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CircRNA Samd4 induces cardiac repair after myocardial infarction by blocking mitochondria-derived ROS output

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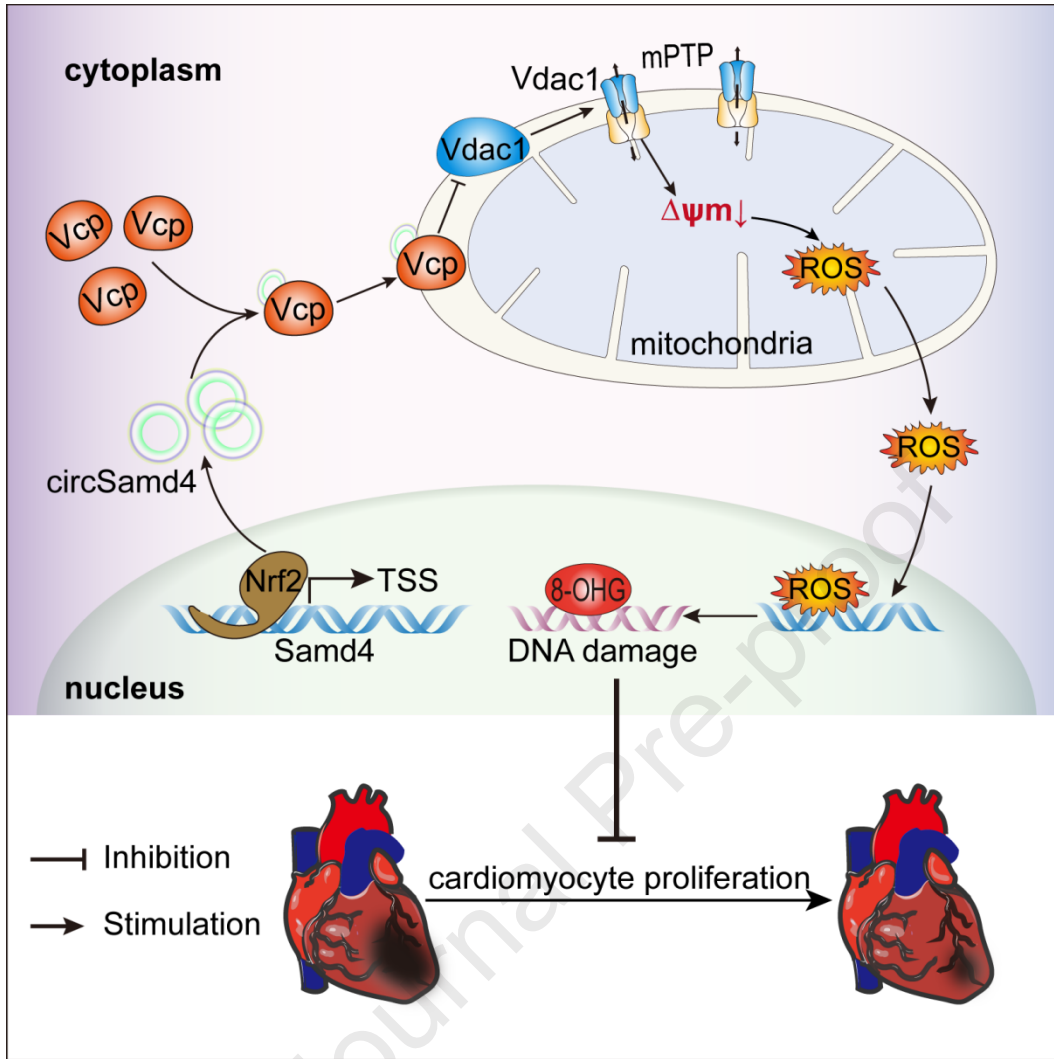
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2 **mitochondria-derived ROS output**

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20 **Running title:** Overexpression of circRNA Samd4 induces cardiac regeneration.

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1 Abstract

2 Reactive oxygen species (ROS) derived from oxygen-dependent mitochondrial
3 metabolism are the essential drivers of cardiomyocyte (CM) cell cycle arrest in
4 adulthood. Mitochondria-localized circular RNAs (circRNAs) play important roles in
5 regulating mitochondria-derived ROS production, but their functions in cardiac
6 regeneration are still unknown. Herein, we investigated the functions and underlying
7 mechanism of mitochondria-localized circSamd4 in cardiac regeneration. We found
8 that circSamd4 was selectively expressed in fetal and neonatal CMs. The transcription
9 factor Nrf2 controlled circSamd4 expression by binding to the promoter of circSamd4
10 host gene. CircSamd4 overexpression reduced while circSamd4 silencing increased
11 mitochondrial oxidative stress and subsequent oxidative DNA damage. Moreover,
12 circSamd4 overexpression induced CM proliferation and prevented CM apoptosis,
13 which reduced the size of the fibrotic area and improved cardiac function after
14 myocardial infarction (MI). Mechanistically, circSamd4 reduced oxidative stress
15 generation and maintained mitochondrial dynamics by inducing the mitochondrial
16 translocation of the Vcp protein, which downregulated Vdac1 expression and
17 prevented the mitochondrial permeability transition pore (mPTP) from opening. Our
18 findings suggested that circSamd4 is a novel therapeutic target for heart failure after
19 MI.

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22

1 **Introduction**

2 Reactive oxygen species (ROS) signaling is a critical regulator of cardiac regeneration
3 that potentially offers innovative strategies for preventing or treating heart failure after
4 myocardial infarction (MI). One of many factors shared by organisms capable of
5 cardiac regeneration is a hypoxic environment accompanied by relatively low reactive
6 oxygen species (ROS) levels in cardiomyocytes (CMs).¹ In contrast, adult mammalian
7 CMs exist in an oxygen-rich environment, which triggers an increase in intracellular
8 ROS production.² Excessive ROS are thought to cause cellular toxicity by inducing
9 oxidative damage to DNA, such as oxidized bases and DNA strand breaks, leading to
10 adult CM cell cycle arrest and even CM death or senescence. The systemic
11 scavenging of ROS or DNA damage response DDR (DDR) inhibition has been
12 confirmed to induce postnatal CM cell cycle re-entry, indicating the therapeutic
13 potential of targeting ROS metabolism to induce cardiac repair.^{2,3} However, clinical
14 studies on general antioxidant therapies have failed to reveal any beneficial effects in
15 that regard. Thus, it is necessary to explore novel targets for antioxidant therapy to
16 effectively restore cardiac function and prevent heart failure post-MI.

17 It is known that intracellular ROS are generated in multiple compartments via the
18 contributions of various macromolecules. Mitochondria, the core organelles of cell
19 metabolism and redox balance, are a major source of ROS production in adult CMs.⁴
20 Nearly 90% of ROS, including mitochondrial superoxide, which is believed to be
21 largely responsible for DNA damage, are estimated to be generated by the leakage of
22 electrons from mitochondria. Excessive mitochondrial ROS generation in CMs has

1 been causally linked to cell cycle arrest and disease pathophysiology. Interestingly,
2 accumulating evidence shows that other sources of ROS could also play protective
3 and signaling roles in the heart. A recent study reported that nicotinamide adenine
4 dinucleotide phosphate (NADPH) oxidases from the epicardium produce H_2O_2 to
5 recruit inflammatory leukocytes during heart regeneration.⁵ Additionally,
6 NADPH-induced oxidative stress in endothelial cells mediates Vegf-induced
7 endothelial cell activation and angiogenesis.⁶ The different functions of mitochondrial
8 ROS and other sources of ROS indicate that precisely scavenging
9 mitochondria-derived ROS might improve the efficacy of antioxidant therapy and
10 promote cardiac regeneration. Despite the clinical significance, scavengers targeting
11 mitochondria-derived ROS for cardiac regeneration are still lacking.

12 Circular RNAs (circRNAs) are a newly identified class of noncoding RNAs that
13 circularize by joining at their 3' and 5' ends.⁷ It is known that circRNAs are more
14 likely to be stable than their parent linear transcripts due to their structure.⁸ Emerging
15 studies have proposed that circRNAs have fundamental roles in physiological and
16 pathophysiological processes in the heart. Several circRNAs have also been reported
17 to regulate CM cell cycle progression, apoptosis and survival by affecting oxidative
18 stress injury after MI.^{9,10} Recent studies have demonstrated that
19 mitochondria-localized circRNAs act as key regulators of mitochondrial function and
20 dynamics. They also regulate mitochondrial ROS production by controlling
21 mitochondrial membrane potential, indicating their potential role in alleviating the
22 mitochondrial ROS burden.^{11,12} Moreover, these regulators tend to have few effects on

1 nonmitochondrial ROS due to their special localization in cells.¹¹ Therefore, targeting
2 mitochondria-localized circRNAs might be an attractive strategy for promoting
3 cardiac regenerative repair due to their unique effect on mitochondrial ROS. Herein,
4 we hypothesized that mitochondria-localized circRNAs highly expressed in neonatal
5 CMs promote cardiac regeneration by precisely scavenging mitochondria-derived
6 ROS in CMs.

7 In line with this hypothesis, we first identified the mitochondria-localized circRNA
8 Samd4 (circSamd4) as an important regulator of mitochondrial oxidative stress in
9 CMs. Crucially, circSamd4 overexpression increased CM proliferation and induced
10 structural and functional recovery after MI. Our results indicate that circSamd4 may
11 serve as a valuable therapeutic target for promoting heart regenerative repair and
12 improving the outcome after MI.

13 14 **Results**

15 **CircSamd4 is located in CM mitochondria and involved in the antioxidant** 16 **response.**

17 It is known that the heart of neonatal mouse possesses the ability to regenerate lost
18 myocardium, partially mediated by the activation of antioxidant response.³ The
19 comparison of transcriptome between neonatal CMs and adult CMs is likely to
20 uncover the antioxidant factors involved into cardiac regeneration. To identify the
21 potential circRNAs associated with antioxidant response during cardiac regeneration,
22 we used previously published transcriptome datasets from mammalian hearts¹³ and

1 selected 87 circRNAs that are homologously conserved and have more than 30 reads
2 supporting a circular junction (Supplemental Fig. 1A). Among them, 13 circRNAs
3 were differentially expressed in neonatal and adult mouse hearts (Fig. 1A). The
4 distinct tendencies of circular and linear transcripts suggest the important biological
5 roles of circRNAs in cells.¹⁴ We further filtered 4 circRNAs with expression profiles
6 that were opposite those of the linear transcripts, including circSamd4, circHipk3,
7 circRyr2 and circStrn3 (Fig. 1A). With the combination of differential expression
8 between adult and neonatal hearts, we speculated these 4 circRNAs were involved
9 into antioxidant response during cardiac regeneration. Accordingly, we detected their
10 expression in CMs treated with H₂O₂. We found that circSamd4 and circRyr2 were
11 upregulated in CMs after treatment with 20 μM H₂O₂ (Fig. 1B). We also investigated
12 the expression profiles of these circRNAs. Notably, qRT-PCR analyses showed that
13 compared to other circRNAs, circSamd4 and circRyr2 were highly expressed in
14 mouse hearts (Fig. 1C, Supplemental Fig. 1B-1D). Moreover, compared to the other
15 circRNAs, circSamd4 was the most abundant circRNA in the mouse heart
16 (Supplemental Fig. 1E). We also investigated the expression of these circRNAs in
17 different types of heart cells, revealing that circSamd4 and circRyr2 were highly
18 expressed in CMs (Supplemental Fig. 1F). In addition, we isolated cytosol, nucleus
19 and mitochondria fraction, and then investigated the subcellular distribution of
20 circSamd4 in CMs by qRT-PCR assays. CircHipk3, circRyr2 and circStrn3 was also
21 detected in cytoplasm and mitochondria extract of CM. Interestingly, we revealed the
22 predominant expression of circSamd4 in the mitochondria of CMs (Fig. 1D,

1 Supplemental Fig. 1G). We also performed *in situ* hybridization staining of circSamd4,
2 circHpk3, circRyr2 and circStrn3 in CMs *ex vivo*. It was observed the colocalization
3 of circSamd4 and mitochondria in CMs (Fig. 1E, Supplemental Fig. 1H-1I). The *in*
4 *situ* hybridization staining of circSamd4 in heart tissue also revealed the
5 colocalization of circSamd4 and mitochondria (Supplemental Fig. 1J). These results
6 suggest that circSamd4 is a mitochondria-localized circRNA and that its expression is
7 associated with the antioxidant response in CMs. We subsequently focused on
8 circSamd4 for detailed expression profiling and functional investigation.

9 CircSamd4 is derived from the third exon of the Samd4 gene (Supplemental Fig. 2A).

10 The sequence of circSamd4 is highly conserved among the human, mouse and rat
11 genomes (Supplemental Fig. 2A-2C). The circRNA ID of circSamd4 in the circBase
12 database (<http://www.circbase.org/>) is mmu_circ_0000529. The predicted head-to-tail
13 junction of circSamd4 was validated in CMs by a PCR assay and subsequent Sanger
14 sequencing (Fig. 1F, Supplemental Fig. 2A). RNase R digestion analysis also
15 supported the circularized structure of circSamd4 (Fig. 1G). In accordance with the
16 RNA-seq results, qRT-PCR assays confirmed that circSamd4 expression is
17 significantly decreased during heart development (Fig. 1H). We then isolated CMs
18 from P1, P7 and adult hearts and found that the circSamd4 expression in P1 CMs was
19 higher than that in P7 or adult CMs (Fig. 1I). Furthermore, cardiac circSamd4
20 expression was validated in rats and mice of different ages via ISH assays. We found
21 that circSamd4 expression was approximately 3-fold higher in the neonatal stage than
22 in the adult stage (Fig. 1J). These results also indicate that the expression level of

1 circSamd4 might be dynamically downregulated in CMs over time.

2 It is known that posttranslational modifications of histones mediate several important
3 biological processes, including chromatin modification, which affects the expression
4 or repression of target genes.¹⁵ The dynamics of H3K27ac in the DNA regulatory
5 element are a fundamental issue controlling the precise timing and level of gene
6 transcription. Moreover, a previous study revealed that H3k27ac could be a marker
7 for active promoters, which supports the transcriptional process and the high
8 expression of circRNAs.¹⁶ Consistent with the reduced circSamd4 expression in the
9 myocardium with age, both ChIP-seq and ChIP-PCR assays showed that the levels of
10 the active histone marker H3k27ac at the Samd4 promoter were obviously decreased
11 (Fig. 1K-1L). A previous study confirmed that the transcription factor Nrf2 is
12 involved in the antioxidant response during cardiac repair.³ qRT-PCR analysis
13 revealed that circSamd4 was downregulated in CMs after Nrf2 knockdown (Fig. 1M).
14 We also detected the changes of circSamd4 pre-mRNA and Samd4 mRNA expression
15 after Nrf2 knockdown (Figure 1M). We found that Nrf2 knockdown significantly
16 reduced the expression level of circSamd4 pre-mRNA and Samd4 mRNA, which
17 indicated that Nrf2 might bind to the promoter of circSamd4 host gene and controls
18 the Samd4 transcription, thereby regulating the expression the circSamd4 indirectly.

19 Bioinformatics analysis using the JASPAR program (<http://jaspar.genereg.net>)
20 revealed two binding sites of Nrf2 on the circSamd4 promoter (Fig. 1N). ChIP-PCR
21 assays further confirmed the interaction between Nrf2 and the circSamd4 host gene
22 promoter (Fig. 1O). Then, we detected the expression level of Nrf2 in CMs of

1 different developmental stages. The Western blot results revealed that the Nrf2
2 expression level was significantly higher in P1 CMs than in P7 or adult CMs
3 (Supplemental Fig. 3A). To further investigate the effect of Nrf2 overexpression,
4 AAV9-Nrf2 was injected into the myocardial tissue of adult mice (Supplemental Fig.
5 3B). Nrf2 overexpression increased the circSamd4 expression and the extent of CM
6 proliferation (Supplemental Fig. 3C-3E). These results suggest that circSamd4
7 expression is controlled by Nrf2.

8

9 **CircSamd4 reduces oxidative stress and promotes CM proliferation *in vitro*.**

10 To explore the effect of circSamd4 on the antioxidant response, we designed two
11 siRNAs specifically targeting the circSamd4 back-splicing site. qRT-PCR assays
12 confirmed that the transfection of the siRNAs significantly reduced circSamd4
13 expression (Supplemental Fig. 4A), while had no obvious effect on Samd4 mRNA
14 expression. The intracellular ROS production in CMs transfected with circSamd4
15 siRNAs was significantly upregulated compared with that in CMs transfected with the
16 control siRNA (Fig. 1P). We also quantified cellular oxidative stress by DCFH-DA
17 assay using flow cytometry, and the results also revealed that circSamd4 knockdown
18 increased oxidative stress in CMs (Supplemental Fig. 4B). ROS levels have been
19 shown to influence the expression of key genes involved in regulating cellular and
20 systemic oxidative stress.¹⁷ We performed QRT-PCR assays to investigate the
21 expression of these genes after circSamd4 knockdown, including Sod1, Sod2, Ucp3,
22 Catalase, Ant1 and Gpx1. We found that circSamd4 knockdown significantly

1 increased the expression of these genes (Supplemental Fig. 4C). Moreover, western
2 blotting assay and immunofluorescence assay was used to detect pATM, an index of
3 DNA damage caused by oxidative stress. It was shown that circSamd4 knockdown
4 also significantly increased pATM level (Supplemental Fig. 4D-4F).
5 Immunofluorescence assay showed that another index of DNA damage, 8-OHG, was
6 also significantly elevated in CMs after circSamd4 down-regulation (Supplemental
7 Fig. 4G). Previous studies demonstrated that mitochondria-localized circRNAs play
8 important roles in mitochondrial functions and dynamics.¹¹ We also found an
9 increased mitochondria-derived ROS level after circSamd4 downregulation in CMs
10 (Fig. 1Q). We then investigated the effect of circSamd4 downregulation on mPTP
11 opening, which is crucial for MMP maintenance and ROS generation in CMs. We
12 recorded the mPTP opening after circSamd4 downregulation *in vitro* by detecting
13 alterations in the JC-1 fluorescence signal over time (Supplemental Fig. 5A). The
14 opening status of the mPTP was also assessed by the calcein-AM/CoCl₂ method
15 using confocal microscopy (Supplemental Fig. 5B). Downregulation of circSamd4
16 promoted mPTP opening in CMs (Supplemental Fig. 5A-4B). We also transduced
17 CMs with an adenovirus (Adv) vector to overexpress circSamd4 (Supplemental Fig.
18 6A and 6B). Northern blot analysis was performed to prove the successful formation
19 of the back-splicing junction after transfection of circSamd4 overexpression vector. It
20 was observed that Adv-circSamd4 transfection increased circSamd4 that was resistant
21 to RNase R in CMs (Supplemental Fig. 6C). We also performed PCR amplification
22 using the divergent primers, and the products were detected by agarose gel

1 electrophoresis. The results also indicated the successful formation of back-splicing
2 junction after circSamd4 overexpression plasmid transfection (Supplemental Fig. 6D).
3 Functionally, circSamd4 overexpression significantly decreased the level of total
4 cellular ROS as well as mitochondria-derived ROS production (Fig. 2A-2C). We also
5 examined the mitochondrial DNA content in CMs after circSamd4 overexpression.
6 qRT-PCR assays showed that the value obtained by dividing the mitochondrial DNA
7 copy number by the nuclear DNA copy number was increased after H₂O₂ exposure,
8 which was reversed by circSamd4 overexpression (Fig. 2D). Next, we detected the
9 mitochondrial crista density, a predictor of the oxygen uptake rate and metabolic
10 power in mitochondria.¹⁸ Transmission electron microscopy revealed that the
11 mitochondrial crista density was increased after H₂O₂ exposure but decreased after
12 circSamd4 overexpression (Fig. 2E). It is widely known that ROS are likely to cause
13 widespread damage to nucleic acids. We therefore assessed and quantified oxidative
14 DNA base modifications by detecting 8-OHG. We found that the nuclear 8-OHG level
15 was significantly increased when CMs were treated with H₂O₂ and decreased by
16 ectopic circSamd4 expression (Fig. 2F). In concert, phosphorylated ataxia
17 telangiectasia mutated (pATM), an index of DDR activation, was lower in the CMs
18 overexpressing circSamd4 in combination with H₂O₂ than in those exposed to H₂O₂
19 only (Fig. 2G). H₂O₂ induced mPTP opening and decreased the MMP, which was
20 reversed by circSamd4 overexpression (Fig. 2H, Supplemental Fig. 6E). Moreover,
21 TUNEL staining, trypan blue exclusion and flow cytometry analysis of Annexin
22 V-FITC/PI staining further indicated that circSamd4 overexpression could mitigate

1 H₂O₂-induced apoptosis (Supplemental Fig. 6F-6H). These findings indicate that
2 circSamd4 reduces mitochondrial ROS generation and protects CMs from oxidative
3 injury.

4 Next, we assessed the effect of circSamd4 on CM proliferation by detecting several
5 cell cycle markers, including EdU, Ki67, pH3 and Aurora B. First, we explored
6 whether circSamd4 is required for P1 CM proliferation *in vitro*. We found that
7 circSamd4 knockdown decreased the proportion of P1 CMs expressing EdU, Ki67
8 and pH3 (Supplemental Fig. 7A-7F). Then, we investigated whether circSamd4
9 overexpression could protect CMs from cell cycle arrest under oxidative stress. H₂O₂
10 significantly decreased the proportion of P1 CMs expressing EdU, Ki67, pH3 and
11 Aurora B, while circSamd4 overexpression reversed this repression (Fig. 3A-3D).
12 Importantly, the mitochondrial dye tetramethylrhodamine ethyl ester (TMRE) was
13 used to capture time-lapse images of CMs after treatment. In the H₂O₂-only group,
14 CMs hardly underwent cell division. In contrast, CMs overexpressing circSamd4 in
15 combination with H₂O₂ underwent karyokinesis and cytokinesis (Fig. 3E,
16 Supplemental Videos 1-4). Additionally, flow cytometry assays revealed that H₂O₂
17 prevented P1 CMs from entering the S and G2/M phases, while circSamd4 increased
18 the percentage of CMs in the S and G2/M phases (Fig. 3F). CircSamd4 also reversed
19 the negative effect of Nrf2 downregulation on CM proliferation (Supplemental Fig.
20 3F-3G). Moreover, circSamd4 overexpression significantly increased the proportion
21 of P7 CMs expressing EdU, Ki67, pH3 and Aurora B (Supplemental Fig. 8A-8D).

22 The Adv vectors encoding for Samd4 mRNA and Samd4 pre-mRNA fragment were

1 also used as the control. After transfection of these vectors, we detected circSamd4
2 and Samd4 mRNA level (Supplemental Fig. 9A). It was observed that vector
3 encoding for circSamd4 specially overexpressed circSamd4 but not Samd4 mRNA,
4 and vector encoding for Samd4 mRNA specially overexpressed Samd4 mRNA
5 (Supplemental Fig. 9B). The vector with Samd4 pre-mRNA fragment containing
6 exon3 and its flanking sequences also successfully overexpressed circSamd4 but not
7 Samd4 mRNA (Supplemental Fig. 9B). Interestingly, we found that transfection of
8 vector for Samd4 pre-mRNA fragment could reduce intracellular oxidative stress and
9 increase cell cycle markers, while transfection of vector for Samd4 mRNA have no
10 significant effect (Supplemental Fig. 9C-9G). These results further indicated that
11 circSamd4, but not Samd4 mRNA, play an important role on antioxidative stress role
12 in cardiac regeneration.

13

14 **CircSamd4 overexpression induces adult CM proliferation *in vivo*.**

15 To investigate the pro-proliferative effect of circSamd4 *in vivo*, AAV9-circSamd4 was
16 delivered to the myocardial tissues of adult mice to overexpress circSamd4
17 (Supplemental Fig. 10A-10B). We also performed Northern blot analysis and found
18 that AAV9-circSamd4 infection increased circSamd4 that was resistant to RNase R in
19 heart tissue (Supplemental Fig. 10C). By detecting GFP- and cTnT-colocalized cells,
20 we found that AAV9 targeted CM with high efficiency and specificity, as determined
21 by an exclusive GFP signal in approximately 80% of cells that were assigned as a CM
22 by morphology and cTnT signals (Supplemental Fig. 10D). To further determine the

1 rate of AAV9 infection in non-CMs, we isolated CMs and non-CMs from adult mouse
2 hearts 4 weeks after AAV9-GFP injection, as described in a previous study.¹⁹
3 Immunostaining of cTnT, CD31 and Vimentin was used to determine the individual
4 cell types. The proportion of GFP+ CMs was approximately 40%, while that of GFP+
5 endothelial cells and fibroblasts was less than 10% (Supplemental Fig. 10E-10G). Our
6 results are consistent with previous studies reporting the CM-specific transduction of
7 AAV9.²⁰ To determine whether AAV9-circSamd4 induced CM-specific circSamd4
8 overexpression, we further detected circSamd4 expression in CMs and non-CMs
9 isolated from the AAV9-circSamd4 and AAV9-NC groups. The qRT-PCR assay
10 results confirmed that AAV9-circSamd4 specifically increased circSamd4 expression
11 in CMs but not in non-CMs (Supplemental Fig. 10H). Immunofluorescence assays
12 revealed that circSamd4 overexpression significantly increased the proportion of adult
13 CMs expressing proliferative markers, including Ki67, pH3 and Anillin both *in vivo*
14 and *ex vivo* (Fig. 4A-4C, Supplemental Fig. 11A-11C). Moreover, circSamd4
15 overexpression decreased the intracellular ROS level and the number of 8-OHG sites
16 (Fig. 4D-4E). Quantification analysis revealed an increase in the total number of CMs
17 after circSamd4 overexpression (Supplemental Fig. 11D). We detected heart
18 weight/body weight ratios at 4 weeks after the injection of AAV9-circSamd4 or
19 AAV9-NC. Consistently, circSamd4 overexpression significantly increased heart
20 weight/body weight ratios (Supplemental Fig. 11E). In addition, WGA staining
21 revealed that circSamd4 overexpression had no effect on cardiac hypertrophy (Fig.
22 4F).

1 On the basis of these *in vivo* results, we explored whether circSamd4 overexpression
2 induces adult cardiac repair after MI. We injected AAV9-circSamd4 and AAV9-NC
3 into the peri-infarcted areas of MI model animals to overexpress circSamd4
4 (Supplemental Fig. 12A). CircSamd4 overexpression significantly increased the
5 proportion of CMs expressing Ki67 and pH3 (Fig. 4G-4H). Moreover, upregulation of
6 circSamd4 reduced the intracellular ROS production, the number of 8-OHG sites and
7 the DDR in the CMs of the infarct border zone (Fig. 4I-4J, Supplemental Fig. 11B).
8 TUNEL staining revealed that circSamd4 also protected CMs from apoptosis
9 (Supplemental Fig. 11C). We also found that circSamd4 overexpression significantly
10 increased the heart weight/body weight ratios after MI (Supplemental Fig. 11D).
11 We also used AAV9 to overexpress Samd4 mRNA and Samd4 pre-mRNA fragment in
12 myocardium tissue. Similar to the results from Adv transduction, it was observed that
13 vector encoding for circSamd4 specially overexpressed circSamd4 but not Samd4
14 mRNA, and vector encoding for Samd4 mRNA specially overexpressed Samd4
15 mRNA (Supplemental Fig. 13A). The vector with Samd4 pre-mRNA fragment
16 containing exon3 and its flanking sequences also successfully overexpressed
17 circSamd4 but not Samd4 mRNA (Supplemental Fig. 13A). We subsequently studied
18 the effect of circSamd4 overexpression on cardiac function improvement post-MI. It
19 was found that AAV9 overexpressing circSamd4 and AAV9 overexpressing Samd4
20 pre-mRNA fragment significantly restored cardiac functional post-MI (Figure 5A and
21 Supplemental Figure 13B). Furthermore, quantitative assessment of cardiac perfusion
22 by PET/CT revealed that the infarct size in the AAV9-circSamd4 group was

1 significantly reduced compared to that in the control group at four weeks after MI
2 (Fig. 5B). Histological examination by Masson trichrome staining revealed that
3 compared to the control treatment, circSamd4 overexpression significantly reduced
4 the size of the cardiac fibrotic area after MI (Fig. 5C).²¹ TTC staining further
5 confirmed that the proportion of viable myocardium was higher in the
6 AAV9-circSamd4 group than in the AAV9-NC group (Fig. 5D).²²
7 Collectively, these results indicate that circSamd4 overexpression could inhibit
8 cardiac remodeling and improve the recovery of cardiac performance after MI.

10 **CircSamd4 is required for cardiac regeneration in neonatal mice.**

11 To determine the important role of circSamd4 in cardiac regeneration *in vivo*, we
12 injected Adv encoding an shRNA into the myocardia of P1 neonatal mice
13 (Supplemental Fig. 14A). By detecting GFP fluorescence in isolated CMs, we
14 confirmed that Adv infected the myocardial tissues of P1 neonatal mice with high
15 efficiency (Supplemental Fig. 14B). Both qRT-PCR assays and ISH assays confirmed
16 that compared with mice treated with Adv-shRNA-control, neonatal mice treated with
17 Adv-shRNA-CircSamd4 had successful downregulation of circSamd4 in the
18 myocardial tissues (Supplemental Fig. 14C-14D).

19 To determine whether the increase in ROS resulting from circSamd4 knockdown
20 contributed to damaged cardiogenesis, we administered an ROS inhibitor,
21 N-acetyl-L-cysteine (NAC), to circSamd4-knockdown neonates after MI. CircSamd4
22 knockdown led to the upregulation of oxidative stress and DNA damage levels in the

1 peri-infarcted zone of neonates (Fig. 6A and 6B). Accordingly, the number of
2 proliferative CMs in the peri-infarcted area was reduced in the
3 Adv-shRNA-circSamd4 group compared with the Adv-shRNA-control treated group
4 (Fig. 6C and 6D). Moreover, echocardiographic analysis showed that circSamd4
5 knockdown impaired cardiac functional recovery after MI (Fig. 6E). Masson
6 trichrome staining also revealed an increase in the size of the fibrotic area after
7 circSamd4 knockdown (Fig. 6F). Collectively, these findings demonstrate that
8 circSamd4 functions in endogenous cardiac regeneration by regulating the antioxidant
9 pathway.

10

11 **CircSamd4 reduces mitochondria-derived ROS by recruiting Vcp to the**
12 **mitochondria**

13 Mitochondria-derived circRNAs have been reported to regulate mitochondrial
14 function by interacting with proteins.¹¹ Thus, we conducted RNA pulldown assays to
15 detect potential circSamd4-binding proteins in CMs using a biotin-labeled
16 circSamd4-specific probe. The results of mass spectrometry analysis showed that Vcp
17 is the most abundant protein which has been reported to affect mitochondrial
18 functions in heart. Previous studies confirmed that it could be recruited to
19 mitochondria and participate in several mitochondrial functions, such as promoting
20 mitochondrial permeability transition pore (mPTP) opening, increasing mitochondrial
21 respiration capacity, leading to ATP synthesis and so on.^{23,24} Moreover, functional
22 Inhibition of Vcp induced cardiac dilation and dysfunction, indication the important

1 role of Vcp in maintaining cardiac function and homeostasis.²⁵ All things considered,
2 we speculated that Vcp might be the downstream protein of circSamd4. The following
3 western blotting assays confirmed that Vcp interacts with circSamd4 (Fig. 7A,
4 Supplemental Table 3). Moreover, RIP assays showed that anti-Vcp antibodies
5 enriched circSamd4 in CMs, further demonstrating the direct interaction between
6 circSamd4 and the Vcp protein (Fig. 7B). To identify the specific region of circSamd4
7 that directly bound to Vcp, various region of circSamd4 were subcloned into
8 pcDNA3.1 plasmid to generate missing constructs, which was separately transfected
9 into CMs (Supplemental Fig. 15A). RNA pull-down assays revealed that deletion of
10 circSamd4 184-320bp, but not circSamd4 47-133bp, 376-519bp, disrupted the
11 interaction between the circSamd4 and Vcp, indicating that circSamd4 184-320bp
12 may play a critical role in the circSamd4-Vcp bond (Supplemental Fig. 15A)

13 Next, we explored the biological effects of the interaction between circSamd4 and the
14 Vcp protein. First, we detected the expression level of Vcp after circSamd4
15 interference. qRT-PCR assays and Western blotting assays revealed that circSamd4
16 knockdown had no significant effect on the protein or mRNA expression of Vcp (Fig.
17 7C and 7D). Subsequently, we investigated whether circSamd4 affects the subcellular
18 location of the Vcp protein. H₂O₂ promoted the mitochondrial translocation of the
19 Vcp protein in CMs (Fig. 7E), which was consistent with the results of previous
20 studies.^{26,27} We found that circSamd4 overexpression promoted the mitochondrial
21 translocation of the Vcp protein, while circSamd4 silencing repressed this
22 phenomenon (Fig. 7F-7G and Supplemental Fig. 16), indicating that circSamd4 acted

1 as a mitochondrial recruitment factor for Vcp in CMs. Previous studies demonstrated
2 an important role of the Vcp protein in mitochondrial function and dynamics.^{26,28}
3 Indeed, once cells are subjected to DNA damage, cell cycle checkpoint pathways are
4 activated to arrest cells in the G1 or G2 phase.²⁹ Recent studies have revealed that
5 Wee1 is the key negative regulator of the G2-to-M transition and prevents cycle entry
6 into mitosis in response to ROS-induced DNA damage in CMs.² Inactivation of
7 Wee1-dependent signaling is sufficient to promote CM proliferation and cardiac
8 regeneration.² Thus, we hypothesized that Wee1 is the essential effector that functions
9 downstream of the circSamd4-induced antioxidant response and cardiac regeneration.
10 The Wee1 expression level might be negatively correlated with the oxidation status
11 and regenerative capacity of the heart. Importantly, we observed that the expression
12 level of Wee1 was also upregulated by circSamd4, which was reversed by a selective
13 ATP-competitive inhibitor of VCP, ML240 (Supplemental Fig. 15B). To explore the
14 role of Vcp, we overexpressed Vcp in CMs using an Adv vector and detected the Vcp
15 protein level (Supplemental Fig. 15C). The increase in the proportion of proliferative
16 CMs after Vcp overexpression further suggested a link between the Vcp protein and
17 CM proliferation (Fig. 7H and 7I). We also observed that Vcp overexpression
18 inhibited mPTP opening and MMP decline in CMs upon H₂O₂ treatment (Fig. 7J).
19 Moreover, we found that Vcp overexpression reduced the intracellular ROS levels and
20 subsequent DNA damage in H₂O₂-treated CMs (Fig. 7K and 7L, Supplemental Fig.
21 15D and 15E).

22 *In vivo* studies were then performed to determine the role of Vcp in

1 circSamd4-mediated cardiac repair. The administration of the Vcp inhibitor ML240
2 significantly reversed the circSamd4-induced reduction in oxidative stress and DNA
3 damage in the adult myocardium post-MI (Fig. 7M and 7N). Consistently, ML240
4 administration alleviated circSamd4-induced CM proliferation and fibrosis reduction
5 post-MI (Fig. 7O-7P and Supplemental Fig.17). In addition, we investigated the effect
6 of ML240 on neonatal CM and observed that the administration of ML240 promoted
7 mPTP opening in CMs *in vitro* (Supplemental Fig. 15F-15G). Furthermore, ML240
8 treatment *in vivo* increased ROS production, induced oxidative DNA damage, and
9 even repressed CM proliferation in the neonatal myocardium (Supplemental Fig.
10 15H-15K). Collectively, the effects of circSamd4 on CM proliferation and cardiac
11 regeneration are likely mediated by the Vcp protein.

12

13 **Vcp protein attenuates mPTP opening by decreasing Vdac1 expression**

14 Next, we further investigated the underlying mechanism by which Vcp regulates
15 mPTP opening in CMs. Previous studies demonstrated that mPTP opening and MMP
16 maintenance in CMs is regulated by Vdac1, a channel protein located in the outer
17 mitochondrial membrane.³⁰ We performed Co-IP and RIP assays using myocardial
18 tissues isolated from the border zones of infarcted hearts. Co-IP assays using an
19 antibody against Vcp revealed that Vcp bound to Vdac1 (Supplemental Fig. 18A), and
20 the subsequent Co-IP assay using an antibody targeting Vdac1 also showed an
21 interaction between Vdac1 and Vcp (Supplemental Fig. 18B). The following RIP
22 assays using an antibody targeting Vcp revealed that Vcp effectively

1 immunoprecipitated with circSamd4 (Supplemental Fig. 18C). These results
2 confirmed the ternary interaction of circSamd4/Vcp/Vdac1 in the peri-infarcted zone.
3 As Vdac1 was reported to be degraded in a ubiquitin-dependent manner, we assumed
4 that Vcp might promote the degradation of the Vdac1 protein. We assessed the
5 expression level of Vdac1 after Vcp or circSamd4 interference. As expected, Western
6 blotting assays showed that Vcp or circSamd4 overexpression decreased Vdac1
7 expression (Fig. 8A, Supplemental Fig. 18D), while inhibition of Vcp or circSamd4
8 downregulation increased Vdac1 expression (Fig. 8B, Supplemental Fig. 18E).
9 Moreover, the application of the Vcp inhibitor ML240 reversed the effect of
10 circSamd4 overexpression on Vdac1 (Fig. 8C). We also performed qRT-PCR assays
11 to assess the mRNA expression of Vdac1 in CMs transduced with Adv-Vcp or
12 Adv-NC. The Vdac1 mRNA levels did not significantly differ between the Adv-Vcp
13 group and Adv-NC group, indicating that Vcp overexpression had no effect on the
14 transcription of Vdac1 (Supplemental Fig. 18F). Next, we investigated the effect of
15 Vdac1 on MMP and mPTP opening, which was determined by the JC-1 fluorescence
16 signal. We found that treatment with VBIT-12, a VDAC1 oligomerization inhibitor,³¹
17 suppressed mPTP opening and the decrease in the MMP (Fig. 8D-E). Additionally,
18 VBIT-12 treatment reduced the DDR (Fig. 8F-G) and promoted CM proliferation (Fig.
19 8H-I). Moreover, VBIT-12 treatment reversed the inhibitory effect of ML240
20 treatment on mPTP opening, ROS generation, oxidative DNA injury and CM
21 proliferation (Fig. 8J-N). Taken together, these results demonstrate that the
22 circSamd4/Vcp/Vdac1s pathway plays an essential role in reducing oxidative stress

1 and inducing CM proliferation (Fig. 8O).

2 We also performed RNA-seq to understand the global effect of circSamd4
3 overexpression. The expression levels of 189 genes were significantly increased and
4 those of 616 genes were significantly reduced after circSamd4 overexpression
5 (Supplemental Fig. 19A, 19B and Table 4). EnrichedGO analysis of these
6 differentially upregulated genes revealed processes related to cell proliferation, such
7 as the cell cycle, cell division and mitosis (Supplemental Fig. 19C). Further qRT-PCR
8 assays validated the expression levels of several genes involved in cell cycle
9 progression (Supplemental Fig. 19D). The results of RNA-seq analysis also
10 demonstrated the important role of circSamd4 in inducing CM proliferation.

11

12 **Discussion**

13 In this study, we originally proposed a novel antioxidant therapeutic strategy to
14 achieve cardiac regeneration by targeting mitochondria-localized circSamd4 in CMs.
15 *In vivo* and *in vitro* studies demonstrated that circSamd4 synergistically promoted CM
16 proliferation and CM apoptosis inhibition, thereby effectively restoring cardiac
17 function and reducing the size of the fibrotic area after MI. These findings suggested
18 that circSamd4 is a valuable therapeutic target to improve the outcome of MI.

19 The current study revealed the potential of circSamd4 as a crucial regulator of cardiac
20 regeneration by alleviating mitochondrial oxidative injury. Mitochondria-derived ROS
21 are thought to be important signals that block CM cell cycle reentry in adulthood.² We
22 confirmed the mitochondrial localization of circSamd4 and found that circSamd4

1 exerted a marked effect on maintaining the mitochondrial membrane potential and
2 preventing mPTP opening. By scavenging CM mitochondrial ROS, circSamd4
3 overexpression reduced oxidative DNA damage and subsequent DDR pathway
4 activation while increasing DNA synthesis and mitosis markers in CMs both *in vitro*
5 and *in vivo*. The proliferative rate of CMs was similar to that seen in previous studies
6 using other strategies, indicating the efficacy of mitochondria-based strategies.²
7 Moreover, we found that circSamd4 overexpression prevented CMs from undergoing
8 apoptosis, which is also essential for regenerative repair after MI. The beneficial
9 effects of circSamd4 overexpression work together to decrease the size of the infarct
10 area and restore cardiac function after MI, indicating that circSamd4 might be an
11 effective target for cardiac regeneration. Furthermore, conservation of the circSamd4
12 sequence among different species indicates the clinical translational value of
13 circSamd4.

14 In this study, we demonstrated that circSamd4 reduces mitochondria-derived ROS by
15 recruiting Vcp to mitochondria. A previous study reported that cytoplasmic circRNAs
16 function by redistributing proteins from the nucleus to the cytoplasm.³² Our studies
17 revealed a novel mechanism of action via which mitochondria-localized circRNAs
18 promote protein mitochondrial translocation. As a member of the AAA+ (ATPase
19 associated with diverse cellular activities) family of proteins, Vcp is ubiquitously
20 expressed in mammals and can be detected in multiple tissues and cells.³³ Prior
21 research has demonstrated that the Vcp protein is expressed in various subcellular
22 organelles and cell type.³⁴⁻³⁶ The Vcp protein is reported to have many biological

1 functions, including playing roles in protein degradation and homeostasis, the
2 assembly of organelle membranes, cell proliferation and apoptosis.³⁴⁻³⁶ In our study,
3 Vcp was recruited by circSamd4 and translocated to mitochondria, thereby regulating
4 mPTP opening and mitochondrial ROS generation. Our results were consistent with
5 those of previous studies reporting that Vcp participates in mitochondrial functions
6 and combats oxidative stress in CMs.^{26,28} Thus, Vcp might serve as the main
7 downstream effector of circSamd4 during cardiac regeneration.

8 We also revealed the mechanism by which Vcp exerted its functions in CMs. The
9 downregulation of Vdac1 was further found to be responsible for the antioxidant
10 activity of Vcp. The Vdac1 protein is also ubiquitously expressed in the heart,
11 duodenum and other tissues.³⁷ This protein functions as the gatekeeper on the outer
12 mitochondrial membrane and regulates the flow of ions, nucleotides, and
13 metabolites.³⁸ Previous studies have confirmed that Vdac1 plays essential roles in
14 controlling cell fate transition by mediating metabolism and energy cross-talk in
15 cells.³⁹ It was reported that Vdac1 participates in the process of cell apoptosis by
16 permitting the release of apoptotic proteins and mitochondrial DNA from
17 mitochondria.³⁹ Moreover, it also maintains the mitochondrial membrane potential,
18 cristae organization, and mitochondrial morphology, thereby controlling
19 mitochondrial ROS generation.⁴⁰ We assumed that the effect of Vcp on Vdac1
20 expression was mediated by ubiquitin-dependent degradation. Previous studies also
21 reported that targeting Vdac1 could restore the dissipated mitochondrial membrane
22 potential and decrease ROS production.⁴⁰ Taken together, the results of these

1 mechanistic studies confirm that the circSamd4/Vcp/Vdac1 pathway plays an
2 antioxidative stress role in cardiac regeneration.

3 There are several limitations to the current study. First, this study focused on the
4 association between circSamd4 and CM proliferation, but circSamd4 might also
5 function in non-CMs. Considering the indispensable role of non-CMs in cardiac
6 regeneration, the effect of circSamd4 on non-CM ROS generation should be explored
7 in the future. Second, in our study, overexpression and knockdown of circSamd4
8 expression in the heart was conducted via AAV9 injection, which has been widely
9 applied in gene therapy. Although circSamd4 expression was significantly changed
10 after AAV9 administration, there are still several limitations affecting the results, such
11 as the instability of AAV transduction, the potential effect on inflammation, and safety
12 concerns.⁴¹ The application of CM-specific knockout and overexpression transgenic
13 mice might be helpful for determining the role of circSamd4 in cardiac regeneration.

14 In conclusion, Mitochondria-localized circRNA Samd4, regulated by the transcription
15 factor Nrf2, reduced CM mitochondrial oxidative stress and induced cell cycle
16 progression by promoting Vcp mitochondrial translocation and reducing Vdac1
17 expression. CircSamd4 overexpression triggered cardiac repair and decreased the
18 infarct size after MI, which significantly improved cardiac function. Our findings
19 suggest that circSamd4 is a promising therapeutic target for heart failure after MI.

20

21 **Methods and materials**

22 **MI model establishment**

1 C57BL/6 mice were obtained from Guangdong Medical Laboratory Animal Center.
2 Experiments were approved by the Animal Research Committee of Southern Medical
3 University and performed in accordance with the National Institutes of Health Guide
4 for the Care and Use of Laboratory Animals. MI of neonatal and adult mouse hearts
5 was performed according to previous studies.^{16,42} Briefly, adult mice (8-10 weeks old,
6 male) were anesthetized by inhalation of isoflurane (5% for induction and 2% for
7 maintenance). The mice were also endotracheally intubated and ventilated using a
8 volume-controlled ventilator to maintain the airway. Neonatal mice were anesthetized
9 by hypothermia with an ice box. MI was conducted by ligation of the left coronary
10 artery (LAD) at a level of 1 mm below the left atrium. An analogous surgical
11 operation was performed without occlusion of the LAD for sham-operated animals.

12

13 **Transthoracic echocardiography**

14 Transthoracic echocardiography was conducted under light anesthesia with inhaled
15 isoflurane (0.4–1.5%). A Vevo 2100 high-resolution imaging system equipped with a
16 30-MHz transducer (RMV-707B, VisualSonics, Toronto, ON, Canada) was utilized as
17 previously reported.¹⁶

18

19 **Histology**

20 Hearts were isolated and fixed in 4% paraformaldehyde for 48 h at room temperature
21 and then processed for paraffin sectioning. Masson's trichrome staining was
22 performed according to the manufacturer's instructions (Cat# MST-8004, MXB

1 Biotechnologies). TdT-mediated dUTP nick-end labeling (TUNEL) staining was
2 performed using an In-Situ Cell Death Detection Kit (Cat# 11684795910, Roche).
3 ImageJ Software (NIH, Bethesda, MD) was used to measure percent area of fibrosis
4 in left ventricular.²¹

5

6 **Cell isolation and culture**

7 Neonatal CMs from 1-day-old (P1) and 7-day-old (P7) C57BL/6 mice were isolated
8 as previously described.^{32,42} Briefly, the ventricles of the mice were isolated, cut into
9 pieces, digested with 0.25% trypsin (Sigma) at 4 °C for approximately 12 h and
10 digested again with type II collagenase (Roche) at 37 °C for 15 min two to three times.
11 The supernatant was collected and centrifuged, and the collected cells were
12 resuspended in DMEM/F12 medium (Life Technologies) supplemented with 10%
13 FBS. The cell suspension was subsequently plated onto uncoated 100-mm plastic
14 dishes for 90 min at 37 °C and 5% CO² in a humidified atmosphere. Due to the
15 differential adhesion between CMs and fibroblasts, the supernatant, composed mostly
16 of CMs, was then collected, resuspended in the aforementioned culture medium, and
17 seeded at the appropriate density.

18 Adult CMs were isolated from 56- to 70-day-old mice as previously described.^{32,42}
19 Briefly, adult hearts were isolated, placed on a Langendorff apparatus, and perfused
20 with calcium-free perfusion buffer for 5 min and digestion buffer for 10 min. The
21 perfusion buffer contained NaCl (113 mM), KCl (4.7 mM), KH₂PO₄ (0.6 mM),
22 Na₂HPO₄ (0.6 mM), MgSO₄ (1.2 mM), Na-HEPES (10 mM), NaHCO₃ (12 mM),

1 KHCO₃ (10 mM), phenol red (0.032 mM), taurine (30 mM), BDM (10 mM), and
2 glucose (5.5 mM, pH 7.0). The digestion buffer contained 15,000 U of type II
3 collagenase (Roche) and 50 µM CaCl₂. Then, the hearts were cut into small pieces
4 and triturated with a Pasteur pipette to separate individual cells. The adult CMs were
5 then centrifuged at a low speed and seeded onto culture slides coated with laminin (10
6 µg ml⁻¹, Life Technologies, 23017015) in DMEM/F12 medium (Life Technologies)
7 supplemented with 10% FBS. Primary mouse cardiac endothelial cells and fibroblasts
8 were obtained from Cell Biologics, Inc. (C57-6024 and C57-6049, respectively) and
9 handled according to the company's instructions. Descriptions of the isolation and
10 quality control analyses of these cells are presented at the company's website
11 (<https://cellbiologics.com/>).

12

13 **qRT-PCR assays**

14 RNA isolation, reverse transcription and qRT-PCR assays were performed as
15 previously described.^{16,32,42} The primers used for qRT-PCR assays were present in
16 Supplemental Table 1.

17 For mitochondrial DNA (mtDNA) quantification, mtDNA was extracted from samples
18 with a Cell Mitochondria Isolation Kit (#C3601, Beyotime). Mitochondrial DNA
19 (mtDNA) was quantified with qRT-PCR with primers using SYBR Green PCR
20 Master Mix. The relative mtDNA copies were calculated from the ratio of mtDNA
21 copies to nuclear DNA copies.

22

1 **Small interfering RNA (siRNA), adenovirus (Adv) and adeno-associated virus 9**
2 **(AAV9) transduction**

3 SiRNAs were purchased from RiboBio Co., Ltd. (Guangzhou, China). The target
4 sequences of the siRNAs are presented in Supplementary Table 2. A scrambled siRNA
5 was used as a negative control (NC). Transfections were conducted with a LipoFilter
6 Kit (MicroAire). SiRNAs were transfected at a final concentration of 50 nM, and
7 subsequent experiments were performed 48 h after transfection.

8 The Adv and AAV9 vectors for gene knockdown or overexpression were purchased
9 from Vigene (Shandong, China). Neonatal CMs were transduced with Adv for 48 h.

10 The multiplicity of infection (MOI) ranged from 10 to 20. The delivery of Adv and
11 AAV9 vectors to mice was conducted as previously reported.^{16,32,42} Briefly, adult mice
12 were intramyocardially injected at 3–5 sites with AAV9 vectors expressing circSamd4
13 or an empty vector (AAV9-circSamd4 or AAV9-vector) at a dose of 10^{11} viral
14 genome particles per mouse (approximately 30 μ l) using an insulin syringe with a
15 30-gauge needle. For the infarcted mice, AAV9 was injected into myocardial tissue
16 close to the infarct zone immediately after left anterior descending coronary artery
17 ligation. Myocardial tissues were collected 28 days after injection. In P1 neonatal
18 mice, an Adv vector expressing a shRNA targeting circSamd4 or an empty vector
19 (Adv-shcircSamd4 or Adv-NC) was injected into 3 sites in the left ventricle at a dose
20 of 5×10^9 viral genome particles per animal (approximately 5 μ l) by using an insulin
21 syringe with a 30-gauge needle. Adv was also injected into the peri-infarcted
22 myocardium immediately after LAD ligation. The hearts of the mice were isolated 1

1 week after the Adv injection. Real-time polymerase chain reaction (qRT-PCR) assays
2 were used to determine successful transduction.

3

4 **Subcellular fractionation**

5 To determine subcellular distribution of circRNAs, three distinct fractions were
6 isolated from CMs: mitochondria, cytoplasmic, and nuclear, using a Cell
7 Fractionation Kit (#9038, Cell Signaling Technology) according to the manufacturer's
8 instructions.

9

10 **Immunostaining**

11 Immunostaining assays were performed as previously described.⁴³ The primary
12 antibodies used were as follows: anti-Ki67 antibody (ab15580, Abcam), anti-p-histone
13 H3 (pH3) antibody (ab170904, Abcam), anti-c-TnT (ab33589, Abcam), anti-aurora B
14 (ab2254, Abcam), anti-Anillin (ab211872, Abcam), anti-CD31 (AF3628, R&D),
15 anti- α -actinin (ab68167, Abcam), anti-Vimentin (60330-1-Ig, Proteintech), anti-p-Atm
16 (sc-47739, Santa Cruz) and anti-8-OHG (ab62623, Abcam). To detect EdU
17 incorporation, cells were stained with a Click-it EdU Imaging Kit (Life Technologies,
18 #C10638) according to the manufacturer's instructions. Image acquisition was
19 performed using confocal laser scanning microscopy (Leica).

20

21 ***In situ* hybridization (ISH)**

22 ISH assays were performed as previously reported.^{16,32,42} Briefly, samples were

1 incubated in 3% pepsin diluted in fresh citrate buffer at 37 °C for 30 min and then
2 prehybridized in prehybridization solution for 2 h at 37 °C. DIG-labeled RNA probes
3 (Biosense Bioscience Co., Ltd., Guangzhou, China) were used for hybridization at
4 37 °C overnight. Samples were then washed with a graded series of SSC buffer,
5 blocked with 3% BSA, and incubated with alkaline phosphatase-conjugated sheep
6 anti-DIG Fab fragments.

8 **ROS detection**

9 Total cellular ROS and mitochondria-derived ROS were detected by DCFH-DA (Cat#
10 D6883, Sigma–Aldrich) and MitoSOX (Cat# M36008, Invitrogen), respectively. CMs
11 at a density of 10^5 cells/well in a confocal dish were cultured in 10 mM DCFH-DA
12 or 5 mM mitoSOX working solution at 37 °C for 30 min in the dark. Images were
13 captured by Zeiss LSM 800 confocal microscopy. The fluorescent density was also
14 detected via a flow cytometry as previously described.⁴⁴

16 **RNase R digestion**

17 RNase R digestion was performed as previously reported.⁴⁵ Briefly, 2 µg of RNA was
18 incubated with or without 3 U of RNase R (Sigma) at 37 °C for 30 min. Then, the
19 treated RNA was purified using the RNeasy MinElute Cleanup Kit (Qiagen).

21 **Drug treatments**

22 A Vcp inhibitor (ML240, MedChemExpress) was dissolved in diluted DMSO. *In vitro*,

1 CMs were incubated with 5 μ M ML240 for 24 hours; *in vivo*, mice were injected with
2 ML240 (1.2 mg/kg) intraperitoneally every 2 days from Day 1 to Day 28. A VDAC1
3 inhibitor (VBIT-12, 20 μ M, Selleck Chemicals) was added to CMs for 2 hours. To
4 explore the effect of H₂O₂ on CMs, CMs were transduced with Adv for 24 h and then
5 incubated with H₂O₂ (20 μ M, Sigma) for 8 hours as indicated.

6

7 **Western blot analysis**

8 Total cells or tissue extracts were lysed by RIPA lysis buffer (BestBio) containing
9 Protease Inhibitor Cocktail Set I (BestBio). Proteins were resolved in 10%
10 SDS-PAGE buffer, fractioned by polyacrylamide gels, and transferred onto
11 nitrocellulose membranes. The following primary antibodies were used: anti-Vcp
12 (ab11433, Abcam), anti-Vdac1 (55259-1-AP, Proteintech), anti-Wee1 (ab137377,
13 Abcam), and anti-Nrf2 (ab19867, Abcam), anti-Sod2 (ab137037, Abcam), and
14 anti-Gpx1 (29329-1-AP, Proteintech). A rabbit anti-Gapdh antibody (1:1000 dilution;
15 bs-0755R, Bioss) and anti-Cox4 antibody (11242-1-AP, Proteintech) were used as
16 controls. Alexa Fluor 680-conjugated anti-mouse IgG (1:10000 dilution; Abcam, USA)
17 was used as the secondary antibody. The signal was detected by an Odyssey detection
18 system (LI-COR Biosciences, Lincoln, NE, USA).

19

20 **RNA Fluorescence *in situ* hybridization (RNA-FISH)**

21 Cy3-labeled RNA probes against the circSamd4 back-splice sequence were purchased
22 from RiboBio Co., Ltd. (Guangzhou, China). Cultured cells and isolated tissues were

1 permeabilized with 0.5% Triton X-100 and then incubated with RNA probes in
2 hybridization buffer (RiboBio, Guangzhou, China). Nuclei were stained with DAPI.

3

4 **RNA pulldown assays**

5 RNA pulldown assays were performed as previously reported.⁴⁶ Briefly, cell lysates
6 were incubated with biotinylated DNA oligo probes at room temperature for 4 h. Then,
7 cell lysates were incubated with washed streptavidin-coated magnetic beads (SA1004;
8 Invitrogen) at room temperature for one hour. The precipitated components were
9 extracted by SDS-PAGE and then subjected to silver staining and mass spectrometry
10 analysis. The biotin-labelled probe targeting circSamd4 back-splicing sequence was
11 used for RNA pulldown assay, and the probe without biotin labeling was used as the
12 control group.

13

14 **Triphenyltetrazolium chloride (TTC) staining**

15 TTC assays were performed as previously reported.^{16,32,42} Briefly, the mouse hearts
16 were isolated and sectioned at a thickness of 3 mm. The slices were incubated in 1%
17 TTC buffer (Sigma Aldrich) diluted in PBS for 30 min at room temperature. Then, the
18 slices were washed with PBS and photographed. Image-Pro Plus 6.0 (Media
19 Cybernetics, Bethesda, MD, USA) was utilized to calculate the infarcted area.

20

21 **Chromatin immunoprecipitation (ChIP) assay**

22 ChIP assays were performed as reported previously.^{16,32,42} An EpiQuik Chromatin

1 Immunoprecipitation Assay Kit (EpiGentek, Brooklyn, NY) was used according to the
2 manufacturer's recommendations. An anti-Nrf2 (#12721, CST) antibody or IgG
3 antibody was used for immunoprecipitation. Then, qRT-PCR and PCR gel
4 electrophoresis were performed to detect the enrichment of DNA fragments at
5 potential Nrf2 binding sites.

6

7 **Coimmunoprecipitation (Co-IP) assay**

8 Co-IP assays were performed according to a previous study.¹⁶ Briefly, cell lysates
9 were collected and incubated with antibodies at 4 °C for 12 h. Then, they were
10 incubated with protein A/G magnetic beads (Thermo Scientific, USA). Finally, the
11 beads were collected and subjected to Western blotting.

12

13 **RNA immunoprecipitation (RIP) assay**

14 RIP assays were performed according to previous studies.^{16,32,42} Briefly, a Magna
15 RIP™ RNA-binding Protein Immunoprecipitation Kit (Millipore, Stafford, VA) was
16 used according to the manufacturer's instructions. We used an anti-Vcp antibody
17 (ab11433, Abcam) to precipitate RNA, and PCR assays were then performed to assess
18 the binding of RNA to Vcp.

19

20 **Mitochondrial membrane potential (MMP) measurement**

21 MMP changes were measured by a mitochondrial membrane potential assay kit with
22 JC-1 (Beyotime) according to the manufacturer's instructions. Briefly, CMs were

1 harvested and washed with PBS. Then, these CMs were resuspended in a mixture of
2 culture medium and JC-1 staining fluid for 20 min in the dark at 37 °C. Next, CMs
3 were washed with cold staining buffer prior to flow cytometry (BD, America). The
4 MMP was indicated by the ratio of red to green fluorescence intensity. When the
5 MMP low in cells, JC-1 remains in the monomeric form and emit a green
6 fluorescence. While in cells with high MMP, JC-1 forms complexes known as
7 J-aggregates. Aggregates of JC-1 emit an orange-red fluorescence. The higher ratios
8 of red to green fluorescence were correlated with higher mitochondrial membrane
9 polarization.

10

11 **Annexin V-FITC/PI staining**

12 The apoptosis ratio of CMs was assessed by the Annexin V-FITC/PI apoptosis
13 detection kit according to the manufacturer's instructions. Briefly, CMs were
14 resuspended in 0.1 mL of binding buffer and then incubated with Annexin V-FITC/PI
15 buffer in the dark for 10 minutes. The fluorescence intensity was determined by flow
16 cytometry (FACScan, BD Biosciences), and the data were analyzed by FlowJo 7.6.1.

17

18 **Trypan Blue Exclusion**

19 Trypan blue exclusion was used to determine cell viability. Cells were trypsinized,
20 pelleted, resuspended in a 1:1 mixture of PBS and 0.5% trypan blue, and manually
21 counted using a hemocytometer. The number of dead (blue stained) cells was then
22 expressed as a ratio of the total (stained and unstained) cells counted.

1

2 Calcein-AM/CoCl₂ assays

3 Mitochondrial permeability transition pore (mPTP) opening was assessed by
4 Calcein-AM/CoCl₂ assays as described previously.⁴⁷ Briefly, the isolated CMs were
5 incubated with 5 μM calcein-AM for 30 min at 37 °C and then incubated with 40 μM
6 CoCl₂ for 20 min at 37 °C. When the mPTP was unopened, calcein fluorescence was
7 maintained in mitochondria in the presence of CoCl₂. When the mPTP opened, calcein
8 was lost from the mitochondria. Fluorescence intensities were determined by confocal
9 microscopy.

10

11 PET/CT imaging

12 MicroPET-CT was used to investigate glucose uptake in myocardial tissue, according
13 to previous studies.^{48,49} Briefly, ¹⁸F-fluorodeoxyglucose (¹⁸F-FDG) was
14 intraperitoneally injected into animals. Then, the mice were scanned by using a Focus
15 220 microPET scanner (Siemens Medical Solutions USA, Inc., Knoxville, TN, USA).
16 Dynamic scans were conducted for 1 h. PET images were reconstructed using the
17 microPET-CT manager (Siemens Medical Solutions USA, Inc.).

18

19 Northern blot

20 Northern blots were performed as previously described.¹¹ Briefly, RNA with or
21 without RNase R treatments was added into agarose gels, and electrophoresis for 3 hr.
22 Then, RNA was transferred to a nylon membrane and UV-crosslinked for 2 min. After

1 hybridization with digoxigenin labeled Oligonucleotide probes overnight, membrane
2 was incubated in anti-digoxigenin-AP and exposed onto chemiluminescence solution.
3 The images were captured by a Typhoon 9500 scanner (GE Healthcar). The sequence
4 of the probe is
5 ATTCCTGTTGCCATTGGTTAATGATTCTCGTGCTTGCGCCGGTTCCAATCGT
6 GT.

8 **RNA Isolation and Library Preparation**

9 Total RNA was extracted using TRIzol reagent according to the manufacturer's
10 protocol. RNA purity and quantification were evaluated using a NanoDrop 2000
11 spectrophotometer (Thermo Scientific, USA). RNA integrity was assessed using the
12 Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Then,
13 libraries were constructed using the TruSeq Stranded mRNA LT Sample Prep Kit
14 (Illumina, San Diego, CA, USA) according to the manufacturer's instructions.
15 Transcriptome sequencing and analysis were conducted by OE Biotech Co., Ltd.
16 (Shanghai, China).

18 **RNA Sequencing and Differentially Expressed Gene Analysis**

19 The libraries were sequenced on an Illumina HiSeq X Ten platform, and 150 bp
20 paired-end reads were generated. Raw data (raw reads) in the fastq format were first
21 processed using Trimmomatic.⁵⁰ And the low-quality reads were removed to obtain
22 clean reads. The clean reads were mapped to the mouse genome (mm10) using

1 HISAT2.⁵¹ The fragments per kilobase of exon model per million mapped fragments
2 (FPKM)⁵² values of each gene were calculated using Cufflinks.⁵³ And the read counts
3 of each gene were obtained by HTSeq count.⁵⁴ Differential expression analysis was
4 performed using the DESeq R package.⁵⁵ The adjusted *P* value < 0.05 and a fold
5 change > 2 or < 0.5 were set as the thresholds for significantly different expression.
6 Enriched Gene Oncology (GO) analysis of differentially expressed genes was
7 performed using R based on the hypergeometric distribution.⁵⁶

8

9 **Data availability**

10 CircRNA and gene expression profiles in adult and neonatal hearts were obtained
11 from previous studies.¹³ The thresholds for up- and downregulated circRNAs and
12 genes were a fold change ≥ 2 and an FDR < 0.05.

13 The ChIP-seq data are available through the Gene Expression Omnibus (GEO) under
14 accession numbers GSM1264370 (embryonic Day 11.5, E11.5 mouse heart H3K27ac),
15 GSM1264378, (postnatal Day 0, P0 mouse heart H3K27ac) and GSM1264384
16 (postnatal Day 56, P56 mouse heart H3K27ac).

17

18 **Statistical analysis**

19 The results were statistically analyzed using SPSS 22.0 software. All data are shown
20 in graphs as the mean \pm SD. For the statistical comparison of 2 groups, an unpaired,
21 2-tailed Student's *t* test was utilized. One-way ANOVA, followed by Bonferroni's
22 multiple comparisons test, was used for the comparison of more than 2 groups. The *P*

1 value was calculated, and $P < 0.05$ indicated statistical significance.

2

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13

14 **Author Contributions**

15 J.B., S.H., and H.Z. conceived the study. S.H. wrote the original manuscript. S.H.,
16 H.Z., and J.B. reviewed and edited the manuscript. H.Z., G.W., and Y.S.
17 performed *in vitro* experiments. H.Z., C.L., and Y.C. performed *in vivo* experiments.
18 X.S., Z.T., X.L., and Y.C. analyzed the data. Y.L., W.L., and J.B. supervised the
19 experiments. All authors read and approved the final manuscript.

20

21 **Declaration of Interests**

22 The authors declare no competing interests.

1

2 **Key words**

3 CircRNA; Cardiac regeneration; Mitochondria; ROS; Myocardial infarction

4 **References**

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1 **Figure legends**

2 **Fig. 1 A mitochondria-localized circRNA, circSamd4, is highly expressed in the**
3 **neonatal heart.** (A) Heatmap of conserved circRNAs that were differentially
4 expressed in adult and neonatal rat hearts and their linear transcripts. Changes in the
5 expression profile of circRNAs were opposite those in the linear transcripts. (B) The
6 relative expression levels of the indicated circRNAs in P1 CMs treated with 20 μ M
7 H₂O₂ or PBS for 8 hours; * P <0.05, n=6. (C) qPCR analysis showing the circSamd4
8 expression levels in multiple tissues of neonatal mice; * P <0.05 vs. heart, n=6. (D)
9 The indicated circRNAs were detected in cytoplasm and mitochondria extract of CM.
10 Gapdh and mt-Co2 was used as the cytoplasm and mitochondria marker, respectively.
11 * P <0.05, n=6. (E) RNA-FISH assay of circSamd4 and coimmunostaining of Cox4 in
12 P1 CMs. (F) Amplification of circSamd4 in cDNA but not genomic DNA using
13 divergent primers; gDNA, genomic DNA. (G) Comparison of circSamd4 and Samd4
14 mRNA expression levels by RNase R digestion analysis; * P <0.05 vs. mock treatment,
15 n=6. (H) Detection of the expression levels of circSamd4 in neonatal and adult mouse
16 hearts using qRT-PCR assays; * P <0.05, n=6. (I) Detection of the expression levels of
17 circSamd4 in adult, P7 and P1 mouse CMs using qRT-PCR assays; * P <0.05, n=6. (J)
18 ISH assays showing circSamd4 expression in the rat and mouse hearts; * P <0.05, n=6.
19 (K) ChIP-seq analysis of H3k27ac at the promoter of the circSamd4 host gene. TSS,
20 transcription start site. (L) ChIP-PCR assays detecting the H3k27ac levels in the
21 promoter of the circSamd4 host gene; * P <0.05 vs. anti-IgG, n=6. (M) Detection of the
22 expression levels of the circSamd4 pre-mRNA and Samd4 mRNA in P1 CMs after

1 Nrf2 knockdown or control treatment using qRT-PCR assays; * $P < 0.05$ vs. si-NC,
2 $n = 6$. (N) The predicted TF motif of Nrf2 and binding sites on the circSamd4 host gene
3 promoter, which was provided by the JASPAR database. (O) ChIP-PCR assays
4 detecting the binding of Nrf2 to the promoter of the circSamd4 host gene; * $P < 0.05$ vs.
5 anti-IgG, $n = 6$. (P-Q) Detection of cytosolic (P) and mitochondrial (Q) ROS in P1 CMs
6 by confocal microscopy; * $P < 0.05$ vs. si-NC, $n = 6$.

7
8 **Fig. 2 CircSamd4 reduces mitochondrial ROS and oxidative injury in CMs.** (A-C)

9 CircSamd4 reduced the total cellular ROS (A) and mitochondrial ROS (B-C) levels in
10 CMs. P1 CMs were transduced with Adv for 24 h and then incubated with H_2O_2 (20
11 μM) for 8 hours. Total cellular ROS were detected by DCFH-DA staining.
12 Mitochondrial ROS was detected by mitoSOX staining. * $P < 0.05$, $n = 6$. (D)
13 CircSamd4 decreased the mitochondrial DNA content in P1 CMs treated with H_2O_2 .
14 * $P < 0.05$, $n = 6$. (E) CircSamd4 repressed mitochondrial crista maturation in P1 CMs
15 treated with H_2O_2 . The mitochondrial ultrastructure was detected by transmission
16 electron microscopy. * $P < 0.05$, $n = 6$. (F-G) CircSamd4 alleviated DNA damage (F)
17 and the DDR (G) induced by H_2O_2 . * $P < 0.05$, $n = 6$. (H) CircSamd4 overexpression
18 repressed the H_2O_2 -induced decrease in the MMP. * $P < 0.05$, $n = 6$. JC-1 monomer:
19 green; J-aggregate: red.

20
21 **Fig. 3 CircSamd4 induces CM cell cycle progression and proliferation *in vitro*.** (A)

22 Detection of P1 CM proliferation by an EdU incorporation assay. P1 CMs were

1 transduced with Adv for 24 h and then incubated with H₂O₂ (20 μM) for 8 hours.
2 EdU+ CMs are indicated by arrows, **P*<0.05, n=6. (B) Evaluation of P1 CM
3 proliferative activity by Ki-67 immunostaining. Ki67+ CMs are indicated by arrows,
4 **P*<0.05, n=6. (C) Evaluation of P1 CM proliferative activity by pH3 immunostaining.
5 **P*<0.05, n=6. (D) Evaluation of P1 CM proliferative activity by Aurora B
6 immunostaining. **P*<0.05, n=6. (E) Representative images obtained from a time-lapse
7 video of CM cell division. The arrows indicate CMs undergoing division events.
8 Immunostaining of cTnT was then performed to determine whether the proliferating
9 cells were CMs. **P*<0.05, n=6. (F) Flow cytometry analysis of CMs undergoing cell
10 cycle events. **P*<0.05, n=6.

11
12 **Fig. 4 CircSamd4 overexpression reduces oxidative injury and promotes CM**
13 **proliferation *in vivo*.** (A) Evaluation of adult CM proliferation by Ki67
14 immunostaining. CMs were isolated from adult mouse hearts 14 days after
15 AAV9-circSamd4 or AAV9-NC injection. **P*<0.05, n=6. (B) Evaluation of adult CM
16 proliferation by pH3 immunostaining. **P*<0.05, n=6. (C) Evaluation of adult CM
17 proliferation by Anillin immunostaining. **P*<0.05, n=6. (D) Detection of intracellular
18 ROS levels in the myocardium 14 days after AAV9-circSamd4 or AAV9-NC injection;
19 **P*<0.05, n=6. (E) Detection of oxidative DNA damage in the adult myocardium 14
20 days after AAV9-circSamd4 or AAV9-NC injection; **P*<0.05, n=6. (F) WGA staining
21 of adult mouse hearts 14 days after AAV9-circSamd4 or AAV9-NC injection. (G)
22 Detection of Ki67+ adult CMs in the peri-infarcted area 14 days post-MI. Ki67+ CMs

1 are indicated by arrows, $*P<0.05$, $n=6$. (H) Detection of pH3+ adult CMs in the
2 peri-infarcted area 14 days post-MI; $*P<0.05$, $n=6$. (I) Detection of intracellular ROS
3 levels in the peri-infarcted area 14 days post-MI; $*P<0.05$, $n=6$. (J) Detection of
4 oxidative DNA damage in adult CMs 14 days post-MI; $*P<0.05$, $n=6$.

5

6 **Fig. 5 CircSamd4 overexpression restores cardiac function after MI.** (A)

7 Evaluation of the left ventricular ejection fraction (LVEF), left ventricular
8 end-systolic dimension (LVESD), left ventricular end-diastolic dimension (LVEDD)

9 and fractional shortening (FS) post-MI after circSamd4 overexpression. The

10 echocardiography analysis was performed on Days 1, 7, 14 and 28 after surgery.

11 $*P<0.05$ vs. the sham group, $\#P<0.05$ vs. the AAV9-NC group, $\&P<0.05$ vs. the

12 AAV9-Samd4 group, $n=6$. (B) PET-CT of the adult mouse heart 28 days post-MI;

13 $*P<0.05$ vs. the AAV9-NC group, $n=6$. (C) Masson trichrome staining of cross

14 sections from adult mouse hearts at 28 days post-MI. $*P<0.05$ vs. the AAV9-NC

15 group, $n=6$. (D) TTC staining of cross sections from adult mouse hearts at 28 days

16 post-MI. $*P<0.05$ vs. the AAV9-NC group, $n=6$.

17

18 **Fig. 6 CircSamd4 downregulation increases oxidative injury and impairs**

19 **regenerative repair in the neonatal mouse heart post-MI.** (A) Detection of

20 intracellular ROS levels in the myocardium with dihydroethidium (DHE) 7 days

21 post-MI; $*P<0.05$, $n=6$. (B) Detection of oxidative DNA damage in neonatal CMs

22 with 8-OHG staining 7 days post-MI; $*P<0.05$, $n=6$. (C) *In vivo* evaluation of CM

1 proliferation by Ki67 immunostaining 7 days post-MI. Ki67+ CMs are indicated by
2 arrows; * P <0.05, n=6. (D) *In vivo* evaluation of CM proliferation by pH3
3 immunostaining 7 days post-MI. pH3+ CMs are indicated by arrows; * P <0.05, n=6.
4 (E) Evaluation of left ventricular function 7 days post-MI; * P <0.05, n=6. (F)
5 Evaluation of the fibrotic area 7 days post-MI; * P <0.05, n=6.

6

7 **Fig. 7 CircSamd4 decreases mitochondria-derived ROS by recruiting Vcp to the**

8 **mitochondria.** (A) The proteins precipitated by a specific biotin-labeled circSamd4

9 probe were resolved by SDS-PAGE; bottom, the Vcp protein was detected by

10 Western blotting. CircSamd4 probe: biotin-labelled probe targeting circSamd4

11 back-splicing sequence; control probe: non-labelled probe targeting circSamd4

12 back-splicing sequence. (B) RIP assays revealed that the Vcp protein enriched

13 circSamd4 in P1 CMs; * P <0.05, n=6. (C) qPCR assays were performed to detect the

14 Vcp mRNA expression levels after circSamd4 knockdown; n=6. (D) Western blot

15 analysis of the Vcp protein levels in CMs. P1 CMs were transduced with Adv for 24 h

16 and then incubated with H₂O₂ (20 μM) for 8 hours, * P <0.05, n=6. (E) Western blot

17 analysis of the Vcp protein levels in the cytoplasmic and mitochondrial fractions of

18 CMs. * P <0.05, n=6. (F) Western blot analysis of the Vcp protein levels in the

19 cytoplasmic and mitochondrial fractions of CMs. * P <0.05, n=6. (G) Western blot

20 analysis of the Vcp protein levels in the cytoplasmic and mitochondrial fractions of

21 CMs. * P <0.05, n=6. (H) Evaluation of P1 CM proliferative activity by Ki67

22 immunostaining after Adv-Vcp or Adv-NC transduction. Ki67+ CMs are indicated by

1 arrows, * P <0.05, n =6. (I) Evaluation of P1 CM proliferative activity by pH3
2 immunostaining after Adv-Vcp or Adv-NC transduction. pH3⁺ CMs are indicated by
3 arrows, * P <0.05, n =6. (J) Evaluation of the MMP in P1 CMs after Adv-Vcp or
4 Adv-NC transduction. * P <0.05, n =6. JC-1 monomer: green; J-aggregate: red. (K)
5 Evaluation of mitochondria-derived ROS levels in P1 CMs after Adv-Vcp or Adv-NC
6 transduction. * P <0.05, n =6. (L) Evaluation of oxidative DNA damage in P1 CMs
7 after Adv-Vcp or Adv-NC transduction. * P <0.05, n =6. (M) Detection of intracellular
8 ROS levels in the myocardium 14 days after AAV9-circSamd4 transduction or
9 ML240 treatment; * P <0.05, n =6. (N) Detection of oxidative DNA damage in adult
10 CMs 14 days after AAV9-circSamd4 transduction or ML240 treatment; * P <0.05, n =6.
11 (O) Evaluation of CM proliferation by pH3 immunostaining 14 days after
12 AAV9-circSamd4 transduction or ML240 treatment; * P <0.05, n =6. (P) Masson
13 trichrome staining of cross sections from adult mouse hearts 28 days after
14 AAV9-circSamd4 transduction or ML240 treatment; * P <0.05, n =6.

15
16 **Fig. 8 Vcp attenuates mPTP opening by reducing Vdac1 expression.** (A) Changes
17 in the expression level of the Vdac1 protein after Vcp overexpression; * P <0.05, n =6.
18 Mitochondria were extracted from P1 CMs, and western blotting was then performed
19 to detect the Vdac1 protein. (B) Changes in the expression level of the Vdac1 protein
20 after 5 μ M ML240 treatment; * P <0.05, n =6. (C) Changes in the expression level of
21 the Vdac1 protein after circSamd4 overexpression and/or 5 μ M ML-240 treatment;
22 * P <0.05, n =6. (D) Detection of the MMP in P1 CMs treated with VBIT-12 using the

1 flow cytometry assay. P1 CMs were incubated with VBIT-12 (20 μ M) for 24 h and
2 then incubated with H₂O₂ (20 μ M) for 8 hours. JC-1 monomer: green; J-aggregate:
3 red. **P*<0.05, n=6. (E) Detection of the MMP in P1 CMs treated with VBIT-12 using
4 an immunofluorescence assay. (F) Detection of mitochondrial ROS in CMs treated
5 with VBIT-12. **P*<0.05, n=6. (G) Detection of DDR activation in CMs treated with
6 VBIT-12. **P*<0.05, n=6. (H) Detection of Ki67+ CMs after VBIT-12 treatment.
7 Ki67+ CMs are indicated by arrows, **P*<0.05, n=6. (I) Detection of pH3+ CMs after
8 VBIT-12 treatment. **P*<0.05, n=6. (J) Detection of the MMP in P1 CMs after
9 circSamd4 overexpression, 5 μ M ML-240 and/or 20 μ M VBIT-12 treatment using the
10 flow cytometry assay. The CMs were treated with 20 μ M H₂O₂ prior to detection.
11 **P*<0.05, n=6. (K) Detection of the MMP in P1 CMs after circSamd4 overexpression,
12 5 μ M ML-240 and/or 20 μ M VBIT-12 treatment using the immunofluorescence assay.
13 JC-1 monomer: green; J-aggregate: red. (L) Detection of mitochondrial ROS after
14 circSamd4 overexpression, 5 μ M ML-240 and/or 20 μ M VBIT-12 treatment. **P*<0.05,
15 n=6. (M) Detection of DDR activation after circSamd4 overexpression, 5 μ M ML-240
16 and/or 20 μ M VBIT-12 treatment. **P*<0.05, n=6. (N) Detection of pH3+ P1 CMs after
17 circSamd4 overexpression, 5 μ M ML-240 and/or 20 μ M VBIT-12 treatment. **P*<0.05,
18 n=6. (O) Schematic illustrating the main finding of this study: Nrf2-upregulated
19 circSamd4 binds to the Vcp protein and promotes Vcp mitochondrial translocation,
20 thereby reducing Vdac1 expression and attenuating mPTP opening. CircSamd4
21 overexpression decreases oxidative stress production and triggers CM proliferation,
22 leading to cardiac repair after MI.

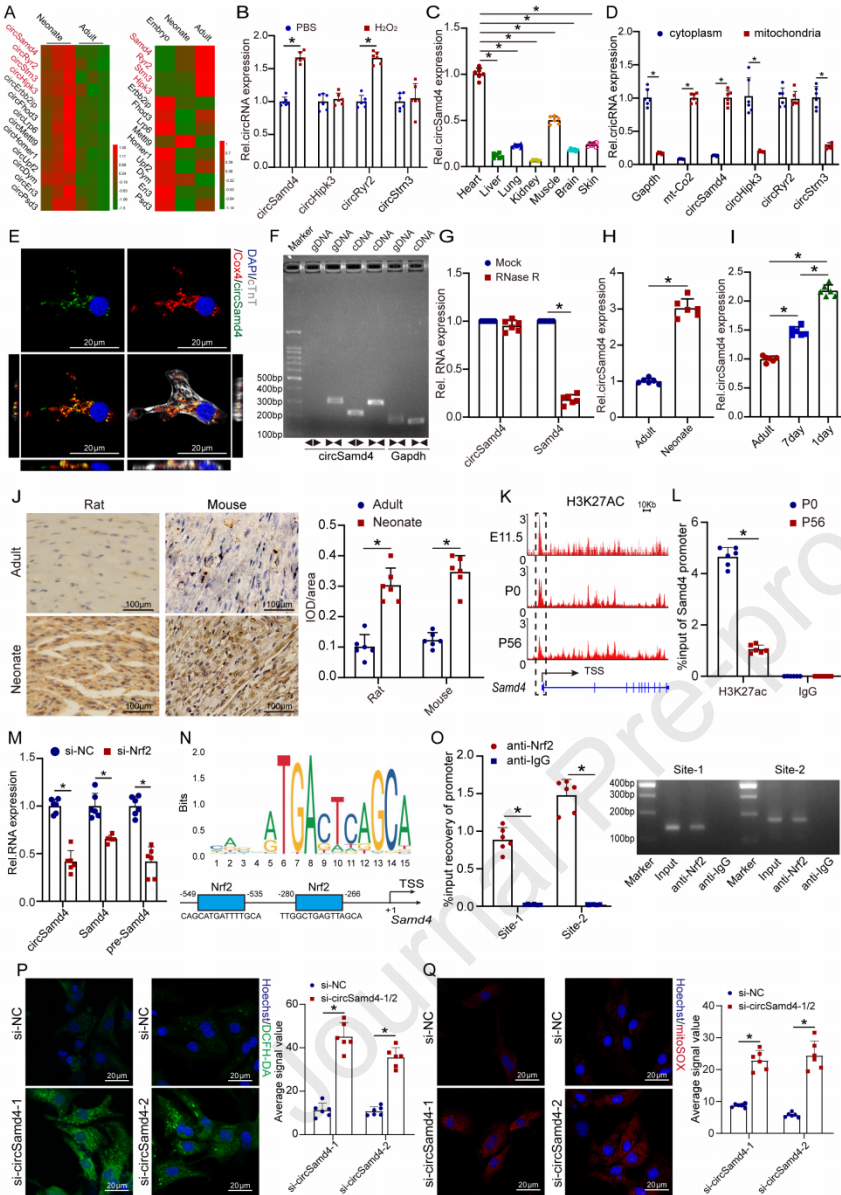
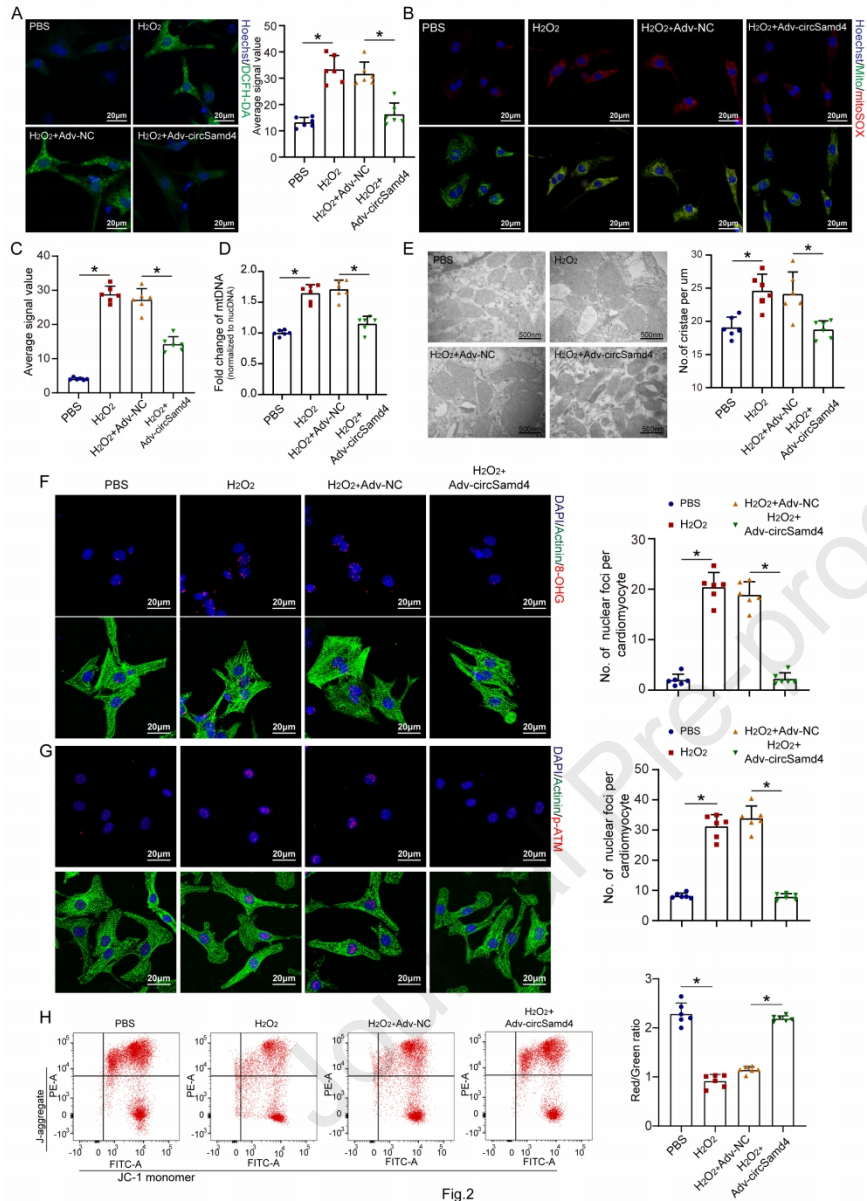


Fig.1



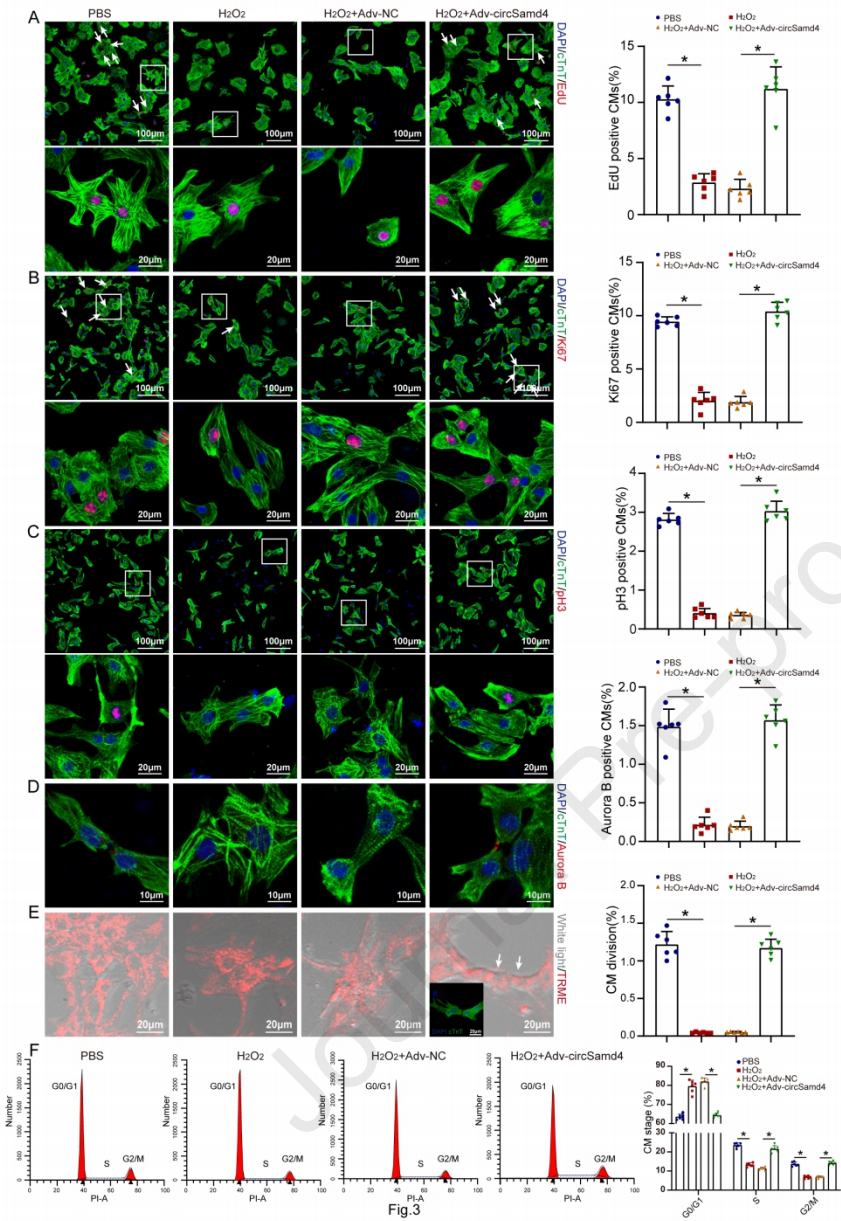


Fig.3

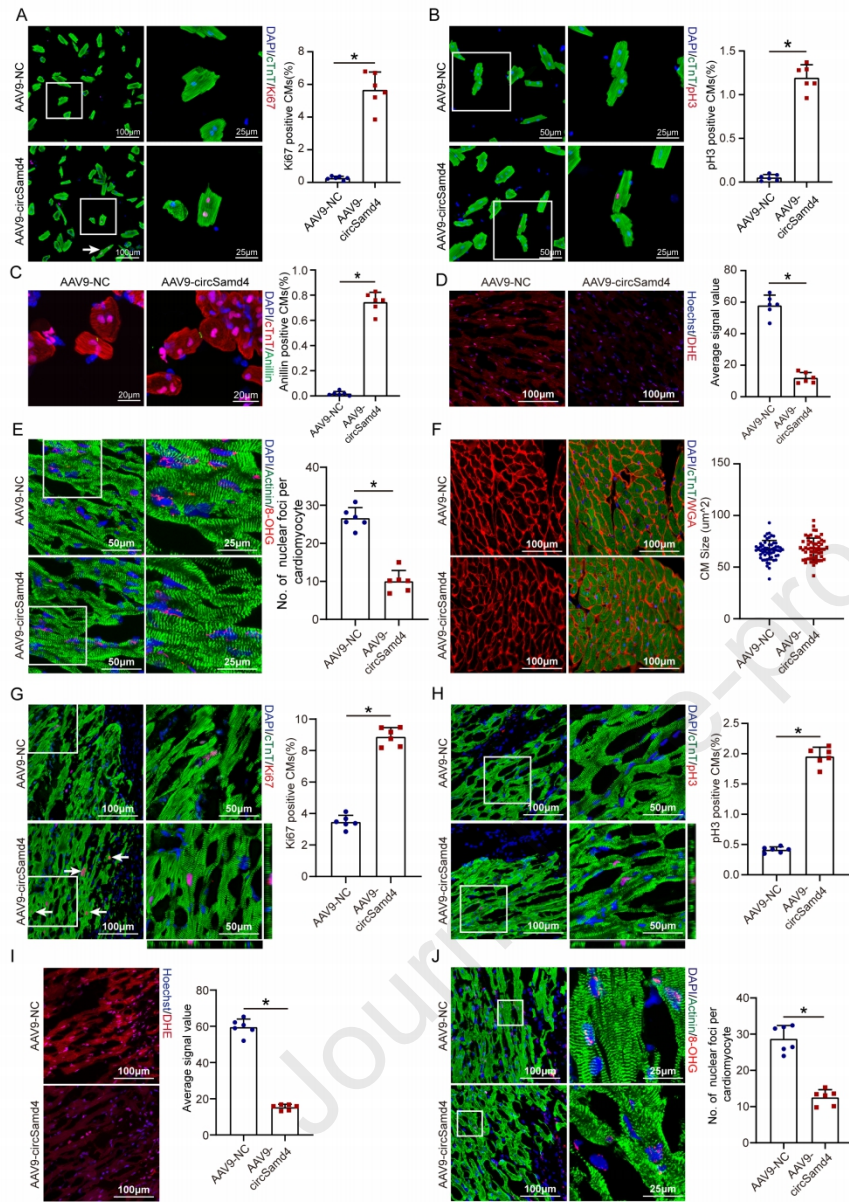
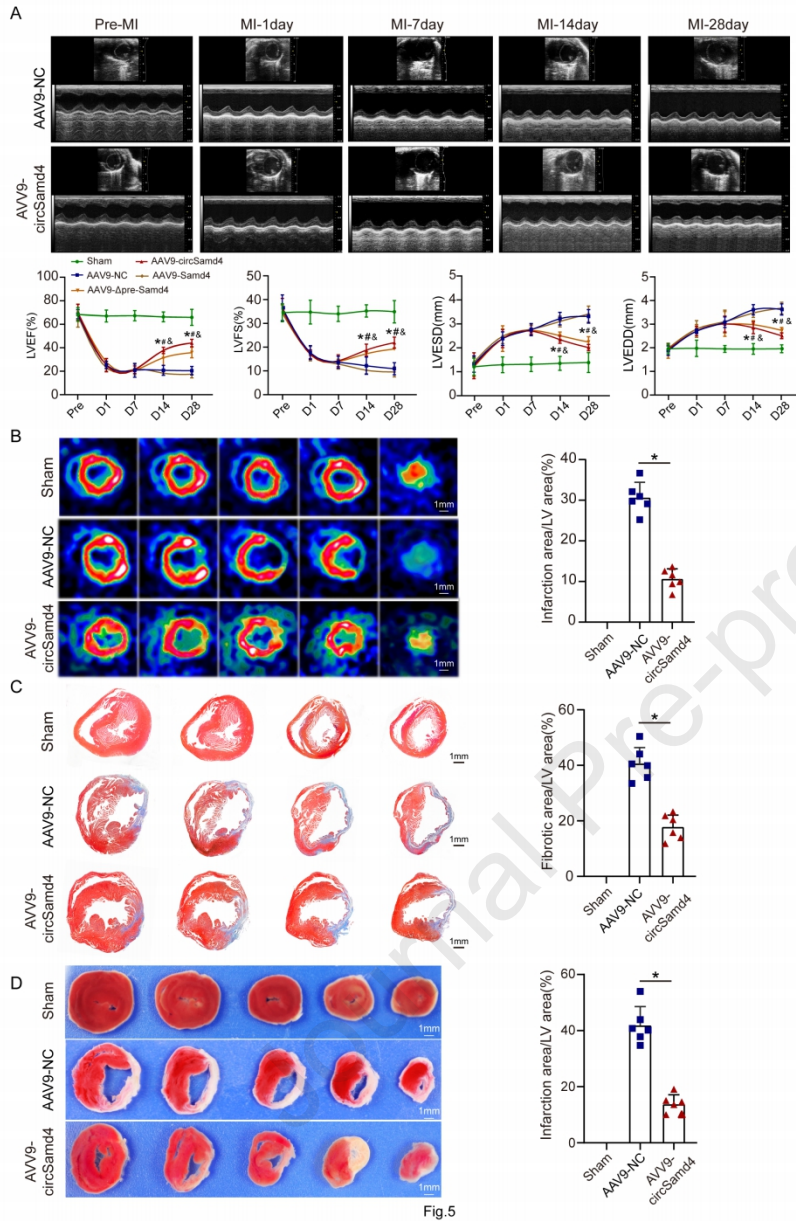


Fig.4



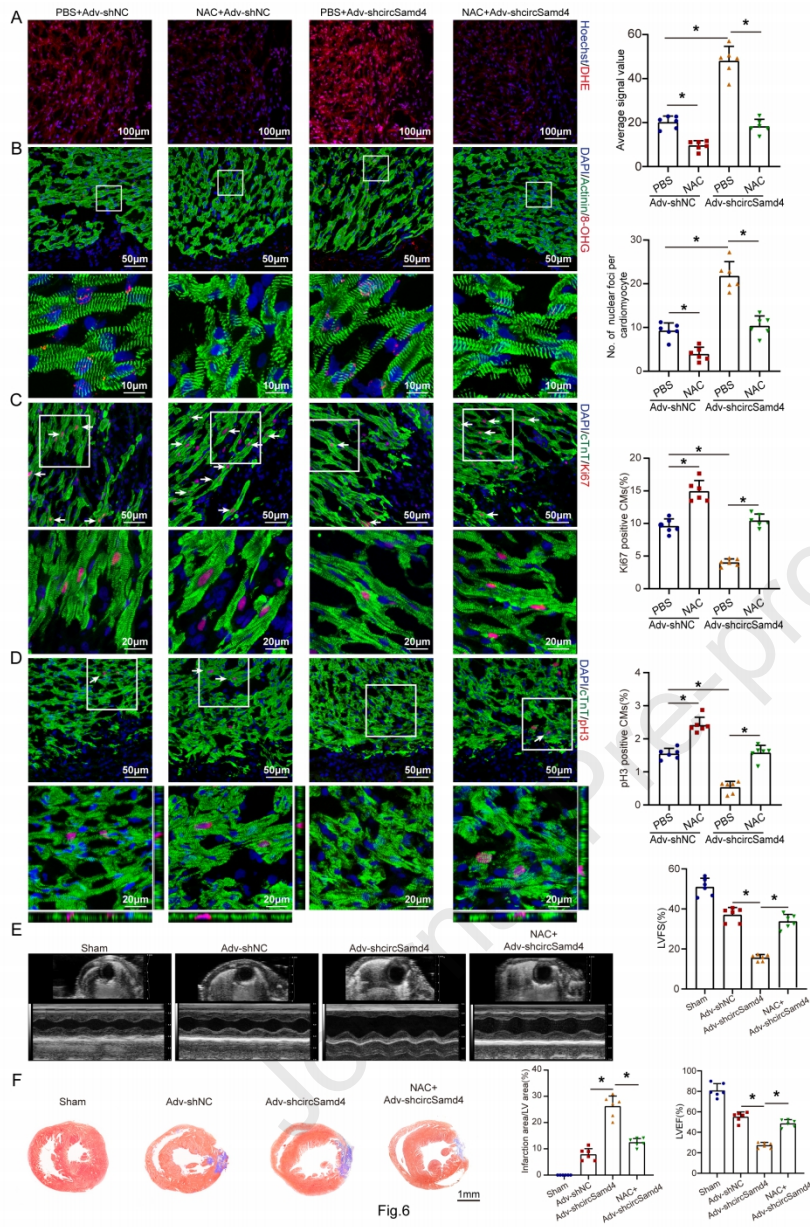


Fig 6

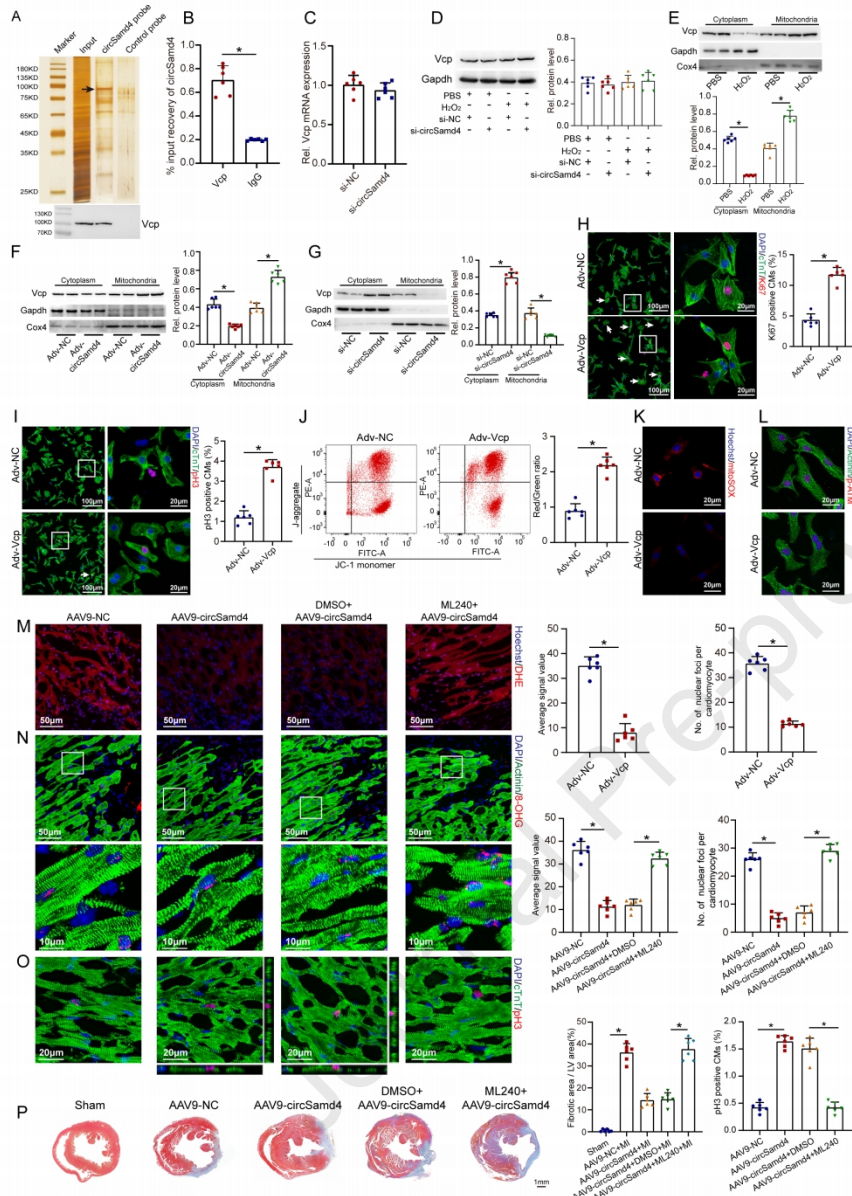


Fig.7

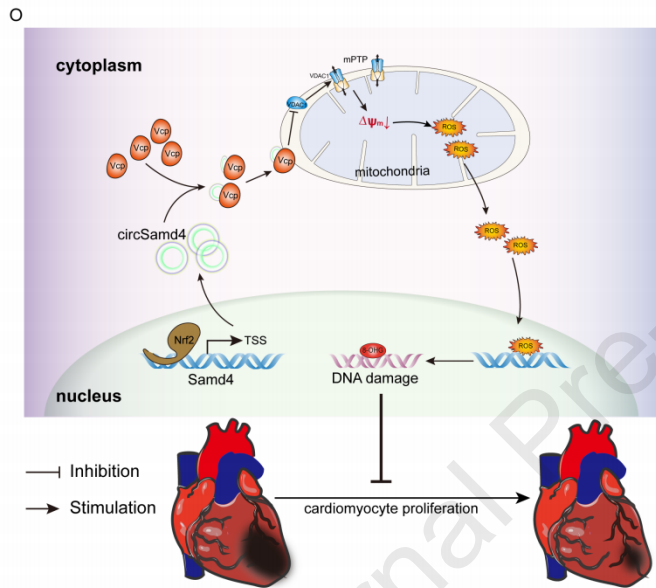
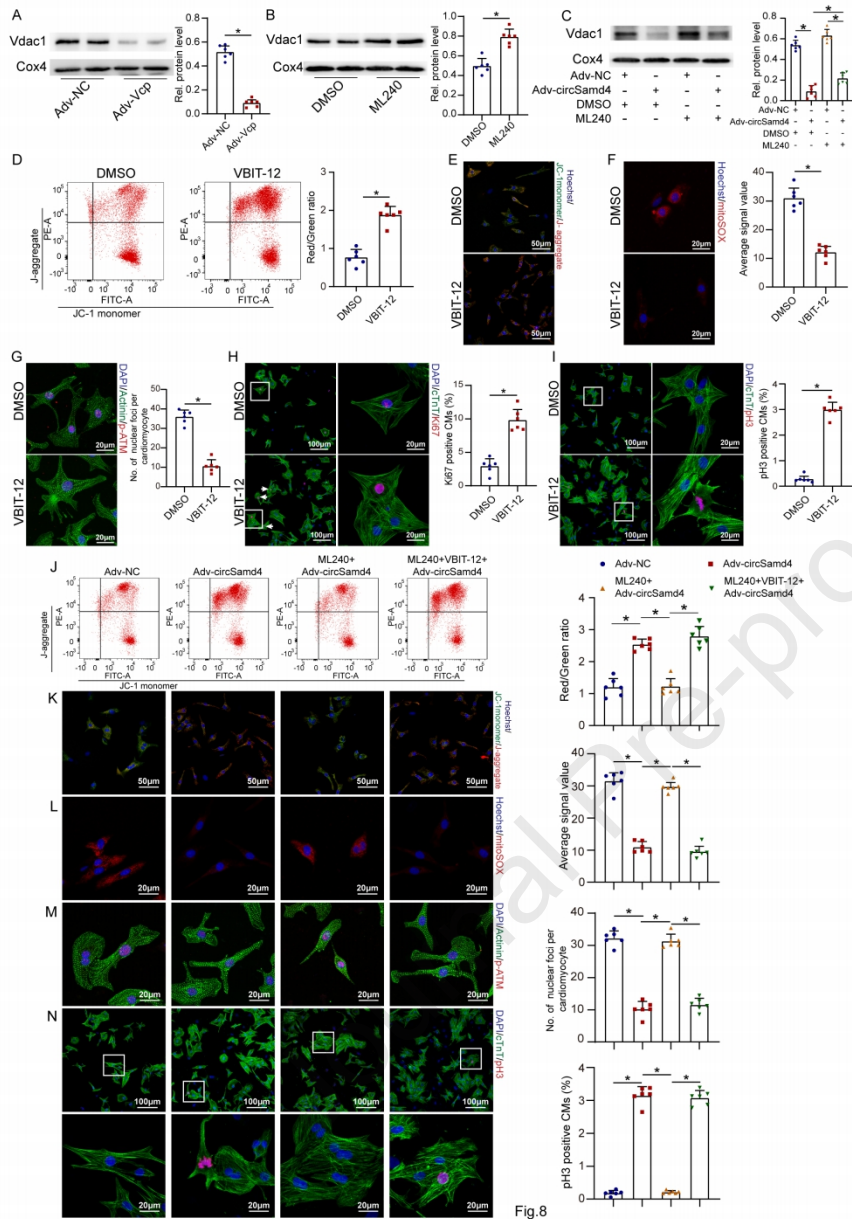


Fig.8 continued



A mitochondria-located circRNA Samd4 promotes CM cell-cycle progression and inhibits CM apoptosis by preventing the mitochondrial permeability transition pore from opening and repressing mitochondrial ROS production in CMs, providing a valuable therapeutic target for improving prognosis after myocardial infarction.

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