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

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Abstract

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 *Six1*, which functions in cardiac progenitors but is stably silenced upon cardiac differentiation[10]. Ezh2 deletion in cardiac progenitors caused postnatal myocardial pathology and destabilized cardiac gene expression with activation of Six1-dependent skeletal muscle genes. Six1 induced cardiomyocyte

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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 prior to publication; X.L. and A.T. provided mouse lines; D.C. generated and analyzed RNAseq 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.

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The authors declare no competing interests.

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 postnatal heart gene expression and homeostasis. Our results suggest that epigenetic dysregulation in embryonic progenitor cells predisposes to adult disease and dysregulated stress responses.

Cardiac hypertrophy results from integrating numerous signalling pathways that culminate in well-described transcriptional networks[11]. In humans, variation in hypertrophy is induced by similar stimuli across populations. While genetic variation contributes[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 descendents[3,4], and is likely critical for establishing and maintaining gene expression and stress responses throughout life. Polycomb complexes are candidates for stabilizing cardiac gene expression. They control cell identity and epigenetic memory in other contexts[5]. Ezh2, the major histone methyltransferase of the polycomb repressor complex 2 (PRC2), tri-methylates 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[14,15] are expressed in cardiomyocytes (Supplementary Fig. 1), and H3K27me3 levels increase 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 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 (*Ezh2*$^{fl/lf}$)[6] were crossed with mice expressing Cre recombinase under the *Mef2cAHF* enhancer (*Mef2cAHF::Cre*), which is active from embryonic day 7.5 (E7.5)[16]. AHF progenitors with inactivated *Ezh2* failed to increase H3K27me3 levels as they differentiated into cardiomyocytes (Supplementary Fig. 2c–h), resulting in decreased Ezh2 and over 80% of right, but not left, ventricular cardiomyocytes with decreased H3K27me3 (Supplementary Fig. 3a,b), 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 heart weight to tibia length ratios (Fig. 1a,b). Cardiac enlargement was restricted to the right ventricular wall, in which 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 pulmonary stenosis (Supplementary Table 1). Cardiomyocytes were already enlarged in E20 fetuses (Supplementary Fig. 5a), 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[12,17]. 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[18,19] *Nppa, Nppb*, and *Myh7*, as well as the profibrotic genes *Tgfß3, Postn* and *Spp1* were highly upregulated (Fig. 2a,b). Immunofluorescence detected low levels of Tgfβ3 in the perinuclear region of wild-type cardiac myocytes, while it 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 function were epigenetic, a sustained requirement for its activity would be anticipated. To address this, *Ezh2* was deleted in differentiating ventricular myocytes using ventricular myocyte-specific *Nkx2.5::Cre* transgenic mice[20,21], which express Cre only after cardiomyocytes begin to differentiate. *Ezh2$^{fl/fl}$;Nkx2.5::Cre* mice had mild cardiomyocyte hypertrophy and increased *Myh7* mRNA levels (Supplementary Fig. 6b,e), perhaps due to reduced *Nkx2.5::Cre*-mediated recombination efficiency[20,21] (Supplementary Fig. 3c,d), or to a critical window of activity of Ezh2 during early cardiac progenitor differentiation. Thus, Ezh2 functions in cardiomyocyte progenitors, with long-term consequences.

We hypothesized Ezh2-mediated H3K27me3 directly represses fetal genes, and reactivation in Ezh2-deficient hearts results from loss of epigenetic repression. At the *Nppa* promoter[19], occupancy of the PRC2 core subunit suppressor of zeste 12 homolog (Suz12) and H3K27me3 was decreased in Ezh2 mutant hearts, and acetylated histone H3 (AcH3, activating mark) and RNA polymerase II (PolII) were increased (Fig. 2d, Supplementary Fig. 7). These results suggest *Ezh2* directly represses fetal gene expression to maintain cardiac homeostasis.

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

In pluripotent cells, Polycomb complexes repress transcription factors regulating cell differentiation and development[5]. We hypothesized Ezh2-mediated repression of key transcription factors is essential to stabilize cardiac gene expression. Our analyses showed increased expression of the homeodomain transcription factor *Six1*[22] and its coactivators, *Eya1, Eya2*, and *Eya4*[23,24] (Fig. 2e; Supplementary Table 2). *Six1* functions in non-cardiac cell types, including skeletal muscle. Indeed, many ($P<2\times10^{-8}$) genes misregulated in Ezh2-

deficient hearts are direct Six1 targets in differentiating skeletal myoblasts (Fig. 2e)[25]. Six1 targets were preferentially misregulated over non-Six1 targets (Fig. 2g). Levels of *Six1*, *Eya1*, and some skeletal muscle genes were also increased in *Ezh2$^{fl/fl}$;Nkx2.5::Cre* hearts (Supplementary Fig. 6e). *Six1* functions in early cardiac progenitors, but not in differentiating cardiomyocytes[10] and remains silent in developing and postnatal heart (Fig. 3b,c; 6d; Supplementary Fig. 8). Upon loss of Ezh2, Six1 might activate transcription of a skeletal muscle genes subset 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 (QRTPCR) confirmed *Six1* de-repression in early developing ventricular myocardium (E10) and exclusively in the adult *Ezh2*-deficient right ventricle (Fig. 3a), consistent with specific repression by *Ezh2*. At E13.5, in newborn, and adult hearts, *Six1* expression was increased in *Ezh2*-deficient right ventricular myocardium (Fig. 3b,c; Supplementary Fig. 8), and *Eya1* was derepressed exclusively in adult cardiomyocytes (Fig. 3b). Furthermore, upon loss of Ezh2, Six1 was expressed in nuclei of adult cardiomyocytes that concomitantly expressed the skeletal muscle-specific Six1 target *Actn3* (Fig. 3c).

To determine if *Six1* and *Eya1* are targets for PRC2-mediated repression, we performed chromatin immunoprecipitation. In Ezh2-deficient adult hearts, H3K27me3 and Suz12 were depleted at the *Six1*, but not *Eya1*, promoter (Fig. 3d, not shown). PolII and acetylated histone H3 (AcH3) were enriched, consistent with *Six1* derepression. Thus, PRC2 epigenetically represses *Six1* expression.

Six1 expression is rapidly extinguished upon differentiation of cardiac progenitors[10], but its derepression in *Ezh2$^{fl/fl}$;Mef2cAHF::Cre* hearts suggests an expression potential in differentiated myocardium. Computational analysis of the *Six1* locus identified a highly conserved sequence in the *Six1* 5' non-coding region with putative binding motifs for cardiac development regulators Nkx2-5, GATA4 and Mef2c (Fig. 4a). To test its potential to drive cardiac expression, this conserved element was fused to a *LacZ*-reporter driven by the *Hsp68* promoter, and the resulting reporter construct (Fig. 4b) was injected in mouse zygote pronuclei. About 50% of transient transgenic embryos (6/12) showed β-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 agrees with suppression of a cardiac expression-potential of the *Six1* locus 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 fetal cardiac and skeletal muscle gene expression (Fig. 5b; Supplementary Fig. 9).

To determine if *Six1* derepression causes the phenotype of mice with *Ezh2*-deficient hearts, we reduced *Six1* levels. *Ezh2$^{fl/fl}$;Mef2cAHF::Cre* mice were bred onto a heterozygous *Six1-LacZ* knock-in background[23], and cardiomyocyte hypertrophy and fibrosis were analyzed in

adult mice. Reducing levels of *Six1* by 50% in *Ezh2*-deficient hearts significantly normalized heart size, as measured by heart weight to tibia length ratios (Fig. 6a). Furthermore, cardiomyocyte size and the levels of Tgfβ3 and fibrosis were almost completely rescued (Fig. 6b,c; Supplementary Fig. 10). Skeletal muscle genes, the hypertrophy marker *Myh7*, and the profibrosis factors *Tgfß3* and *Postn*, also returned to normal expression levels (Fig. 6d; 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 rule out secondary effects due to outflow tract pressure overload. *Six1* and its target *Actn3* are coexpressed in Ezh2-deficient cardiomyocytes (Fig. 3c), and Six1 occupied the *Nppa*, *Myl1* and *Tgfß3* locus in Ezh2-deficient hearts (Supplementary Fig. 11). In addition, mild thickening of the pulmonary valve leaflets in *Ezh2$^{fl/fl}$;Mef2cAHF::Cre* adult hearts (Supplementary Fig. 12) was not present in already hypertrophied fetal hearts (Supplementary Fig. 5b), and was not rescued upon decreasing *Six1* levels (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 the *Ezh2*-deficient heart.

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 repressing fetal gene expression, which is reinforced by repression of *Six1*. (Supplementary Fig. 13). This represents a novel pathway for regulating cardiac growth: neither Ezh2 nor *Six1* is upregulated in hearts subjected to hypertrophic stimuli (Supplementary Fig. 14). Repressing *Six1* is required to stabilize cardiac gene expression by preventing activation of skeletal muscle genes in cardiac muscle. Ezh2 loss is likely insufficient to activate this program merely acting as a “brake”, but reactivation of *Six1* (the “gas pedal”) and expression of its targets results in pathologic cardiac remodelling with hypertrophy and fibrosis. Mutations in *EYA4* cause cardiomyopathy in humans[24], strengthening the idea that control of Six-Eya function is critical for normal cardiac homeostasis. Our results suggest Ezh2-mediated *Six1* repression 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

Mice

The following mice strains were used: *Ezh2$^{fl/fl}$*, *Mef2cAHF::Cre*, *Nkx2-5::Cre*, and *Six1$^{Lacz/+}$*. All animal experiments were conducted following guidelines established and approved by the UCSF 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/kg* body weight) was administered for 14 days in 9 weeks old mice using ALZET osmotic minipumps, which were surgically implanted subdermally. As control mice were implanted with PBS-filled minipumps. 5 mice per genotype were treated.

Protein analysis

Immunostaining and *in situ* hybridization procedures were carried out by standard protocols. For immunostaining, cryosections were incubated with the next primary antibodies: anti-trimethyl-Histone H3 (Lys 27) (Millipore 07-449, 1:3000), histone H3 trimethyl Lys9 (39161 Active Motif, 1:1000), alpha tropomyosin (CH1 monoclonal, Hybridoma bank, 1:100), cardiac troponin t (Thermo Scientific,1:1000) Pab to Vimentin (GP53 Progen, 1:500), Tgfβ3 (sc-83 Santa Cruz Biotechnology, 1:100), Alpha-actinin 3 (Epitomics, 1:100), Six1[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 next primary antibodies: β-galactosidase (Abcam, 1:100) and anti-V5 (R960-25 Invitrogen, 1:100). After incubation with primary antibodies, the sections were washed 3× with PBS, incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen, 1:500), washed and mounted with Prolong gold with 4,6-diamidino-2-phenylindole (Invitrogen) to counterstain nuclei. Fluorescent signal for vimentin and Tgfβ3 was quantified as Mean Gray Value with ImageJ. Multiple sections from at least three hearts per genotype were analyzed. The next antibodies were used for Western blot: anti-Ezh2 (Cell Signalling, 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 DNaseI. cDNA was prepared with the SuperScript III First Strand Synthesis Kit (Invitrogen). 10 pg of cDNA were used for quantitative real-time amplification with the next TaqMan probes. *Nppa* Mm01255747_g1, *Nppb* Mm00435304_g1, *Myh7* Mm00600555_m1, *Tgfβ3* Mm01307950_m1, *Postn* NM_015784.2, *Spp1* Mm00436767_m1, *Six1* Mm00808212_m1, *Eya1* Mm00438796_m1, *Myh8* Mm01329494_m1, *Myl1* Mm00659043_m1, *Myl2* Mm00440384_m1, *Myl4* Mm00440377_m1, *Mylpf* Mm00443940_m1, *Tnni1* Mm00502426_m1, *Actn3* Mm00496495_m1 and *Tnni2* Mm00437157_g1. *In situ* hybridization on whole mount embryos and on 10 um paraffin sections was performed as previously reported. RNA antisense 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 ug/ul wheat germ agglutinin, Alexa Fluor® 594 conjugate (W11262 Invitrogen) for 10 min and washed 3× ten min each with PBS. Sections were then mounted with Prolong gold with 4,6-diamidino-2-phenylindole (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 alpha 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 *Ezh2*$^{fl/fl}$ and five *Ezh2*$^{fl/fl}$;Mef*2cAHF::*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 *Ezh2*$^{fl/fl}$;Mef*2cAHF::*Cre *vs Ezh2*$^{fl/fl}$ 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). Genes with adjP<0.05 were considered differentially expressed. RNAseq was performed as previously described in RNA from the right ventricle of five hearts per genotype[26]. Gene ontology analysis was performed using GO Elite.

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 analized 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 Cardiomycyte Isolation System (Cellutron) as recommended by the manufacturer.

Cardiomyocytes were seeded in 8 well chamberslides 48 h before infection. Cells were infected with 5.5×10^8 IFU of *Six1*-encoding adenovirus and 1.2×10^8 IFU of *Eya1*-encoding adenovirus per well. Cells were fixed and stained 48 h post infection.

For induction of cadiomyocyte hypertrophy, 0.2 mM of Endothelin 1 (Sigma) was added to the culture media. 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 tittered using the AdEasy Viral Titer Kit (Agilent Technologies).

Statistical analysis

Data are presented as mean +/− S.D. The P-value was determined by two-tailed T-test. A *P*-value <= 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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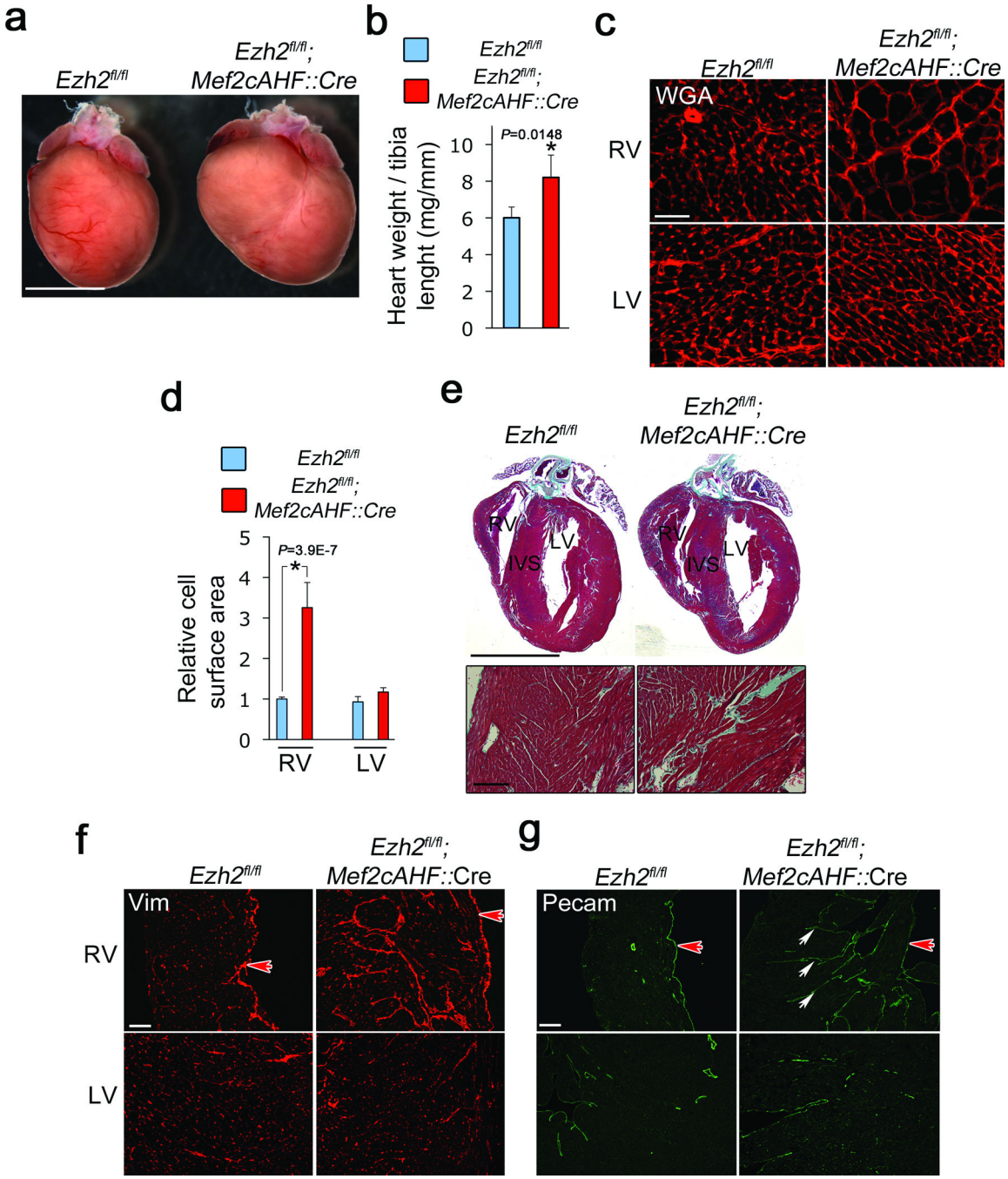

Figure 1.

Ezh2 limits cardiac growth and fibrosis. (**a**) Adult mice in which Ezh2 was deleted in second heart field progenitors (*Ezh2$^{fl/fl}$; Mef2cAHF::Cre*) present hypertrophied right ventricle, with increased (**b**) heart weight to tibia length ratio (mg/mm), as compared to controls (*Ezh2$^{fl/fl}$*). *Ezh2$^{fl/fl}$* (blue bars) and *Ezh2$^{fl/fl}$; Mef2cAHF::Cre* (red bars). (**c**) Wheat germ agglutinin (WGA) staining of heart sections in control (*Ezh2$^{fl/fl}$*) or Ezh2-deficient (*Ezh2$^{fl/fl}$; Mef2cAHF::Cre*) right ventricle (RV) and left ventricle (LV). (**d**) Cardiomyocyte cell surface area in *Ezh2$^{fl/fl}$* (blue bars) or *Ezh2$^{fl/fl}$; Mef2cAHF::Cre* (red bars) hearts. (**e**) Tissue

disorganization and fibrosis, as revealed with Masson's trichrome staining. In **b** and **d**, bars represent the mean +/− S.D. of measurements from at least three hearts per genotype. * indicates $P < 0.05$. (**f**) Vimentin staining of RV and LV of *Ezh2*$^{fl/fl}$ or *Ezh2*$^{fl/fl}$*; Mef2cAHF::Cre* hearts. (**g**) Pecam staining showing invaginations of endocardium into myocardium (arrows). In **f,g**, the red arrow points to endocardial lining of the RV lumen. Reference bars in (**a**) and (**e**) (upper panel) = 0.5 cm; (**e**) (lower panel) = 100 μm; (**c**) = 25 μm; (**f**) and (**g**) = 100 μm.

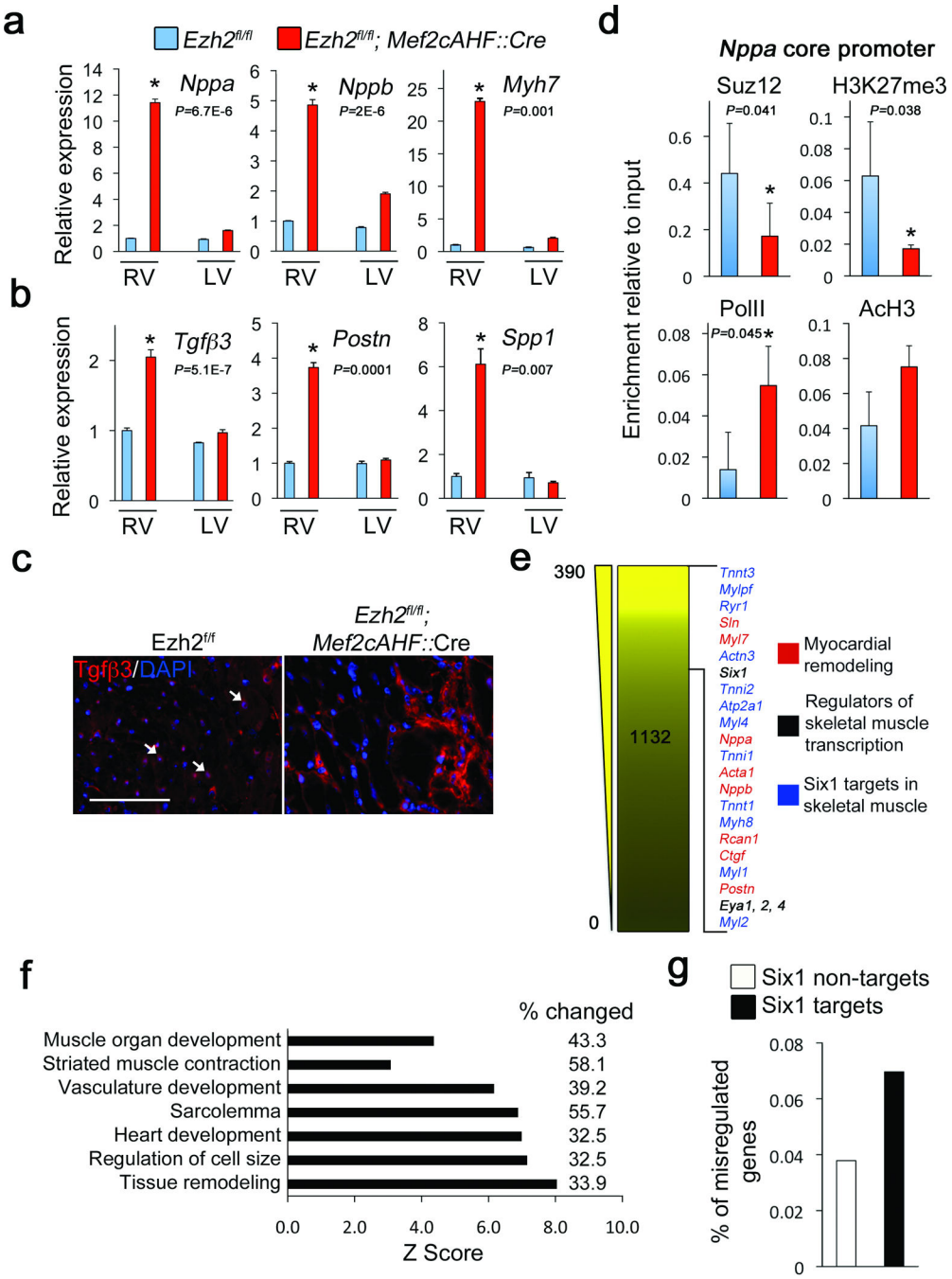

Figure 2.

Ezh2 represses expression of fetal genes, profibrosis factors and *Six1*. (**a**) QRTPCR showing relative expression of *Nppa, Nppb* and *Myh7* mRNA. *Ezh2*$^{fl/fl}$ (blue bars) and *Ezh2*$^{fl/fl}$*; Mef2cAHF::Cre* (red bars). (**b**) QRTPCR showing expression of *Tgfβ3, Postn* and *Spp1*. (**c**) Immunofluorescence showing low levels of perinuclear Tgfβ3 in control cardiomyocytes (arrows), and strong extracellular staining in the extracellular region in *Ezh2*$^{fl/fl}$*; Mef2cAHF::Cre* right ventricle (RV). Nuclei were counterstained with DAPI. (**d**) Chromatin immunoprecipitation for Suz12, H3K27me3, RNA PolII, and acetylated histone H3 (AcH3)

on the *Nppa* core promoter in Ezh2-deficient Hearts (red bars), as compared with controls (blue bars). **e**, Heat map of 1132 upregulated genes identified by RNA-seq comparing Ezh2-deficient hearts to control hearts. Numbers at left indicate fold increase. Indicated genes were amongst the most upregulated. (**f**) Analysis of functional categories identified a high percentage of genes regulating skeletal muscle and tissue remodelling. (**g**) RNA-seq analyses showed a higher percentage of deregulated genes targeted by Six1, as compared to deregulated genes not targeted by Six1, in Ezh2-deficient hearts. Reference bars = 25 μm. Bars in panels (**a**), (**b**) and (**d**) represent the mean +/− S.D. from at least three biological replicates. * Indicates $P < 0.05$.

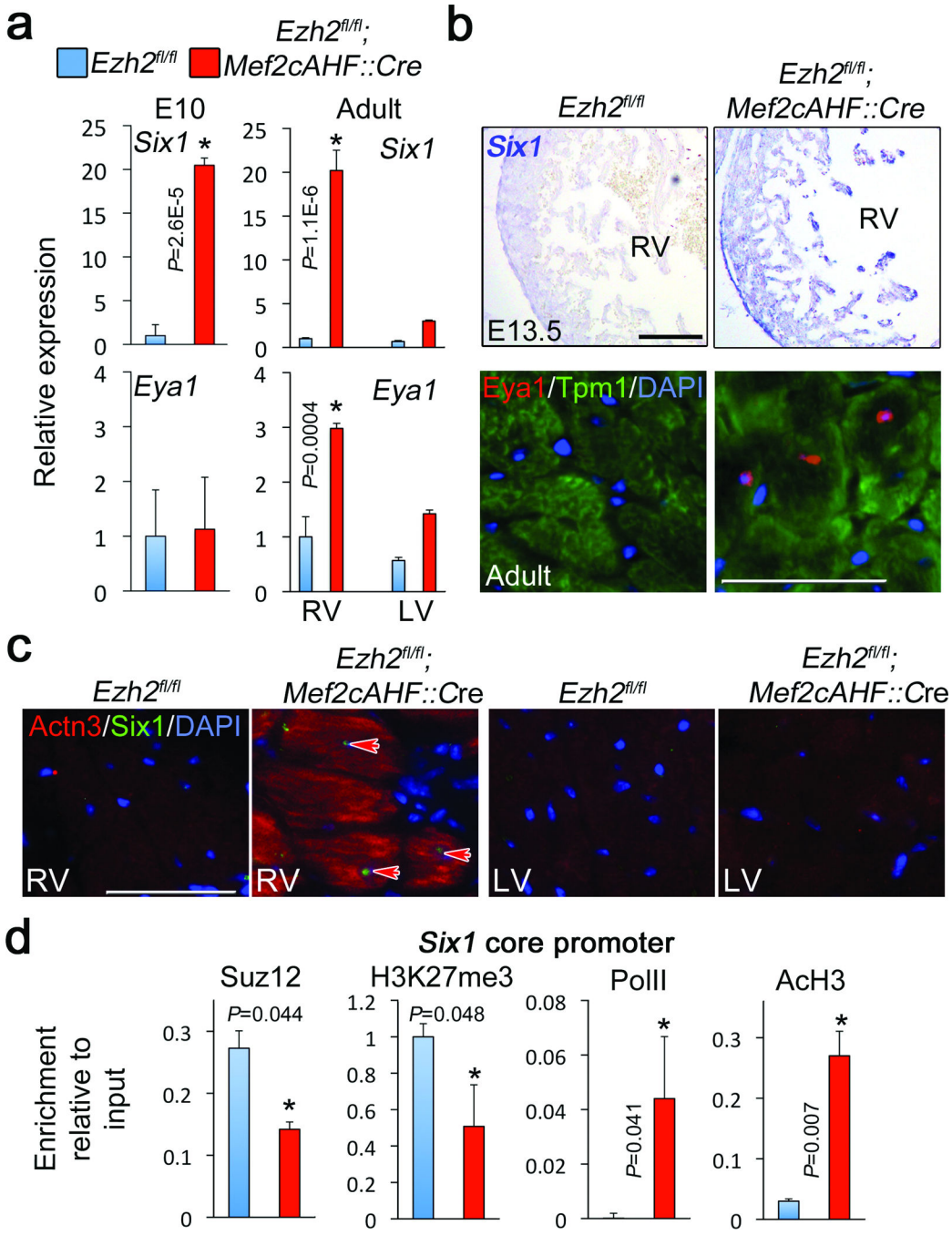

Figure 3.

Six1 is epigenetically repressed by PRC2. (**a**) QRTPCR showing expression of *Six1* and *Eya1* in embryonic and adult myocardium. (**b**) Upper panel: *In situ* hybridization showing *Six1* mRNA in E13.5 Ezh2-deficient (*Ezh2$^{fl/fl}$; Mef2cAHF::Cre*) right ventricle (RV). Reference bar = 200 μm. Lower panel. Immunofluorescence for Eya1. Cardiomyocytes were co-stained with an anti tropomyosin (Tpm1) antibody, and nuclei with DAPI. (**c**) Immunofluorescence for Six1 and alpha-actinin 3 (Actn3) in control (*Ezh2$^{fl/fl}$*) and *Ezh2$^{fl/fl}$; Mef2cAHF::Cre* adult hearts. Only Ezh2-defficient right ventricular (RV) cardiomyocytes

co-expressed Six1 and Actn3. Arrows point to Six1-possitive cardiomyocyte nuclei. LV: left ventricle. Reference bar = 25 μm. (**d**) Chromatin immunoprecipitation for Suz12, H3K27me3, RNA PolII, and acetylated histone H3 (AcH3) on the *Six1* core promoter in Ezh2-deficient Hearts (red bars), and controls (blue bars). Bars in panels (**a**) and (**d**) represent the mean +/− S.D. of at least three biological replicates. * Indicates $P < 0.05$.

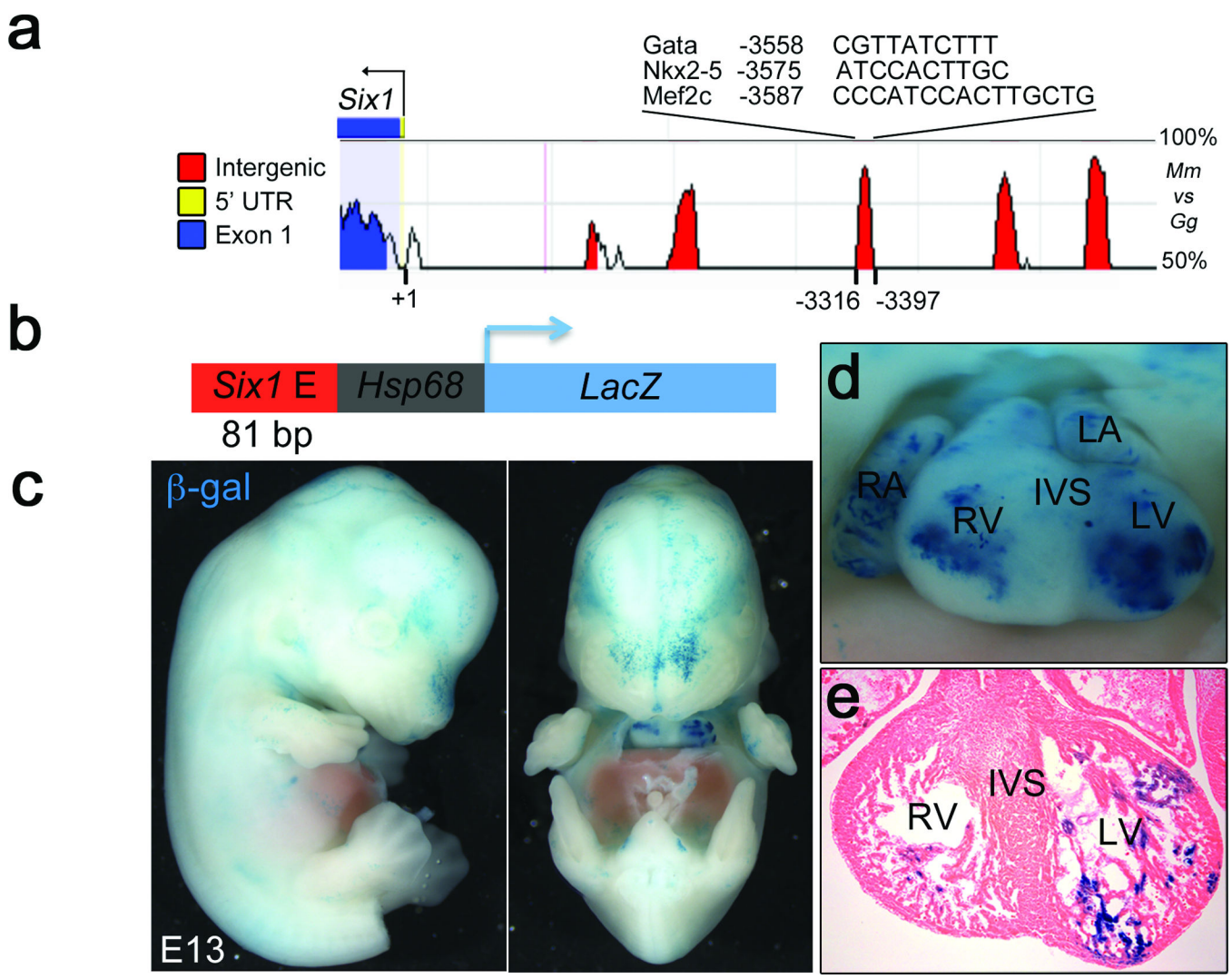

Figure 4.

Six1 has differentiated myocardium expression potential. (**a**) rVista plot of the percentage of conservation between the mouse (*Mm*) and chicken (*Gg*) *Six1* upstream regulatory region. The indicated peak represents a highly conserved element including potential Nkx2-5, Mef2c and Gata binding motifs. (**b**) A LacZ reporter driven by the Hsp68 promoter and a 80bp DNA fragment including such a conserved element was injected into mouse blastocysts. (**c**) Lateral and frontal views of whole mount transient transgenic *Six1-hsp68-lacZ* embryos at E11.5 that were stained for β-galactosidase (β-gal) activity. (**d**) Close up of the heart from the same embryo. (**e**) Section from heart in g showing b-gal staining in ventricular myocardium. RA, LA, RV and LV: right and left atria, and ventricles, respectively.

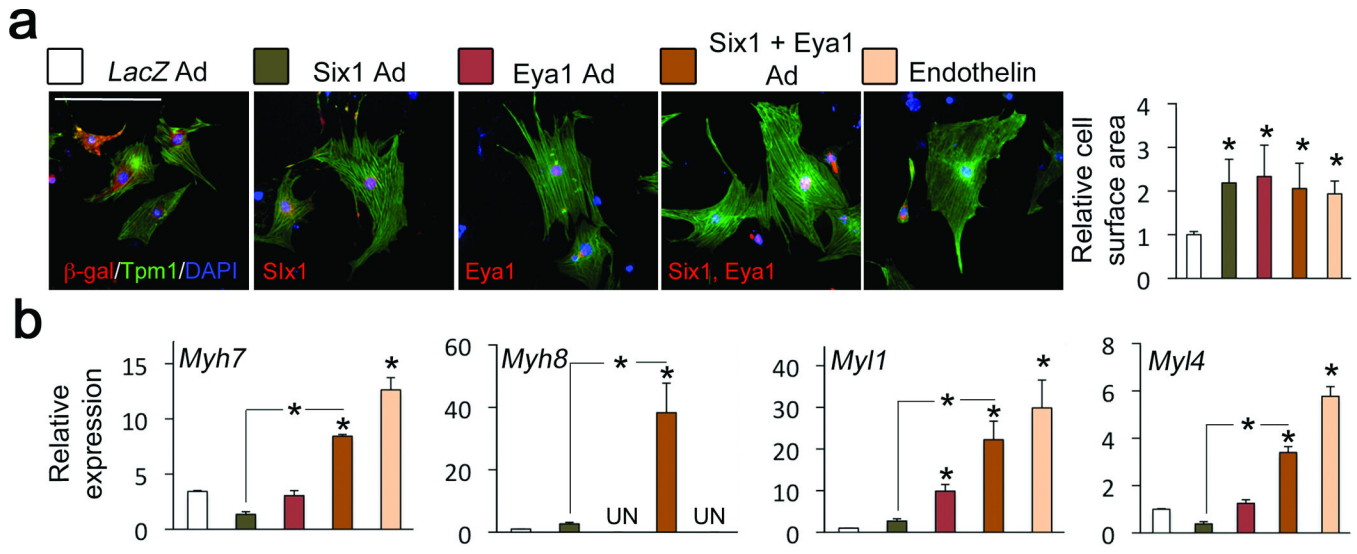

Figure 5.

Six1 induces cardiac hypertrophy and skeletal muscle gene expression in cardiomyocytes. (**a**) Cardiac myocytes infected with adenoviruses (ad) expressing control cDNA (lacZ), *Six1*, or *Eya1*, or treated with Endothelin-1. Expression of *LacZ*, *Six1* and *Eya*, was confirmed with staining for β-galactosidase (β -gal), and V5-tagged Six1 and Eya1. Cardiomyocytes were stained for tropomyosin, and nuclei counterstained with DAPI. Graph shows average cell surface area. Bars represent the mean +/− S.D. from at least 100 myocytes measured. Reference bars = 10 μm. (**b**) QRTPCR of skeletal muscle genes on RNA from infected cardiomyocytes. Bars in (**a**,**b**) represent the mean +/− S.D. from at least three independent infections. * Indicates $P < 0.05$. LV: left ventricle

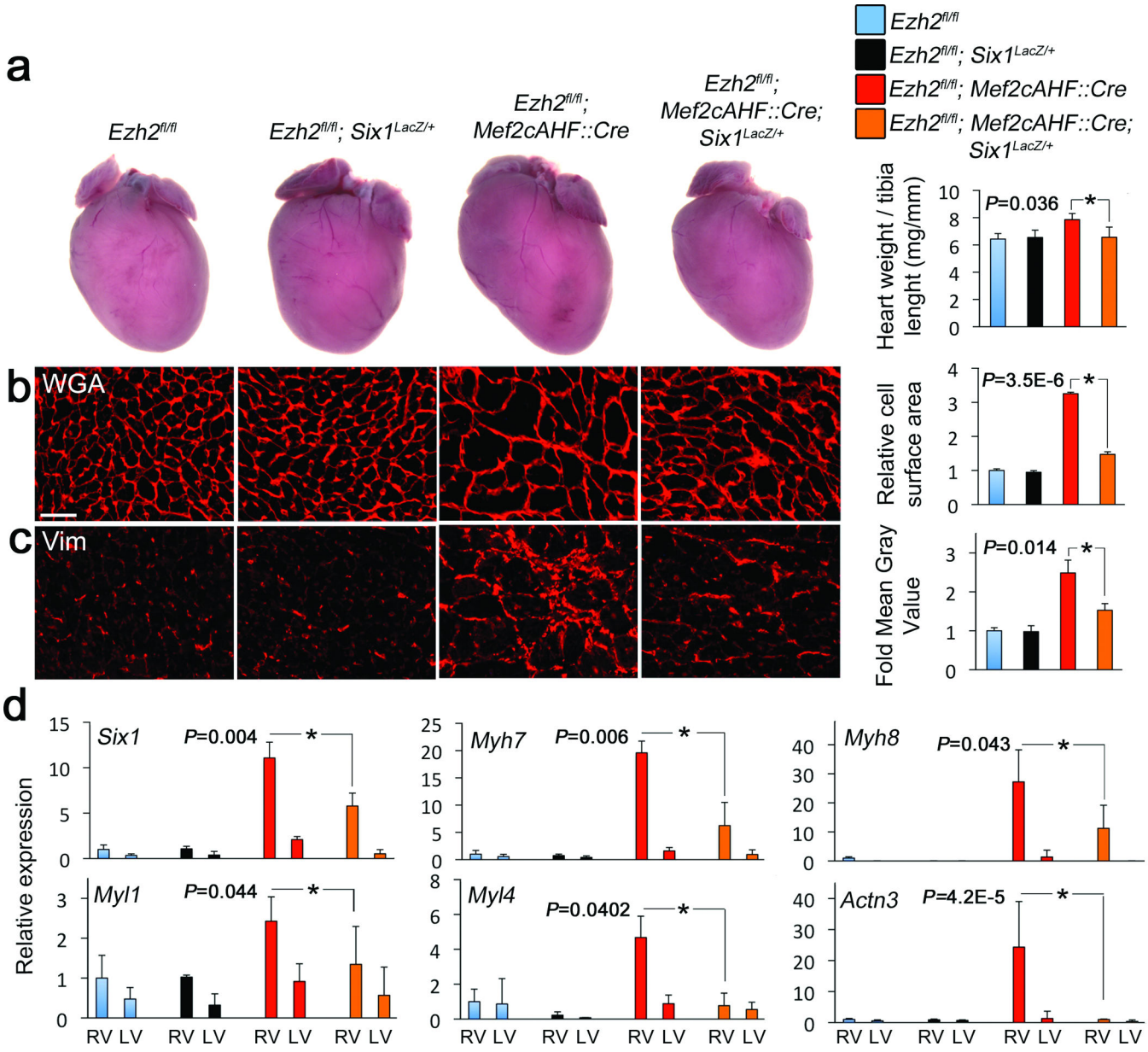

Figure 6.

Ezh2 limits cardiac hypertrophy and fibrosis by repressing *Six1*. (**a**) Whole heart images and heart mass, as represented by heart weight to tibia length ratio of *Ezh2* floxed (*Ezh2*$^{fl/fl}$), *Six1* heterozygous (*Ezh2*$^{fl/fl}$*; Six1*$^{LacZ/+}$), *Ezh2*-deficient mice (*Ezh2*$^{fl/fl}$*; Mef2c::Cre*), and *Ezh2*-deficient mice with decreased levels of Six1 (*Ezh2*$^{fl/fl}$*; Mef2c::Cre;Six1*$^{LacZ/+}$) (**b**), Heart sections from mice with the same genotypes as in (**a**) stained with wheat germ agglutinin (WGA) and (**c**) vimentin. Graphs show the respective quantitations. (**d**) QRTPCR for *Six1*, *Myh7*, *Myh8*, *Myl1*, *Myl4* and *Actn3*. Reference bars = 10 μm. Bars represent the mean +/− S.D. from at least three hearts per genotype. * indicates $P < 0.05$.