Divergent Requirements for EZH1 in Heart Development Versus Regeneration

Shanshan Ai,* Xianhong Yu,* Yumei Li,* Yong Peng, Chen Li, Yanzhu Yue, Ge Tao, Chuanyun Li, William T. Pu, Albin He

Rationale: Polycomb repressive complex 2 is a major epigenetic repressor that deposits methylation on histone H3 on lysine 27 (H3K27me) and controls differentiation and function of many cells, including cardiac myocytes. EZH1 and EZH2 are 2 alternative catalytic subunits with partial functional redundancy. The relative roles of EZH1 and EZH2 in heart development and regeneration are unknown.

Objective: We compared the roles of EZH1 versus EZH2 in heart development and neonatal heart regeneration.

Methods and Results: Heart development was normal in Ezh1−/− (Ezh1 knockout) and Ezh2fl/fl::cTNTCre (Ezh2 knockout) embryos. Ablation of both genes in Ezh1−/−::Ezh2fl/fl::cTNTCre embryos caused lethal heart malformations, including hypertrabeculation, compact myocardial hypoplasia, and ventricular septal defect. Epigenome and transcriptome profiling showed that derepressed genes were upregulated in a manner consistent with total EZH dose. In neonatal heart regeneration, Ezh1 was required, but Ezh2 was dispensable. This finding was further supported by rescue experiments: cardiac myocyte-restricted re-expression of EZH1 but not EZH2 restored neonatal heart regeneration in Ezh1 knockout. In myocardial infarction performed outside of the neonatal regenerative window, EZH1 but not EZH2 likewise improved heart function and stimulated cardiac myocyte proliferation. Mechanistically, EZH1 occupied and activated genes related to cardiac growth.

Conclusions: Our work unravels divergent mechanisms of EZH1 in heart development and regeneration, which will empower efforts to overcome epigenetic barriers to heart regeneration. (Circ Res. 2017;121:106-112. DOI: 10.1161/CIRCRESAHA.117.311212.)

Key Words: lysine ■ methylation ■ myocardium ■ regeneration ■ polycomb repressive complex 2

Adult mammalian cardiac myocytes (CMs) have limited capacity to proliferate, and as a result, the adult human heart has little capacity to regenerate from heart injury.1,2 Murine heart regeneration potential remains robust into the first postnatal week of life because hearts injured by apical resection or myocardial infarction (MI) on postnatal day 1 (P1) recover with little scar through proliferation of preexisting CMs.3 This regenerative capacity is lost rapidly because similar injury at P7 induces scar rather than myocardial regeneration. The mechanisms responsible for CM cell cycle exit remain incompletely understood.3 Reactivation of some transcriptional regulatory networks essential for heart development, such as those controlled by YAP and TBX20, has been shown to promote adult CM cell cycle activity.4,5 However, clinically meaningful strategies are still lacking. The barriers likely reside in the intrinsically distinct chromatin states and epigenetic pathways of adult compared with fetal and neonatal CMs, which repress critical regulatory genomic loci required for effective regeneration.6

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Polycomb repressive complex 2 (PRC2) is a major epigenetic repressive complex, the core of which is composed of EED, SUZ12 and a catalytic subunit, either EZH1 or EZH2. PRC2 trimethylates histone H3 on lysine 27 (H3K27me3), an epigenetic mark associated with transcriptional repression.7 Recently, EZH1 was also found to form a distinct complex containing SUZ12 but not EED that activates genes in association with increased H3K27me1 and H3K4me3.8,9 In postnatal CMs, Ezh1/2 markedly decreases coincident with loss of regenerative
Novelty and Significance

What Is Known?

- Polycomb repressive complex 2 subunits EZH1 and EZH2 catalyze trimethylation of histone H3 lysine 27 (H3K27me3) to repress gene expression.
- Polycomb repressive complex 2 regulates chromatin function during normal heart development.
- Developmental pathways are often reactivated to drive organ regeneration.

What New Information Does This Article Contribute?

- EZH1 and EZH2 are functionally redundant in differentiated cardiac myocytes for heart development.
- EZH1 but not EZH2 is essential for neonatal heart repair.
- EZH1 promotes heart repair by activating genes involved in cardiac growth.

The adult mammalian heart has limited regenerative capacity after injury. Epigenetic marks such as covalent histone modifications are important regulators of gene expression. However, little is known about the role of the epigenetic regulators in heart development versus regeneration. We investigated the function of EZH1 and EZH2, alternative subunits of polycomb repressive complex 2, a major epigenetic repressor complex, in heart development and regeneration. Our data show that in developing heart, EZH1 and EZH2 function redundantly to silence ectopic gene transcription by depositing repressive histone mark H3K27me3. Surprisingly, EZH1, but not EZH2, was required for heart regeneration after neonatal myocardial infarction. Moreover, overexpression of EZH1 but not EZH2 improved myocardial function after myocardial infarction at postnatal stages with poor innate regenerative capacity. EZH1 activated cardiac growth genes to promote heart repair by enhancing the deposition of active histone marks. These findings suggest that heart regeneration requires epigenetic regulators and mechanisms that differ from those involved in heart development.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>CM</th>
<th>cardiac myocyte</th>
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<tbody>
<tr>
<td>DKO</td>
<td>double knockout</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>E1KO</td>
<td>Ezh1 knockout</td>
</tr>
<tr>
<td>E2KO</td>
<td>Ezh2 knockout</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>histone H3 on lysine 27</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day</td>
</tr>
<tr>
<td>PRC2</td>
<td>polycomb repressive complex 2</td>
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Potential and reduced CM cell cycle activity, leading us to hypothesize that Ezh1/2 is required for heart regeneration.

In this work, we studied the relative contribution of Ezh1 versus Ezh2 to heart development and regeneration. We show that Ezh1 and Ezh2 function redundantly to silence ectopic gene transcription in fetal CMs by depositing H3K27me3 in an EZH dose-dependent manner. Surprisingly, Ezh1 but not Ezh2 was required for neonatal heart regeneration. Moreover, overexpression of Ezh1 but not Ezh2 improved myocardial function after MI at postnatal stages with poor innate regenerative capacity. Thus, our results indicate that heart regeneration requires a distinct set of epigenetic regulators from heart development.

Methods

A detailed Materials and Methods used in this study are available in the Online Data Supplement.

Results

Cardiac Ezh1/2 Double Knockout Disrupted Heart Development and Reduced CM Proliferation in an EZH Dose-Dependent Manner

Consistent with previous studies, constitutive Ezh1 knockout (E1KO) mice were phenotypically normal, viable, and fertile and showed no abnormalities in postnatal growth and heart function (Figure 1A; Online Figure 1A). To further investigate the effect of PRC2 catalytic subunit EZH1 and EZH2 in early heart development, we inactivated an Ezh2 floxed allele (Ezh2fl/fl) in CMs using cTNTCre (Ezh2 knockout [E2KO]).13 which is active in differentiated CMs by embryonic day 8.5 (E8.5).14 By crossing Ezh1−/−; Ezh2fl/fl; cTNTCre− and Ezh1−/−; Ezh2fl/fl mice, we generated compound double-knockout (DKO) embryos (Ezh1−/−; Ezh2fl/fl; cTNTCre−). By embryonic day E12.5, DKO CMs exhibited markedly reduced Ezh1 and Ezh2 expression (Online Figure IB). E12.5 and E16.5 DKO embryos were present at the expected Mendelian ratio but exhibited abnormal heart development (Figure 1A; Online Figure IC). Unlike E1KO and E2KO, a majority of DKO mice died perinatally (Figure 1A). By E12.5, DKO mutant hearts exhibited hypoplasia of the compact myocardium of both left and right ventricles (Figure 1B through 1D). Both ventricles also had excessive trabeculation.

Hypoplasia of compact myocardium suggested reduced CM proliferation. We stained histological sections of E13.5 DKO and control hearts for phosphorylated histone H3, a cell cycle M-phase marker. Dual inactivation of Ezh1 and Ezh2 caused a 2.5-fold decrease in phosphorylated histone H3−positive CMs (P<0.05; Online Figure ID and IE). In line with decreased proliferation, the expression of cell cycle inhibitors Ink4a and Ink4b (Mouse Genome informatics: Cdkn2a/b), which we previously showed were repressed by PRC2 in fetal CMs, was dramatically upregulated in E12.5 DKO ventricles (Online Figure IF), although the extent of upregulation was less in E2KO, coinciding with its normal heart development.

Next, we investigated the molecular regulation of gene expression in DKO hearts. By immunostaining, we found markedly reduced H3K27me3 in E13.5 DKO CMs (Online Figure IG). Western blotting also showed the global reduction in H3K27me3 in E16.5 DKO heart (Figure 1E). Interestingly, H3K27me3 was also significantly reduced in E2KO, but not to the same degree as DKO, whereas it was normal in E1KO.
These data suggest that Ezh2 is the main catalytic subunit of PRC2 in fetal CMs, but Ezh1 provides partial functional compensation after Ezh2 ablation. This level of compensation is sufficient to support normal heart development. We also found that H3K27me1 and H3K27me2, also deposited by PRC2, were reduced (Figure 1E) in DKO. Interestingly, H3K27me2 was not different between E2KO and DKO, suggesting that Ezh1 does not compensate for its global level, and that global H3K27me2 level is not critically linked to normal heart development.

We globally measured gene expression by RNA analysis by next-generation sequencing in E1KO, E2KO, and DKO. A large number of genes (399) were uniquely upregulated in DKO, and 212 genes were shared between DKO and E2KO (Figure 1F). Quantitative analysis of gene expression levels showed that the extent of gene dysregulation was much greater in DKO compared with E1KO or E2KO (Figure 1G). The promoters (TSS ±5 kb) of the majority of these differentially expressed genes were occupied by H3K27me3 in controls (Figure 1G). Further analysis of the direction of differential expression (up- or downregulated) and the presence of H3K27me3 at the promoter indicated that upregulated genes were significantly enriched for H3K27me3 (Figure 1H). These genes are ordered by decreasing promoter H3K27me3 signal. 

**Figure 1.** Derangement of heart development and transcriptional regulation in cardiac Ezh1 and Ezh2 double knockout (DKO).

A, Survival analysis of mutants at indicated developmental stages. A majority of DKO died perinatally. Numbers next to bars indicate sample size. Pearson χ² test was used for P value calculation. B, Cardiac abnormalities of embryonic: d 12.5 (E12.5), E16.5, and postnatal d 0 (P0) embryos. Representative HE stained transverse sections revealing thinning of compact myocardium (black arrowheads), excessive myocardial trabeculation (green arrowheads), and ventricular septal defect (double-headed arrow). Scale bar=100 µm. C and D, Quantification showing decreased compact myocardial thickness (C) and increased myocardial trabecular area (D). E, Western blotting and quantification showing downregulation of bulk H3K27me1/2/3 in DKO. Heart apex of embryos at E16.5 was used for protein extraction. F, Venn diagram showing differentially expressed genes in Ezh1 knockout (E1KO), Ezh2 knockout (E2KO), and DKO compared with Ctl (Ezh1−/+ or Ezh2fl/+; cTNTCre). Differentially expressed genes identified by Cuffdiff (P<0.05 and log2(fold change) >0.5 or <−0.5).

G, Heat map showing RNA analysis by next-generation sequencing and histone H3 on lysine 27 (H3K27me3) at ±5 kb of TSS of genes in F. Genes are ordered by decreasing promoter H3K27me3 signal. H, Bar graph showing association of differentially expressed genes with H3K27me3 enrichment at ±5 kb of TSS. *, P<0.05; **, P<0.01; ***, P<0.001 compared with control. CM indicates cardiac myocyte; LA, left atrium; LV, left ventricle; RA, right atrium; and RV, right ventricle.
results suggest that most genes upregulated in DKO are normally directly repressed by PRC2. Gene ontology term analysis of upregulated genes showed that these upregulated genes are enriched for functional terms related to development, cell proliferation, and noncardiac gene expression programs (Online Figure II). Together, these results show that EZH1 and EZH2 function redundantly and regulate heart development by depositing H3K27me3 and repressing noncardiac gene programs.

**EZH1 but Not EZH2 Was Essential for Heart Regeneration**

Reactivation of PRC2 has been linked to proliferation of tumor cells, and PRC2 is downregulated in postnatal CMs coincident with loss of cardiac regenerative capacity. These observations led us to hypothesize that Ezh1 or Ezh2 is required for neonatal heart regeneration. To test this hypothesis, we studied the effect of Ezh1 or Ezh2 depletion on the neonatal heart’s capacity to regenerate after injury by induction of MI by ligation of the left anterior descending coronary artery at postnatal day 2 (P2; Figure 2A). At 3 weeks after MI, both control (Ezh2fl/fl) and E2KO hearts had little fibrotic scar (Figure 2B and 2C), indicating that Ezh2 is dispensable for neonatal heart regeneration (data not shown). In contrast, E1KO mice had markedly increased fibrotic scar from 1 to 3 weeks after MI (Figure 2B and 2D). Consistent with impaired myocardial regeneration, E1KO mice had severely depressed heart function, as measured by echocardiography (Figure 2E). CM proliferation, measured by EdU incorporation and staining of transverse tissue sections, was clearly reduced in the border zone of E1KO (Online Figure III). These results indicate that Ezh1 but not Ezh2 is essential for cardiac regeneration after neonatal MI.

To confirm the differential requirement of Ezh1 and Ezh2 in this context, we performed a rescue experiment in which AAV9-cTNT-Ezh1, -Ezh2, or -GFP (control) were delivered to E1KO mice at P5, when MI was also induced (Figure 3A; Online Figure IVA). We validated overexpression of EZH1, EZH2, and GFP proteins in CMs (Online Figure IVB through IVD). CM-specific re-expression of Ezh1, but not Ezh2 or GFP, dramatically reduced fibrosis area of injured E1KO hearts (Figure 3B and 3C) and improved heart function (Figure 3D). We also examined CM proliferation in the rescue experiment. Ezh1 overexpression increased the incidence of EdU+ CMs by nearly 7-fold relative to Ezh2 or GFP overexpression (Online Figure III). Unlike EZH1, overexpression of EZH2 in E1KO did not rescue fibrosis or heart function (Figure 3B through 3D). These results confirm the key role of Ezh1 in heart regeneration and show that Ezh2 cannot functionally replace Ezh1 in cardiac regeneration. Furthermore, because AAV9 with Tnt2 promoter selectively drives gene expression in CMs,15

![Figure 2. Ezh1 was required for neonatal heart regeneration after myocardial infarction.](image-url)
these data demonstrate that CMs are the cell type that requires Ezh1 for effective cardiac regeneration. Collectively, these data support the crucial role of EZH1 in neonatal heart regeneration through modulating CM proliferation.

**EZH1 Overexpression Prolongs the Heart Regeneration Window**

Because Ezh1 played a crucial role in both heart morphogenesis and regeneration, we next tested the hypothesis that Ezh1 might be sufficient to prolong the window during which the neonatalmurine heart remains competent for regeneration. By P7, murine hearts have lost regenerative capacity, and as a result, myocardium lost from myocardial infarction is replaced by fibrotic scar. We studied the effect of overexpression of AAV9-expressed Ezh1, Ezh2, or GFP on heart regeneration after MI induced at P10, well outside the innate regenerative window (Figure 4A). Histological evaluation 3 weeks after MI showed that AAV-Ezh1 markedly and significantly reduced fibrotic scar area compared with AAV-GFP, whereas AAV-Ezh2 had no significant effect (Figure 4B and 4C). Echocardiography demonstrated that AAV-Ezh1 significantly improved heart function compared with AAV-GFP, whereas AAV-Ezh2 was ineffective (Figure 4D).

To examine the cellular mechanism, we measured CM proliferation by EdU staining. We found that EdU+ CM frequency in the border zone of AAV-Ezh1 hearts was significantly greater than AAV-GFP, and AAV-Ezh2 did not have a significant effect (Online Figure VIA and VIB). To study the effect of Ezh1 overexpression on apoptosis, we measured the fraction of CMs positive for TUNEL staining, a marker for apoptosis. Compared with the AAV-Ezh2 group, TUNEL+ CMs were 10-fold less frequent in the AAV-Ezh1 group (Online Figure VIC and VID), suggesting that EZH1 also enhances CM survival. Border zone CMs in AAV-Ezh1 hearts were significantly smaller than in AAV-GFP or AAV-Ezh2 (Online Figure VIE and VIF), which likely reflects amelioration of compensatory hypertrophy in AAV-Ezh1, as well as smaller size of proliferative CMs.

To dissect the molecular mechanism underlying enhanced CM proliferation and survival induced by Ezh1 overexpression, we profiled gene expression by RNA analysis by next-generation sequencing and genomic occupancy of EZH1, H3K27me3, H3K27ac, and H3K4me3 by chromatin immunoprecipitation followed by next-generation sequencing. Compared with AAV-GFP mice, 597 and 182 differentially expressed genes were significantly up- and downregulated, respectively, in AAV-Ezh1 heart after MI (Online Figure VIIA). Upregulated genes were enriched in GO terms related to embryonic morphogenesis and cardiac muscle growth (Figure 4E), whereas downregulated genes did not yield any significantly enriched terms. Quantitative RT-PCR validated 7 of 11 upregulated genes involved in fetal heart development (Online Figure VIIIB).

EZH1 deposits H3K27me3, canonically a repressive histone mark. A distinct EZH1-containing protein complex has been shown to activate genes in association with accumulation
of H3K4me3 and H3K27me1,10,11 histone marks which have been linked to gene activation.16 To examine the mechanism of gene dysregulation, we measured H3K27me1/2/3 bulk levels by immunoblotting. Surprisingly, compared with AAV-GFP treatment, AAV-Ezh1 caused a reduction in overall H3K27me3 and a clear increase in H3K27me1 (Figure 4F). This result was further reinforced by genome-wide profiling of EZH1, H3K27me3, and H3K27ac chromatin occupancy by chromatin immunoprecipitation followed by next-generation sequencing. This showed that overexpressed EZH1 was found at the TSS of most upregulated genes (Figure 4G). Consistent with bulk levels, H3K27me3 occupancy of many upregulated genes was reduced by AAV-Ezh1 (Figure 4G). Furthermore, only 5535 of 31224 (17.8%) EZH1 peaks overlapped with H3K27me3 peaks (Online Figure VIIC). These data suggest that EZH1 has alternate mechanism that is not dependent on deposition of H3K27me3. In agreement with the reported activation role of EZH1,10,11 we observed substantial overlap between EZH1 and H3K4me3 occupied chromatin regions (Online Figure VIIID), including at promoters (±5 kb of TSS) of upregulated genes in AAV-Ezh1 compared with AAV-GFP. Scale bar=500 µm; *P<0.05. There were 4 to 6 replicates per group. Numbers in parentheses indicate nonsignificant P values. FS% indicates fractional shortening; LV, left ventricle.

Discussion

Recent studies highlight strategies that redeploy developmental signaling pathways to achieve the goals of cardiac regeneration18 and point to epigenetic barriers to effective regeneration in mammalian hearts17 Previous work by us and others suggested that Ezh1 is functionally redundant with Ezh2 in differentiated CMs. Here, we provide direct evidence that
Ezh1 and Ezh2 complement each other in heart development. Furthermore, we extended the analysis of Ezh1 and Ezh2 function to heart regeneration and made the surprising discovery that these genes have distinct activities in regeneration compared with development. Specifically, whereas Ezh2 was functionally dominant in development but dispensable for regeneration, Ezh1 contributed to heart development and was essential for heart regeneration. Indeed, Ezh1 was sufficient to enhance regeneration in 10-day-old hearts, which normally lack regenerative capacity. It will be important to evaluate the ability of Ezh1 overexpression to improve myocardial outcome after adult heart injury.

The molecular mechanisms underlying EZH1/2 activity in development and regeneration were also distinct. As shown in He et al13 and this study, during development, Ezh2 and, to a lesser (but functionally sufficient) degree Ezh1, deposited H3K27me3 and repressed noncardiac gene programs. In regeneration, likely through globally increasing H3K27me1, EZH1 directly targeted and upregulated genes, related to cardiac regeneration, likely through globally increasing H3K27me1, and associated with H3K27me3 and repressed noncardiac gene programs. In contrast, EED that has been linked to transcriptional activation was lacking EED, and this study, during development, Ezh2 in He et al13 and this study, during development, Ezh1 and Ezh2 complement each other in heart development.

Collectively, our work illuminates a novel role for EZH1 in heart regeneration and illustrates the divergent role of epigenetic regulators in heart development and regeneration, as summarized in the working model (Online Figure VIII). Further interrogation of the factors that enhance or decrease competence for neonatal cardiac regeneration may provide a framework for resetting the epigenetic landscape to enhance myocardial outcome after heart injury.

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Disclosures

None.

References
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Online Data Supplement

SUPPLEMENTAL MATERIALS AND METHODS

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Online Table I. Antibodies used in this study.
Online Table II. Primers used in this study.
Supplemental Materials and Methods

Mice

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Peking University. Ezh2\(^\text{II}\) and cTNT-Cre were described previously\(^1\text{-}^3\), and the Ezh1\(^-\) allele was provided by Dr. Thomas Jenuwein\(^4\).

The mice were on a mixed C57BL6/J and 129 genetic background. Noon of the day of a vaginal plug was defined as day 0.5 (E0.5). Mice were injected with AAV9 (1x10\(^{12}\) viral particles/gram body weight) by intraperitoneal injection. Echocardiography was conducted with a VisualSonics Vevo2100. M-mode images were acquired from awake mice and used to calculate fractional shortening.

Neonatal MI

Neonatal MI surgery were performed on neonatal mice as previously described\(^4,\text{5}\). Briefly, following anesthetizing neonatal pups on ice for about 5-12 min, lateral thoracotomy was performed at the fourth intercostal space and a 7-0 prolene suture was ligated through the left anterior descending coronary artery(LAD) to induce infraction. After ligation, incisions were sutured with a 7-0 prolene suture, and the mice were allowed to recover under a heat lamp for several minutes. Sham-operated neonatal mice underwent the same procedure except for ignoring the step of LAD ligation. Mice were subjected to echocardiography, and then euthanized at 1, 2 and 3 weeks after occlusion.

Histology analysis

For Hematoxylin & Eosin (H & E) staining, Masson's staining, embryos were fixed in 4% PFA overnight at room temperature, dehydrated in ethanol, and embedded in paraffin. Sections of 5 \(\mu\)m in thickness were deparaffinized in xylene and rehydrated through the graded ethanol series (100%, 95%, 75%, 50%), then stained for H & E or Masson's Trichrome. In morphometric measurements, wall thickness was calculated as the ventricular compact myocardial thickness divided by its outer circumference. Trabecular and myocardial area were measured in Adobe Photoshop after selection of
the image areas with myocardial color range. To determine the infarct size following Masson's trichrome staining as labeled by blue area in the sections, percentage of the infarcted LV wall was calculated by using MIQuant as previously described. For immunofluorescent staining, fixed embryos or hearts were infused with 30% sucrose overnight at 4°C and embedded in OCT medium (Leica #14020108926) and sectioned in 10 μm. Immunostaining was performed with antibodies listed in Online Table I. Primary antibodies were visualized by staining with Alexa-conjugated secondary antibodies: Alexa Fluor 488 donkey anti-goat and Alexa Fluor 555 donkey anti-rabbit (1:200 Invitrogen). All the slides were mounted in VECTASHIELD hardset antifade mounting medium (Vector Laboratories) and imaged on Zeiss Axio Vert.A1 microscope or Olympus FV1000 Confocal microscope.

**Edu incorporation**

For P12 mice, 4 mg/kg of Edu was injected subcutaneously 12 h before heart harvest. For P15 mice, 5 mg/kg of Edu was injected subcutaneously 36 h before heart harvest. After dissection, hearts were fixed with 4% PFA, infused with 30% sucrose overnight at 4°C and embedded in OCT medium. Transverse sections in 10 μm were made for following staining. Edu incorporation was detected using Click-it Edu imaging Kit (Life Technologies, #C10638). Tissue slides were imaged with Zeiss Axio Vert.A1 microscope or Olympus FV1000 Confocal microscope.

**RNA expression**

Total RNA was extracted from all samples using Trizol (Invitrogen) according to the manufacturer's instruction. For Quantitative reverse transcription-PCR (qRT-PCR), total 500 ng RNA was reverse transcribed using cDNA synthesis kit (Vazyme R223-01). qRT-PCR was performed with the ABI Power SRBR Green qPCR master mix (Applied Biosystems). Gene expression was normalized to actb or 18s rRNA. Primers for qRT-PCR are provided in Online Table II.

Genome-wide expression profiling was performed on E12.5 heart apex and P13 heart infarct and border zones by RNA-seq, as described previously but steps for library normalization were omitted. Briefly, polyadenylated RNA was isolated from total RNA using two rounds of selection on oligo-dT dynabeads(Invitrogen). RNA was
converted to cDNA with Superscript III (Invitrogen) and random hexamer primers. After
RNaseH treatment and PolI catalyzed second strand cDNA synthesis, DNA end repair
performed with End-It DNA End-Repair Kit (Epicentre). DNA was then A-tailed with Exon
(-) Klenow (NEB), and adapters were ligated using T4 quick ligase. Fragments 150-300
bp were size-selected by agarose gel electrophoresis. Recovered DNA was PCR
amplified using Phusion DNA polymerase (NEB), multiplexing PCR primer 1.0, and one
indexed primer. The library was quantitated using the QuantIT DNA quantitation kit
(Invitrogen). Library quality control was performed using an Agilent Bioanalyzer. Paired
end 150 bp reads were obtained using an Illumina Hiseq 2500. Primer sequences are
provided in Online Table II.

PE150 RNA-seq reads were filtered by cutadapt (v1.10) and then aligned to the
mm10 mouse genome using TopHat2 (v2.1.1, -N 10 --read-gap-length 3 --read-edit-dist
10 -a 8 -m 0 --no-coverage-search --segment-length 30 --segment-mismatches 2). The
mapped reads were further filtered to only keep uniquely mapped reads. Cufflinks (-G --b)
was used to estimate gene expression level which is measured by fragments per
kilobase per million fragments mapped (FPKM). Cuffdiff (-b --library-norm-method
classic-fpkm --total-hits-norm) module in Cufflinks was used to determine differentially
expressed genes. The significant differentially expressed genes were determined by p-
value less than 0.05 and the absolute log2 fold change greater than 0.5. Gene Ontology
(GO) terms enrichment analysis was determined using Metascape with all mouse genes
as background.

All up-regulated genes in AAV-cTNT-Ezh1 hearts were filtered to keep genes if
FPKM > 1 in at least 1 sample of both replicates. FPKM was further log10 transformed
for hierarchical clustering by samples. Clustering was generated using the “complete”
agglomeration method, in which the distance metric was measured by “euclidean”.

**Western blotting**

Mice heart apex was homogenized with a T10 homogenizer (IKA) and lysed in
Nuclear Lysis buffer (NLB, 10% glycerol, 20 mM Tris-Hcl pH 8.0, 137 mM NaCl, 1%
Nonidet P-40 (NP40), 10 mM EDTA, 1 mM EGTA and fresh 1 mM PMSF and protease
inhibitor cocktail (PIC)) for 30 min before 0.2% SDS was added to lysates and
incubated on ice for 15 min. Samples were centrifuged at 4°C, 1300 rpm for 15 min and supernatant was saved for protein concentration measurement and Western blotting. Equal amounts of proteins were resolved on 7.5% or 10% or 14% SDS polyacrylamide gels and immunoblotted with primary antibodies listed in Online Table I.

**ChIP-seq**

Chromatin immunoprecipitation followed by parallel next-generation sequencing (ChIP-seq) was performed as previously described using antibodies listed in Online Table I. Briefly, ventricular heart apex was minced to 1–2 mm³ and immediately fixed in 1% V/V formaldehyde at room temperature for 15 min. Formaldehyde was neutralized with 2.5M glycine. Tissue was homogenized with a T10 homogenizer (IKA). Samples were then placed in hypotonic lysis buffer and dounce homogenized for 10 strokes. DNA was then fragmented in a Misonix 4000 sonicator to an average size of 150-500 bp. Chromatin was then precleared with protein G Dynabeads (Invitrogen), immunoprecipitated with antibodies (Online Table I, The Ezh1 antibody was a kind gift from Dr. Vittorio Sartorelli) and protein G Dynabeads. After extensive washing, complexes were eluted and reverse crosslinked overnight in ChIP elution buffer (1% SDS, 50 mM Tris-Hcl pH 8.0, and 10 mM EDTA) at 70°C. DNA was purified using the QIAquick PCR purification kit (Qiagen).

ChIP-seq libraries were prepared using the NEB Next DNA sample preparation kit (NEB, E7370) and primers were listed in Online Table II. Libraries were sequenced (paired end 150 bp) on an Illumina Hiseq 2500. Reads were filtered by cutadapt (v1.10) (http://dx.doi.org/10.14806/ej.17.1.200) and then aligned to the mm10 mouse genome using bwa (v0.7.13-r1126) BWA-MEM algorithm. PCR duplicates were removed using samtools (v1.3.1) rmdup command and uniquely mapped reads with map quality greater than 30 are used to call peaks by MACS2 (v2.1.1) with parameters (-f BAMPE -g 1.87e9 -q 0.05). ChIP-seq signals used to generate heat maps were processed as following. The fragment coverage of ChIP and input samples were calculated by deeptools (v2.4.2) and normalized to 10 million fragments. ChIP subtracted by input was log2 transformed with a pseudocount 1 added. The color codes in all ChIP-seq signal heat maps were performed by rescaling the value in each single heat map in the
range of 0 to 1. The overlap of ChIP-seq peaks were calculated by bedtools (v2.20.1) multiinter and then generated venn diagram by R package “eulerr”.

**Statistical analysis**

qRT-PCR, ChIP-qPCR and protein quantification of Western blotting results were expressed as mean ± SEM. Two group comparisons were performed with Student’s *t*-test with *P* values less than 0.05 taken as statistically significant. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001.

**ACCESSION CODES**

The high throughput data used in this study have also been deposited at GEO,https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=gdwzimsvlebtez&acc=GSE97733.

**SUPPLEMENTAL REFERENCES**


Online Figure I. Characterization of heart development of E1KO, E2KO and DKO mice. A. Echocardiography measurement of E1KO heart function. FS%, fractional shortening. B. qRT-PCR validation of genes encoding core PRC2 subunits. C. Gross morphology of hearts of DKO and control (Ctl) mice. Scale bar = 200 μm. D. Immunostaining (D) and quantification (E) of PH3+ CMs in DKO and Ctl hearts at E13.5. Scale bar = 20 μm. F. qRT-PCR validation of selected differentially expressed genes. *, P<0.05; **, P<0.01; ***, P<0.001; NS, not significant. G. Immunostaining of H3K27me3 in E13.5 heart. Scale bar = 20 μm.
Online Figure II. Heat map showing GO terms enriched in upregulated genes. Hierarchical clustering of \(-\log_{10}(P\text{ value})\) of enriched GO terms in upregulated genes. Terms related to development are highlighted in red.
Online Figure III. EdU labeling in hearts of mice post MI. A. Immunostaining of TNNI3 and EdU in hearts of E1KO and control (Ezh1+/−) one week post MI. B. Quantification of EdU labeled cardiomyocytes. Numbers in bars indicate number of mice in each group. **, P<0.01; Scale bar = 20 μm
Online Figure IV. Protein expression of AAV-cTNT-Ezh1, -Ezh2 or -GFP in cardiomyocytes.

A. Diagram of AAV9 constructs. B. Western blotting validation of transgene expression. Arrow indicates the bands for EZH1 or EZH2 proteins. C. Immunostaining validation of AAV9 transgene expression in cardiomyocytes. D. Brightfield and GFP fluorescent signals in hearts of mice injected with AAV9-GFP at P2, subjected to myocardial infarction (MI) surgery at P10, and harvested one week post MI. Scale bar = 20 μm (C) and 500 μm.
Online Figure V. Examination of cardiomyocyte proliferation by EdU staining. A. Representative EdU immunostaining of heart sections from mice injected with indicated AAV9 at postnatal day 2 (P2) and harvested one week post MI. EdU was injected 24 hrs before collection. Dotted white line demarcates infarct and border zone. White arrows indicate EdU+ non-myocytes while yellow arrowheads indicate EdU+ cardiomyocytes. *, P< 0.05; Scale bar = 20 μm. B. Quantification of EdU-labeled cardiomyocytes stained as in A.
Online Figure VI. Functional characterization of Ezh1 and Ezh2 gain-of-function in hearts post MI. Mice were injected with indicated AAV9 at P3 and harvested one week after P10 MI. EdU was injected 24 hrs before collection. A,B. EdU immunostaining (A) and quantification (B) of White arrows indicate EdU positive non-myocytes while yellow arrowheads indicate EdU positive cardiomyocytes. C, D. TUNEL (apoptosis) staining (C) and quantification (D). White arrows indicates TUNEL positive non-myocytes while yellow arrowheads indicate TUNEL positive cardiomyocytes; Dotted white line demarcating infarct and border zones; Scale bar = 20 μm; *, P<0.05; **, P<0.01.
Online Figure VII. Association of differentially expressed genes with histone modifications in AAV-Ezh1-treated hearts following MI at P10.  

A. Volcano plot of differentially expressed genes in hearts treated with AAV-Ezh1 versus AAV-GFP 3 days post MI.  

B. qRT-PCR validation of selected differentially expressed genes.  

C. Venn diagram of H3K27me3 and EZH1 peaks as indicated.  

D. Venn diagram of EZH1 and H3K4me3 peaks found in AAV-Ezh1 treated heart three days after MI. * P<0.05; ** P<0.01; NS, not significant.
Online Figure VIII. Working model manifests divergent mechanisms of EZH1/2 regulation of heart development versus regeneration. During heart development, EZH1 and EZH2 function redundantly to deposit H3K27me3 for transcriptional repression of non-cardiac and potent cell cycle inhibitor genes. To prolong the window of heart regeneration, EZH1 but not EZH2 reduces repressive marks H3K27me3, increases active marks H3K27me1 and activates cardiac growth genes in association with promoter-promixal H3K4me3 enrichment.