

**Figure 2 | Loss of *Brg1* leads to heart development defects in the zebrafish embryos.** (a, b) Lateral views at 5 dpf of WT (a) and *brg1*<sup>s481</sup> (b) zebrafish embryos. Arrowhead in b shows pericardial oedema. (c, d) Frontal views of WT or *brg1*<sup>s481</sup> zebrafish embryos at 48 hpf, showing myocardium, labelled with *cmlc2:eGFP* (c), and endocardium, labelled with *flk1:eGFP* (d). Original magnification:  $\times 100$ . (e) Cardiac gene expression in WT and *brg1*<sup>s481</sup> zebrafish embryos for indicated transcripts (left panels, top to bottom: *cmlc2*, *amhc*, *bmp4* and *notch1b*; right panels, top to bottom: *vmhc*, *nppa*, *tbx2b* and *ncx*). White arrow shows normal absence of *nppa* at the atrioventricular (AV) junction, grey triangles show staining of pacemaker cells, red brackets show normal and expanded domains of AV canal markers (*bmp4* and *tbx2b*). Original magnification:  $\times 200$ .

expressed normally (Fig. 1d), indicating deregulation of a specific programme in *Nkx2-5::Cre;Brg1*<sup>fl/fl</sup> hearts.

We conclude that *Brg1* regulates specific programmes of gene expression in the developing heart that are critical for differentiation of cardiac myocytes and cardiac morphogenesis.

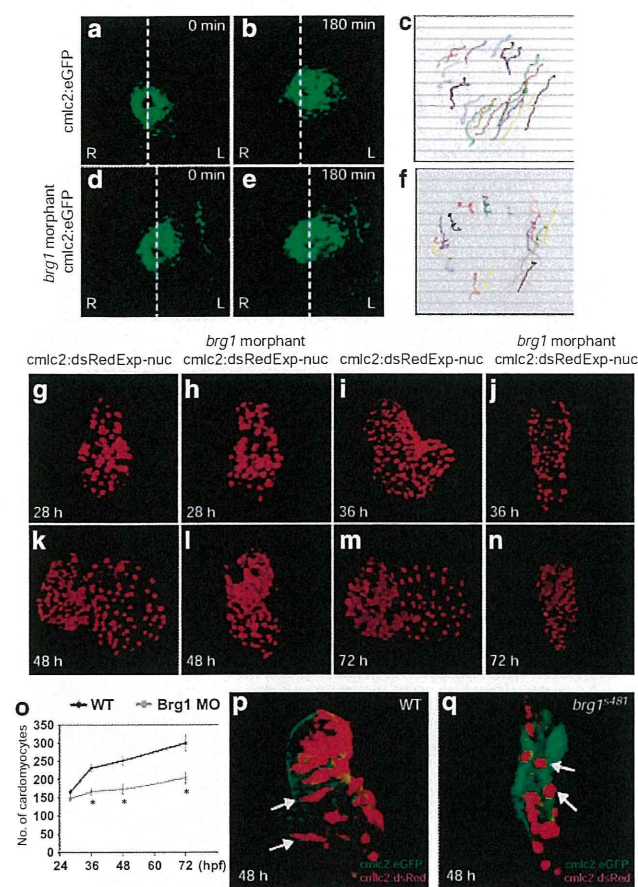
***Brg1* is critical for zebrafish heart development.** The BAF complex is conserved throughout evolution<sup>10</sup>. Zebrafishes have a single BAF complex ATPase, *brg1*. *young*, a loss-of-function mutation of *brg1* (refs 18, 19), results in defects in retinal neurogenesis and pericardial oedema, which often indicates a defective heart function. We isolated a new mutation in *brg1*, *brg1*<sup>s481</sup>, which is predicted to be a null allele (Supplementary Fig. S2); this mutation creates a premature stop codon, predicting a truncation at amino-acid

residue 252 (of 1,627), deleting all functional domains including the ATPase/SNF2 domain and the bromodomains. This mutation fails to complement the published *ying* allele<sup>18,19</sup> and is phenocopied by morpholino oligonucleotide (MO) treatment (see below), consistent with a null allele. These mutants formed a heart; however, after 48 h of development, the heart became hypoplastic and had severe arrhythmias with sporadic arrests in contraction (Fig. 2a,b; Supplementary Movies 1 and 2). The survival of *brg1* mutant embryos to a late stage is likely related to the presence of maternal *brg1* transcripts<sup>19</sup>. To knock down *brg1* in other transgenic lines, translation-inhibiting MOs<sup>20</sup> were injected. Knockdown of *brg1* by MO does not alter endocardial differentiation, and vascular development occurred normally (Supplementary Fig. S3). However, the heart chamber displayed severe stenosis (Fig. 2c,d). Co-injection of an MO targeting *p53* was used to investigate a role for cell death in the *brg1* cardiac phenotype<sup>21</sup>. Injection of *p53* MO into *brg1* mutant embryos resulted in an identical cardiac phenotype to that observed in uninjected mutant siblings (Supplementary Fig. S3). Gene expression analysis (Fig. 2e) demonstrated that, although differentiation of both heart chambers occurred properly, *brg1*<sup>s481</sup> zebrafish had lost the regionalization of *nppa* expression, which marks ‘working’ myocardium in fish and mice<sup>22</sup>; this is reminiscent of the loss of *Brg1* in the mouse. Expression of atrioventricular canal-specific genes, including *bmp4*, *tbx2b* and *notch1b*, was abnormal in *brg1*<sup>s481</sup> embryos, suggesting patterning abnormalities in *brg1* mutant zebrafish embryos. Expression of the Na<sup>+</sup>/Ca<sup>++</sup> exchanger (*Ncx*) was elevated in *brg1*<sup>s481</sup> embryos, which could explain contractility defects in *brg1*<sup>s481</sup> embryos<sup>23,24</sup>. We conclude that, in zebrafish, as in the mouse, *brg1* is required for a specific programme essential for cardiac morphogenesis and patterning.

To uncover the cellular mechanisms underlying loss of *brg1* in zebrafish, cardiomyocyte migration, proliferation and shape were analysed. During cardiac cone tilting, an early event in the formation of the zebrafish heart tube, atrial myocardium undergoes left-directed anterior migration<sup>25</sup> (Fig. 3a–c; Supplementary Movie 2). In *brg1* morphants, anterior cardiomyocytes, especially those on the right side of the heart, displayed randomized trajectories (Fig. 3d–f; Supplementary Movie 3), resulting in the failure of proper heart jogging, which is indispensable for subsequent heart looping and chamber formation. We monitored the growth of the heart by counting the number of cardiomyocytes at different time points (Fig. 3g–n). At early stages, cell numbers between control and *brg1* morphants were comparable (at 28 hpf,  $166 \pm 12$  ( $n=4$ ) cardiomyocytes in wild type (WT) hearts versus  $145 \pm 14$  in *brg1* morphants ( $n=5$ )). As development proceeded, however, growth in myocardial cell number obviously lagged in *brg1* morphants and appeared to halt by 48 hpf ( $251 \pm 13$  ( $n=6$ ) in WT hearts versus  $173 \pm 14$  in *brg1* morphants ( $n=4$ ); Fig. 3o; Supplementary Movies 4 and 5).

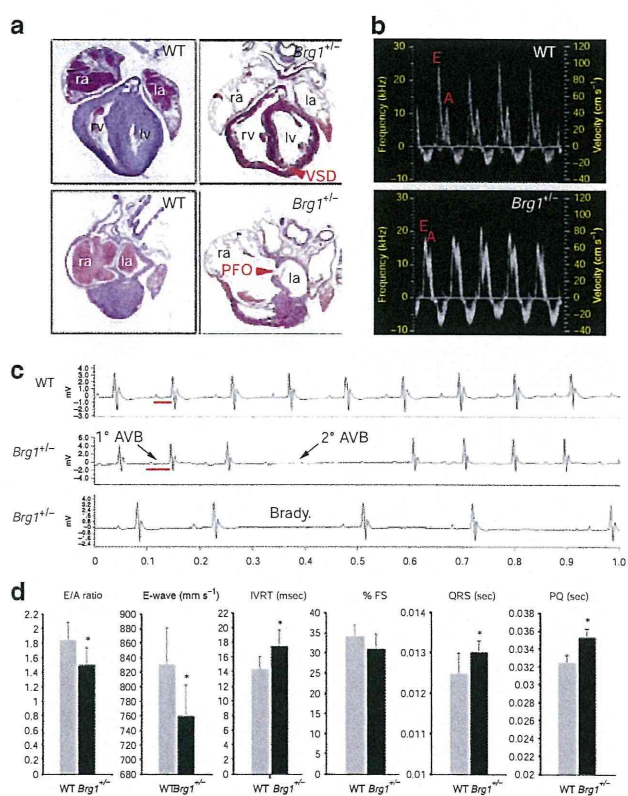
During zebrafish heart development, confined myocardial cell-shape changes are a key parameter in cardiac morphogenesis<sup>22</sup>. In WT embryos, outer curvature cardiomyocytes became flattened and elongated and were aligned relative to each other (Fig. 3p; Supplementary Movie 6). In *brg1*<sup>s481</sup> embryos, cardiomyocytes had a cuboidal shape throughout the heart (Fig. 3q; Supplementary Movie 7). Because of the altered and variable cell shape, it is difficult to assess cell size, and thus we have not quantified this parameter; however, cell size did not appear to be grossly altered. As the changes in cell shape require a balance between extrinsic (blood flow) and intrinsic (contractility) biomechanical forces<sup>22</sup>, we examined the circulation in *brg1*<sup>s481</sup> embryo (Supplementary Movie 8). The circulation in mutant embryos was slower than that in WT embryos at 36 hpf, but still robust, suggesting that reduced circulation did not cause the abnormal myocyte shapes. Rather, the cell-shape changes in *brg1* mutants may reflect either an intrinsic defect in myocardial morphogenesis and/or be secondary to abnormal heart contractility.





**Figure 3 | Defective cardiomyocyte migration and cell shape in zebrafish *brg1* mutants.** (a–f) Imaging of atrial cell migration in WT embryos (a–c) and *brg1* morphants (d–f). (a, b, d, e) Dorsal views of heart in Tg(*cmlc2:eGFP<sup>vu34</sup>*) embryos between 18 and 21 hpf; dotted white lines indicate the embryonic midline. (c, f) Arrows of different colours indicate the trajectories of individual cells. Original magnification:  $\times 200$ . (g–o) Measurement of cardiomyocyte numbers. Three-dimensional reconstructions of the nuclear DsRed signal from Tg(*cmlc2:dsRedExp-nuc<sup>hsc4</sup>*) embryos are shown (g–n). The 28 hpf embryos (g, h) are shown in dorsal view, the 36 hpf (i, j), 48 hpf (k, l) and 72 hpf (m, n) embryos are shown in anterior views. (o) Quantitation of cardiomyocyte cell numbers. Data are mean  $\pm$  s.d.,  $n = 5$ –8 embryos;  $*P < 0.05$  by *t*-test. Original magnification:  $\times 400$ . (p, q) Three-dimensional assessment of cell morphologies in Tg(*cmlc2:eGFP<sup>vu34</sup>*)-expressing hearts that exhibit mosaic expression of Tg(*cmlc2:dsRedExp-nuc<sup>hsc4</sup>*). Arrows point to representative cells. (p) WT cells transplanted; (q) *brg1<sup>ts481</sup>* cells transplanted. Original magnification:  $\times 400$ . A, atrium; L, left; R, right; V, ventricle.

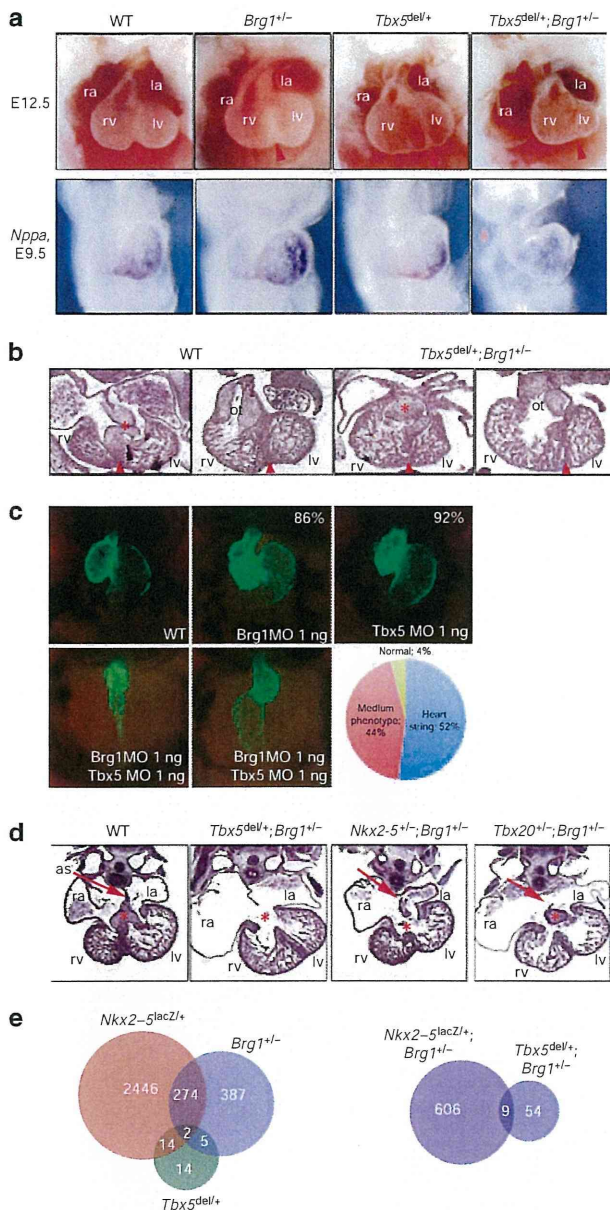
**CHDs in *Brg1* haploinsufficient mice.** The defects upon cardiac-specific deletion of *Brg1* and the phenotype of *brg1* zebrafish show that *Brg1* is critical for specific aspects of cardiac gene expression. As *Brg1<sup>+/-</sup>* mice are underrepresented after birth<sup>13</sup>, we hypothesized that the partially penetrant lethality in *Brg1<sup>+/-</sup>* mice might be due to CHDs. We found that 50% of *Brg1<sup>+/-</sup>* mice died before 3 weeks of age (compared with 0% in WT controls), and in neonatal mice ( $n = 10$ ) we found CHDs such as dilated hearts (5/10), muscular ventricular septal defects (2/10) and incomplete closure of the atrial septum (4/10), a condition known as patent foramen ovale (Fig. 4a). Specific heterozygous deletion of *Brg1* in ventricular myocytes (with *Nkx2.5::Cre*) and cardiac precursors (with *Mef2cAHF::Cre<sup>26</sup>*) also led to CHDs, indicating that these are not due to nonspecific



**Figure 4 | CHDs in *Brg1* heterozygous null mice.** (a) Histology of postnatal day 0 WT (left panels) and *Brg1<sup>+/-</sup>* hearts (right panels), showing dilated chambers, muscular ventricular septal defect (VSD) and patent foramen ovale (PFO) in *Brg1<sup>+/-</sup>* hearts. Top and bottom panels are planes of section of the same heart at the level of the outflow tract (top panels) and at the level of the atrial septum (bottom panels). Original magnification:  $\times 50$ . (b) Doppler waveforms of flow at the mitral valve of adult WT and *Brg1<sup>+/-</sup>* mice, showing altered E and A wave amplitudes in *Brg1<sup>+/-</sup>* mice. (c) ECG telemetry in WT and *Brg1<sup>+/-</sup>* mice, showing prolonged PQ interval, sinus pause and second-degree atrioventricular block in *Brg1<sup>+/-</sup>* mice. (d) Quantitation of selected parameter in WT (grey bars) and *Brg1<sup>+/-</sup>* (black bars) mice. Units of measure are indicated in parentheses next to the graphed metric title. Data are mean  $\pm$  s.d.;  $n = 5$ ;  $*P < 0.05$ . la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle.

effects of loss of *Brg1* outside the heart (Supplementary Fig. S4). The lack of severe defects in *Nkx2.5::Cre;Brg1<sup>fl/+</sup>* mice (in which heterozygous loss of *Brg1* is only in ventricular myocytes) indicate that the defects in *Brg1<sup>+/-</sup>* mice are likely to result from combined defects in multiple cardiac cell types. *Brg1<sup>+/-</sup>* mice that survived the neonatal period had structurally normal hearts, but we also identified specific abnormalities in heart function in adult *Brg1<sup>+/-</sup>* mice, as determined by high-frequency ultrasound and electrical function (Fig. 4b–d). Haemodynamic anomalies consisted of predominantly impaired cardiac relaxation, as determined primarily by a decrease in E-wave amplitude (Fig. 4b,d). Defects in cardiac electrical function were also found in surviving *Brg1<sup>+/-</sup>* mice by telemetry electrocardiogram (ECG), which included atrioventricular block, mild prolongation of the QRS complex (indicative of slowed conduction) and occasional sinus node dysfunction (resulting in an irregular heart beat; Fig. 4b–d). We conclude that *Brg1* haploinsufficiency predisposes mice to CHDs and abnormal heart function. This important result identifies a dosage requirement for BAF complexes in specific aspects of cardiac morphogenesis and functional maturation, and suggests a potential mechanistic link to disease-causing mutations in human CHD.





**Brg1 genetically interacts with transcription factor genes.** The morphological and physiological heart defects in *Brg1*<sup>+/-</sup> mice are reminiscent of those in *Tbx5* haploinsufficient mice<sup>4,27,28</sup>. We hypothesized that *Brg1* might be a critical determinant of the dosage sensitivity of *Tbx5*. To look for a genetic interaction between *Tbx5* and *Brg1*, we generated mice heterozygous for both *Tbx5* and *Brg1* (*Brg1*<sup>+/-</sup>;*Tbx5*<sup>del/+</sup> mice). Compared with *Brg1*<sup>+/-</sup> or *Tbx5*<sup>del/+</sup> mice, which had normal heart morphologies at E12.5, all *Brg1*<sup>+/-</sup>;*Tbx5*<sup>del/+</sup> mice (*n*=6) had severe defects in heart formation at this stage, including hypoplastic left ventricle and dilated atria (Fig. 5a,b). Although *Brg1*<sup>+/-</sup>;*Tbx5*<sup>del/+</sup> hearts appeared relatively normal at E11.5, this genetic interaction was evident at the level of *Nppa* gene expression at E9.5, which was expressed at normal levels in *Brg1*<sup>+/-</sup> mice, at slightly reduced levels in *Tbx5*<sup>del/+</sup> mice, but was undetectable in *Brg1*<sup>+/-</sup>;*Tbx5*<sup>del/+</sup> mice (Fig. 5a). Trivial explanations might be that, in *Brg1*<sup>+/-</sup>;*Tbx5*<sup>del/+</sup> mice, the expression of *Tbx5* is much lower than in *Tbx5*<sup>del/+</sup> or *Brg1*<sup>+/-</sup> mice or that expression of its interacting partners (*Nkx2-5*, *Gata4*) is lower, predisposing the mice to more severe CHDs; this mechanism was discounted by

**Figure 5 | Genetic interactions between *Brg1* and cardiac transcription factor genes.** (a) *Brg1* and *Tbx5* genetically interact. The top row shows an external view of hearts from E12.5 WT, *Brg1*<sup>+/-</sup>, *Tbx5*<sup>del/+</sup> and *Brg1*<sup>+/-</sup>;*Tbx5*<sup>del/+</sup> embryos; the bottom row shows expression of *Nppa* at E9.5 for the same genotypes. Original magnification: ×50. (b) Histology of E11.5 WT and *Brg1*<sup>+/-</sup>;*Tbx5*<sup>del/+</sup> embryos. *Brg1*<sup>+/-</sup> or *Tbx5*<sup>del/+</sup> embryos are indistinguishable from WT. Asterisk indicates atrioventricular cushion and arrowhead indicates interventricular septum. Original magnification: ×100. (c) *Brg1* and *Tbx5* interactions in zebrafish. Tg(*cmlc2:eGFP*<sup>mw34</sup>) control (WT) or MO-injected embryos are shown in ventral-anterior views at 72 hpf. *Brg1*MO: MO directed against *Brg1*; *Tbx5*MO: MO directed against *Tbx5*. All MOs were injected at 1 ng. Percentages in the *Brg1*MO and *Tbx5*MO show the percentage of normal hearts. The graph shows the percentage of phenotypes observed in double knockdown experiments (*n* = 74, 91 and 126 for *brg1*, *tbx5* and *brg1* + *tbx5* morpholino injections, respectively). Original magnification: ×400. (d) *Brg1* genetically interacts with *Tbx5*, *Nkx2-5* and *Tbx20*. Histology of E11.5 hearts shows specific defects in *Brg1*<sup>+/-</sup>;*Tbx5*<sup>del/+</sup>, *Brg1*<sup>+/-</sup>;*Nkx2-5*<sup>+/-</sup> and *Brg1*<sup>+/-</sup>;*Tbx20*<sup>+/-</sup> embryos, compared with WT. *Nkx2-5*<sup>+/-</sup> and *Tbx20*<sup>+/-</sup> hearts are structurally identical to WT hearts. Asterisk indicates atrioventricular cushion and arrow indicates atrial septum (as). Original magnification: ×100. (e) Summary of microarray analysis performed on E11.5 hearts of the indicated genotypes. Venn diagrams show the number of altered transcripts for each genotype, using a statistical cutoff of *P* < 0.01 and fold change > 0.3. la, left atrium; lv, left ventricle; ot, outflow tract; ra, right atrium; rv, right ventricle.

quantitative reverse transcription-PCR and microarray analysis that showed similar levels of *Tbx5*, *Nkx2-5* and *Gata4* messenger RNA (mRNA) in *Brg1*<sup>+/-</sup> and *Brg1*<sup>+/-</sup>;*Tbx5*<sup>del/+</sup> hearts at E11.5 (Supplementary Fig. S5 and Supplementary Data 1).

In zebrafish, mutation of *tbx5* in the *heartstrings* mutant<sup>29</sup> leads to defects in heart looping and progressive deterioration of the heart. The similarity between *tbx5* and *brg1* mutants in the fish raised the possibility that genetic interactions between these two genes may exist also in zebrafish. We performed double knockdown of *brg1* and *tbx5* via MOs. When 1 ng *brg1* or *tbx5* morpholino was injected, the heart developed normally in all cases (Fig. 5c). In contrast, co-injection of *brg1* and *tbx5* morpholino (1 ng each) resulted in 52% of embryos displaying a severe *heartstrings*-like phenotype and 44% of embryos having a milder unlooped, tube-like heart phenotype (*n* = 120; Fig. 5c), demonstrating a clear and conserved genetic interaction between *brg1* and *tbx5*.

We utilized the variable dose dependency of other critical cardiac transcription factors in the mouse to test further genetic interactions with *Brg1*. Although *Tbx5* haploinsufficiency in the mouse mimics CHDs in humans<sup>4</sup>, *Nkx2-5* heterozygous mice display only very mild aspects of CHDs caused by human *NKX2-5* mutations<sup>30,31</sup>, and *Tbx20* haploinsufficiency does not result in any detectable heart defects<sup>32</sup>. We generated compound heterozygous mice for *Brg1* and for either *Tbx5*, *Nkx2-5* or *Tbx20*; all mouse mutations are loss-of-function alleles with no known dominant activity. In all three cases, dramatic and specific defects in heart formation were observed in all compound heterozygous mice (*n* = 4–8) at E12.5 (Fig. 5d), and no compound heterozygous mice survived past E14.5. Defects included severe loss of cardiac cushion formation (*Tbx5*<sup>+/-</sup>;*Brg1*<sup>+/-</sup> and *Brg1*<sup>+/-</sup>;*Nkx2-5*<sup>+/-</sup>), thinned atrial walls (*Tbx5*<sup>+/-</sup>;*Brg1*<sup>+/-</sup>, *Tbx20*<sup>+/-</sup>;*Brg1*<sup>+/-</sup>), hypoplastic ventricles (*Tbx5*<sup>+/-</sup>;*Brg1*<sup>+/-</sup>, *Nkx2-5*<sup>+/-</sup>;*Brg1*<sup>+/-</sup>) and thinned ventricular walls (*Nkx2-5*<sup>+/-</sup>;*Brg1*<sup>+/-</sup>, *Tbx20*<sup>+/-</sup>;*Brg1*<sup>+/-</sup>). *gata5* mutant fishes also have heart defects<sup>33</sup>, and mouse *Gata4*, the functional homologue of zebrafish *gata5*, interacts with BAF complexes<sup>7,8</sup>; we also observed an interaction of *brg1* and *gata5* low-dose MOs, supporting a more general importance of dose-dependent interactions between *Brg1* and cardiac transcription factors (Supplementary Fig. S2).



These strong genetic interactions between *Brg1* and cardiac transcription factor genes in mouse and zebrafish demonstrate a finely regulated interdependency between cardiac DNA-binding transcription factors and BAF chromatin remodelling complexes.

#### Programmes regulated by *Brg1* and cardiac transcription factors.

The genetic interaction between *Brg1* and cardiac transcription factor genes could be a result of a genetic programme common to all transcription factors that is sensitive to *Brg1* levels or, alternatively, *Brg1* may potentiate each transcription factor's specific genetic programme. To address this question, we performed transcriptional profiling of E11.5 hearts from mice heterozygous for deletions of *Brg1*, *Tbx5* or *Nkx2-5*, and mice that were compound heterozygotes for *Brg1* and each transcription factor gene. We observed varying degrees of overlap between the sets of genes that were altered by decreased dosages of *Brg1*, *Tbx5* and *Nkx2-5*, indicating some specificity to the programmes that are regulated by these factors (Fig. 5e and Supplemental Data 1, and quantitative RT-PCR (QRT-PCR) validation in Supplementary Fig. S5). Clustering analysis defined specific groups of genetic interactions (Fig. 6a,b), indicating considerable complexity to the genetic interactions between *Nkx2-5* or *Tbx5* and *Brg1*. For example, some genes were altered mostly in compound heterozygous hearts (clusters a, m, g, t). These genes are more resistant to an imbalance between *Brg1* and the DNA-binding factors, but still rely on the balance of *Brg1* and *Tbx5* or *Nkx2-5* for their normal regulation. Imbalance in the expression of these sets of genes is likely to be key for the compound heterozygous phenotype observed between *Brg1* and *Tbx5* or *Nkx2-5*.

Perhaps more surprisingly, many genes that were altered in single heterozygous (*Nkx2-5<sup>lacZ/+</sup>*, *Brg1<sup>+/-</sup>* or *Tbx5<sup>del/+</sup>*) hearts compared with WT hearts were not significantly altered in compound heterozygous (*Nkx2-5<sup>lacZ/+</sup>;Brg1<sup>+/-</sup>* or *Tbx5<sup>del/+</sup>;Brg1<sup>+/-</sup>*) hearts (clusters d, e, l, n, o and u in Fig. 6a,b). One possible interpretation of this striking interaction is that the relative levels of *Nkx2-5* (or *Tbx5*) and *Brg1* need to be maintained for normal expression of this set of genes, and that loss of one allele of either *Nkx2-5* or *Brg1* in single heterozygotes upsets this balance, leading to an allelic ratio of 2:1 or 1:2; however, in *Nkx2-5<sup>lacZ/+</sup>;Brg1<sup>+/-</sup>* hearts the balance is restored (a 1:1 allelic ratio). This implies that the relative levels between the DNA-binding factors (*Tbx5* and *Nkx2-5*) and *Brg1* is critical for a large set of genes.

The types of genes identified in the transcriptional profiling assays were from a wide range of functional classes, as exemplified from statistical exploration of Gene Ontology classifications that revealed broad classes of transcripts enriched in specific clusters (Fig. 6). Several genes important for heart development were identified in the different clusters of differentially regulated genes, including several previously identified as altered in *Nkx2-5*- or *Tbx5*-deficient hearts<sup>31,34,35</sup>. One particular novel set of transcripts was of interest: in *Nkx2-5<sup>lacZ/+</sup>* and *Brg1<sup>+/-</sup>* hearts, we measured a clear increase in expression of several genes associated with the haematopoietic programme, including key regulators of this programme such as *Gata1*, *Klf1* and *Tal1*. This implies that *Nkx2-5/Brg1* suppresses haematopoietic differentiation in the embryonic heart.

It is difficult to precisely link the contribution of the different genetic programmes regulated by individual factor dosage and combinatorial dosage to the heart defects in each genetic modification, especially with only one snapshot in developmental time. Furthermore, many of the affected transcripts are likely to be due to indirect effects. However, it is clear that overlapping programmes are sensitive to the dosage of *Brg1* and the cardiac transcription factor genes.

**Reduced promoter occupancy by *Brg1* in mutant hearts.** Genetic interactions between *Brg1* and cardiac transcription factors may reflect a direct physical interaction that is sensitive to the dosage of each factor, in one or multiple cell types, as well as interactions

between various cell types in which one factor is haploinsufficient, and another cell type in which the interacting factor is haploinsufficient. To address the former possibility, we performed chromatin immunoprecipitation (ChIP) for *Brg1* on E9.5 hearts from WT, *Tbx5<sup>del/+</sup>*, *Brg1<sup>+/-</sup>* and *Tbx5<sup>del/+</sup>;Brg1<sup>+/-</sup>* embryos at the cardiomyocyte-specific *Nppa* and *Gja5* promoters (Fig. 7a). We found that at the *Nppa* gene, *Brg1* occupancy was reduced in *Brg1<sup>+/-</sup>* hearts, consistent with a limiting amount of *Brg1*, and that at both *Nppa* and *Gja5*, *Brg1* occupancy was reduced in *Tbx5<sup>del/+</sup>* hearts, indicating that reduced levels of *Tbx5* directly result in reduced occupancy of *Brg1* (Fig. 7b). *Brg1* occupancy on both promoters was further reduced in *Tbx5<sup>del/+</sup>;Brg1<sup>+/-</sup>* embryos, suggesting that the interdependency of *Tbx5* and *Brg1* is partly compensated by interactions with other factors, perhaps other transcription factors that can interact with BAF complexes. These results provide a mechanism for some of the dosage-related relationships between *Tbx5* and *Brg1* on *Tbx5* target genes, and support the notion that at least some of the effects of the genetic interaction between *Tbx5* and *Brg1* reflect cell-autonomous direct interactions.

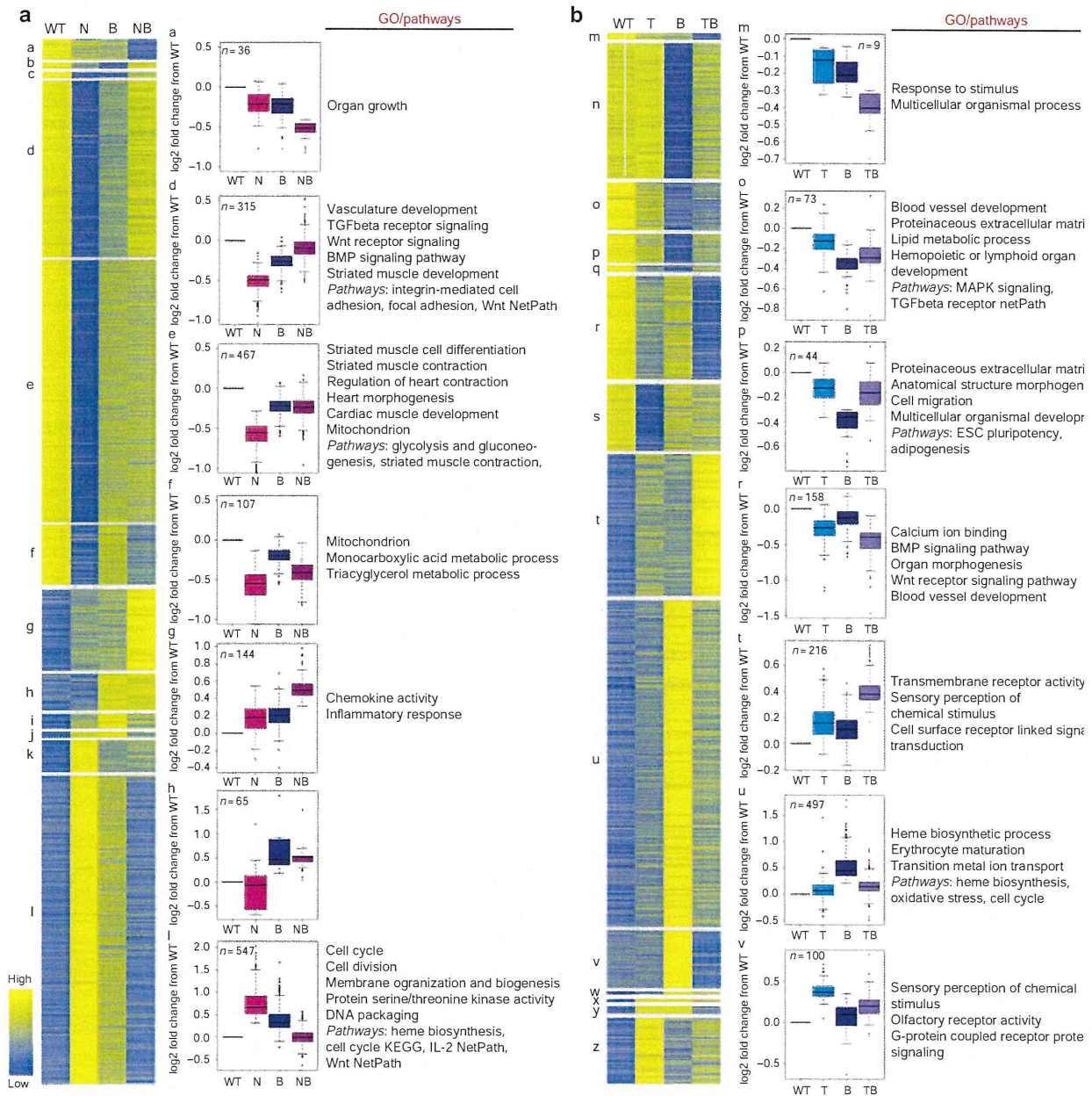
***Brg1*-dependent transcriptional activation *in vitro*.** We investigated whether transcriptional activation of cardiac genes directly requires the function of *Brg1*. We previously showed that *Tbx5*, *Nkx2-5*, *Gata4* and the BAF complex subunit *Baf60c* can activate cardiac gene expression in non-cardiac mesoderm<sup>8</sup>. We found that co-transfection of NIH3T3 fibroblasts with *Tbx5*, *Nkx2-5* and *Gata4* expression constructs could robustly induce *de novo* the cardiac-specific gene *Nppa*, a marker of differentiated cardiomyocytes, the promoter of which is well characterized as a target of these transcription factors (Fig. 7c) (refs 4, 36). As *Baf60c* is expressed in NIH3T3 fibroblasts, exogenous *Baf60c* did not enhance the effect (data not shown). Other cardiac genes examined were not induced by this combination of factors. To determine the requirement for *Brg1* in the *de novo* activation of *Nppa*, we depleted *Brg1* with endoribonuclease prepared small interfering RNAs (esiRNAs)<sup>37</sup>. esiRNAs directed against *Brg1* resulted in an ~50% decrease in *Brg1* protein level (Fig. 7d). Decreased levels of *Brg1* completely prevented *Nppa* activation by cardiac transcription factors (Fig. 7e,f), suggesting that this transcriptional activation is dependent on limiting amounts of functional *Brg1*-containing BAF complexes.

#### Discussion

We demonstrated a critical, conserved and dosage-sensitive role for the BAF chromatin remodelling complex ATPase *Brg1* in vertebrate heart development. Our results further show an important interdependency between BAF complexes and disease-related cardiac transcription factors, which suggests a mechanism for CHDs caused by mutations in these transcription factor genes and implications for multigenic inheritance of CHDs.

In mouse and zebrafish, *Brg1* is required for important and specific aspects of heart development. In particular, chamber morphogenesis is disrupted in both mice and fish lacking *Brg1*, and proliferation of cardiac progenitors is reduced. A key subset of cardiac genes was affected by the loss of *Brg1*, consistent with specific roles for BAF complexes in differentiation of other cell types<sup>9,14,19,38–42</sup>. In many of these cases, *Brg1* is required for differentiation after specification. Our results support a clear role for *Brg1* in cardiac differentiation. As the function of *Gata4*, *Nkx2-5* and *Tbx5* relies on BAF complexes, in part via *Baf60c*<sup>8</sup>, loss of *Brg1* likely affects target genes primarily by reducing the activation potential of cardiac transcription factors. Loss of *brg1* in zebrafish demonstrates a strikingly conserved role for BAF complexes in vertebrate cardiac development. The morphogenetic and gene expression defects in *brg1* mutant fish closely resemble that seen in mice lacking *Brg1*, suggesting that the gene networks regulated by BAF complexes are well conserved in vertebrate evolution.



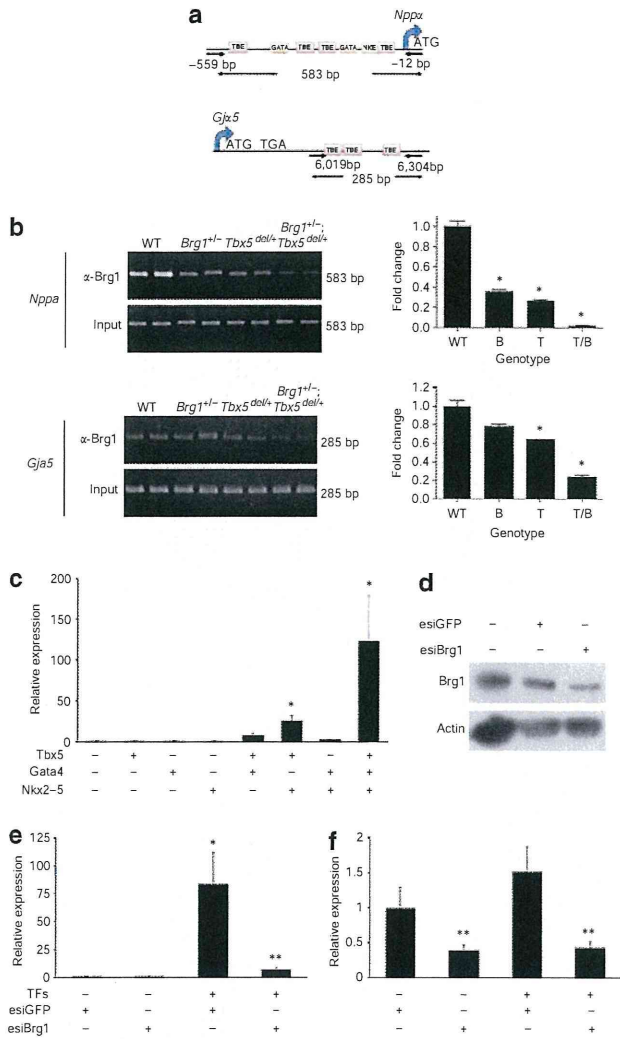


**Figure 6 | Clustering analysis of altered gene expression.** (a) Clustering analysis of altered transcripts from *Nkx2-5*<sup>+/+</sup> (N), *Brg1*<sup>+/-</sup> (B) and compound heterozygotes, *Nkx2-5*<sup>+/-</sup>;*Brg1*<sup>+/-</sup> (NB) E11.5 hearts. Heart maps for all statistically significant clusters indicate the average value for each group. All biological replicates within groups were included in cluster analyses; figures were made by taking the mean for each group. Box plots for a selection of clusters show the median (middle line), third and first quartile (top and bottom (hinges) of the boxes); the 'whiskers' extend 1.5× the length of the box, with other points (outliers) plotted as circles. Statistically significantly overrepresented Gene Ontology (GO) categories (adjusted *P* < 0.01) are shown. Genotypes are shown on the x axis; y axis shows log<sub>2</sub> fold change from WT. (b) Clustering analysis as in (a), but for transcripts from *Tbx5*<sup>del/+</sup> (T), *Brg1*<sup>+/-</sup> (B) and compound heterozygotes, *Tbx5*<sup>del/+</sup>;*Brg1*<sup>+/-</sup> (TB) E11.5 hearts.

Notably, mice heterozygous for a deletion of *Brg1* have heart defects. This important finding indicates that *Brg1* is haploinsufficient in the heart. Haploinsufficiency of *Brg1* has been reported in the brain<sup>13</sup> and immune system<sup>45</sup>, indicating that certain cell types are sensitive to the dosage of *Brg1*. The important dose dependency of *Brg1* function in cardiogenesis shows that, as for DNA-binding transcription factors, normal formation of the heart relies on precise levels of functional BAF complexes. Furthermore, the genetic interaction between *Brg1* and several cardiac transcription factor genes

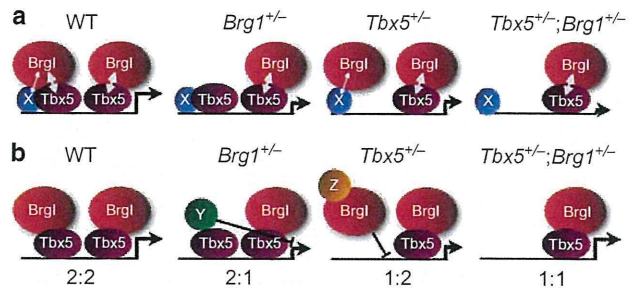
shows that transcription factor function is intimately linked to levels of *Brg1*. The resulting CHDs are likely to reflect these interactions in multiple cell types. Our promoter occupancy data indicate that, for at least a portion of co-regulated genes, a direct cell-autonomous dosage-related interaction exists. Specific genetic programmes are sensitive to the dosage of each transcription factor, and there is considerable complexity in the genomic regulation of gene expression by *Brg1* and cardiac transcription factors. In some cases, a clear interrelationship exists between the dosage effects of either factor,





**Figure 7 | Direct interactions between Brg1 and cardiac transcription factors.** (a) Diagram indicating the location of primer pairs for amplification of immunoprecipitated chromatin-associated DNA for *Nppa* and *Gja5*. Locations of conserved T-box-binding elements (TBE), GATA factor-binding sites (GATA) and Nkx2-5-binding sites (NKE) are shown. ATG: start codon; TGA: translational stop codon. Nucleotide positions shown are relative to start codon. (b) ChIP of Brg1 at the *Nppa* or *Gja5* promoters from E9.5 hearts of the indicated genotypes. On the left are EtBr-stained gels showing amplification, and quantitation by qPCR is shown in the graphs on the right. Data are mean  $\pm$  s.e.;  $n=3$ ;  $*P<0.05$ . (c) Quantitation of *Nppa* mRNA in NIH3T3 fibroblasts transfected with combinations of Tbx5, Nkx2-5 and Gata4. Data are mean  $\pm$  s.e.;  $n=3$ ;  $*P<0.05$  versus mock transfection. (d) Western blot to measure reduction of Brg1 protein levels by anti-Brg1 esiRNAs. Actin is a loading control. (e) Quantitation of *Nppa* mRNA in NIH3T3 fibroblasts transfected with Tbx5, Nkx2-5 and Gata4, with or without esiRNAs targeting Brg1, or control esiRNAs. TFs = Tbx5, Gata4 and Nkx2-5. Data are mean  $\pm$  s.e.;  $n=3$ ;  $*P<0.05$  versus mock transfection;  $**P<0.05$  esiBrg1<sup>+</sup> versus esiBrg1<sup>-</sup>. (f) Brg1 mRNA levels in the same samples as in c. Data are mean  $\pm$  s.e.;  $n=3$ ;  $**P<0.05$  esiBrg1<sup>+</sup> versus esiBrg1<sup>-</sup>.

resulting in a more profound effect in embryos haploinsufficient for *Brg1* and *Nkx2-5* or *Tbx5*. In other words, diminution of levels of one factor will reduce that factor's chance of interaction with BAF complexes, and this can be accentuated when Brg1 levels are also lower. Our molecular data supported a model (Fig. 8, model a) for



**Figure 8 | Models for the interaction between DNA-binding transcription factors and Brg1.** Two models for the interaction between DNA-binding transcription factors and Brg1. Model (a) is based on the dosage-sensitive interrelationship between DNA-binding transcription factors (represented by Tbx5) and Brg1. In *Brg1*<sup>+/-</sup> hearts, some Tbx5-binding events are not accompanied by concomitant Brg1 binding, resulting in decreased gene expression. In *Tbx5*<sup>+/-</sup> hearts, Brg1 can only interact with one of two potential Tbx5-binding events, but may still interact more weakly with other DNA-binding transcription factors (X). In *Tbx5*<sup>+/-</sup>;*Brg1*<sup>+/-</sup> hearts, all quantitative interactions are impaired, leading to further reduction in target gene expression. Model (b) is based on the normalization of gene expression in *Tbx5*<sup>+/-</sup>;*Brg1*<sup>+/-</sup> and *Nkx2-5*<sup>+/-</sup>;*Brg1*<sup>+/-</sup> hearts, and is based on the allelic balance between the two genes. In *Brg1*<sup>+/-</sup> hearts, the ratio of Tbx5 to Brg1 alleles is now 2:1, which allows some Tbx5-binding events to interact with other factors, including a hypothetical repressor protein (Y). In *Tbx5*<sup>+/-</sup> hearts, the ratio of Tbx5 to Brg1 alleles is now 1:2, which allows some Brg1-binding events to interact with other factors, including a hypothetical repressor or sequestering protein (Z). In *Tbx5*<sup>+/-</sup>;*Brg1*<sup>+/-</sup> hearts, the balance is restored to 1:1, eliminating the potential for other interactions.

direct interactions between Brg1 and cardiac transcription factors, and imply that reduced recruitment of BAF complexes may be a significant molecular mechanism underlying transcription factor haploinsufficiency in congenital heart disease.

The observation that expression levels of several transcripts that are altered in heart heterozygous for a null allele of *Nkx2-5*, *Tbx5* or *Brg1* are restored to normal or near-normal levels in *Nkx2-5*<sup>lacZ/+</sup>; *Brg1*<sup>+/-</sup> or *Tbx5*<sup>lacZ/+</sup>;*Brg1*<sup>+/-</sup> compound heterozygous hearts indicates that for some genes, the relative allelic balance of *Nkx2-5* (or *Tbx5*) and *Brg1* may be more important than their absolute levels. The mechanism underlying this dosage interdependence is not known, but may include increased potential for interaction with other proteins that would alter the functional output on target genes (Fig. 8, model b). Thus, haploinsufficiency of cardiac transcription factors in CHDs is predicted to result in an imbalance between transcription factors and BAF complexes, likely resulting in impaired transcriptional activation at loci sensitive to this balance. The reason for distinct dosage-sensitive responses from one group of genes to the next is not known, but perhaps the chromatin status of some genes requires different dosage-related interactions between BAF complexes and DNA-binding transcription factors.

We cannot discern which specific group of genes, between those that respond more in the compound heterozygotes, those that return to WT levels in compound versus single heterozygotes, or combinations thereof, is responsible for the phenotypic output of transcription factor or *Brg1* haploinsufficiency. It is likely that deregulation of many different genes in various functional classes would be responsible for the complex altered morphology and function that we observe.

In conclusion, we demonstrated a critical requirement for Brg1 in the development of the vertebrate heart, and importantly showed that there exists a fine balance between Brg1 levels and those of cardiac transcription factors that have been implicated in human CHD. The genetic interactions between *Brg1* and cardiac transcription factor



genes predict that, in the developing heart, maintaining relative levels of BAF complexes and transcription factors is critical for the timely and precise activation of groups of genes during development. In human congenital heart disease, disruption of these dosage-sensitive interactions would predict the impaired activation of specific gene networks that are essential for specific aspects of cardiac morphogenesis and function. We propose that an imbalance in this relationship is a molecular basis underlying CHDs caused by mutations in cardiac transcription factors. Although such a finely regulated interrelationship is perhaps more prone to disruption, it confers significant advantages in the fine quantitative regulation of transcript levels, which is an essential component of complex morphogenesis. These results further underscore the potential for multigenic effects on dominant mutations, and suggest that polymorphisms in BAF complex subunit genes, including but not restricted to *Brg1*, may modulate the penetration and phenotypic consequence of disease-causing mutations in transcription factor genes in human populations.

## Methods

**Mouse strains and embryology.** The following mouse strains were used: *Brg1*<sup>12/14</sup>, *Nkx2-5::Cre*<sup>15</sup>, *Mef2cAHF::Cre*<sup>26</sup>, *Tbx5<sup>del/+</sup>*, *Nkx2-5<sup>loxZ/+35</sup>*, *Tbx20<sup>+/-32</sup>*, *Z/EG<sup>44</sup>* and *RYR<sup>45</sup>*. *Brg1<sup>+/-</sup>* mice were generated by crossing *Brg1<sup>12/-</sup>* mice with E11a-Cre mice<sup>46</sup>. For all experimental analyses, at least three embryos per genotype were examined. All animal experiments were conducted following guidelines established and approved by the UCSF Institutional Animal Care and Use Committee, and in accordance with best practices outlined by the Canadian Council on Animal Care and licensed by Lab Animal Services at the Hospital for Sick Children.

**Analysis of protein and mRNA expression.** Immunostaining and *in situ* hybridization procedures were carried out by standard protocols. For immunostaining, cryosections were incubated with primary antisera against enhanced green fluorescent protein (EGFP; Abcam ab13970, 1:300) and with alpha-tropomyosin (CH1 monoclonal, Hybridoma bank, 1:50), followed by incubation with Alexa Fluor 488-coupled goat-anti-chicken immunoglobulin (Ig) G (Invitrogen A11039, 1:200) and Alexa Fluor 594-coupled goat anti-mouse IgG (Invitrogen A110320), and were mounted with Prolong gold with 4,6-diamidino-2-phenylindole (Invitrogen), to stain nuclei. *In situ* hybridization by whole mount or on 10 µm paraffin sections was performed using previously described protocols<sup>47</sup>. RNA probes were labelled with digoxigenin-labelled UTP, hybridized to samples and after washes were incubated with alkaline phosphatase-coupled anti-digoxigenin antisera (Roche); colorimetric detection was carried out by incubation with BM purple (Roche). Optical projection tomography (OPT) was performed as described<sup>7</sup> using embryos labelled for alpha-cardiac actin (*Actc*) by fluorescent *in situ* hybridization (using the TSA amplification system, Amersham). OPT images were acquired in the GFP channel for tissue autofluorescence, and in the red channel for *Actc* fluorescence. Three-dimensional reconstructions were analysed and rendered in Amira 4.0 (Visage Imaging). QRT-PCR was performed with Taqman probe sets (Supplementary Table S1).

**Cardiac physiology.** Cardiac function was assessed by high-frequency ultrasound and telemetry ECG<sup>42,47</sup>. For ultrasound assessment of cardiac function, a high-frequency ultrasound imaging system (Vevo 660, VisualSonics) with a 30 MHz transducer was used for *in vivo* evaluation of cardiac morphology and function as previously described in detail<sup>27</sup>. Mice were anaesthetized with isoflurane at 1.5% by face mask, and body temperature was maintained at ~37°C. An M-mode recording of the left ventricle was made in long-axis view and analysed for wall thickness and chamber dimension. Fractional shortening was also calculated as a measure of left ventricular systolic function. Mitral Doppler flow spectrum was recorded in apical four-chamber view, with the Doppler sample volume placed at the centre of mitral orifice and at the tip level of the valves for the highest velocities. The peak velocities of the early ventricular filling wave (E wave) and the late ventricular filling wave caused by atrial contraction (A wave) were measured, and their ratio was calculated. The left ventricular systolic and diastolic time intervals were also measured, including the isovolumic relaxation time, ventricular diastolic filling time, isovolumic contraction time and ventricular ejection time. All parameters were averaged for three cardiac cycles.

For ECG telemetry, telemetry devices (DSI) were implanted subcutaneously dorsally under anaesthesia (sodium pentobarbital, 0.033 mg g<sup>-1</sup> i.p.), and the leads were placed in lead II conformation. Following a 72 h recovery period, mice housed in individual cages were placed on telemetry detection platforms and continuous ECG tracings were acquired for 24 h. ECGs were manually examined for arrhythmias. Ultrasound measurements and ECG intervals were calculated during periods of regular sinus rhythm.

**Zebrafish embryology and genetics.** The *brg1<sup>481</sup>* allele was identified in a diploidENU mutagenesis screen for mutations affecting endodermal organ morphogenesis<sup>48</sup>. A C-to-T base-pair change at position 754 in the *brg1* (*smarca4*) coding sequence

creates a premature STOP codon at amino acid 252. MOs targeting *brg1* and *p53* have been previously published and validated<sup>20,21</sup>. RNA *in situ* hybridization probes for *myl7/cmlc2*, *nppa*, *bmp4*, *tbx2b* and *notch1b* have been characterized<sup>49,50</sup>. For the *ncx1h* (*slc8a1a*) probe, a 560 bp PCR product amplified from cDNA was used as a template.

For mosaic labelling of cardiomyocytes, embryos from Tg(*cmlc2:eGFP<sup>uvr54</sup>*)<sup>+/-</sup> in-crosses were injected at the one-cell stage with 10 pg of pB1SceI-cmlc2:dsRed plasmid DNA with I SceI enzyme (NEB) in I SceI buffer.

For time-lapse analysis, Tg(*cmlc2:eGFP*) control embryos or morphants were mounted in agarose, and imaged at 28.5°C using a 25× water objective. To count cardiomyocytes at various stages, we used the Tg(*cmlc2:dsRedExp-nuc<sup>54</sup>*) line. Mounted embryos were imaged with a 40× water immersion lens. Sequential confocal images were taken with a standardized step size of 0.65 µm in the z-direction. Quantification of myocardial cells, cell migration tracking and three-dimensional reconstructions of confocal stacks were carried out using Velocity (Improvision).

**Microarrays and statistical analysis.** Affymetrix mouse Gene ST 1.0 arrays were hybridized and scanned according to the manufacturer's recommendations. Raw intensities from CEL files were analysed using Affymetrix Power Tools (APT, version 1.10.1) to generate robust multiarray average<sup>51</sup> expression intensities on a log-2 scale for each probe set and various quality metrics. The probe intensities were background-corrected, quantile-normalized and summarized for each probe set using a robust fit of linear models. Linear models were fitted for each gene to estimate genotype effects and associated significance using the limma package<sup>52</sup> in R/Bioconductor. Because sample sizes are often small in microarray experiments, the eBayes function in limma computes s.e. that are moderated across all genes on the array. The interpretation of the moderated t-statistics is the same as an ordinary t-statistics. *Nkx2-5/Brg1* and *Tbx5/Brg1* experiments were analysed separately using 2×2 factorial models. Linear contrasts were used to extract comparisons of interest. P-values were adjusted for multiple testing by controlling for false-discovery rate using the Benjamini-Hochberg method<sup>53</sup>. Genes differentially expressed > 1.3-fold with adjusted *P* < 0.01 were considered significantly different between groups. QRT-PCR and ChIP data were analysed by analysis of variance, followed by *post hoc* Tukey's test; *P* < 0.05 was considered significant. Pairwise comparisons were made using *t*-test; *P* < 0.05 was considered significant.

**Clustering and pathway analysis.** Genes that were declared to be differentially expressed were subjected to unsupervised cluster analysis with the R package HOPACH<sup>54</sup>. All biological replicates within groups were included in cluster analyses. Pearson's correlations between genes were used as the distance metric, and default settings were used for other parameters. Clusters with similar expression patterns between genotypes were collapsed for further analysis. GOElite ([www.genmapp.org/go\\_elite/go\\_elite.html](http://www.genmapp.org/go_elite/go_elite.html)) was used to identify over-represented gene ontology terms and pathways among the sets of differentially expressed genes. All genes that were subjected to expression analysis were used as the 'denominator' gene list for detecting overrepresentation. Null distributions were derived by permutation (*n* = 2000). Significance was assessed using false-discovery rate-adjusted *P*-values.

**Cell culture and ChIP.** NIH/3T3 mouse embryonic fibroblasts were seeded in a 24-well format at 40,000 cells per well; 2 days after seeding, cells were transfected using Lipofectamine 2000 reagent (Invitrogen) with a combination of HA-Tbx5, myc-GATA4 and FLAG-Nkx2-5 overexpression plasmids (200 ng) or empty pcDNA3.1<sup>+</sup> (600 ng total). esiRNAs were generated as described<sup>37</sup>; briefly, regions of EGFP or *Brg1* were amplified by PCR (using the following primers: eGFP\_esi1 (forward): 5'-TAATACGACTCACTATAGGGCGTAAACGGCCACAAAGTTCA-3'; eGFP\_esi2 (reverse): 5'-TAATACGACTCACTATAGGGATGGGGGTGTTCTGCTGGTA-3'; *Brg1* esiRNA (forward): 5'-GGGCGGGTGTGTCCCTGTACAACAACAACC-3'; *Brg1* esiRNA (reverse): 5'-GGGCGGGTCTGCAGCTCTTGAAGATAGTGG-3'). *Brg1* PCR products were further amplified with an adaptor primer containing T7 sequences (5'-TAATACGACTCACTATAGGGAGACCAGGGCGGGT-3'). EGFP and *Brg1* products were then purified using a Qiagen PCR Clean Up kit and were *in vitro* transcribed using T7 RNA polymerase (Roche). Transcribed RNA was annealed to yield double stranded RNA. After purification (Roche; Quick Spin Columns), 10 µg of double stranded RNA was subjected to digestion using purified glutathione S-transferase (GST)-RNase III fusion protein and purified by ethanol precipitation to generate pools of 21 bp esiRNAs.

In knockdown experiments, 450 ng of either esiGFP or esiBrg1 was transfected. Relative expression was determined by quantitative PCR using TaqMan probes against *Nppa* and *Brg1*; expression was normalized to β-actin (probe-set details in Supplementary Table S1). Each reaction was performed in technical triplicate.

ChIP was performed as previously described<sup>8</sup>, using chromatin isolated from E12.5 embryonic hearts, with anti-Brg1 antiserum (Upstate 07-478) or normal rabbit IgG (Santa Cruz 2027), using 1 µg of antiserum. PCR was performed using the following primers: *Nppa*-595F: 5'-TCTTTCACCTGACTGCTAAACA-3', *Nppa*-12R: 5'-AGCATCTCCCGTTTTTATAG-3'; *Gja5*-F1: 5'-ACGACTGTGGCAGCAGGTGTCTGC-3', *Gja5*-R1: 5'-AGAACCAGCAGGTGCCCTTCCCT-3'. Amplicons were 573 bp long for *Nppa* and 583 bp long for *Gja5*. Quantification was performed using the same primers and SYBR green quantification.



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### Author contributions

J.K.T. and B.G.B. designed the study. J.K.T. performed most of the work on mice. X.L. carried out most of the work on zebrafish, under the direction of I.C.S. C.M. isolated the zebrafish *brg1* mutation under the direction of D.Y.R.S. J.M.A. and P.D.-O. performed cell culture assays. H.S. performed chromatin immunoprecipitation under the direction of J.K.T. A.K.H., R.-F.Y. and K.S.P. designed and performed bioinformatics analysis of array data. RNA isolation and preparation were performed by A.D.M. and J.N.W. R.P.H., D.M. and P.C. provided genetically modified mouse strains. Y.Z. and Y.-Q.Z. performed mouse physiological measurements under the direction of B.G.B. and R.M.H., respectively. B.G.B. wrote the paper with contributions from all authors.

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# Epigenetic factors and cardiac development

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## Abstract

Congenital heart malformations remain the leading cause of death related to birth defects. Recent advances in developmental and regenerative cardiology have shed light on a mechanistic understanding of heart development that is controlled by a transcriptional network of genetic and epigenetic factors. This article reviews the roles of chromatin remodelling factors important for cardiac development with the current knowledge of cardiac morphogenesis, regeneration, and direct cardiac differentiation. In the last 5 years, critical roles of epigenetic factors have been revealed in the cardiac research field.

## Keywords

Epigenetics • Chromatin remodelling factors • Baf60c • Cardiac transcription factors • Cardiac cell fate • Cardiac reprogramming

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## 1. Introduction: cardiac morphogenesis and diseases

The heart is the first functional organ to form in developing embryos, and cardiogenesis takes place in a highly conserved manner from insects to vertebrates. After the formation of three germ layers (ectoderm, endoderm, and mesoderm), pre-cardiac mesodermal cells arise bilaterally from the nascent mesoderm. These cells migrate into the midline and differentiate, giving rise to the contractile heart.

In mammals and birds, the bilateral cardiogenic mesodermal cells migrate and merge at the anterior midline to generate the cardiac crescent, a crescent-shaped heart-forming region at the cranial border of the embryonic disc.<sup>1–3</sup> Upon folding of the embryonic disc, the cardiac crescent positions towards a developing neck area of the embryo and the edges of the cardiac crescent migrate to fuse and form the primitive heart tube.<sup>4</sup> The resulting heart tube undergoes the process of looping and chamber formation, accompanied by the activation of specific cardiac gene expression programmes required for the differentiation and maturation of pre-cardiac cells to generate the myocardium of the atrial and ventricular chambers, as well as the inflow tract, atrioventricular canal, and outflow tract.<sup>5,6</sup>

Two distinct pools of pre-cardiac fields were identified, both of which contribute to heart formation.<sup>7</sup> The first heart field (FHF) cells are derived from the cardiac crescent and give rise to the

formation of the left ventricle and a part of the atria.<sup>8</sup> Cells from the second heart field (SHF), described by Kelly *et al.*,<sup>9</sup> are located in the pharyngeal mesoderm dorsal to the heart tube, giving rise to the outflow tract and right ventricle myocardium at the arterial pole of the heart.<sup>9–11</sup>

The induction, expansion, and differentiation of pre-cardiac cells are controlled by various signalling molecules, including bone morphogenetic proteins (BMPs),<sup>12</sup> fibroblast growth factors (FGFs),<sup>8</sup> and wingless-related MMTV integration site (Wnt) proteins.<sup>13</sup> BMP and FGF signals are important for the induction and differentiation of cardiogenesis.<sup>12,14,15</sup> BMPs play a key role in the specification of FHF cells by activating the expression of cardiac transcription factors such as Nkx2–5, Gata4, and Tbx5.<sup>16,17</sup> SHF progenitors, on the other hand, require Wnt/ $\beta$ -catenin signalling for their proper development.<sup>13,18,19</sup> Wnt/ $\beta$ -catenin signals positively regulate the expansion of SHF progenitors and affect the expression of *Islet1* (*Isl1*), a marker for multi-potent cardiac progenitor cells (CPCs).<sup>18,20,21</sup>

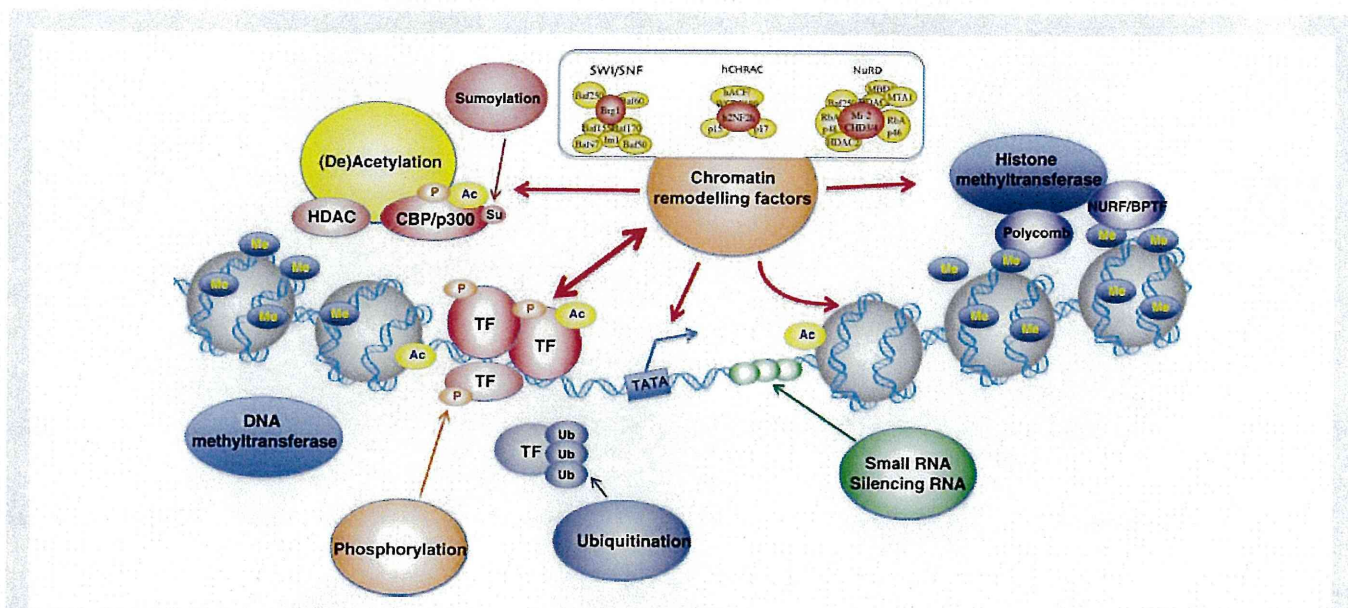
The mammalian heart consists of various cell types, including atrial and ventricular cardiomyocytes, fibroblasts, endocardial cells, epicardial cells, cells from the conduction system (sinoatrial node, atrioventricular node, Purkinje fibers), smooth muscle cells making up the aorta and (coronary) arteries, and cells from the autonomous nervous system. Formation of the functional heart requires proper development of these cardiac cells through tight transcriptional regulation of cardiac genes. The fact that congenital cardiac anomalies occur at a high

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**Figure 1** Chromatin structure and remodelling factors. DNA is organized in chromatin composed of condensed nucleosomes: units of 146 bp of DNA wrapped twice around an octamer of two copies of each histone protein H2A, H2B, H3, and H4. The flexible amino-terminal tails of the histones, protruding outward from the nucleosome, allow for post-translational modifications through (de)acetylation, phosphorylation, ubiquitination, methylation, and sumoylation. Such covalent modifications alter DNA–histone interactions, affecting accessibility of transcription factors.

frequency [ $\sim 1$ – $2\%$  of human live births suffer from a form of congenital heart defects (CHDs)] and mutations in numerous transcription factors can cause CHDs indicates the complexity of cardiac development.<sup>22–24</sup> At the epigenetic level, transcription factors are regulated by the assembly of DNA in higher-order chromatin structures (Figure 1). In this review, we will focus on epigenetic chromatin remodelling factors that are important for cardiac development, and discuss how these factors can be exploited to regulate the directed differentiation of non-cardiac cells towards fully functional cardiomyocytes in the search for new therapies against human CHDs.

## 2. Epigenetic factors and their roles in cardiac development

### 2.1 Epigenetic regulation: chromatin remodelling and DNA methylation

Eukaryotic development requires epigenetic mechanisms to control gene transcription for cell specification and differentiation. Chromatin remodelling is one of the essential epigenetic mechanisms for gene regulation (Figure 1). Chromatin is a multifaceted complex that serves to efficiently pack the large amount of DNA in the  $5\ \mu\text{m}$  cell nucleus and to regulate gene transcription.<sup>25–27</sup> It consists of nucleosomes that are formed by wrapping of DNA around a core of histones.<sup>28</sup> Condensation of nucleosomes enables the packing of all the genomic molecules into the relatively small nucleus.<sup>26</sup> This compact, higher-order organization of chromatin requires regulatory mechanisms to allow the access of transcription factors to the DNA.<sup>29–32</sup> The chromatin state often determines gene activation and repression. ‘Open chromatin’ (euchromatin) refers to a lightly packed form of DNA that allows active gene transcription, whereas

‘closed chromatin’ (heterochromatin) is a tightly packed form of DNA in which transcription is repressed.<sup>28,33,34</sup>

The state of chromatin structure can be regulated by ATP-dependent chromatin remodelling complexes or modifications of histone tails.<sup>32,35</sup> The ATP-dependent chromatin remodelling complexes use the energy of ATP hydrolysis to modify chromatin structure. They can be classified into the complexes of SWI/SNF, ISWI, nucleosome remodelling and deacetylase complex (NuRD), and INO80 on the basis of their catalytic ATPase subunits.<sup>31,35–38</sup> Modification of histone tails is often enzymatically reversible<sup>39–41</sup> and results in an alteration of the interaction between chromatin and DNA. These modifications include acetylation,<sup>42</sup> methylation,<sup>43</sup> phosphorylation,<sup>44</sup> sumoylation,<sup>45</sup> and ubiquitination.<sup>46</sup>

Another epigenetic mechanism that regulates gene transcription, besides histone modification, is DNA methylation.<sup>47,48</sup> DNA methylation typically occurs at CpG sites that contain cytosine-guanine nucleotides in a linear sequence. CpG-rich islands, short stretches of DNA with a relatively high frequency of CpG sites,<sup>48</sup> are often found at promoters of mammalian genes, and the extent of methylation at these sites is well correlated with the transcription status of corresponding genes. DNA methylation functions to stably silence gene transcription.<sup>47</sup>

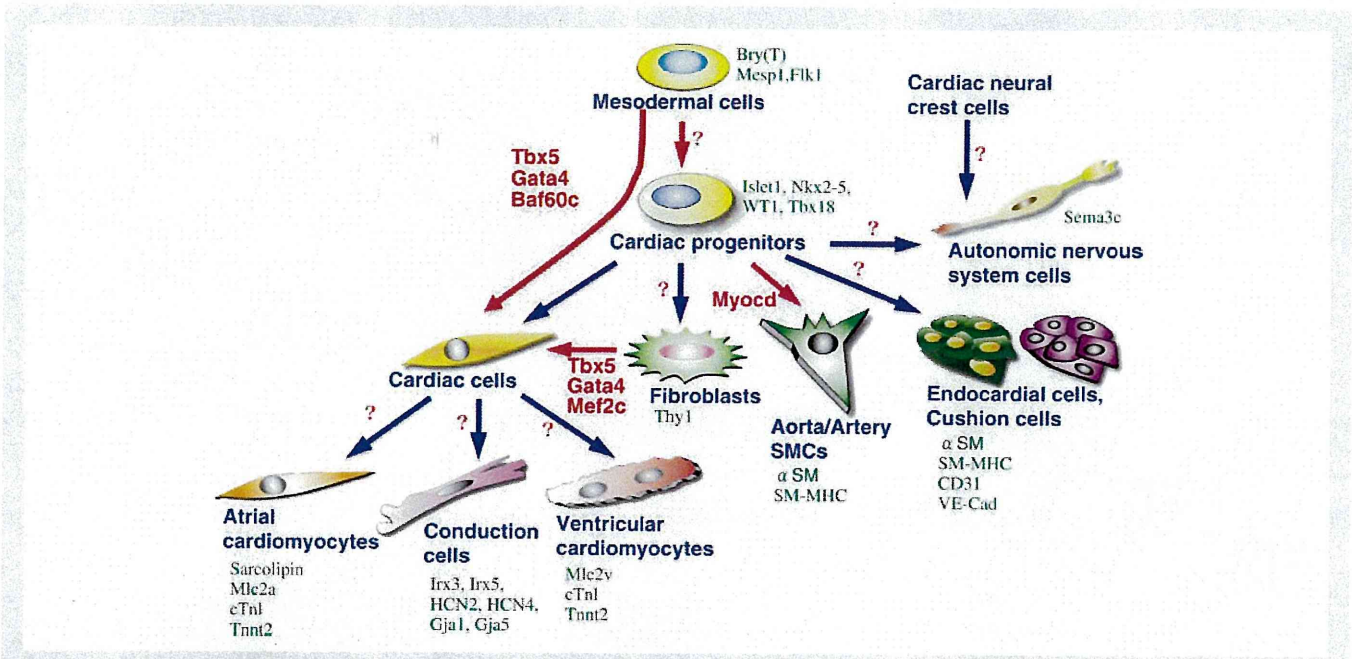
### 2.2 Chromatin remodellers for cardiac development and CHDs

#### 2.2.1 Brg1/Brm-associated factor complex

The Brg1/Brm-associated factor (BAF) chromatin remodelling complex is the mammalian SWI/SNF complex composed of at least 11 subunits, and their variable arrangements contribute to distinct functions during development.<sup>31,49</sup>

The ATPase subunit of the BAF complex is encoded either by homologous genes *Brg1* (Brahma-related gene 1) or *Brm*, but *Brg1* is the





**Figure 2** Cardiac cell types derived from multipotent progenitors. Differentiated cardiac cell types are marked by indicated genes (green). Recently, several factors have been defined as master regulators for cardiomyogenesis (red). The combination of Tbx5, Gata4, and Baf60c induces direct differentiation of mesodermal cells into ectopic beating myocytes, bypassing the cardiac progenitor state.<sup>55</sup> Tbx5, Gata4, and Mef2c together can also induce cardiomyocytes from fibroblasts.<sup>91</sup> Factors for direct induction of other cardiac cell types are currently unknown (question marks).

indispensable ATPase of the BAF complex.<sup>31,49,50</sup> Brg1 acts in the BAF complex to increase promoter accessibility for transcription factors, but it can also directly bind to transcription factors such as Gata proteins to regulate gene transcription.<sup>51</sup> Mice heterozygous for *Brg1* deletion exhibit cardiac morphogenetic defects, suggesting haploinsufficiency of *Brg1* in heart development.<sup>52</sup> It turns out that the proper dosage of *Brg1* is critical for normal heart development, as the disruption of the balance between *Brg1* and CHD-causing cardiac transcription factors such as Tbx5, Tbx20, and Nkx2-5 leads to severe cardiac anomalies.<sup>52</sup> In mouse embryos, *Brg1* activates β-myosin heavy chain (β-MHC, expressed primarily in foetal myocytes) while repressing α-MHC expressed in adult myocytes.<sup>53</sup> Although silenced in adult mice, *Brg1* is reactivated upon cardiac stress in adult myocytes and induces an α-MHC to β-MHC shift, suggesting its role in maintaining myocytes in an embryonic state.<sup>53,54</sup>

Baf60c is the cardiac-specific subunit of the BAF complex during early development and required for the ectopic induction of cardiomyocyte differentiation in combination with Gata4 and Tbx5.<sup>55</sup> (Figure 2, Figure 3A and B). Baf60c is encoded by the gene *Smarcd3*, whose mRNA is initially restricted to the developing heart from mouse embryonic day (E) 7.5.<sup>50,56</sup> Its subfamily members, *Smarcd1* and *Smarcd2*, which encode Baf60a and Baf60b, respectively, are not expressed in the developing heart at these stages, indicating the tissue specificity of Baf60c in embryonic development (Figure 3C and D).<sup>50,57</sup> Baf60c cooperates with Tbx5 to initiate their target gene activation for FHF formation.<sup>55,58,59</sup> Baf60c deficiency leads to outflow tract shortening, hypoplastic right ventricles and atria, and lack of atrioventricular canal.<sup>56,57</sup> Baf60a plays a role in linking the glucocorticoid receptor to the BAF complex, and is involved in c-fos/c-jun-mediated transcriptional activity. The precise role of Baf60b is

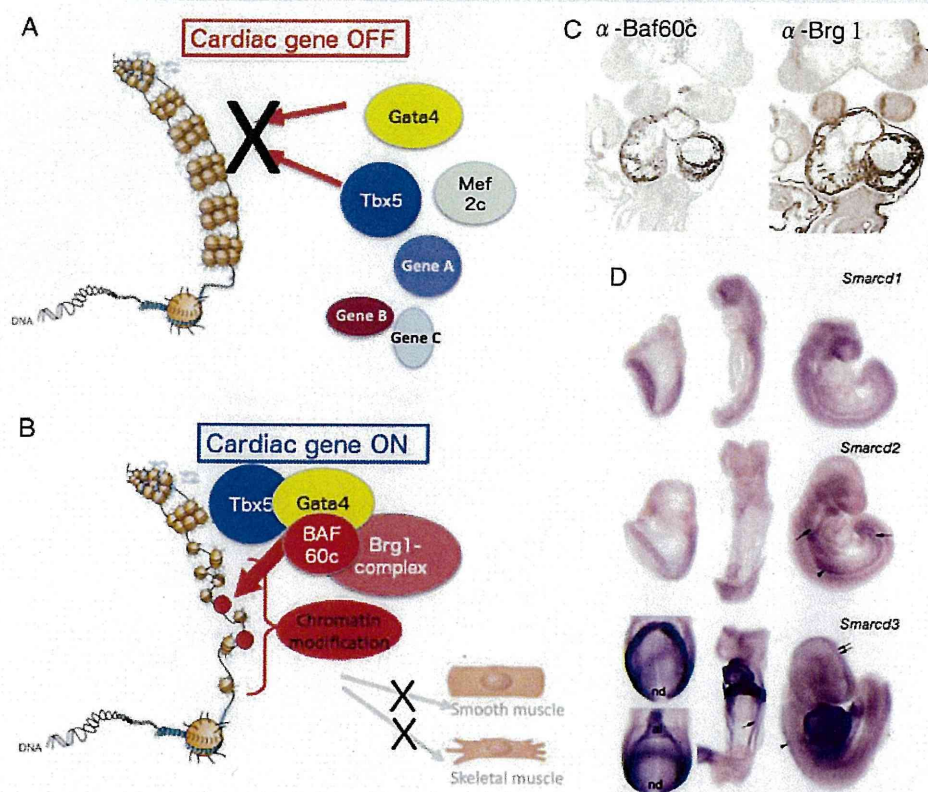
unclear, but it is specifically ubiquitinated by Unkempt, a RING finger protein partner of Rac GTPase. Although the significance of this ubiquitination is not completely understood, it is thought to be involved in maintaining the stoichiometry of the SWI/SNF complex.

Polybromo (BAF180), the prominent subunit of the BAF-related PBAF complex, is also involved in cardiogenesis by potentiating transcriptional activation mediated by nuclear receptors, such as RXRα, VDR, and PPARγ.<sup>60</sup> Deletion of Baf180 does not lead to early embryonic lethality, but, similar to RXRα deletion, results in a very thin cardiac wall with diminished trabeculae.<sup>61</sup> BAF180 is expressed in the epicardium and holds a non-redundant function to that of Baf60c in the respect that it mediates late aspects of cardiac chamber maturation and coronary development.<sup>60</sup> Ablating other subunits of the BAF complex (Baf47, Baf155, or Baf250) cause embryonic lethality at pre-implantation (Baf47, 155) or E6.5 (Baf250) in mice, indicating an essential role of the BAF complex for early embryonic development.<sup>31,62,63</sup>

**2.2.2 NuRD and histone deacetylase complex**

The NuRD complex contains histone deacetylases that function as transcriptional repressors.<sup>64</sup> Similar to the BAF complex, the NuRD complex exhibits diverse functions as a result of variable assemblies. Their ATPase activity resides in the two Mi-2 proteins, CHD3 and/or CHD4.<sup>65</sup> NuRD complexes mediate gene repression and regulate cell patterning and differentiation during early development.<sup>66,67</sup> The NuRD complex associates with Whsc1 (Wolf-Hirschhorn syndrome candidate 1) methyltransferase<sup>68</sup> and interacts with the Spalt-family zinc-finger transcription factor Sall4,<sup>69</sup> which is involved in inter-ventricular septum development,<sup>70</sup> suggesting that the complex may play a role in heart development.





**Figure 3** Chromatin remodelling factor-mediated regulation of cardiac transcription factors. (A) In the absence of Baf60c, the cardiac transcription factors Gata4, Mef2c, and Tbx5 may not have access to their target genes. The highly condensed euchromatin in which the DNA is tightly wrapped around histones makes transcription factors inaccessible to regulatory DNA, thereby represses transcription. (B) Chromatin remodelling factors modify chromatin organization by unwinding DNA from the histones, making target sequences accessible for transcription factors. Baf60c acts as a bridge to bring the Brg1 complex together with the transcription factors Tbx5 and Gata4 in a tissue-specific manner. (C) Heart-restricted expression of Baf60c and Brg1 shown by immunostaining. (D) Expression of *Smarcd3* (encoding Baf60c) restricted to cardiogenic mesoderm and the developing heart shown by *in situ* hybridization (adapted from Lickert et al.<sup>56</sup>).

### 2.2.3 Histone methyltransferase

Whsc1 is a histone methyltransferase that regulates activation of Nkx2–5, a homeobox protein critical for cardiac morphogenesis.<sup>68</sup> Whsc1 physically associates with Nkx2–5 and is required for the negative regulation of Nkx2–5 and its target genes, possibly through histone H3 trimethylation at lysine 36 H3K6me3.<sup>68</sup> Similar to Nkx2–5 mutations, Whsc1 mutations cause CHD, including atrial and ventricular septum defects in mice and human.<sup>68,71</sup>

Smyd1 (SET and MYND domain 1), a member of the lysine methyltransferase family, is specifically expressed in muscle tissue and acts as a transcriptional repressor by catalysing histone methylation through the SET domain.<sup>72,73</sup> Smyd1-deleted mouse embryos exhibit severe cardiac defects, including loss of the right ventricle with disrupted cardiomyocyte maturation.<sup>72</sup> In zebrafish, Smyd1 is essential for cardiac muscle contraction and myofiber maturation,<sup>74</sup> suggesting a conserved role of Smyd1 for cardiac development.

### 2.2.4 High mobility group chromatin protein

The high mobility group (HMG) of nuclear proteins exerts its function by architectural remodelling of the chromatin structure and by forming multi-protein complexes with promoter/enhancer sites, leading to transcriptional activation of their target genes.<sup>75</sup> The cardiac HMG member,

HMGA2, was shown to play important roles for cardiac differentiation *in vitro* and *in vivo*. Overexpression or siRNA-mediated knockdown of HMGA2 enhances or blocks cardiomyocyte differentiation *in vitro*, respectively. In *Xenopus* embryos, normal heart formation is blocked upon morpholino-mediated knockdown of HMGA2.<sup>75</sup> The fact that 'HMGA2 is abundantly expressed during embryogenesis, whereas its expression is almost undetectable in adult tissues' further indicates its role for embryonic heart development.<sup>75</sup> Furthermore, Nkx2–5 appears to be a target of HMGA2; in the presence of BMP, HMGA2 forms a protein complex with Smad1/4 and synergistically up-regulates promoter activity of Nkx2–5 in the presence of BMP stimulation through Smad- and HMGA2-binding elements. Moreover, promoter activity of Nkx2–5 requires a conserved HMGA2-binding site.<sup>75</sup>

## 3. Cell fate specification and epigenetic signalling

### 3.1 Transcription factors with instructive roles for cardiac differentiation

Cardiac transcription factors play critical roles in the early processes of cardiac cell specification and lineage determination. A number of



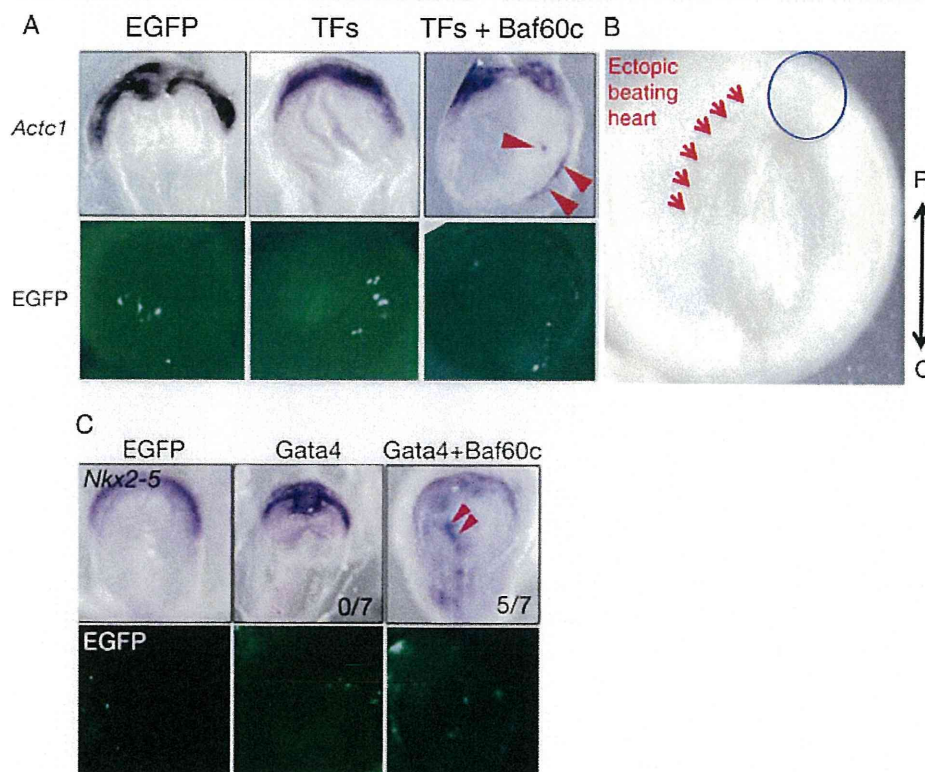
gain-of-function experiments have been carried out to identify factors to induce cardiac differentiation. For instance, the ectopic overexpression of Gata5, a zinc-finger transcription factor essential for proper heart and endoderm development, induces the expression of several cardiac genes (*Nkx2-5*, *Gata4*, *Gata6*) in zebrafish.<sup>76</sup> Gata4 possesses a similar potential in *Xenopus*.<sup>77</sup> However, the observed ectopic heart tissues appear to be formed as a secondary effect, as the overexpression of Gata genes causes additional axis-formation along the rostro-caudal axis.<sup>76,77</sup> Conditional deletion of  $\beta$ -catenin in the early endoderm layer leads to ectopic heart formation with *Nkx2-5* expression, and this phenotype is attributed to blockage of the inhibitory role of the Wnt pathway on cardiac differentiation.<sup>78,79,80</sup> In *Xenopus*, overexpression of myocardin was sufficient to induce ectopic expression of  $\alpha$ -SMA,  $\alpha$ -cardiac actin, and *Nkx2-5*. However, myocardin alone appears to be insufficient for establishing beating heart cells.<sup>81</sup> One of the key regulators for early heart development is Tbx5, a T-box transcription factor.<sup>58,82,83</sup> Tbx5 specifies the left-right identity of the cardiac chambers and the development of the ventricular septum.<sup>84</sup> Mice heterozygous for Tbx5 exhibit malformed cardiac chambers with an abnormal inter-ventricular septum, and homozygous deletion of Tbx5 alleles results in the absence of the left ventricle.<sup>58,59</sup> Similarly, human mutations in Tbx5 cause the Holt-Oram syndrome, which is characterized by atrial septal defects, upper

limb defects, and anomalies of the digits.<sup>58,85,86</sup> The importance of Tbx5 in heart development is also exemplified by the fact that its role is evolutionarily conserved among species.<sup>70,87</sup> Although overexpression of Tbx5 affects cardiac septum morphogenesis, it is not enough to induce cell differentiation into cardiomyocytes. Given that no single transcription factor so far has been shown to sufficiently induce cardiomyocytes, the developmental programme of cardiogenesis might be activated through multiple factors.

### 3.2 Master regulators for cardiomyogenesis

'Master regulators' control multiple genes to direct cell differentiation and are sufficient to activate an entire developmental programme. In 1988, Davis and colleagues demonstrated that overexpression of MyoD, a basic-helix-loop-helix (bHLH) transcription factor, is sufficient to convert fibroblasts to skeletal muscle cells.<sup>88,89</sup> Similarly, another bHLH-type transcription factor, myocardin, is sufficient to activate the developmental programme of smooth muscle differentiation.<sup>81,90</sup> However, as described earlier, no single transcription factor is known to act as a master regulator for cardiomyogenesis.

Recently, various combinations of cardiac transcription factors were used in an attempt at the directed transdifferentiation of non-cardiac cells into the cardiomyocyte lineage.<sup>55,57</sup> In this study, developmentally critical cardiac transcription factors (*Gata4*, *Nkx2-5*,



**Figure 4** Ectopic induction of cardiomyogenesis by defined factors (Tbx5, Gata4, and Baf60c). (A) Ectopic induction of cardiac tissues by co-overexpression of TFs (Tbx5, *Nkx2-5*, and Gata4) and Baf60c. The early cardiomyocyte marker *Actc1* was used to monitor the induction of cardiomyocytes. The chromatin remodelling component Baf60c is required for the induction. (B) Beating heart tissues (arrowheads) are observed in non-cardiogenic mesoderm upon overexpression of Tbx5, Gata4, and Baf60c. At this stage, the endogenous heart cells do not beat, indicating accelerated cardiac differentiation by the defined factors. (C) Whole-mount *in situ* hybridization showing that Gata4 requires Baf60c to induce ectopic expression of *Nkx2-5*. EGFP expression indicates transfected cells (adapted from Takeuchi and Bruneau<sup>55</sup>).



**Table 1** Combinatorial activation of beating heart by Gata4, Tbx5, and Baf60c +, transfection of DNA; O, cardiac marker induction or beating heart induction

Actc1, Myl7 induction	Beating heart induction	Tbx5	Gata4	Gata1	Nkx2-5	Baf60c	Baf60b
x	x	+	-	-	-	-	
x	x	-	+	-	-	-	
x	x	-	-	+	-	-	
x	x	+	+	-	-	-	
x	x	+	-	+	-	-	
x	x	-	-	-	+	-	
x	x	+	-	-	+	-	
x	x	-	+	-	+	-	
x	x	+	+	-	+	-	
O	x	-	+	-	-	+	-
O	x	-	+	-	-	-	+
O	x	-	-	+	-	+	-
x	x	-	-	+	-	-	+
O	O	+	+	-	-	+	-
O	O	+	+	-	+	+	-

and Tbx5) were introduced into mesodermal cells of developing mouse embryos in different combinations. However, any combination of Gata4, Nkx2-5, and/or Tbx5 did not fully induce cardiomyocyte differentiation, suggesting that these transcription factors are not sufficient for cardiomyogenesis (Figure 4A, Table 1). Surprisingly, the addition of Baf60c, a cardiac-specific subunit of BAF chromatin remodelling complexes,<sup>56</sup> led to ectopic differentiation of mesodermal cells into beating cardiomyocytes.<sup>55</sup>

Chromatin modification is a dynamic process required for the proper function of transcription factors, allowing them to have access to their target loci (Figure 3A and B). Genome-wide screening revealed the presence of cardiac-specific chromatin remodelling factors,<sup>56</sup> indicating their potential involvement in directed transdifferentiation. Indeed, expression dosage of Baf60c allowed Gata4 to access its target genes by modifying their chromatin structures, leading to the ectopic expression of cardiac genes. Tbx5 overexpression promoted cardiomyogenesis by repressing the activation of non-cardiac mesodermal genes.<sup>55</sup> Chromatin immunoprecipitation assays confirmed these findings by showing the presence of the heart-specific Baf60c-remodelled chromatin. Furthermore, the binding of Gata4 and Tbx5 to the cTnT promoter region required Baf60c-mediated chromatin remodelling, suggesting that the combination of Gata4, Tbx5, and Baf60c acts as a master regulator for cardiomyocyte differentiation from mesodermal cells (Figure 3).<sup>55</sup>

More recently, Ieda et al.<sup>91</sup> demonstrated that combinatorial overexpression of developmentally critical transcription factors is sufficient to the direct reprogramming of cardiac fibroblasts into functional cardiomyocytes. Interestingly, Gata4 and Tbx5 were also required for the reprogramming, although Mef2c was used instead of Baf60c (Figure 2). The induced cardiomyocytes expressed the cardiac-specific markers Actc1, Myh6, Ryr2, and Connexin43, whereas Col1a2—a marker for fibroblasts—was markedly down-regulated. Strikingly, they exhibited a global gene expression profile similar to that of cardiomyocytes, with cardiomyocyte-like chromatin patterns on several genes, indicating epigenetic resetting. H3K27me3 (trimethylated histone H3 of lysine 27) and H3K4me3 (trimethylated

histone H3 of lysine 4) mark transcriptionally inactive or active chromatin, respectively.<sup>92</sup> Further methylation analyses of induced cardiomyocytes revealed decreased levels of H3K27me3 and increased levels of H3K4me3 in the promoters of cardiomyocyte genes.<sup>91</sup>

Curiously, Baf60c was not required for the reprogramming of cardiac fibroblasts. This is likely due to cell-type differences between embryonic mesodermal cells and fibroblasts. It is reasonable to speculate that cardiac or dermal fibroblasts share similar chromatin patterns with cardiogenic cells, so that overexpression of cardiac chromatin remodellers may not be necessary for the event. Also, Mef2c may regulate expression of chromatin remodelling factors required for cardiac reprogramming.

### 3.3 Approaches for cardiac regeneration

CHDs are the most common birth defects in humans, and heart disease remains the leading cause of human death worldwide. The high morbidity and mortality is largely attributed to the limited regenerative capacity of the heart. Recent research has focused on developing new strategies, especially cell-mediated therapies, to treat damaged hearts. One approach is to utilize pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) or induced PSCs (iPSCs). These cells are highly plastic and can expand and differentiate into most of existing cells, including functional cardiomyocytes.<sup>75,93,94</sup> After birth, the heart itself is insufficient in its regenerative response upon damage, such as from infarcts, as most cardiomyocytes are terminally differentiated and do not proliferate. Therefore, ESCs or iPSCs may hold potential for treating cardiac defects. In addition, iPSC transplantation is advantageous over whole-organ transplantation in that these cells can be directly obtained from patients to avoid immune rejection.<sup>95</sup> However, transplantation of undifferentiated iPSCs into the mouse heart has resulted in teratoma formation.<sup>96</sup> Analysis of these teratomas revealed cell types from all three embryonic germ layers, indicating that existing cardiac cells do not guide iPSCs to differentiate into cardiac cells.<sup>96,97</sup> As described earlier, cardiogenesis takes place in a highly coordinated fashion by interactions of multiple factors,<sup>12,98-101</sup> and it will therefore be



important to understand the cardiogenic mechanisms of these factors for iPSC-mediated cardiac therapy.

CPC-based therapy offers a better approach for heart regeneration. CPCs are committed pre-cardiac cells with a potential to differentiate into multiple cardiac cell types, including cardiomyocytes, smooth muscle cells, and endothelial cells.<sup>20,21,102</sup> The identification of Isl1<sup>+</sup> cells led to the discovery of the undifferentiated CPCs, which has advanced our knowledge of multi-potent CPCs.<sup>20,103</sup> They are also marked by Nkx2-5 or Flk1 and can be isolated from early developing embryos or differentiating pluripotent cells.<sup>21,102,104</sup> A recent trial of embryonic CPC transplantation in post-myocardial infarcted hearts of non-human primates showed successful engraftment with myocardial differentiation,<sup>105</sup> suggesting that CPCs can be used as an effective source for heart regeneration. Understanding the mechanisms of lineage-specific differentiation of CPCs will accelerate the CPC-mediated cardiac therapeutics.

Hattori *et al.*<sup>106</sup> recently introduced a novel approach to isolate cardiomyocytes. By use of tetramethylrhodamine ethyl ester perchlorate (a fluorescent dye specific to mitochondria), they successfully isolated embryonic and neonatal cardiomyocytes (>99% purity) by fluorescence-activated cell sorting. Moreover, transplantation of these purified cardiomyocytes did not induce teratoma formation, and their aggregation resulted in long-term survival of the transplanted myocytes *in vivo*.<sup>106</sup> Further studies will be necessary to test their effects on damaged hearts and large animals. Induced cardiomyocytes from directed differentiation also have tremendous therapeutic potential to treat heart disease. However, the differentiation method will need to be optimized before a clinical trial. For example, the differentiation efficiency needs to be improved with quantitative studies and more rigorous functional assays should be carried out *in vitro* and *in vivo*. In addition, it will be important to test whether endogenous cells such as cardiac fibroblasts can be directly differentiated into cardiomyocytes *in vivo*.

## 4. Future perspectives

Numerous genetic and epigenetic factors regulating cardiac morphogenesis, differentiation, and maturation have been identified through decades of progress in developmental cardiology. The knowledge gained from the developmental studies led to the recent breakthrough discoveries of defined factors, whose co-overexpression is sufficient to instruct non-cardiac cells to convert into cardiomyocytes. The defined factors (Gata4 and Tbx5 with Baf60 or Mef2c), also essential for cardiac development, are transcription and chromatin remodelling factors that act cooperatively with others, highlighting the importance of a mechanistic understanding of transcriptional and chromatin regulation. It would be interesting to see whether direct differentiation of other cardiac lineages such as smooth muscle, endothelial, or conduction cells also occurs through defined factors. As illustrated in Figure 2, different types of cardiac cells express distinct gene products, but it is mostly unknown if these cell types can be directly obtained from multi-potent progenitors by defined factors. It would be important to identify factors that can activate the programmes for individual cardiac lineage determination.

Although cardiac transcription factors have been extensively studied for their roles and targets, the mechanisms by which chromatin remodellers modulate activation or repression of specific signalling and transcriptional networks are not well understood. Further investigation will be required to elucidate the roles of cardiac epigenetic

factors for a better understanding of the process of cardiogenesis, as well as directed cardiac differentiation or reprogramming.

Given that the mammalian heart has limited regeneration capacity, direct reprogramming is emerging as a novel form of potential cardiac therapeutics along with CPC-mediated transplantation.<sup>105,106,107</sup> We are rapidly entering into a new era of cardiac regenerative medicine that combines knowledge from the diverse fields of heart biology, including developmental, molecular and cellular cardiology, and cardiac physiology. This integrative approach and effort should accelerate novel discoveries for future cardiac therapeutics as well as preventive strategies for CHD.

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