toward PRC2-mediated gene silencing of GATA4 target genes. Moreover, it is possible that GATA4 is not an isolated example and PRC2 has other non-histone substrates that remain to be identified.

PRC2 accessory component: Jumonji/Jarid2

Jumonji (Jmj, also known as Jarid2) is the founding member of the Jumonji family that consists of 27 proteins in humans. Many of Jumonji family proteins are predicted to be histone lysine demethylases (KDM) (reviewed in Cloos *et al.*, 2008). However, Jmj itself is predicted to be enzymatically inactive because it carries several amino acid substitutions in the cofactor Fe(II) binding region that is essential for KDM activity (Klose *et al.*, 2006). Jmj is associated with PRC2 in ES cells and several other cell types and tissue (Landeira *et al.*, 2010; Li *et al.*, 2010a; Pasini *et al.*, 2010; Peng *et al.*, 2009; Shen *et al.*, 2009). Consistent with this association, Jmj and PRC2 co-localize at many chromatin regions and each promotes the efficient recruitment of the other to target chromatin. However, it is uncertain whether Jmj enhances or inhibits the HMTase activity of PRC2, and it is possible that Jmj can do both depending on its protein level relative to PRC2. Finally, Landeira *et al.* showed that Jmj is required for maintaining PRC2 target promoters in a “primed” state, bound by RNA polymerase II phosphorylated at Ser 5 (the “poised” RNA pol II) and ready to be activated when differentiation cues are received.

Jmj plays critical roles in cardiac development. *Jmj* expression in the ventricles initiates in the trabecular layer at E10.5 and expands into the compact layer by E12.5; it is broadly expressed in both layers by E18.5 and in postnatal stages (Toyoda *et al.*, 2003). The effect of *Jmj* mutation is highly influenced by genetic background. $Jmj^{-/-}$ in a C3H/He background died by around E11.5 with hyper-proliferation in the trabecular layer, which could have prevented blood circulation and caused lethality (Takeuchi *et al.*, 1999). On the other hand, $Jmj^{-/-}$ embryos in a C57BL/6 background survived to perinatal stages and exhibited double-outlet right ventricle (DORV), ventricular septal defects (VSD) and severe noncompaction of the ventricular wall (Lee *et al.*, 2000).

While the molecular mechanism of Jmj function during cardiac development is far from well-understood, a number of studies have shed light on it in various ways. First of all, Jmj interacts with two important cardiac transcription factors, Nkx2.5 and GATA4, and inhibits the activation of Nkx2.5 and GATA4 target promoters (Kim *et al.*, 2004). This may explain why $Jmj^{-/-}$ hearts failed to down-regulate atrial natriuretic factor (ANF, encoded by the *Nppa* gene), which is a known target of Nkx2.5 and GATA4 (Lee *et al.*, 2000). Secondly, Jmj regulates cardiomyocyte cell cycle in two ways (Fig. 5A): on the one hand, it represses *cyclin D1* expression (and hence inhibits Rb phosphorylation); on the other hand, it interacts with Rb and functions as an Rb co-repressor (Toyoda *et al.*, 2003; Jung *et al.*, 2005). Both will result in repression of E2F target genes, which are required for cell cycle progression. Jmj is able to bind to the *cyclin D1* promoter in transfected cells and recruit H3K9 methylation activity (Shirato *et al.*, 2009), thus directly repressing *cyclin D1*. Thirdly, in the endocardium, Jmj is a negative regulator of Notch signaling, which serves as a mitogenic signal to the myocardium and regulates the trabeculation process (Fig. 5) (for a review of Notch signaling in trabeculation, see High and Epstein, 2008). Jmj is associated with a conserved region in the *Notch1* locus, and Notch1 protein level is significantly elevated in $Jmj^{-/-}$ hearts (Mysliwiec *et al.*, 2011). Remarkably, an endothelial-specific knockout of *Jmj*, which deleted Jmj in the endocardium and other cells of the endothelial lineage, recapitulated most of the cardiac phenotypes of $Jmj^{-/-}$ mice (Mysliwiec *et al.*, 2011). This suggests that Notch and possibly other signals originating in the endocardium mediate a significant portion of Jmj function, in a cell non-autonomous manner. Finally, *Jmj* is

required for the expression of differentiation markers, such as *Myh6* (encoding α-MHC), *α-cardiac actin*, and *actinin*, in fetal cardiomyocytes (Takeuchi *et al.*, 1999; Nakajima *et al.*, 2011). However, this may be an indirect effect stemming from Jmj function in regulating proliferation versus differentiation. Increased expression of cyclin D in *Jmj*$^{-/-}$ hearts causes hyper-proliferation and prevents the expression of GATA4, which is a transcriptional activator of these differentiation genes (Nakajima *et al.*, 2011).

Polycomb Repressor Complex 1 (PRC1) core component: Phc1

Phc1 (also known as Rae28) is a mammalian homolog of the *Drosophila* PcG protein Polyhomeotic (Ph). Phc1 and Ph are components of the mammalian and *Drosophila* PRC1 complex, respectively. *Phc1* mutant embryos are defective in an early and important step of cardiac morphogenesis - cardiac looping - which takes place between E8.5 and E9.5 (Shirai *et al.*, 2002). While the mutant heart is able to form chambers, it displays VSD and other cardiac abnormalities (Takihara *et al.*, 1997; Shirai *et al.*, 2002). These defects appear to stem from a loss of expression of the cardiac transcription factor *Nkx2.5* (Shirai *et al.*, 2002). *Phc1* is not required for the initiation of *Nkx2.5* expression, but is required for its continued expression. Given that PcG proteins generally function to silence genes, regulation of *Nkx2.5* by Phc1 may be indirect, but the exact mechanism remains to be determined.

Interestingly, transgenic studies showed that while ubiquitous expression of a *Phc1* transgene could restore *Nkx2.5* expression and rescue cardiac morphogenesis defects in *Phc1*$^{-/-}$ embryos, cardiomyocyte-specific expression could not (Shirai *et al.*, 2002; Koga *et al.*, 2002). This suggests that normal cardiac morphogenesis requires the function of Phc1 in a cell population other than cardiomyocytes. Cardiomyocyte-specific over-expression of *Phc1* neither rescued congenital heart defects in *Phc1*$^{-/-}$ embryos nor disrupted cardiac morphogenesis in embryos of wild-type background. However, continued expression of *Phc1* in adult cardiomyocytes is deleterious and leads to disorganization of sarcomeres, cardiomyocyte apoptosis, chamber dilation and heart failure (Koga *et al.*, 2002). Because PcG proteins function in multi-subunit complexes, the activity of a complex – such as PRC1 – is likely influenced by both subunit composition and stoichiometry. Thus, it is difficult to predict whether constitutive expression of *Phc1* in adult cardiomyocytes would boost or interfere with PRC1 activity. Nonetheless, we can conclude from the *Phc1* transgenic studies that the fine regulation of PcG activity in adult stages is essential for the maintenance of cardiomyocyte function. It awaits future studies to decipher the molecular function(s) of PRC1 in the adult heart.

BAF complex core component: Brg1/Smarca4

The *Drosophila* TrxG protein Brm is a core component of the BRM complex, which mediates ATP-dependent chromatin remodeling (Papoulas *et al.*, 1998). In humans, the BRG1-associated-factor (BAF) complex shares multiple conserved subunits with the *Drosophila* BRM complex (Wang *et al.*, 1996). The ATPase core of hBAF can be either hBRG1 (also known as SMARCA4) or hBRM (SMARCA2), both of which are homologous to *Drosophila* Brm.

In the mouse, *Brg1* is widely expressed in embryonic heart, and its expression in different regions appears to serve different roles. *Brg1* expression in the endocardium is crucial for trabeculation in the ventricular myocardium (Stankunas *et al.*, 2008) (Fig. 5B). During the process of trabeculation, signaling between the endocardium and the myocardium induces myocardial cells to form finger-like projections, or trabeculae. Trabeculation is a temporally regulated process that initiates at ~E9.0, slows down around E12.5 and completes by E14.5. An appropriate degree of trabeculation is critical for normal contraction and hemodynamics

of the embryonic heart, and is essential for the survival of the embryo. The extracellular matrix between endocardium and myocardium, known as cardiac jelly, plays important roles in trabeculation by affecting the diffusion and function of signaling molecules and by providing a microenvironment that supports the extensive cellular movements needed for trabeculae formation (for a comprehensive review of the role of ECM in cell signaling, see Kim *et al.*, 2011). Brg1 is required for the repression of ADAMTS1, a secreted matrix metalloproteinase that degrades Versican and possibly other proteoglycans in the cardiac jelly, and thereby terminates the trabeculation process (Stankunas *et al.*, 2008). When *Brg1* is deleted in the endocardium, ADAMST1 becomes de-repressed prematurelly, resulting in early degradation of cardiac jelly and hypo-trabeculation. A small-molecule inhibitor of ADAMST1 can rescue the hypo-trabeculation phenotype in cultured *Brg1* mutant embryos, suggesting that the main function of Brg1 in the trabeculation process is to regulate ADAMST1.

Brg1 is also expressed throughout the embryonic myocardium, and this expression is required for normal proliferation and differentiation of cardiomyocytes (Hang *et al.*, 2010). Myocardium-specific deletion of *Brg1* resulted in significantly reduced cardiomyocyte proliferation, reduced expression of Bmp10 (a cardiomyocyte growth factor) and increased expression of $p57^{kip2}$ (a cyclin-dependent kinase inhibitor) (Fig. 5A). In addition, $Brg1^{-/-}$ cardiomyocytes exhibited premature formation of organized sarcomeres, elevated expression of the “adult” MHC isoform α-MHC, and reduced expression of β-MHC (encoded by the *Myh7* gene), the “fetal” isoform that is primarily expressed by embryonic hearts.

In addition to being required for heart development, Brg1 also has roles in heart disease in the adult. With the exception of a small number of non-cardiomyocyte cells, the adult heart does not express *Brg1*. However, *Brg1* can be reactivated by stress signals. The reactivation of *Brg1* is essential for the development of hypertrophy in TAC-operated mice (Hang *et al.*, 2010) (Fig. 6). The adult murine heart predominantly expresses α-MHC. One of the hallmark events during the hypertrophic process is α/β-MHC isoform switching: the re-activation of *Myh7*, and often a concurrent repression of *Myh6*. Consistent with its role in the embryonic heart, Brg1 is required for *Myh7* activation and *Myh6* repression. This may partially explain why Brg1 is required for hypertrophy, though additional mechanism(s) may also be at work.

While the recruitment of PcG proteins to target chromatin sites is thought to involve specialized regulatory elements (reviewed in Ringrose and Paro, 2007) and/or long non-coding RNA (Kotake *et al.*, 2011; Pandey *et al.*, 2008; Rinn *et al.*, 2007; Plath *et al.*, 2003; Zhao *et al.*, 2008), a large number of studies suggest that transcription factors play important roles in the recruitment of BAF and other chromatin remodeling complexes (reviewed in Peterson and Workman, 2000; Sudarsanam and Winston, 2000). Consistent with this view, Brg1 and the BAF complex functionally interact with multiple cardiac transcription factors (Lickert *et al.*, 2004; Lou *et al.*, 2011; Takeuchi and Bruneau, 2009; Takeuchi *et al.*, 2011) (Fig. 7). In the mouse, *Brg1* genetically interacts with *Tbx5*, *Nkx2.5* and *Tbx20* (Takeuchi *et al.*, 2011). Double heterozygotes of *Brg1* and any of these transcription factors die before E14.5 and exhibit various cardiac morphogenic defects, demonstrating mutual genetic enhancement between mutations in *Brg1* and in these transcription factors. Functional interaction has also been observed between BAF complex and GATA4 in both mouse and zebrafish (Lickert *et al.*, 2004; Lou *et al.*, 2011; Takeuchi and Bruneau, 2009; Takeuchi *et al.*, 2011).

BAF complex core component: Baf60c/Smarcd3

Baf60c (also known as Smarcd3) is another core component of the BAF complex. Its yeast homolog has been shown to be essential for the activity of the SWI/SNF complex, which is

the yeast counterpart of BAF (Cairns *et al.*, 1996). There are three Baf60 paralogues in the mammalian genome, Baf60a, b and c. Among the three, Baf60c is the only one that is expressed in the developing heart (Lickert *et al.*, 2004).

In contrast to the broad expression of *Brg1*, the expression pattern of *Baf60c* is highly tissue-specific. When *Baf60c* expression initiates at ~E7.5, it is restricted to the cardiac crescent. It continues to be expressed at high levels in the heart tube and chambered heart. By E9.5, expression is also detected in the somites, dorsal neural tube and limb bud. Using transgenic mice expressing siRNA against *Baf60c*, Lickert *et al.* showed that *Baf60c* was particularly important for development of the outflow tract (OFT), right ventricle (RV) and atrium, all of which are derivatives of the SHF (Lickert *et al.*, 2004).

When Baf60c and GATA4 were co-transfected into wild-type E6.5–E8.75 mouse embryos, the early cardiac marker *Actc1* was ectopically induced in normally non-cardiogenic mesoderm tissues (Takeuchi and Bruneau, 2009). Addition of Tbx5 to the transfection mixture allowed further differentiation into beating cardiomyocytes. These results suggest that Baf60c may have a central function in the specification of cardiac fate in addition to its later role in cardiac morphogenesis. This function was not uncovered in the *siBaf60c* model, possibly because RNAi did not completely eliminate *Baf60c* expression (Lickert *et al.*, 2004).

Although Brg1 and Baf60c are both core components of the BAF complex, *Brg1*- and *Baf60c*-deficient mice exhibited distinct, albeit overlapping, phenotypes. Both are required for trabeculation (Lickert *et al.*, 2004; Stankunas *et al.*, 2008). On the other hand, neither epicardium-specific nor myocardium-specific deletion of *Brg1* exhibited gross defects in the SHF (Stankunas *et al.*, 2008; Hang *et al.*, 2010). The disparity in *Brg1*- and *Baf60c*-deficiency phenotypes may result from differences in the timing of *Brg1* or *Baf60c* loss in the respective mouse models: a role for Brg1 in SHF development may have been missed in the conditional knockouts, in which deletion of *Brg1* occurs after E9.5. Alternatively, it may be due to the distinct roles that the two proteins play within the BAF complex. In reporter assays, Baf60c potentiates the activity of Tbx5, Nkx2.5, and GATA4 by promoting the interaction between these cardiac transcription factors and Brg1 (Lickert *et al.*, 2004) (Fig. 7). This suggests that Baf60c functions as a bridge between the BAF complex and select cardiac transcription factors. In addition to bringing chromatin remodeling activity contained in Brg1, Baf60c may allow its partner transcription factors to access other activities via the BAF complex, such as histone H2B ubiquitinase activity (Li *et al.*, 2010b) or interaction with the basal transcription machinery (Cho *et al.*, 1998; Lemieux and Gaudreau, 2004; Neish *et al.*, 1998; Wilson *et al.*, 1996). Thus, different target genes may exhibit individual requirements for Brg1 and Baf60c, depending on whether their activation requires chromatin remodeling and/or other activities mediated by BAF.

Baf60c not only is important for the recruitment of Brg1 and other BAF-associated activities, but also is required for detectable binding of GATA4 to two of its target loci (Takeuchi and Bruneau, 2009). A role for BAF in the recruitment of its interacting transcription factors has been previously observed. For example, SWI/SNF (the yeast counterpart of BAF) stimulates nucleosome binding by transcription factors Sp1, USF, and NF-κB *in vitro* (Utley *et al.* 1997). *In vivo*, SWI/SNF is required for the efficient binding of GAL4 to low affinity, nucleosomal sites, but not for GAL4 binding to high affinity sites or nucleosome-free low affinity sites (Burns and Peterson, 1997). It has been proposed that BAF may be recruited by a transcription factor binding to a high affinity site, and in turn promotes the recruitment of other transcription factors that bind to weaker sites. Alternatively, BAF may interact with the latter transcription factor in solution before both are recruited to the target site (Peterson and Workman, 2000; Sudarsanam and Winston,

2000). Thus, Baf60c may be recruited by another factor and permits subsequent GATA4 binding, or it may be co-recruited with GATA4 and stabilizes GATA4-chromatin association that is otherwise weak or transient. Whether the recruitment of Baf60c/BAF is dependent on GATA4 or any of its interacting transcription factors needs to be directly tested by future experiments.

Perspectives

During embryonic development, the process of heart morphogenesis involves multiple groups of cells whose specification, proliferation, migration, differentiation and interaction must be precisely regulated both temporally and spatially. During adult life, cardiomyocytes need to maintain their identity and function for many years while having the capacity to respond to physiological changes. PcG and TrxG proteins are well suited for the regulation of both embryonic heart development and adult heart function. By creating repressive and activating chromatin structures, respectively, PcG and TrxG proteins play unique roles in the maintenance of lineage identity and cellular memory. The studies reviewed here have demonstrated important functions for several PcG and TrxG proteins in the heart. However, many questions remain to be answered. While we know PcG and TrxG proteins play critical roles in multiple steps of cardiac development, there are conspicuous gaps in our knowledge: we still know nothing about their function in the development of cardiac neural crest and the proepicardium, both of which make essential contributions to the heart (reviewed in Gittenberger-de Groot *et al.*, 2010; Stoller and Epstein, 2005). While PcG and TrxG proteins have been shown to regulate the hypertrophic response in the adult heart, we need a deeper understanding of their functional mechanisms to address whether cardiac hypertrophy involves a modification of “cellular memory” and whether the normal cellular memory, once modified, can be restored. While functional interactions between PcG/TrxG proteins and cardiac transcription factors have been reported, we are still a long way from integrating PcG and TrxG function into the overall picture of cardiac transcriptional regulation. Finally, to translate findings from basic research to medicine, we need to address whether mutations in human *PcG* and *TrxG* genes are associated with heart disease. As we gain a better knowledge of the roles and functional mechanisms of PcG and TrxG proteins in the heart, we will move closer to harnessing epigenetic mechanisms in the prevention and/or treatment of heart disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AHF	Anterior heart field
ANF	atrial natriuretic factor
BAF	BRG1-associated-factor
CHD	congenital heart defects
DORV	double-outlet right ventricle

ES cells	embryonic stem cells
H3K4	histone H3 lysine 4
H3K4me3	trimethylated histone H3 lysine 4
H3K27	histone H3 lysine 27
H3K27me3	trimethylated histone H3 lysine 27
HDAC	histone deacetylase
HMTase	histone methyltransferase
Hox genes	homeotic genes
KDM	lysine demethylases
LV	left ventricle
OFT	outflow tract
PcG	Polycomb Group
PRC1	Polycomb Repressor Complex 1
PRC2	Polycomb Repressor Complex 2
PR-DUB	Polycomb Repressor Deubiquitinase
RV	right ventricle
SHF	second heart field
TAC	tranverse aortic constriction
TrxG	Trithorax Group
uH2A	mono-ubiquitinated H2A
VSD	ventricular septal defect

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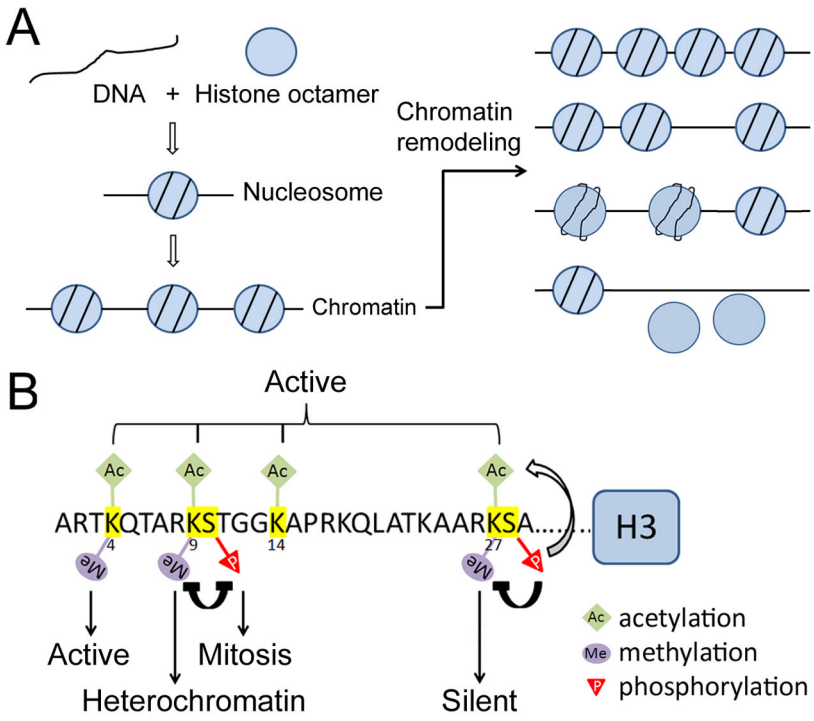

Figure 1. Eukaryotic genomic DNA is packaged into chromatin

(A) Nucleosomes are the basic subunits of chromatin. Each nucleosome consists of ~146 bp of DNA wrapped approximately twice around a histone octamer (the individual monomers and histone tails are not shown). ATP-dependent chromatin remodeling can change nucleosome density, the position of nucleosome(s), nucleosome-DNA affinity, and the integrity of nucleosome(s). (B) Major modification sites on histone H3 tail. Different modifications have different effects on chromatin structure and transcriptional activity. For example, methylation of K4 and K27 is associated with transcriptionally active and silent chromatin, respectively. Modification of one residue can also promote or inhibit the modification of another residue. For example, methylation of K9 and phosphorylation of S10 inhibits each other.

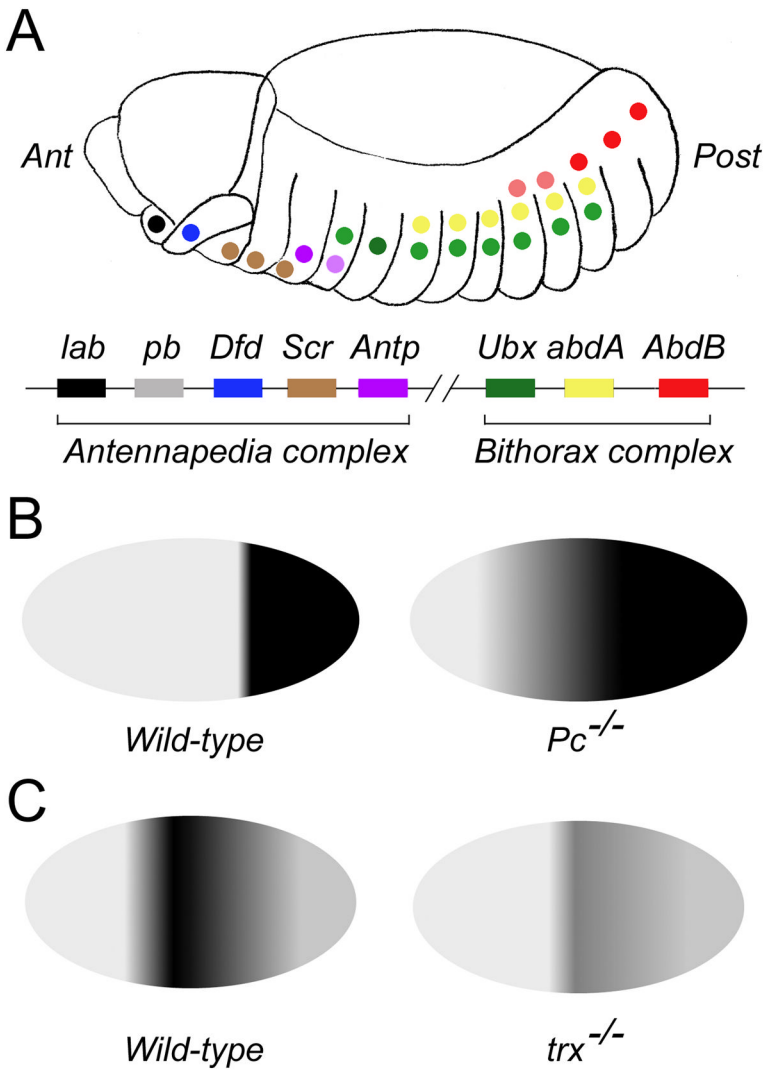

Figure 2. Regulation of *Hox* genes expression by *PcG* and *TrxG* genes in *Drosophila*

(A) Staggered expression of *Drosophila Hox* genes along the anterior-posterior axis of the embryo. The *Drosophila* genome contains seven *Hox* genes arranged in two clusters: the Antennapedia complex and the Bithorax complex. The genes and their respective expression domains in the *Drosophila* embryo are color-coded in this diagram. Ant: anterior; Post: posterior. (B) PcG genes are required to repress *Hox* genes outside their normal expression domains. The diagram shows expression domain of the *Hox* gene *AbdB* in wild-type (left) vs. $Pc^{-/-}$ (right) embryos. *AbdB* is normally expressed in the posterior segments of wild-type embryos but expands anteriorly in $Pc^{-/-}$ embryos. (C) *TrxG* genes are required to maintain *Hox* gene expression. The diagram shows expression of the *Hox* gene *Ubx* in wild-type (left) vs. $trx^{-/-}$ embryos (right). *Ubx* expression is greatly reduced in $trx^{-/-}$ embryos.

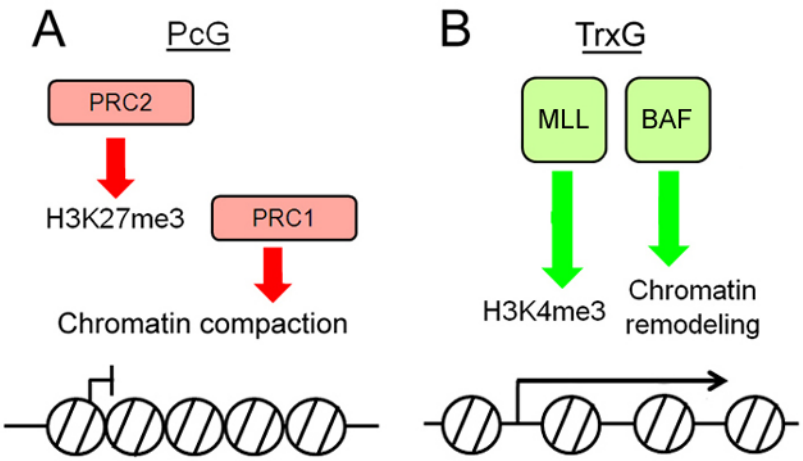

Figure 3. PcG and TrxG proteins function at the level of chromatin

(A) Biochemical functions of the PcG complexes PRC2 and PRC1. PRC2 mediates the trimethylation of histone H3K27, a repressive histone mark. PRC1 binds H3K27me3 and compacts chromatin. (B) Biochemical functions of TrxG complexes MLL and BAF. MLL mediates trimethylation of histone H3K4. BAF has chromatin remodeling activity.

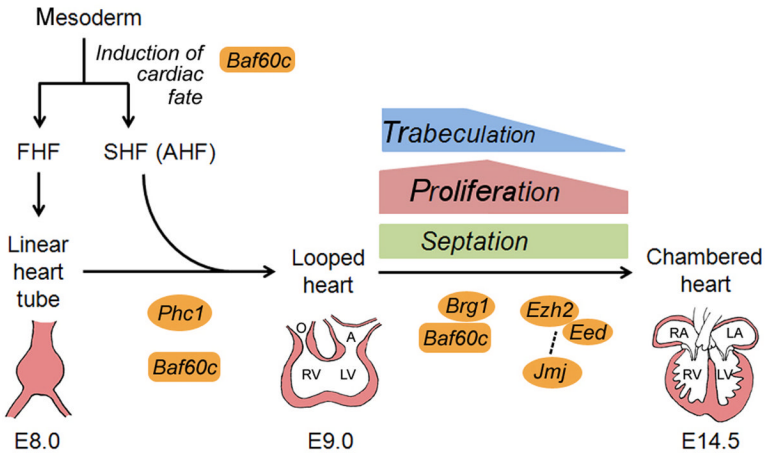

Figure 4. Multiple steps of cardiac development require PcG/TrxG function

Precardiac mesoderm give rise to cardiac progenitors in the first heart field (FHF) and second heart field (SHF, also known as anterior heart field or AHF). The TrxG protein Baf60c likely regulates the induction of cardiac fate. Cells in the FHF form the linear heart tube, which gives rise to the bulk of the left ventricle (LV) and also serves as a scaffold for subsequent heart growth. As the heart tube loops, cells in the SHF migrate to join the linear heart tube and give rise to the outflow tract (O), right ventricle (RV) and atria (A). Both Baf60c and the PcG protein Phc1 have been shown to regulate this early phase of cardiac development. The formation of the chambered heart from the looped heart involves a number of morphogenic processes such as trabeculation, proliferation and septation. Multiple PcG and TrxG proteins, including Brg1, Baf60c, Ezh2, Eed and Jmj, have been shown to regulate these processes. The dashed line between Jmj and Ezh2/Eed represents possible functional interaction. In addition to the FHF and SHF, cells from cardiac neural crest and proepicardium also contribute to the heart (not diagrammed).

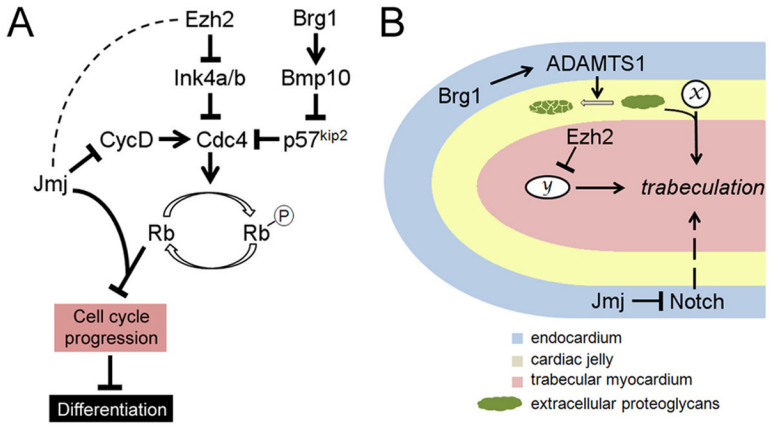

Figure 5. Roles of Ezh2, Brg1 and Jmj in cardiomyocyte proliferation and trabeulation

(A) Pathways by which Ezh2, Brg1 and Jmj regulate fetal cardiomyocyte proliferation. Cyclins (such as cyclin D) activates cyclin-dependent kinases (such as Cdc4), which phosphorylate Rb and relieve Rb repression of a number of genes essential for cell cycle progression. Ezh2 promotes fetal cardiomyocyte proliferation by direct repression of the cyclin-dependent kinase inhibitor Ink4a/b. Brg1 also promotes fetal cardiomyocyte proliferation, and it does so by activating Bmp10, which in turn represses another cyclin-dependent kinase inhibitor $p57^{kip2}$. Jmj inhibits fetal cardiomyocyte proliferation by repressing *cyclin D* and by acting as a co-repressor for Rb. It is unclear whether Jmj and Ezh2 functionally interact with each other in the repression of *cyclin D* and *Ink4a/b*, and if they do, whether Jmj promotes or inhibits PRC2 activity in these contexts (hence dashed line between Jmj and Ezh2). Over-proliferation of fetal cardiomyocytes may result in delayed differentiation. (B) Regulation of trabeculation by Ezh2, Brg1 and Jmj. The diagram shows a trabecula. Formation of these finger-like trabeculae is induced by signaling between the endocardium and the myocardium. Proteoglycans in the cardiac jelly modulates the trabeculation process by modulating the function of signaling molecules (*x*). Ezh2 expression in the myocardium is required for trabeculation, possibly by repressing an as-yet unidentified downstream effector (*y*). Brg1 expression in the endocardium promotes termination of trabeculation by activating the secreted proteinase ADAMTS1, which mediates the degradation of extracellular proteoglycans. Jmj expression in the endocardium negatively regulates trabeculation by repressing Notch.

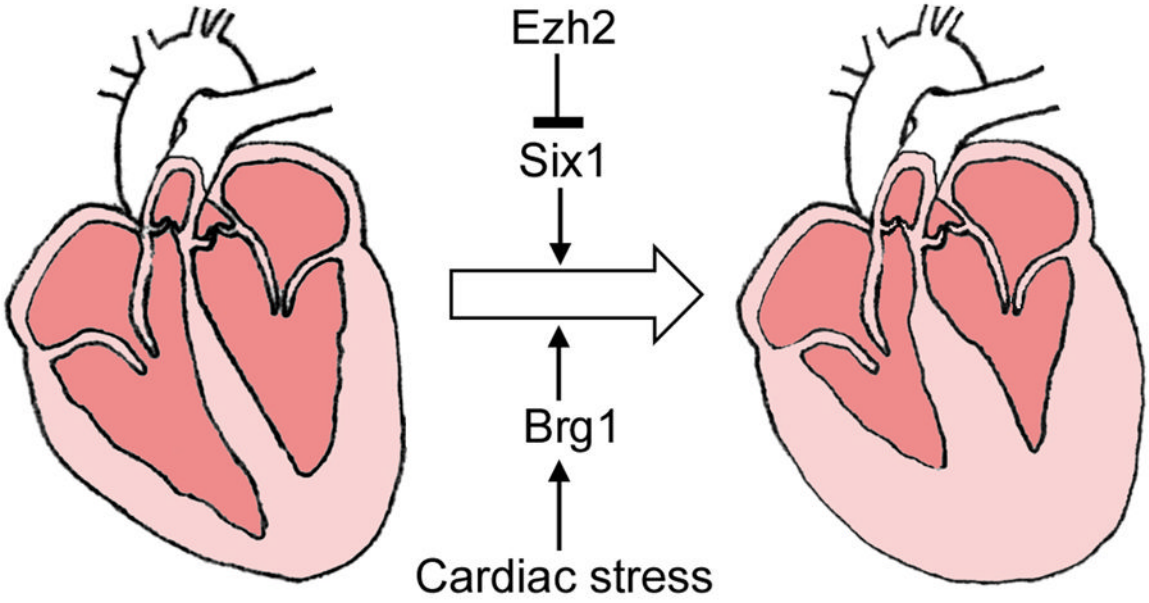

Figure 6. Opposing roles of Ezh2 and Brg1 in the regulation of hypertrophic response

The adult heart responds to stress, such as pressure overload or β-adrenergic stimulation, by hypertrophic growth of cardiomyocytes. This results in thickened myocardial walls and smaller ventricular chamber(s). The PcG protein Ezh2 represses cardiac hypertrophy through a Six1-dependent pathway. The TrxG protein Brg1 is required for development of the hypertrophic response.

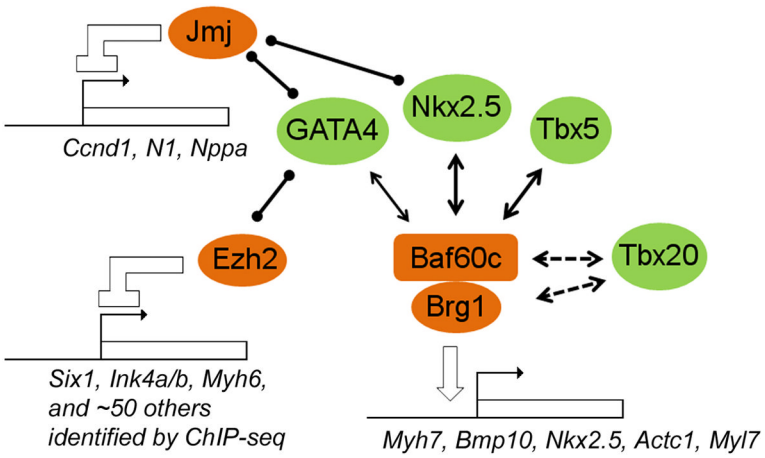

Figure 7. Known interactions between PcG/TrxG proteins and cardiac transcription factors

The TrxG complex BAF physically interacts with cardiac transcription factors GATA4, Nkx2.5 and Tbx5 and potentiates their activity on target promoters. A genetic interaction between Brg1 and the transcription factor Tbx20 has been shown, but it is unclear whether Tbx20 physically interacts with Brg1, Baf60c, or other subunit(s) of BAF. GATA4 also physically interacts with the PcG protein Ezh2, which methylates GATA4 and inhibits its activity. Jmj interacts with both GATA4 and Nkx2.5 and inhibits their activities through an as-yet unknown mechanism.