Epigenetic repression of cardiac progenitor gene expression by Ezh2 is required for postnatal cardiac homeostasis

Paul Delgado-Olguín1, Yu Huang1, Xue Li2–4, Danos Christodoulou5, Christine E Seidman5,6, J G Seidman5, Alexander Tarakhovsky7 & Benoit G Bruneau1,8–10

Adult-onset diseases can be associated with in utero events, but mechanisms for this remain unknown.1–2. The Polycomb histone methyltransferase Ezh2 stabilizes transcription by depositing repressive marks during development that persist into adulthood.3–9, but its function in postnatal organ homeostasis is unknown. We show that Ezh2 stabilizes cardiac gene expression and prevents cardiac pathology by repressing the homeodomain transcription factor gene Six1, which functions in cardiac progenitor cells but is stably silenced upon cardiac differentiation.10. Deletion of Ezh2 in cardiac progenitors caused postnatal myocardial pathology and destabilized cardiac gene expression with activation of Six1-dependent skeletal muscle genes. Six1 induced cardiomyocyte hypertrophy and skeletal muscle gene expression. Furthermore, genetically reducing Six1 levels rescued the pathology of Ezh2-deficient hearts. Thus, Ezh2-mediated repression of Six1 in differentiating cardiac progenitors is essential for stable gene expression and homeostasis in the postnatal heart. Our results suggest that epigenetic dysregulation in embryonic progenitor cells is a predisposing factor for adult disease and dysregulated stress responses.

Cardiac hypertrophy results from the integration of numerous signaling pathways that culminate in well-described transcriptional networks.11. In humans, variation in hypertrophy is induced by similar stimuli across populations. Although genetic variation contributes to this phenotypic variability,12 epigenetic regulatory mechanisms, particularly during embryonic development, are largely unexplored.1–2. The heart may be particularly sensitive to early events as turnover of human cardiomyocytes is limited over a lifetime.13.

Epigenetic regulation via histone methylation stabilizes transcriptional programs in embryonic progenitors and their differentiated descendants3,4 and is likely to be crucial for establishing and maintaining gene expression and stress responses throughout life. Polycomb complexes are candidates for stabilizing cardiac gene expression as they control cell identity and epigenetic memory in other contexts.5, Ezh2, the major histone methyltransferase of the Polycomb repressor complex 2 (PRC2), trimethylates histone H3 at lysine 27 (H3K27me3). Ezh2 is essential for embryonic development,5–9, but its function in organogenesis or postnatal organ maintenance is unknown. Ezh2 and the related H3K27 methyltransferase Ezh1 (refs.14,15) are expressed in cardiomyocytes (Supplementary Fig. 1), and H3K27me3 increases in cardiac progenitor cells as they differentiate into cardiomyocytes (Supplementary Fig. 2). This suggests that PRC2 functions in the transition from cardiac progenitor to differentiated cardiomyocyte.

To determine the function of PRC2 in the mouse heart, we inactivated Ezh2 by Cre-mediated recombination in a subpopulation of cardiac progenitors known as the anterior heart field (AHF), which contribute to the right ventricle, interventricular septum and outflow tract.16. Mice with loxP sites flanking the catalytic domain–encoding region of Ezh2 (Ezh2i0/+) were crossed with mice expressing Cre recombinase under the control of the Mef2cAHF enhancer (Mef2cAHF::Cre), which is active from embryonic day (E) 7.5 (ref. 16). AHF progenitors with inactivated Ezh2 did not show increased H3K27me3 levels as they differentiated into cardiomyocytes (Supplementary Fig. 2c–h), resulting in decreased Ezh2 and, in more than 80% of right, but not left, ventricular cardiomyocytes, decreased H3K27me3 (Supplementary Fig. 3), suggesting that Ezh2 is the major H3K27 histone methyltransferase in the AHF. H3K27me3 levels were not reduced in endothelial cells (Supplementary Fig. 2d). Mice with Ezh2-deficient AHF cells developed normally structured hearts (Supplementary Fig. 4). However, Ezh2-deficient hearts became enlarged after birth, with increased ratios of heart weight to tibia length (Fig. 1a,b). Cardiac enlargement was restricted to the right ventricular wall, where cardiac myocytes were massively hypertrophied, with an almost fourfold increase in cross-sectional area (Fig. 1c,d). Echocardiography confirmed right ventricular enlargement and showed mild

1Gladstone Institute of Cardiovascular Disease, San Francisco, California, USA. 2Urological Diseases Research Center, Children’s Hospital Boston, Boston, Massachusetts, USA. 3Department of Pathology, Harvard Medical School, Boston, Massachusetts, USA. 4Harvard Stem Cell Institute, Cambridge, Massachusetts, USA. 5Department of Genetics, Harvard Medical School, Boston, Massachusetts, USA. 6Howard Hughes Medical Institute, Division of Cardiovascular Medicine, Brigham and Women’s Hospital, Boston, Massachusetts, USA. 7Laboratory of Lymphocyte Signaling, The Rockefeller University, New York, New York, USA. 8Department of Pediatrics, University of California, San Francisco, San Francisco, California, USA. 9Cardiovascular Research Institute, University of California, San Francisco, San Francisco, California, USA. 10Institute for Regeneration Medicine, University of California, San Francisco, San Francisco, California, USA. Correspondence should be addressed to B.G.B. (bbruneau@gladstone.ucsf.edu).

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pulmonary stenosis (Supplementary Table 1). Cardiomyocytes were already enlarged in E20 fetuses (Supplementary Fig. 5), ruling out altered hemodynamics as a primary cause of hypertrophy. Ezh2-deficient right ventricles were fibrotic (Fig. 1e,f), and the endocardium was invaginated into the ventricular myocardium (Fig. 1g), resembling ventricular non-compaction<sup>12,17</sup>. Thus, loss of Ezh2 in cardiac precursors leads to cardiac hypertrophy and fibrosis.

We examined altered gene expression in Ezh2-deficient adult hearts. The hypertrophy-associated genes<sup>18,19</sup> Nppa, Nppb and Myh7 as well as the profibrotic genes Tgfb3, Postn and Spp1 were highly upregulated...
Supplementary Table 2

| Protein | Enrichment relative to input | P value | Ezh2fl/fl | Ezh2fl/fl; Mef2cAHF::Cre |
|---------|-----------------------------|---------|-----------|-------------------------|
| Suz12   | 0.044                       |         | 0.000     | 0.000                   |
| H3K27me3| 0.048                       |         | 0.000     | 0.000                   |

Figure 3 Six1 is epigenetically repressed by PRC2. (a) qRT-PCR showing expression of Six1 and Eya1 in embryonic and adult myocardium. (b) Upper panels, in situ hybridization showing Six1 mRNA in E13.5 Ezh2-deficient (Ezh2fl/fl; Mef2cAHF::Cre) right ventricle (RV) (scale bar, 200 µm). Lower panels, immunofluorescence for Eya1 (red) (scale bar, 25 µm). Cardiomyocytes were stained with an antibody to tropomyosin (Tpm1; green); nuclei were stained with DAPI (blue). (c) Immunofluorescence for Six1 (green) and Actn3 (red) in control (Ezh2fl/fl) and Ezh2-deficient (Ezh2fl/fl; Mef2cAHF::Cre) adult hearts. Only Ezh2-deficient right ventricular (RV) cardiomyocytes coexpressed Six1 and Actn3. Arrows point to Six1-positive cardiomyocyte nuclei (scale bar, 25 µm). (d) Chromatin immunoprecipitation for Suz12, H3K27me3, RNA Pol II and AcH3 on the Six1 core promoter in Ezh2-deficient hearts (red bars) and control hearts (blue bars). Bars in a. represent the mean ± s.d. of at least three biological replicates. *P < 0.05.

(Fig. 2a,b). Immunofluorescence detected low levels of Tgf-β3 in the perinuclei region of wild-type cardiac myocytes, whereas Tgf-β3 was enriched in the extracellular region in Ezh2-deficient hearts (Fig. 2c). Thus, Ezh2 might suppress gene expression that promotes cardiac hypertrophy and fibrosis.

If Ezh2 acts epigenetically, a sustained requirement for its activity would be anticipated. To address this, we deleted Ezh2 in differentiating ventricular myocytes using ventricular myocyte-specific Nkx2-5::Cre transgenic mice20,21, which express Cre only after cardiomyocytes begin to differentiate. Ezh2fl/fl; Nkx2-5::Cre mice had mild cardiomyocyte hypertrophy and increased Myh7 mRNA levels (Supplementary Fig. 6), perhaps because of reduced Nkx2-5::Cre-mediated recombination efficiency20,21 (Supplementary Fig. 3c,d) or a crucial window of activity of Ezh2 during early cardiac progenitor differentiation. Thus, Ezh2 functions in cardiomyocyte progenitors, with long-term consequences.

We hypothesized that Ezh2-mediated H3K27me3 directly represses fetal genes and that reactivation of these genes in Ezh2-deficient hearts results from loss of epigenetic repression. At the Nppa promoter19, occupancy of the PRC2 core subunit Suppressor of zeste 12 homolog (Suz12) and H3K27me3 was decreased in Ezh2-deficient hearts, and acetylated histone H3 (AcH3, activating mark) and RNA polymerase II (Pol II) were increased (Fig. 2d and Supplementary Fig. 7). These results suggest that Ezh2 directly represses fetal gene expression to maintain cardiac homeostasis.

We determined the global gene-expression profile of adult Ezh2-deficient right ventricles by RNA sequencing (RNA-seq) and DNA microarrays. With a stringent P < 0.001 and fold-change cutoff of >1.4, we found that 1,132 genes were upregulated and 1,800 were downregulated (Supplementary Table 2). Consistent with hypertrophy and fibrosis, myocardial remodeling–associated genes (for example, Nppa and Nppb) and atrial-specific genes (Sln and Myl7) were upregulated in Ezh2-deficient hearts (Fig. 2e). Genes involved in signaling pathways mediating myocardial remodeling were also misregulated (Supplementary Table 3). Gene Ontology classification showed that many misregulated genes encode skeletal muscle–specific contractile proteins and developmental regulators (Fig. 2f), including some that are normally induced during cardiac hypertrophy (for example, Acta1). Thus, Ezh2 is required to stabilize postnatal cardiac gene expression.

In pluripotent cells, Polycomb complexes repress transcription factors regulating cell differentiation and development5. We hypothesized that Ezh2-mediated repression of key transcription factors is required to stabilize cardiac gene expression. Our analyses showed increased expression of the homeodomain transcription factor gene Six1 and its coactivators Eya1, Eya2 and Eya4 (Fig. 2e and Supplementary Table 2)22–24. Six1 functions in non-cardiac cell types, including
skeletal muscle. Indeed, many \(P < 2 \times 10^{-8}\) genes misregulated in Ezh2-deficient hearts are direct targets of Six1 in differentiating skeletal myoblasts (Fig. 2e). Six1 targets were preferentially misregulated over non-Six1 targets (Fig. 2g). Expression of Six1, Eya1 and some skeletal muscle genes was also increased in Ezh2-deficient, Nkx2-5::cre hearts (Supplementary Fig. 6e). Six1 functions in early cardiac progenitors but not in differentiating cardiomyocytes and remains silent in the developing and postnatal heart (Fig. 3 and Supplementary Fig. 8). Upon loss of Ezh2, Six1 might activate transcription of a subset of skeletal muscle genes in cardiomyocytes, contributing to instability of cardiac gene expression.

We addressed the relevance of increased Six1 expression in deregulating gene expression in Ezh2-deficient hearts. Quantitative real-time PCR (qRT-PCR) confirmed Six1 derepression in early developing ventricular myocardium (E10) and exclusively in the adult Ezh2-deficient right ventricle (Fig. 3a), consistent with specific repression by Ezh2.

Six1 expression is rapidly extinguished upon differentiation of cardiac progenitors\(^1\), but its derepression in Ezh2-deficient, Mef2cAHF::cre hearts suggests an expression potential in differentiated myocardium. Computational analysis of the Six1 locus identified a highly conserved sequence in the 5' noncoding region of Six1 with putative binding motifs for cardiac development regulators Nkx2-5, Gata4 and Mef2c (Fig. 4a). To test its potential to drive cardiac expression, we fused this conserved element to a lacZ reporter driven by the Hspa8 promoter and injected the resulting reporter construct (Fig. 4b) into mouse zygote pronuclei. About 50% of transient transgenic embryos (6/12) showed \(\beta\)-galactosidase activity in the myocardium (Fig. 4c–e), suggesting that this enhancer is active in the heart when not in its endogenous repressed genomic location. This is consistent with a model whereby the cardiac expression potential of the Six1 locus is suppressed in differentiating cardiac progenitors.

We investigated the effect of persistent Six1 and Eya1 expression in cardiomyocytes on hypertrophy. Overexpression of Six1, with or without Eya1, in cultured neonatal mouse cardiomyocytes resulted in hypertrophy comparable to that induced by the potent hypertrophic agonist endothelin-1 (Fig. 5a). In addition, Six1 and Eya1 synergistically activated expression of fetal cardiac and skeletal muscle genes (Fig. 5b and Supplementary Fig. 9).
To determine whether Six1 derepression causes the Ezh2-deficient heart phenotype, we reduced Six1 levels in this model. We bred Ezh2 knockout, Mef2cAHF::Cre mice onto a heterozygous Six1-LacZ knock-in background and analyzed cardiomyocyte hypertrophy and fibrosis in adult mice. Reducing levels of Six1 by 50% in Ezh2-deficient hearts significantly normalized heart size, as measured by ratios of heart weight to tibia length (Fig. 6a). Furthermore, cardiomyocyte size, Tgfβ3 expression and fibrosis were almost completely rescued (Fig. 6b,c and Supplementary Fig. 10). Skeletal muscle genes, the hypertrophy marker Myh7, and the profibrosis factors Tgfβ3 and Postn also returned to normal expression levels (Fig. 6d and Supplementary Fig. 10). Residual Six1 (Fig. 6d) might account for the persistent phenotype. Evidence points to a direct effect of Six1 misregulation in myocardium and rules out secondary effects caused by an overload of pressure on the outflow tract: Six1 and its target Actn3 are coexpressed in Ezh2-deficient cardiomyocytes (Fig. 3c), and Six1 occupied the Nppa, Myl1 and Tgfβ3 loci in Ezh2-deficient hearts (Supplementary Fig. 11). In addition, mild thickening of the pulmonary valve leaflets in Ezh2−/−; Mef2cAHF::Cre adult hearts (Supplementary Fig. 12) was not present in fetal hearts that were already hypertrophied (Supplementary Fig. 5b) and was not rescued upon decreasing Six1 expression (Supplementary Fig. 12), despite a normalization of hypertrophy and fibrosis (Fig. 6b,c). Thus, myocardial deregulation of Six1 alone is the major cause of myocardial hypertrophy and fibrosis in Ezh2-deficient hearts.

We uncovered a finely regulated epigenetic regulatory mechanism in which Ezh2-mediated stable repression of Six1 in differentiating embryonic cardiac progenitors is essential for normal cardiac growth and stress-responsiveness in the adult. Ezh2 modulates a feed-forward pathway that represses fetal gene expression and is reinforced by repression of Six1 (Supplementary Fig. 13). This represents a previously unknown pathway for regulating cardiac growth: neither Ezh2 nor Six1 is upregulated in hearts subjected to hypertrophic stimuli (Supplementary Fig. 14). Repression of Six1 is required to stabilize cardiac gene expression by preventing activation of skeletal muscle genes in cardiac muscle. Ezh2 loss is probably not sufficient to activate this program by acting merely as a ‘brake’, but reactivation of Six1 (the ‘gas pedal’) and expression of its targets results in pathologic cardiac remodeling with hypertrophy and fibrosis. Mutations in EYA4 cause cardiomyopathy in humans, strengthening the idea that control of SIX1 and EYA function is crucial for normal cardiac homeostasis. Our results suggest that Ezh2-mediated repression of Six1 in cardiac progenitors stabilizes postnatal cardiac gene expression, illustrating the importance of epigenetic regulation early in development and its implications in postnatal organ homeostasis.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession codes. Microarray data can be accessed at Gene Expression Omnibus (GEO) under accession code GSE30076. RNA-seq data can be accessed at GEO under accession code GSE34274.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS
P.D.-O. designed the study with B.G.B. and performed most of the experiments. Y.H. performed echocardiography. X.L. provided essential information before publication. X.L. and A.T. provided mouse lines. D.C. generated and analyzed RNA-seq data with C.E.S. and J.G.S. P.D.-O. and B.G.B. wrote the paper with input from all the authors.

COMPETING FINANCIAL INTERESTS
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ONLINE METHODS

Mice. The following mouse strains were used: Ezh2fl/fl, Mef2cAHF::Cre; Nkx2-5::Cre and Six1lacZ+; All animal experiments were conducted following guidelines established and approved by the University of California, San Francisco Institutional Animal Care and Use Committee.

Plasmids. Six1 and Eya1 expression vectors were constructed by cloning PCR-amplified full-length cDNAs into pCDNA3.1/V5-His TOPO (Invitrogen).

Cardiac hypertrophy induction. Isoproterenol (60 mg per kg body weight) was administered for 14 d in 9-week-old mice using ALZET osmotic mini-pumps, which were surgically implanted subdermally. As a control, mice were implanted with PBS-filled minipumps. Five mice per genotype were treated.

Protein analysis. Immunostaining and in situ hybridization procedures were carried out using standard protocols. For immunostaining, cryosections were incubated with primary antibodies to the following: trimethyl-histone H3 (Lys27) (Millipore 07-449, 1:3000), histone H3 trimethyl Lys9 (39161 Active Motif, 1:1000), α-tropomyosin (CH1 monoclonal, Hybridoma bank, 1:100), cardiac troponin T (Thermo Scientific, 1:1000), pAb to vimentin (GF53 Progen, 1:500), Tgf-β3 (sc-83 Santa Cruz Biotechnology, 1:100), α-actin-3 (Epitomics, 1:100), Six1 (ref. 25, 1:100), Isl1 (Developmental Studies Hybridoma Bank, 1:250), Ezh2 (Active Motif, 1:200) and Ezh1 (Abcam, 1:200). Cultured myocytes were incubated with the following primary antibodies: anti–β-galactosidase (Abcam, 1:100) and anti-V5 (R960-25 Invitrogen, 1:100).

After incubation with primary antibodies, the sections were washed three times with PBS, incubated with Alexa Fluor–conjugated secondary antibodies (Invitrogen, 1:500), washed and mounted with Prolong gold with 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen) to counterstain nuclei. Fluorescent signal for vimentin and Tgf-β3 was quantified as mean Gray value with ImageJ. Multiple sections from at least three hearts per genotype were analyzed. The following antibodies were used for protein blot: anti-Ezh2 (Cell Signaling, 1:1000) and anti-GAPDH (Santa Cruz Biotechnology, 1:1000).

Gene expression analysis. RNA was isolated from the right ventricle and interventricular septum and treated with DNase I. cDNA was prepared with the SuperScript III First Strand Synthesis Kit (Invitrogen). 10 pg of cDNA was used for quantitative real-time amplification with the following TaqMan probes: Nppa (Mm00496495_m1), Nppb (Mm00435304_g1), Myh7 (Mm00600535_m1), Tgf-β3 (Mm01307950_m1), Postn (NM_015784.2), Spp1 (Mm00436767_m1), Six1 (Mm00808212_m1), Eya1 (Mm00438796_m1), Myh8 (Mm01332949_m1), Myl2 (Mm00440384_m1), Myf4 (Mm00440377_m1), Myl2p (Mm00443940_m1), Tnni1 (Mm00502426_m1), Actn3 (Mm00496495_m1) and Tnni2 (Mm00437157_g1). In situ hybridization on whole-mount embryos and on 10-μm paraffin sections was performed as previously reported. Antisense RNA probes were labeled with digoxigenin-coupled UTP. After hybridization, samples were washed and incubated with alkaline phosphatase–coupled anti-digoxigenin antisera (Roche). Signal was developed with BM purple (Roche).

Cell measurements. Cryosections were fixed in 4% PFA for 5 min, incubated with 5 μg/ml wheat germ agglutinin, Alexa Fluor 594 conjugate (W11262, Invitrogen) for 10 min and washed three times for 10 min each with PBS. Sections were then mounted with Prolong gold with DAPI (Invitrogen). Micrographs were taken from right and left ventricles. The cross-sectional area of myocytes was measured using ImageJ. At least 100 cardiomyocytes were measured per image. Multiple sections obtained from at least three hearts per genotype were analyzed. The same method was used to measure α-tropomyosin–stained cardiomyocytes in culture. At least 50 cells were measured from three biological replicates.

Microarray analysis and RNA-seq. RNA was isolated from the right ventricle and interventricular septum of five Ezh2fl/fl and five Ezh2fl/fl; Mef2cAHF::Cre mice. Affymetrix Mouse Gene ST 1.0 arrays were hybridized and scanned according to the manufacturer’s recommendations. Linear models were fitted for each gene on Ezh2fl/fl, Mef2cAHF::Cre versus Ezh2fl/fl samples to derive the mutant effect using the limma package in R/Bioconductor. Moderated t statistics and the associated P values were calculated, adjusting for multiple testing by controlling for false discovery rate with the Benjamini-Hochberg method and for family-wise error rate using the Bonferroni correction (adjP).

Chromatin immunoprecipitation. Chromatin was isolated from the right ventricle and the interventricular septum with the Imprint Chromatin Immunoprecipitation Kit (Sigma) following manufacturer instructions. The immunoprecipitated chromatin, input and IgG-bound chromatin were analyzed by qPCR using TaqMan probes for the Nppa core promoter, Nppa distal promoter, Six1 core promoter, Eya1 core promoter and the Srf core promoter (Supplementary Table 4).

Cardiomyocyte culture and infection. Newborn mouse cardiomyocytes were isolated and maintained using the Neonatal Cardiomyocyte Isolation System (Cellutron) as recommended by the manufacturer. Cardiomyocytes were seeded in eight-well chamber slides 48 h before infection. Cells were infected with 5.5 × 104 infectious units (IFUs) of Six1-encoding adenovirus and 1.2 × 105 IFU of Eya1-encoding adenovirus per well. Cells were fixed and stained 48 h after infection. For induction of cardiomyocyte hypertrophy, 0.2 mM of endothelin-1 (Sigma) was added to the culture medium. Cells were exposed for 48 h.

Adenoviruses. For generation of adenoviral vectors, Six1 and Eya1 cDNAs were cloned into pAd/CMV/V5-DEST (Invitrogen). Viral particles were generated using the ViraPower Adenoviral Expression System. Viral particles were titrated using the AdEasy Viral Titer Kit (Agilent Technologies).

Statistical analysis. Data are presented as mean ± s.d. P values were determined by two-tailed t-test. P ≤ 0.05 was considered statistically significant.

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