REST regulates the cell cycle for cardiac development and regeneration

Donghong Zhang1, Yidong Wang1, Pengfei Lu1, Ping Wang1, Xinchun Yuan2, Jianyun Yan3, Chenleng Cai3, Ching-Pin Chang4, Deyou Zheng5, Bingruo Wu1 & Bin Zhou6,7

Despite the importance of cardiomyocyte proliferation in cardiac development and regeneration, the mechanisms that promote cardiomyocyte cell cycle remain incompletely understood. REI silencing transcription factor (REST) is a transcriptional repressor of neuronal genes. Here we show that REST also regulates the cardiomyocyte cell cycle. REST binds and represses the cell cycle inhibitor gene p21 and is required for mouse cardiac development and regeneration. Rest deletion de-represses p21 and inhibits the cardiomyocyte cell cycle and proliferation in embryonic or regenerating hearts. By contrast, REST overexpression in cultured cardiomyocytes represses p21 and increases proliferation. We further show that p21 knockout rescues cardiomyocyte cell cycle and proliferation defects resulting from Rest deletion. Our study reveals a REST-p21 regulatory axis as a mechanism for cell cycle progression in cardiomyocytes, which might be exploited therapeutically to enhance cardiac regeneration.

1Department of Genetics, Albert Einstein College of Medicine, Bronx, NY 10461, USA. 2Department of Medical Ultrasound, The First Affiliated Hospital of Nanchang University, Nanchang 330006, China. 3Department of Developmental and Regenerative Biology, The Black Family Stem Cell Institute, and The Mindich Child Health and Development Institute, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA. 4Department of Medicine, Indian University School of Medicine, Indianapolis, IN 46202, USA. 5Departments of Genetics, Neurology and Neuroscience, Albert Einstein College of Medicine, Bronx, NY 10461, USA. 6Departments of Genetics, Pediatrics, and Medicine (Cardiology), The Wilf Cardiovascular Research Institute, The Institute for Aging Research, Albert Einstein College of Medicine, Bronx, NY 10461, USA. 7Department of Cardiology of First Affiliated Hospital and State Key Laboratory of Reproductive Medicine, Nanjing Medical University, Nanjing, Jiangsu 210029, China. Correspondence and requests for materials should be addressed to B.Z. (email: bin.zhou@einstein.yu.edu)
Cardiomyocyte proliferation is required for generating myocardial mass and building a functional four-chamber heart during embryonic development\(^1\)-\(^3\). After birth, cardiomyocytes continue to proliferate in a short neonatal period, which is crucial for the final cardiac growth surge as well as for regeneration of injured mouse neonatal hearts\(^4\)-\(^6\). The vast majority of cardiomyocytes then exits the cell cycle and stops proliferating after preadolescence\(^7\)-\(^10\). The inability of cardiomyocytes to proliferate prevents the replenishment of lost or dysfunctional cells in a diseased heart\(^11\). Because heart diseases are the number one cause of death worldwide\(^11\), it is important to identify the regulatory factors of the cardiomyocyte cell cycle, which may be used as therapeutic targets for these devastating conditions.

Several transcription factors, such as GATA4\(^12\), TBX20\(^13\), BRG1\(^14\), YAP\(^15\),\(^16\), ERBB2\(^17\), PITX2\(^18\), and MEIS\(^19\) have been identified as critical for regulating cardiomyocyte proliferation. These factors may serve as therapeutic targets for the treatment of heart diseases.

**Fig. 1** Rest is required for myocardial development and cardiomyocyte proliferation. **a**, **b** Immunostaining and western blot analyses showing the efficient deletion of Rest in cardiomyocytes in TnTCre;Rest\(^{GT/GT}\) (Rest\(^{mKO}\)) hearts. Controls were Rest\(^{GT/GT}\) mice. **c** A summarizing graph of the distribution and number of embryos at different stages indicates death of Rest\(^{mKO}\) embryos between E12.5 and E16.5. **d**, **e** H&E-stained sections of E10.5 and E12.5 hearts indicate thin ventricular wall resulting from Rest deletion. **f** Quantitative immunostaining of cell cycle markers showing reduced proliferation of Rest\(^{mKO}\) cardiomyocytes. \(n=4/\text{group, mean} \pm \text{s.d.}, \*p<0.05\) by unpaired two-tailed Student’s t test. Scale bars = 40 \(\mu\)m.
shown to be essential for cardiomyocyte proliferation during development and for regeneration following neonatal heart injury. Transcriptional repressor element-1 silencing transcription factor (REST), also known as neuron-restrictive silencer factor (NRSF), is widely expressed in the embryonic tissues\(^20,21\). It binds a cis-element of 21 nucleotide base pairs, named RE1 motif\(^22\), to silence the expression of neuronal genes in the non-neuronal cells required for neurogenesis\(^23,24\). We have recently reported REST acting as a transcription repressor in mouse embryonic hearts\(^20\). In this study, we reveal that suppression of REST is required for neonatal cardiomyocyte proliferation and cardiac function.

**Fig. 2** REST is required for neonatal cardiomyocyte proliferation and cardiac function. **a** A graph of experimental design for generating myocardial Rest knockout (Rest\(^{mKO}\)) at different postnatal (P) stages using the inducible Tn\(^{\text{MerCreMer}}\) deleter mice. **b** Western blots showing effective deletion of Rest. **c** Survival curve of control and Rest\(^{mKO}\) mice, \(p < 0.001\), log-rank (Mantel–Cox) testing between mice with Rest deletion at different stages and control (Tam-treated Rest\(^{+/+}\); Tn\(^{\text{MerCreMer/+}}\) mice). **d** Representative H&E staining and heart/body weight ratio revealing underdeveloped Rest\(^{mKO}\) hearts at P3 after Rest deletion at P1. \(n = 5\) group. Scale bar = 100 \(\mu\)m. **e–g** Echocardiography showing structure defects and decreased left ventricle function of P3 Rest\(^{mKO}\). \(n = 6\) for Rest\(^{mKO}\), \(n = 8\) for Control. **h–j** FACS shows reduced percentage of cardiomyocytes isolated from P3 Rest\(^{mKO}\) hearts (h) and arrested cell cycle of the Rest\(^{mKO}\) cardiomyocytes at G0/G1 phase (i, j). **k** Immunostaining for cell cycle markers indicating reduced proliferation of Rest null cardiomyocytes. \(n = 4\) group for FACS and immunostaining. Scale bar = 40 \(\mu\)m. Mean ± s.d., \(* p < 0.05\), unpaired two-tailed Student’s \(t\) test.
the cell cycle inhibitor gene p21 by REST is critically required during cardiac development and regeneration to maintain cardiomyocyte proliferation. We show that REST binds and represses the cell cycle inhibitor gene p21, which is required for mouse cardiac development and regeneration. REST deletion derepresses p21 and inhibits the cardiomyocyte cell cycle and proliferation in embryonic or regenerating mouse hearts. We also show that p21 knockout rescues the cardiomyocyte cell cycle and proliferation defects resulting from REST deletion. By elucidating the REST-p21 genetic mechanism underlying the cell cycle regulation of proliferating cardiomyocytes during cardiac development and regeneration, our study provides an opportunity for developing cell-based therapeutics for heart disease.

Results

REST is required for embryonic cardiomyocyte proliferation. Our recent studies showed that REST represses transcription in mouse embryonic hearts. Based upon this, we did a time course of REST expression levels at various developmental stages. By western blot, we found that REST was downregulated in the ventricles of neonatal hearts (Supplementary Fig. 1a, b). Further, immunostaining showed that REST was expressed in the majority of cardiomyocytes between embryonic day (E) 11.5 and postnatal day (P) 3, whereas the number of REST-expressing cardiomyocytes was drastically reduced from P3 to P28 (Supplementary Fig. 1c, d). The downregulation of REST protein level was not accompanied by a change in mRNA level (Supplementary Fig. 1e), and was possibly due to the REST protein degradation.

Given that downregulation of REST coincided with the cell cycle exit of cardiomyocytes, we examined the relationship of REST expression with cardiomyocyte proliferation by immunostaining. The results revealed that the majority of EdU+ proliferating cells expressed REST (Supplementary Fig. 2a–c). We next inactivated Rest in the myocardium (Rest<sup>mKO</sup>) using Tn<sup>Tcre</sup> and Rest<sup>WT/GT</sup> mice to determine its role in cardiomyocytes and confirmed that Rest was effectively deleted in the myocardium by immunostaining and western blot (Fig. 1a, b). Rest<sup>mKO</sup> embryos were runted and >80% of them were dead by E16.5 (Fig. 1c). Rest<sup>mKO</sup> hearts at E10.5–12.5 had thin ventricular walls and defective trabeculae (Fig. 1d, e). Notably, there was significantly reduced percentage of Rest<sup>mKO</sup> cardiomyocytes that were expressing the cell cycle markers (Ki67 for cell cycle activity, EdU for DNA synthesis, pH3 for mitosis, and Aurora B for cytokinesis) (Fig. 1f). Such proliferation defect was not associated with changes in myocardial differentiation and apoptosis (Supplementary Fig. 3a–g). These observations demonstrate that REST is essential for embryonic cardiomyocyte proliferation and chamber development.

REST is required for neonatal cardiomyocyte proliferation. To determine whether REST is also requirement for neonatal cardiomyocyte proliferation, we deleted Rest at P1, P3, or P7 using an inducible Tn<sup>TMerCreMer</sup> driver (Rest<sup>mKO</sup>, thereafter) (Fig. 2a, b). Rest inactivation at P1, but not at P3 or P7, resulted in the death of ~80% of neonates within 1 week after tamoxifen injection (Fig. 2c). Based upon this, we focused on inactivating Rest at P1 and investigating the resultant changes in cardiac morphology and function at P3. H&E staining showed that the Rest<sup>mKO</sup> hearts were underdeveloped with a reduced ratio of the heart/body weight (Fig. 2d). Echocardiography revealed a noticeable reduction in the left ventricular mass, left ventricular wall thickness, and left ventricular ejection fraction (Fig. 2e, f). These observations demonstrate that REST is also required for neonatal cardiomyocyte proliferation.
and contractility (Fig. 2e–g). Flow cytometry assays further revealed a reduced percentage of cardiomyocytes in RestmKO hearts (Fig. 2h). The null cardiomyocytes were accumulated at the G0/G1 phase of cell cycle, while their presence at the S and G2/M phases was reduced (Fig. 2i, j). Immunostaining for Ki67 and EdU confirmed the cell cycle defect (Fig. 2k). In contrast, Rest deletion at P3 or P7, when cardiomyocytes were exiting the cell cycle, had no effect on cardiac morphology and function (Supplementary Fig. 4a–d). Therefore, Rest is also required for neonatal cardiomyocyte proliferation through maintaining cell cycle progression, and neonatal cardiac growth and function.

REST binds and represses p21 to regulate cardiomyocyte proliferation. To identify the downstream factor(s) of REST involved in the cell cycle regulation, we re-analyzed our previously published RNA-seq data from E12.5 control versus RestmKO hearts\(^\text{20}\) and found that p21 (Cdkn1a) was the only cell cycle inhibitor...
gene significantly upregulated in Rest null hearts, whereas the expression of several cell cycle activators was downregulated (Supplementary Fig. 5a). These findings were then confirmed by qRT-PCR (Supplementary Fig. 5b, c). Notably, p21 was also the only cell cycle inhibitor gene which expression was significantly upregulated in P2 ventricles with myocardial Rest inactivation at P1 (Supplementary Fig. 5d). Immunostaining further showed an increased number of p21-expressing cardiomyocytes in E12.5 and P3 Rest null hearts (Fig. 3a, b). We identified one conserved REST-binding site (RE1 motif) located at the 3′ end of mouse p21 gene by sequence alignment between mouse and human (Fig. 3c).

Luciferase reporter gene assays and mutational analyses revealed that the p21-RE1 motif was required for repressing transcription in cultured P1 mouse cardiomyocytes (Fig. 3d). Quantitative chromatin immunoprecipitation assays (qChIP) showed REST occupancy of the p21-RE1 motif in E12.5 and P1 ventricles, but the occupancy was greatly reduced in the ventricles of P21 when REST level was largely diminished (Fig. 3e). Further, qChIP showed that Rest deletion resulted in an enrichment of H3K4me3 histone marks at the p21-RE1 motif in E12.5 and P1 ventricles, but a reduction of H3K9me3 and H3K27me3 histone marks at the p21-RE1 motif (Fig. 3f). These findings support that REST directly binds p21 and represses its
transcription in cardiomyocytes, possibly through histone methylation changes. To determine whether such repression promoted cardiomyocyte proliferation during cardiac development, we deleted p21 in Rest−/− mice by breeding them with p21 knockout mice. Inactivation of p21 rescued embryonic lethality and cardiac phenotypes of Rest−/− mice at E12.5 (Fig. 4a–c). Inactivation of p21 also restored the levels of Cyclin B, Cyclin E, and CDK1/2 (Fig. 4d), and re-established normal cardiomyocyte cell cycle and proliferation, as indicated by recovered expression of Ki67, PH3, and Aurora B, and EdU incorporation (Fig. 4e).

Double knockout (DKO) mice were survived to birth and appeared healthy postnatally and in adulthood, with normal cardiac morphology, heart/body weight ratio, and cardiac function (Fig. 4f–i). Similarly, inactivation of p21 rescued postnatal lethality of mice caused by inactivation of myocardial Rest at P1 (Fig. 5a). Cardiac phenotypes were rescued, including the cardiac size, heart/body ratio, cardiac function (Fig. 5b–e), as well as the expression of cell cycle activators and markers (Fig. 5f, g). We also examined whether forced overexpression of REST would promote cardiomyocyte proliferation in cultured cardiomyocytes isolated from P1 or P5 hearts. We first treated the culture with siRNAs against β-TrCP that mediates REST degradation via ubiquitination. The results of western blot for REST, qRT-PCR for Rest, and REST-targeted genes and immunostaining for EdU and PH3 showed that downregulation of β-TrCP slowed down the REST degradation (Supplementary Fig. 6a), suppressed the expression of REST target genes (Hcn2, p21, Nppa, and Syn1) (Supplementary Fig. 6b), and upregulated cardiomyocyte proliferation (Supplementary Fig. 6c). Next, we overexpressed REST by transfection of a plasmid (pHR-NRSF-CITE-GFP) containing Rest cDNA and found by western blot and immunostaining for EdU and PH3 that overexpression of REST dose-dependently repressed p21 expression and increased cardiomyocyte proliferation (Supplementary Fig. 6d–f). These findings, therefore, identify that a Rest-p21 axis underlies the...
regulation of cardiomyocyte cell cycle for fetal and neonatal cardiac development.

**Rest is required for regenerative cardiomyocyte proliferation.**

To further investigate whether REST had a role in cardiac regeneration, we investigated Rest expression in repairing neonatal hearts using an apical resection model. Consistent with previous reports, immunostaining for the cell cycle markers showed a significantly increased number of cardiomyocytes that re-entered the cell cycle in the injured region at P7, 3 days after ablation at P4 (Fig. 6a). Notably, the number of REST-expressing cardiomyocytes was also increased in the same region and was correlated with the increased number of cells undergoing DNA synthesis, mitosis, and division (Fig. 6b). The percentage of EdU+ cells that also expressed REST was sustained in the regenerating hearts (Fig. 6c).}

**Fig. 7** Rest is required for regeneration of injured neonatal hearts. **a**, **b** Quantitative Masson’s trichrome staining shows increased cardiac fibrosis in mice with induced myocardial Rest deletion at P3 and injured by apex ablation at P4, n = 5–8/group. Scale bar = 100 μm. **c**, **d** Echocardiography indicating reduced cardiac function of myocardial Rest null mice after injury, n = 6–8/group. **e**–**g** Quantitative immunofluorescence (n = 4/group) showing decreased EdU+ cardiomyocytes in regenerating hearts in mice with Rest deletion. Scale bar = 40 μm. Mean ± s.d. p < 0.05 by two-way ANOVA followed by Sidak’s test in **b**–**d**, unpaired two-tailed Student’s t test in **f**, **g**.
myocardium from P7 to P28 (Fig. 6c). About 90% of REST-expressing cells in the apex undergoing repair were cardiomyocytes; the reminder was epicardial or endocardial cells (Supplementary Fig. 7a–d). These findings indicated that the majority of REST-expressing cells were proliferating cardiomyocytes induced by injury. We deleted Rest in the myocardium at P3 and performed apical resection at P4. The P3 deletion bypassed the death of neonates when Rest was deleted at P1, while apical ablation at P4 was still within the window of myocardial regeneration. We found by Masson’s trichrome staining that the fibrotic scar in the apex of Rest null hearts (RestKO) was not resolved at P14 and P28 compared to the control hearts (Fig. 7a, b). Consistent with the persistent fibrotic phenotype, RestKO hearts had reduced left ventricular function as observed by echocardiography (Fig. 7c, d). Further, immunostaining revealed that the number of EdU+ proliferating cardiomyocytes in the injured apex of RestKO hearts was significantly reduced (Fig. 7e–g). Together, these findings suggest that REST re-expression is necessary for the regeneration of neonatal hearts.

The increased number of REST-expressing cardiomyocytes in the regenerating neonatal hearts was correlated with increased REST occupancy at the p21-RE1 motif (Supplementary Fig. 8a). Concurrently, the regenerating myocardium expressed a low level of p21 mRNA (Supplementary Fig. 8b). Immunostaining also showed that the number of p21-expressing cardiomyocytes was negatively correlated with that of REST-expressing cells in the regenerating myocardium (Supplementary Fig. 8c, d). In contrast, p21 transcription was de-repressed in the injured RestKO apex, which was accompanied with downregulation of the cell cycle activators (Supplementary Fig. 8e, f). To determine whether p21 upregulation was responsible for the reduced proliferation of cardiomyocytes and poor regeneration of myocardium after Rest deletion, we performed the apical resection on the Rest and p21 DKO hearts at P4, 1 day after Rest deletion (Fig. 8a). Like control hearts, p21−/− hearts had normal left ventricular function at P28 (Fig. 8b, c). We noted that p21−/− hearts had less fibrotic scar tissue as compared to controls (Fig. 8d). Importantly, p21 deletion improved regeneration and function of RestKO hearts and restored the number of EdU+ cardiomyocytes in the injured myocardium (Fig. 4e). These results demonstrate that the Rest−p21 axis regulation of cardiomyocyte proliferation also underlies the neonatal cardiac regeneration.

**Discussion**

Overall, our study identifies a molecular mechanism by which REST repression of the cell cycle inhibitor p21 is necessary and sufficient for promoting the cardiomyocyte cell cycle and proliferation during cardiac development and regeneration. p21 is a well known cell cycle inhibitor through interacting with the G1/M and G2/S CDK cyclins and inhibits their function to drive the cell cycle. Of particular note, p21 is the only cell cycle inhibitor gene significantly repressed by REST in the proliferating cardiomyocytes during cardiac development and regeneration, suggesting that REST repression of p21 is a major mechanism underlying the regulation of cardiomyocyte cell cycle. Consistent with this
notion, p21 deletion has been reported to enhance the regeneration of injured appendage and neonatal hearts. Interestingly, GATA4 and MEIS1 also repress p21 transcription in mouse embryonic and injured neonatal hearts, respectively. Since neither GATA4 nor MEIS1 expression is affected by REST inactivation, REST repression of p21 is likely independent of GATA4 and MEIS1. REST thus joins a network of transcription factors or cofactors, including MEIS1, YAP1, GATA4, and MEIS1. REST thus joins a network of transcription factors or cofactors, including MEIS1, YAP1, GATA4, and MEIS1. REST repression of p21 mouse embryonic and injured neonatal hearts, respectively. Since neither GATA4 nor MEIS1 expression is affected by REST inactivation, REST repression of p21 might be one of the limiting factors for sustained proliferation of postnatal cardiomyocytes. Future investigations are needed to determine whether REST is reactivated in adult hearts by injury and if the reactivation promotes adult cardiac regeneration by modulation of the activity of cardiomyocyte cell cycle.

**Methods**

**Mouse strains.** Flanked Rest mice (Rest<sup>GT/GT</sup>), which carries a conditional gene trap (GT) cassette<sup>23</sup>, were bred to Tm<sup>Tcre</sup> or Tm<sup>TMerCreMer</sup> mice to obtain Rest<sup>−/−</sup> deletion in myocardium. Intraperitoneal tamoxifen (TAM) injection at 40 mg/kg was used for inducible inactivation. For rescue experiments, Tm<sup>Tcre</sup> or Rest<sup>GT/GT</sup> mice were crossed to Rest<sup>−/−</sup> mice<sup>27</sup>. All mouse stains were in the C57B6 background. Embryos and neonatal mice were genotyped by PCR using Cre and wildtype primers (Supplementary Table 1). Mouse housing and experiments were performed on postnatal day (P) 4 mice of both sexes as previously described<sup>4</sup>. The yolk sac or tail of mice was used for genotyping by PCR using Cre and wildtype primers (Supplementary Table 1). Mouse housing and experiments were performed on postnatal day (P) 4 mice of both sexes as previously described<sup>4</sup>. The yolk sac or tail of mice was used for genotyping by PCR using Cre and wildtype primers (Supplementary Table 1).

**Neonatal heart apical resection.** Neonatal mouse heart apical resection (AR) was performed on postnatal day (P) 4 mice of both sexes as previously described<sup>3</sup>. Briefly, we induced deletion of Rest in the myocardium at P1 by tamoxifen and performed apical resection at P4. P4 neonates were anesthetized by inhalation of 2% isoflurane and 100% oxygen on ice until the heart rate dropped below 300 bpm, then subjected to intercostal incisions to separate the pericardium and epi-derm. The apex was amputated with microsurgical scissors under a dissection microscope. About 10% of the heart was surgically removed to produce reproducible regeneration. The chest wall was then closed with sutures and the skin incisions were closed using skin adhesive. After the operation, the neonates were placed back with the feeding female mice. Sham-operated mice underwent the same procedure without apical resection.

**Histology.** End-stage injured hearts were fixed in 4% paraformaldehyde (PFA), dehydrated, embedded in paraffin, and sectioned at 6-μm thickness. H&E and Masson’s staining were performed for detecting cardiac structure defects and fibrosis, respectively. The fibrotic area was quantified using the MetaMorph software (Molecular Devices).

**Primary cardiomyocyte cultures.** Cardiomyocytes (CMs) were isolated as described in previous studies<sup>28</sup>. Ventricles were dissected out from P1 or P5 neonates of both sexes, minced and dissociated with 0.05% collagenase I and 0.08% trypsin-EDTA at 37 °C. Non-CMs were removed through adhesive plating. Purified CMs were cultured in 12-well plates for 24 h at 37 °C with 5% CO<sub>2</sub> in Dulbecco’s modified Eagle’s medium’s minimum essential medium (DMEM) supplemented with 10% fetal calf serum.

**siRNA and cDNA transfection.** siRNA against β-TrCP (si-β-TrCP, Santa Cruz Biotechnology) or plasmids containing mouse Rest cDNA (pH-RNR5F-CITE-GFP, Addgene) were transfected into cultured neonatal cardiomyocytes isolated from P1 or P5 neonatal hearts, at 70% confluency. Lipofectamine 3000 Regent (Invitrogen) and si-β-TrCP (40 nM) or REST-cDNA (0.5 or 2 µg/ml) were incubated in OPTIMEM (Invitrogen) for 15 min at RT, and then added to cells and incubated for indicated time. qRT-PCR and western blot were used to confirm the efficiency and effect of transfection.

**ChIP-qPCR.** ChIP-qPCR assays were performed as previously reported<sup>14</sup>. Ventricles (30–40 mg) from indicated stages were treated with 1% formaldehyde at room temperature for 10 min to crosslink protein and DNA complexes before quenched with addition of glycine. After three washes with PBS, tissues were homogenized, lysed, and sonicated to shear DNAs into 100–500 bp fragments. The DNA fragments were incubated with 5 µg antibodies against REST (EMD Millipore, 1:761, 1:100), Tri-Methyl-Histone H3 (Lys4) (CST mAb #13969, 1:100), Tri-Methyl-Histone H3 (Lys27) (CST mAb #9733, 1:100), Tri-Methyl-Histone H3 (Lys4) (CST mAb #9751, 1:100), and Acetyl-Histone H3 (Lys9) (CST mAb #9649, 1:100) overnight at 4 °C using Dynabead Protein G in a final volume of 200 µl and 1% Triton (Starter Pack (Invitrogen, Invitrogen, Invitrogen), 500 µg/ml (pAb, ab171877), 1:1000) were used as control. Immunoprecipitated DNAs were extracted and used for qPCR analysis to determine the enrichment of DNA fragments, containing the p21-RE1 using specific primers (Supplementary Table 1).

**Western blot.** Ventricles were collected at the indicated stages, homogenized in radiomimunoprecipitation assay buffer at 4 °C and quantified by bichinonic acid assay. Equal total proteins (20 µg) were resolved on SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes (Immobilon, Millipore). The membranes were blocked and incubated with primary antibodies at 4 °C overnight before incubated with HRP-conjugated secondary antibodies. Protein bands were detected by enhanced chemiluminescence. Antibodies, including REST (Antibodies-online, ABIN747683, 1:1000), Cdkn1a, ab109199, 1:500, Cyclin E (Santa Cruz, sc-377100, 1:200), Cyclin B (Santa Cruz, sc-166210, 1:1000), CDK1/CDK2 (Santa Cruz, sc-7331, 1:1000), and GAPDH (Thermo Fisher, MA-57783, 1:5000), were used in western blot analysis. Full scans of western blots are shown in Supplementary Figs. 9–11.

**Echocardiography.** Echocardiography was performed using a Vevo 770 ultrasound system (Visual Sonics, Toronto, Canada). Investigators were blinded to genotype and treatment groups. Mice were anesthetized with 1.5% isoflurane and mouse hearts were viewed on two-dimensional short-axis planes and measured using M-mode. Heart rate, left ventricular internal diameter, interventricular septal, and posterior wall (PW) dimensions were measured at end systole and end diastole for all three cardiac cycles. Fractional shortening (FS) were calculated using the average of FS measurements of the left ventricular contraction for five cardiac cycles.

**Quantitative reverse transcription PCR (qRT-PCR).** Total RNAs were extracted from ventricles or cultured CMs using Trizol (Life Technologies). One microgram of total RNAs was reverse transcribed to cDNA using a Superscript II reverse transcriptase kit (Life Technologies). qRT-PCR was performed using Power SYBR Green PCR Master Mix (Life Technologies) on a Bio-Rad CFX96 Fast Real-Time PCR System. Specific primers were listed in Supplementary Table 1. The relative expression of each gene was normalized to the expression of Gapdh and calculated using the 2−ΔΔCt method. Biological replicates were performed using three individual samples of each genotype and technical triplicates were carried out for each run of qPCR.

** Luciferase reporter gene assay.** Transcriptional suppression of p21 via the p21-RE1-pGL3 enhancer by REST was evaluated using luciferase reporter gene assay as described previously<sup>20</sup>. DNA fragment of p21 gene with or without mutation or deletion of the p21-RE1 was PCR cloned into a pGL3 enhancer luciferase reporter vector (Supplementary Table 1). All constructs were confirmed by Sanger sequencing. 0.8 µg of each p21-RE1-pGL3 enhancer construct was co-transfected with 0.2 µg of pRL-SV40 vector (Promega, E2231) into cultured P1 CMs in 96-well plates using Lipofectamine 3000 Reagents (Invitrogen). After 24 h transfection, cell lysates were processed for luciferase activity using the luciferase reporter system (Promega, E1980). Luciferase reporter activity analyses were calculated as firefly luciferase normalized to Renilla luciferase values. Biological triplicates were performed in technical triplicates for each vector.

**Immunostaining.** Immunostaining was performed on sections of paraffin-embedded embryos or hearts, as previously described<sup>29</sup>. Embryos or hearts were collected at the indicated stages and fixed in 4% PFA for 2 h, rinsed in PBS, dehydrated in 15 and 30% sucrose sequentially, embedded in optimal cutting temperature (OCT) compound and sectioned at 8-µm thickness. Heart sections were blocked with 5% horse serum for 1 h at room temperature before being incubated overnight at 4 temperature with the single or double staining with following antibodies diluted in blocking solution: Trt (T) (Fisher Scientific, MS-295-P, 1:100), PECAM1 (Santa, sc-5060, 1:1000), REST (Antibodies-online, ABIN747683, 1:1000), and Kfl7 (Abcam, ab66155, 1:200), P21 (Abcam, ab199199, 1:1000), cleaved caspase-3 (CST, mAb #9664, 1:200), phosphorylated-histone3 (pH3) (Abcam, ab32107, 1:200), or aurora B (Abcam, ab22541, 1:100). After three
washes with PBS for 5 min each, samples were stained for 1 h at room temperature with fluorescent secondary antibodies followed by DAPI (4′,6-diamidino-2-phenylindole, hydrochloride) staining for nuclei visualization. Images were acquired and analyzed using a Zeiss fluorescence microscope with Axiosview image analysis software. For paraffin sections, 6-μm tissue sections were de-waxed in xylene, rehydrated in descending grades of alcohol and antigen-retrieved by incubating in sodium citrate for 10 min at 95 °C. After blocked with 5% horse serum for 60 min, sections were incubated with antibodies against NXX2.5 (Santa, sc-14033, 1:500) overnight at 4 °C, followed by incubation with biotinylated secondary antibodies (Vectorstain, PK-6100) for 1 h and stained with DAB (Vector, SK-4100). Counterstaining with hematoxylin was used for visualization of nuclei. All immunos- taining were performed on four sections per sample, and total four samples of each genotype and treatment group were experimented. Images were taken from six fields per section, processed using ImageJ software and counted blindly to genotype and treatment groups.

**EdU incorporation.** Proliferative cells were pulse labeled for 2 h by intraperitoneal injection of mice with 5-ethyl-2′-deoxyuridine (EdU, 100 μg/kg). Hearts were then collected, fixed in 4% PFA, rinsed in PBS, soaked in 15 and 30% sucrose sequentially, embedded in OCT, and sectioned at 8-μm thickness. Sections were stained with antibodies against TnT (Fisher Scientific, MS-295-P-1, 1:400) or REST (Antibodies-online, ABIN747683, 1:400), followed by EdU staining (Click-iT® EdU Alexa Fluor® 488 Imaging Kit, Life Technologies) and DAPI counterstaining (Vector lab, H12000). Stained sections were photographed under a Zeiss microscope, and images were processed for quantitative analysis as described above.

**Flow cytometry.** P3 ventricles of Rest knockout (Restm119) mice and littermate controls of both sexes were digested into single cell suspension, fixed with 70% ethanol. CMs were stained with TnT antibodies (Fisher Scientific, MS-295-P-1, 1:400) for 2 h at room temperature and then incubated with secondary antibodies (Alexa Fluor™ 488 Tyramide SuperBoost™ Kit, goat anti-mouse IgG). Cell pellets were re-suspended in FcXtreme™ P/I/NAse Staining Solution (Fisher Scientific, FI0797) and analyzed on a FACStar Plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ). An average of 10^5 CMs (FITC+) were collected in the gate from total cells per ventricle using forward and side scatter. We used pulse width versus pulse area as first gate on the single cell population and then applied this gate to the scatter plot to gate out cell debris. The combined gates were applied to the PI histogram plot to obtain the percentage of cells in each cell cycle phase.

**Apoptosis assay.** Apoptotic cells were visualized by TUNEL assay. Fresh sections were stained with TnT antibodies (Fisher Scientific, MS-295-P-1, 1:400) followed by TUNEL staining using a DeadEnd™ Fluorometric TUNEL kit (Promega). After DAPI counterstaining, sections were photographed under a Leica confocal microscope. Stained sections were photographed, and images were processed for quantitative analysis as described above.

**Statistics.** No statistical methods were used to predetermine sample size. Student’s t test (two-tailed) or one-way ANOVA analysis followed by Neuman-Keuls, Sidak’s, or Bonferroni test was used for statistical difference between or among groups. The relationship between REST expression and cell proliferation during cardiac regeneration was analyzed by Pearson correlation coefficient. Survival rate of mice at various stages was calculated using a Kaplan–Meier test. Normality was assumed and variance was computed between or among groups. Sample size was determined based upon previous experience in the assessment of experimental variability. The investigators were not involved to the group allocation during experiments and outcome assessment, unless stated otherwise. We chose the adequate statistic test according to the data distribution to fulfill all statistic assumptions. Statistical comparison for all qualitative assays was performed in at least three independent experiments. All numerical data were presented as mean ± s.d. and p value of <0.05 was considered as significant. Statistical analyses were performed using the SPSS v16.0 software (SPSS Inc., Chicago, IL).

**Data availability.** Data supporting the findings of this study are available within the articles and its supplementary information files. All relevant data are available from the authors on reasonable request. The RNA Seq data have been downloaded from the NIH GEO database with accession code GSE80378.

Received: 4 June 2017 Accepted: 13 November 2017 Published online: 07 December 2017

**References**

1. Soufan, A. T. et al. Regionalized sequence of myocardial cell growth and proliferation characterizes early chamber formation. *Circ. Res.* **99**, 545–552 (2006).

2. von Gise, A. et al. YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *Proc Natl Acad. Sci. USA* **109**, 23859–23864 (2012).

3. Pasumarthi, K. B. & Field, L. J. Cardiomyocyte cell cycle regulation. *Circ. Res.* **90**, 1044–1054 (2002).

4. Porrello, E. R. et al. Transient regenerative potential of the neonatal mouse heart. *Science* **331**, 1078–1080 (2011).

5. Soonpaa, M. H., Kim, K. K., Pajak, L., Franklin, M. & Field, L. J. Cardiomyocyte DNA synthesis and binucleation during murine development. *Am. J. Physiol. Cell. Physiol.* **271**, H2183–H2189 (1996).

6. Porrello, E. R. et al. Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. *Proc. Natl Acad. Sci. USA* **110**, 187–192 (2013).

7. Uygun, A. & Lee, R. T. Mechanisms of cardiac regeneration. *Dev. Cell* **36**, 237–247 (2016).

8. Bergmann, O. et al. Dynamics of cell generation and turnover in the human heart. *Cell* **161**, 1566–1575 (2015).

9. Soonpaa, M. H. et al. Cardiomyocyte cell-cycle activity during Preadolescence. *Circ. Res.* **99**, 1488–1496 (2006).

10. Walsh, S., Ponten, A., Fleischmann, B. K., Hovinge, S. Cardiomyocyte cell cycle control and growth estimation in vivo—analysis based on cardiomyocyte nuclei. *Cardiovasc. Res.* **86**, 365–370 (2010).

11. Xin, M., Olson, E. N. & Rassl, Stub, M. Mending, broken hearts: cardiac development as a basis for adult heart regeneration and repair. *Nat. Rev. Mol. Cell Biol.* **14**, 329–541 (2013).

12. Kikuchi, K. et al. Primary contribution to zebrafish heart regeneration by gata4 (c) cardiomyocytes. *Nature* **464**, 453–456 (2010).

13. Xiang, F. L., Guo, M. S., Futey, K. E. Overexpression of Tbx20 in Adult cardiomyocytes gene expression and improves cardiac function after myocardial infarction. *Circulation* **133**, 1081–1092 (2016).

14. Hang, C. et al. Cardiomyocyte regulation by Brg1 underlies heart muscle development. *Nature* **466**, 62–67 (2010).

15. Heiden, T. Hippo pathway inhibits Wnt signaling to restrain cardiomyocyte proliferation and heart size. *Science* **332**, 458–461 (2011).
34. Malek Mohammadi, M. et al. The transcription factor GATA4 promotes myocardial regeneration in neonatal mice. EMBO Mol. Med. 9, 265–279 (2017).
35. Senyo, S. E. et al. Mammalian heart renewal by pre-existing cardiomyocytes. Nature 493, 433–436 (2013).
36. Wang, Q., Reiter, R. S., Huang, Q. Q., Jin, J. P. & Lin, J. J. Comparative studies on the expression patterns of three troponin T genes during mouse development. Anat. Rec. 263, 72–84 (2001).
37. Wu, B. et al. Inducible cardiomyocyte-specific gene disruption directed by the rat Tnnt2 promoter in the mouse. Genesis 48, 63–72 (2010).
38. Zhang, D. et al. Homocysteine upregulates soluble epoxide hydrolase in vascular endothelium in vitro and in vivo. Circ. Res. 110, 808–817 (2012).
39. Wu, B. et al. Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling. Cell 151, 1083–1096 (2012).

Acknowledgements
We thank B. Morrow, R.N. Kitsis, and N.G. Frangogiannis for critically reading the manuscript. We also thank T. Fless and T. Neshipurova for RestG/rTGT mice. This work was supported by the National Institutes of Health/National Heart, Lung, and Blood Institute grants (R01HL116997 to B.Z.; R01HL133120 to B.Z. and De.Z.; R01HL131735 to C.C.), and an American Heart Association grant (16POST30480021 to Do.Z.; 15GRNT25710153 to C.C.).

Author contributions
Do.Z., Y.W., P.L., X.Y., and B.W. performed most of the experiments. P.W. and De.Z. helped RNA-sequence data analysis. J.Y. and C.C. generated TnTMerCreMer mice. Do.Z. and B.Z. conceptualized the project. B.W. and B.Z. supervised the experiments. C.-P.C. offered help in data analysis and manuscript revision. Do.Z. and B.Z. wrote the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-017-02210-y.

Competing interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.