Runx1 Deficiency Protects Against Adverse Cardiac Remodeling Following Myocardial Infarction

Running Title: McCarroll and He et al.; Runx1 and Myocardial Infarction

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Abstract

Background—Myocardial infarction (MI) is a leading cause of heart failure and death worldwide. Preservation of contractile function and protection against adverse changes in ventricular architecture (cardiac remodeling) are key factors to limiting progression of this condition to heart failure. Consequently, new therapeutic targets are urgently required to achieve this aim. Expression of the Runx1 transcription factor is increased in adult cardiomyocytes following MI; however, the functional role of Runx1 in the heart is unknown.

Methods—To address this question, we have generated a novel tamoxifen-inducible cardiomyocyte-specific Runx1-deficient mouse. Mice were subjected to MI by means of coronary artery ligation. Cardiac remodeling and contractile function were assessed extensively at the whole heart, cardiomyocyte and molecular levels.

Results—Runx1 deficient mice were protected against adverse cardiac remodeling post-MI, maintaining ventricular wall thickness and contractile function. Furthermore, these mice lacked eccentric hypertrophy and their cardiomyocytes exhibited markedly improved calcium handling. At the mechanistic level, these effects were achieved through increased phosphorylation of phospholamban by PKA and relief of sarcoplasmic reticulum calcium pump (SERCA) inhibition. Enhanced SERCA activity in Runx1 deficient mice increased sarcoplasmic reticulum calcium content and sarcoplasmic reticulum-mediated calcium release, preserving cardiomyocyte contraction post-MI.

Conclusions—Our data identified Runx1 as a novel therapeutic target with translational potential to counteract the effects of adverse cardiac remodeling, thereby improving survival and quality of life among patients with MI.

Key Words: cardiac myocyte; infarction; remodeling; calcium regulation; excitation-contraction coupling; Runx1
Clinical Perspective

What is new?

- Our study provides new evidence that Runx1, a gene intensively studied in the cancer and blood research fields, has a critical role in cardiomyocytes following myocardial infarction (MI).
- We provide conclusive evidence that increased Runx1 expression under pathological conditions leads to decreased cardiac contractile function.
- Experiments performed utilizing a newly generated cardiomyocyte-specific Runx1 deficient mouse reveal that reducing Runx1 function preserves myocardial contractility and prevents adverse cardiac remodeling post-MI.

What are the clinical implications?

- Our mechanistic data robustly demonstrate that Runx1 modulates cardiac sarcoplasmic reticulum (SR) calcium uptake and contractile function.
- Reducing Runx1 function drives increased contractility post-MI, thereby preserving LV systolic function and preventing adverse cardiac remodeling.
- Our study therefore importantly identifies Runx1 as a new target holding major promise for limiting the progression to heart failure among patients with MI by preventing adverse cardiac remodeling.
Acute coronary artery blockage leading to prolonged ischemia and subsequent cardiomyocyte death (myocardial infarction; MI) initiates a reparative process in the heart that is associated with the generation of regional infarct tissue composed predominately of fibrillar collagens. The surviving cardiomyocytes undergo eccentric hypertrophy, a process characterized by cardiomyocyte elongation with reduced diameter and impaired calcium handling, in particular decreased sarcoplasmic reticulum (SR)-mediated calcium uptake. These cellular changes are fundamental to adverse cardiac remodeling, which manifests clinically as left ventricular (LV) wall thinning, dilation and reduced contractility. Together with neurohumoral activation, adverse cardiac remodeling post-MI leads to the clinical syndrome of systolic heart failure (HF), which despite optimized medical therapy is associated with extremely high mortality rates. Novel therapeutic strategies to preserve LV contractile function and limit adverse cardiac remodeling are therefore urgently required to treat patients with MI and improve survival rates and quality of life.

The Runx gene family (RUNX1, RUNX2 and RUNX3) encodes DNA-binding α-subunits that partner core binding factor β (CBFβ) to form heterodimeric transcription factors. RUNX proteins act as both activators and repressors of target genes in normal development and disease states. To date, most research has focused on the role of RUNX1 in hematopoiesis owing to the frequent involvement of this gene in leukemic translocations. By contrast, little was known about the role of Runx1 in the heart. This discrepancy is not surprising given that although Runx1 expression is reported in neonatal cardiomyocytes it decreases to minimal levels in adult cardiomyocytes. However, studies have demonstrated that Runx1 is re-activated in cardiomyocytes of the border zone (BZ) region adjacent to the infarct in both patients with MI and experimental animal models. Whether activation of Runx1 in adult cardiomyocytes...
following MI is simply a marker of myocardial damage or actually plays a role in the progression of adverse cardiac remodeling is currently unknown.

We have now addressed this question by inducing MI in a mouse model where Runx1 has been specifically excised in cardiomyocytes. We report that these mice were protected against adverse cardiac remodeling following MI, with markedly preserved LV systolic function through improved SR-mediated calcium uptake. Re-activation of Runx1 following MI therefore plays a crucial role in excitation–contraction (EC) coupling and adverse cardiac remodeling and represents a new therapeutic target with the potential to limit progression to heart failure among patients with MI.

**Methods**

Detailed methods are provided in the Supplemental Material. The care and use of animals was in accordance with the UK Government Animals (Scientific Procedures) Act 1986 (ASPA). All animal procedures were approved by the University of Glasgow ethical review panel and licensed by the Home Office, UK (Project License Number 600/4503).

**Coronary artery ligation**

Mice aged 10–12 wk (25–30g) underwent thoracotomy and left anterior descending (LAD) coronary artery ligation (permanent/temporary) using standard approaches.

**Generation of cardiomyocyte specific Runx1 knock-out mice**

Runx1$^{fl/fl}$ mice described previously, were crossed with mice expressing tamoxifen-inducible Cre recombinase (MerCreMe) under the control of the cardiac specific alpha-myosin heavy chain ($\alpha$MHC) to produce the relevant test and control cohorts (Supplemental Material). PCR of
genomic DNA, RNA isolation, cDNA synthesis, real-time qPCR analysis and immunoblotting are detailed in the Supplemental Material.

Cardiac phenotyping

Echocardiography M-mode measurements were performed before and after LAD ligation and pressure–volume (PV) loop measurements recorded as a terminal procedure using the Scisense/Transonic small animal model PV system.

Histology

Quantification of regional areas and infarct size was performed on picrosirius red/triphenyl tetrazolium chloride (TTC) stained histological sections using Image J and Adobe Photoshop. Cardiomyocyte size was assessed by AlexaFluor-594 conjugated wheat germ agglutinin (WGA; Invitrogen, UK) on adjacent sections. RNAscope using probes to specifically identify cardiomyocyte nuclei (pericentriolar material 1; PCM-1) and Runx1 was performed as detailed in the Supplemental Material. For each heart, positive (PPIB and POLR2A) and negative controls (bacterial dapB) were run (Supplemental Material Figure 1).

Calcium measurements

Cardiomyocytes were isolated as previously described\(^{10}\), loaded with a calcium–sensitive fluorophore (5.0\(\mu\)mol/L Fura-4F AM, Invitrogen) and perfused during field–stimulation (1.0Hz, 2.0ms duration, stimulation voltage set to 1.5 x threshold). The Fura-4F fluorescence ratio (340/380nm excitation) was measured using a spinning wheel spectrophotometer (Cairn Research Ltd.; sampling rate of 5.0kHz) to measure the intra-cardiomyocyte \([\text{Ca}^{2+}]\). Cell-edge detection (IonOptix) was used to measure cell length. Data were analyzed offline as previously described\(^{11}\). Particular experiments utilized pretreatment (30min) and perfusion with the PKA inhibitor H89 (1\(\mu\)mol/L; Tocris Biosciences, Bristol UK) as previously described\(^{12}\).
Adenoviral overexpression of Runx1 in vitro

Adenoviral vectors expressing either enhanced green fluorescent protein (GFP; Ad-GFP) or GFP and Runx1 in a bicistronic configuration (Ad-Runx1) were prepared and titered (see Supplemental Material). Cardiomyocytes isolated from adult New Zealand white rabbits (3kg) were cultured and transduced at a multiplicity of infection (MOI) of 100 for 24h.

Statistics

Data were expressed as mean±SEM. Comparisons between MI and sham hearts were performed using the Student’s t test on raw data before normalization to percentage change. Comparisons between more than two groups were conducted on raw data using ANOVA. In cases where the two control groups were combined, statistics were performed on the pooled raw data of both control groups and compared with Runx1 Δ/Δ mice using the Student’s t test. In experiments where multiple isolated cardiomyocyte observations (n) were obtained from each heart (N), we have firstly ensured normality of data distribution and then determined the differences between control and experimental mice using linear mixed modeling (IBM SPSS Statistics v22) as previously published13.

Results

Expression of Runx1 following MI

Whilst Runx1 expression has previously been shown to increase at 3 wk post-MI it was unknown whether increased Runx1 expression occurred at a later time-point post-MI (e.g. 8 wk post MI). Furthermore, temporal changes in regional Runx1 expression have not previously been investigated. Runx1 expression was therefore quantified in hearts taken from C57BL/6J mice with MI induced by permanent coronary artery ligation and compared with C57BL/6J mice that
had a sham procedure but no coronary artery ligation. PV loop measurements confirmed
C57BL/6J mice with MI had reduced systolic (Supplemental Material Figure 2A-C) and diastolic
function (Supplemental Material Figure 2D&E) with a lower ejection fraction (Supplemental
Material Figure 2F-H).

*Runx1 mRNA and protein levels*

The levels of *Runx1* mRNA increased by 2.5 fold in whole hearts 4 wk post-MI relative to 4 wk
sham hearts (*P*<0.05; Supplemental Material Figure 2I). To determine the contribution of
specific myocardial regions to the observed increase in *Runx1* mRNA level, a separate cohort of
sham and MI hearts were isolated and tissue isolated from four different regions (Supplemental
Material Figure 2J). *Runx1* mRNA levels analyzed using the relative quantification (RQ) method
increased by 5.1 fold and 1.8 fold in the infarct (INF) and BZ regions of 4 wk post-MI hearts
relative to the respective right ventricular (RV) region (*P*<0.05; Supplemental Material Figure
2K). No detectable change was noted in the *Runx1* mRNA levels in the LV region at 4 wk post-
MI and no statistically significant regional differences were detected in the sham hearts
(Supplemental Material Figure 2K).

The levels of Runx1 protein changed in line with levels of *Runx1* mRNA (Supplemental
Material Figure 2L and M). Runx1 protein levels increased by 6.4 fold and 13.0 fold in the BZ
and INF regions, respectively, relative to the LV region in 3 wk post-MI hearts (*P*<0.05).

The pattern of *Runx1* mRNA expression was similar in 8 wk post-MI hearts, with an
increase of 3.7 fold and 2.2 fold in the INF and BZ regions, respectively, relative to the
Corresponding RV region (*P*<0.05; Supplemental Material Figure 3). However, in contrast to 4
wk post-MI hearts, the *Runx1* mRNA levels were increased by 2.7 fold in the LV region relative
to the RV region (*P*<0.05) at the 8 wk time point (Supplemental Material Figure 3).
All of the observed regional changes in Runx1 mRNA expression occurred in the absence of any such changes in the RV region of MI hearts relative to sham hearts.

**Expression of Runx1 in cardiomyocytes following MI**

We next delineated the spatial-temporal expression of Runx1 in cardiomyocytes from other cardiac cell types within different regions of the heart using RNAscope. Runx1 expression was found in 8-13% of cardiomyocytes and 5-7% of non-cardiomyocytes in the four regions (RV, LV and equivalent BZ and INF) of sham hearts (Figure 1A, B and C). Runx1 expression did not significantly differ from sham levels in the RV and LV regions at 1 and 14 day post-MI (Figure 1A, B and C). However, Runx1 expression significantly increased to 43% and 44% of cardiomyocytes within the BZ and INF region respectively at 1 day post MI; a time where whole heart contractile dysfunction was also first observed (Figure 1A and B and Supplemental Material Figure 4). Furthermore, Runx1 expression significantly increased to 59% and 47% of cardiomyocytes within the BZ and INF region respectively at 14 day post MI (Figure 1A and B). With regards to other cardiac cell types, Runx1 expression significantly increased to 14% of non-cardiomyocytes within the INF region at 1 day post MI (Figure 1A and C) and increased further to 35% and 26% of non-cardiomyocytes within the BZ and INF region respectively at 14 day post MI (Figure 1A and C). These data supported separate experiments which found an increased Runx1 level at 1 and 14 days post-MI in cardiomyocytes isolated from whole hearts and separated from other cell types as measured using rtqPCR data even though these data did not provide spatial resolution (Figure 1D).

**Direct assessment of Runx1 function in cardiomyocytes.**

To directly determine the contribution of Runx1 in cardiomyocytes to reduced cardiac function, we generated cardiomyocyte-specific Runx1-deficient mice using Cre-LoxP-based gene targeting
strategies (Supplemental Material and Supplemental Material Figure 5). Three groups of mice were generated: Runx1Δ/Δ mice (aMHC-MerCreMer:Runx1Δ/Δ); litter-mate Runx1Δ/Δ mice controlling for the insertion of the LoxP sites and Runx1wt/wt mice controlling for insertion of the tamoxifen-inducible Cre recombinase (aMHC-MerCreMer:Runx1wt/wt). Cardiomyocyte-specific excision of Runx1 was induced in adult mice by a single i.p. injection of tamoxifen (40mg/Kg). PCR of genomic DNA, rtqPCR and western blot analysis was performed on isolated cardiomyocytes and confirmed successful deletion of the Runx1 gene following injection with tamoxifen (Supplemental Material Figure 5).

**In vivo echocardiographic assessment of Runx1Δ/Δ mice post-MI**

In order to establish whether LV systolic function was altered in Runx1Δ/Δ mice post MI we utilized M-mode echocardiography.

**Cardiac function**

MI was surgically induced in Runx1Δ/Δ, Runx1Δ/Δ and Runx1wt/wt mice 1 wk after tamoxifen injection to all mice. Echocardiography was performed before MI and every 2 wk post-MI to assess cardiac contractile function (Figure 2A). As expected, cardiac systolic function (assessed by fractional shortening) decreased in both groups of control mice (Runx1Δ/Δ and Runx1wt/wt) post-MI (Figure 2B). By contrast, Runx1Δ/Δ mice demonstrated a markedly preserved fractional shortening that was 158% of the control mice at 8 wk post-MI (39.5±0.7 vs. 24.9±1.9%; P<0.05; Figure 2A and B). Runx1Δ/Δ mice undergoing a sham procedure 1 wk post-tamoxifen injection demonstrated no change in fractional shortening over the equivalent 8 wk time course and were not significantly different from the Runx1Δ/Δ MI mice until the 8 wk time point (Figure 2B). The improved fractional shortening in Runx1Δ/Δ mice post-MI was the culmination of substantially improved cardiac contraction as evidenced by the smaller LV internal diameter measured at
systole (LVIDs; Figure 2A and C), which was 77% of the two control groups post-MI (2.5±0.2 vs. 3.3±0.1mm; P<0.05). Runx1<sup>ΔΔ</sup> mice undergoing a sham procedure post-tamoxifen administration demonstrated no change in LVIDs over the equivalent 8 wk time course. The LVID measured at diastole (LVIDd) within the BZ region indicated that the hearts of both control and Runx1<sup>ΔΔ</sup> mice post-MI dilated at this level of the myocardium albeit to a lesser degree in Runx1<sup>ΔΔ</sup> mice (Figure 2D).

**Cardiac structure**

As expected, LV posterior wall thickness during systole (LVPWs) measured at the level of the BZ of control mice (Runx1<sup>fl/fl</sup> and Runx1<sup>wt/wt</sup>) thinned after the 2 wk post-MI time point due to the cardiac remodeling process (Figure 2E). By contrast, Runx1<sup>ΔΔ</sup> mice displayed preserved wall thickness that was 164% of control mice (Runx1<sup>fl/fl</sup> and Runx1<sup>wt/wt</sup>) at 8 wk post-MI (2.07±0.14 vs. 1.27±0.06mm; P<0.05; Figure 2E). Runx1<sup>ΔΔ</sup> mice undergoing a sham procedure post-MI demonstrated no change in LVPWs over the equivalent 8 wk time course. The wall-thickness data were confirmed at diastole 8 wk post-MI (Figure 2F).

These data were confirmed in: (i) a separate blinded study (Supplemental Material Figure 6A-E) where the operator was blinded to the animals undergoing surgery, echocardiography and analysis before MI and at 2 wk post-MI and (ii) at earlier time points at and before 1 wk post-MI (Supplemental Material Figure 7).

**In vivo ventricular luminal volumes and ejection fraction in Runx1<sup>ΔΔ</sup> mice 2 wk post-MI**

LV ventricular luminal volume of the Runx1<sup>ΔΔ</sup> and control mice was assessed *in vivo* at 2 wk post-MI using pressure volume (PV) loops (Figure 2G). The end diastolic volume (EDV) in the Runx1<sup>ΔΔ</sup> mice was reduced to 82% of that in the control mice (Runx1<sup>fl/fl</sup> and Runx1<sup>wt/wt</sup>), indicating a reduction in LV dilation (36.3±3.00 vs. 44.5±2.47 μL; P<0.05; Figure 2G and H).
The end systolic volume (ESV) in Runx1<sup>Δ/Δ</sup> mice was reduced to 54% of that of the control mice, indicating a greater level of emptying of LV blood volume (12.9±2.98 vs. 23.7±2.51 μL; \(P<0.05\); Figure 2G and I). This leftward shift in the PV loop in the Runx1<sup>Δ/Δ</sup> mice resulted in an ejection fraction (EF) that was 138% of the control mice (66.3±5.69 vs. 48.0%±3.18%; \(P<0.05\); Figure 2G and J).

**Histological assessment of Runx1<sup>Δ/Δ</sup> mice 8 wk post-MI**

**Heart structure**

We next investigated whether altered whole heart structure contributed to the preserved cardiac performance of Runx1<sup>Δ/Δ</sup> mice post-MI. Analysis of the different regions of the heart (Figure 3A) using picrosirius red staining of serial sections of hearts from Runx1<sup>Δ/Δ</sup> mice 8 wk post-MI showed that the mean two-dimensional whole heart area (RV+SEP+LV+INF) was 112% of control mice (Runx1<sup>fl/fl</sup> and Runx1<sup>wt/wt</sup>) post-MI (37.4±1.4 vs. 33.5±0.6mm<sup>2</sup>; \(P<0.05\); Figure 3B). This increase in heart area was associated with the LV free wall (Figure 3A; dotted area), which in Runx1<sup>Δ/Δ</sup> mice post-MI was 127% of the control mice post-MI (11.0±0.8 vs. 8.7±0.4mm<sup>2</sup>; \(P<0.05\); Figure 3C). No change was detected in the RV wall area in Runx1<sup>Δ/Δ</sup> mice post-MI (Figure 3D) and therefore further investigation focused on the structure of the LV.

LV wall thickness (measured at the level of the BZ; Figure 3A; arrows) in Runx1<sup>Δ/Δ</sup> mice post-MI was 127% of control mice post-MI (2.07±0.2 vs. 1.63±0.1mm; \(P<0.05\); Figure 3E), a finding that supported the echocardiographic data (Figure 2E). No change was detected in the septal-wall thickness in Runx1<sup>Δ/Δ</sup> mice post-MI (Figure 3F) or overall heart weight (Figure 3G). Infarct thickness and fibrosis (Figure 3H&I) was not different in Runx1<sup>Δ/Δ</sup> mice post-MI versus control mice post-MI. Furthermore, infarct size (32.3±1.5% vs. 32.7±3.1 vs. 31.9±5% of LV; Runx1<sup>Δ/Δ</sup> (N=5) vs. Runx1<sup>wt/wt</sup> (N=7) vs. Runx1<sup>fl/fl</sup> (N=5); \(P>0.05\); Figure 3J) at 8 wks post-MI.
(and the earlier time point of 24 h post-MI; Supplemental Material Figure 8A and B) was not
different in Runx1A/A versus control mice, and therefore did not explain the preserved LV
function observed in vivo (Figure 2B).

Cardiomyocyte size

To investigate why LV free wall thickness was preserved in Runx1A/A mice 8 wk post MI relative
to the wall thinning observed in control mice post-MI (Figures 2E and F), the cardiomyocyte size
in Runx1A/A mice 8 wk post MI was determined using wheat germ agglutinin staining (WGA). As
expected, LV cardiomyocytes from control Runx1fl/fl and Runx1wt/wt mice 8 wk post-MI exhibited
significant cell lengthening to 121% and 118% of Runx1A/A sham mice (111.7±3.8 vs. 108.8±3.7
vs. 92.53±1.04μm; P<0.05; Figure 3K and L). However, cardiomyocyte elongation was absent in
Runx1A/A mice at 8 wk post-MI (Figure 3K and L). An equivalent absence of cardiomyocyte
lengthening was also observed in septal cardiomyocytes (Figure 3M). LV cardiomyocytes from
control Runx1fl/fl and Runx1wt/wt mice 8 wk post-MI exhibited a significant decrease in cell
diameter to 86% and 85% of Runx1A/A sham mice (15.91±0.83 vs. 15.88±0.75 vs. 18.86±0.37μm;
P<0.05; Figure 3N and O). However, cardiomyocyte cell diameter did not decrease in Runx1A/A
mice 8 wk post-MI (Figure 3O). LV cardiomyocytes from control Runx1fl/fl and Runx1wt/wt mice 8
wk post-MI exhibited a significant decrease in cardiomyocyte cross-sectional area to 79% and
76% of Runx1A/A sham mice (350.6±23.9 vs. 338.2±22.6 vs. 440.0±11.5μm; P<0.05; Figure 3N
and P). However, cardiomyocyte cell cross-sectional area did not decrease in Runx1A/A mice 8 wk
post-MI (Figure 3P). No change in septal cardiomyocyte diameter was observed at 8 wk post-MI
in any group (Figure 3Q).
Calcium transients in Runx1<sup>+/−</sup> mice 2 wk post-MI

Although increased fractional shortening paralleled increased wall thickness in Runx1<sup>+/−</sup> mice at 8 wk post-MI (Figure 2B and E), we noted that wall thickness was not significantly different between the three groups at 2 wk post-MI. However, Runx1<sup>+/−</sup> mice still exhibited greater fractional shortening at this time point than was observed for the two control groups. To investigate this dichotomy, we isolated cardiomyocytes from hearts at 2 wk post-MI to characterize calcium handling. This was achieved by measuring the intracellular calcium concentration ([Ca<sup>2+</sup>]), focusing on electrically induced SR-mediated calcium release (calcium transients) into the cytosol, which predominately determines the force of contraction.

Cardiomyocytes isolated at 2 wk post-MI were stimulated at 1.0 Hz to elicit calcium transients and cell shortening (Figure 4A-C). The calcium transient peak (systolic [Ca<sup>2+</sup>]) in Runx1<sup>+/−</sup> mice was 117% and 122% of the control (Runx1<sup>+/+</sup> and Runx1<sup>+/−</sup>) mice (582.8±36.1 vs. 497.4±31.3 nM vs. 477.0±24.5nM [Ca<sup>2+</sup>]; <i>P</i>＜0.05; Figure 4B and D). The calcium transient minimum (diastolic [Ca<sup>2+</sup>]) in Runx1<sup>+/−</sup> mice was 92% and 89% of the control (Runx1<sup>+/+</sup> and Runx1<sup>+/−</sup>) mice (137.8±4.5 vs. 149.8±6.3 vs. 155.1±6.8nM [Ca<sup>2+</sup>]; <i>P</i>＜0.05; Figure 4B and E). The changes in peak and minimum [Ca<sup>2+</sup>] of Runx1<sup>+/−</sup> mice resulted in a calcium transient amplitude which was 128% and 138% of the control (Runx1<sup>+/+</sup> and Runx1<sup>+/−</sup>) mice (445.0±34.3 vs. 347.5±29.0 vs. 321.9±20.7nM [Ca<sup>2+</sup>]; <i>P</i>＜0.05; Figure 4B and F). Furthermore, the time constant of calcium transient decay in the Runx1<sup>+/−</sup> mice was 65% and 55% of the control (Runx1<sup>+/+</sup> and Runx1<sup>+/−</sup>) mice (0.074±0.007 vs. 0.114±0.018 vs. 0.134±0.020s; <i>P</i>＜0.05; Figure 4B and G), suggesting an increased rate of removal of calcium from the cytosol.

The increased calcium transient amplitude in Runx1<sup>+/−</sup> mice post-MI occurred in the absence of any significant changes in calcium entry or action potential duration (as measured
indirectly using the QT interval on the electrocardiogram or directly using voltage measurements on isolated cardiomyocytes at 2 wk post-MI) (Supplemental Material Figure 9).

Caffeine-induced calcium transients and cell shortening

We hypothesized that the lowered time constant of decay detected in the Runx1Δ/Δ mice 2 wk post-MI might reflect either increased SR calcium uptake via SERCA or extrusion from the cell via the sodium calcium exchanger (NCX). To address this issue, we applied a rapid bolus of 10mM caffeine at the end of the protocol to release all of the calcium from the SR into the cytosol. This approach enabled assessment of SR calcium content.

The SR calcium content of the Runx1Δ/Δ mice was 135% and 118% of the control Runx1fl/fl and Runx1wt/wt mice, respectively (842.8±47.2 vs. 624.3±42.2 vs. 712.6±40.0nM [Ca²⁺]; P<0.05; Figure 4H). SERCA-mediated calcium uptake is bypassed during application of 10mM caffeine and cytosolic calcium removal occurs predominately via NCX. The activity of NCX, as assessed by the time constant of caffeine-induced calcium transient decay, was not different between the three groups (Figure 4I).

The increased SR calcium content observed in Runx1Δ/Δ mice might reflect enhanced SERCA activity (K_{SERCA}). Therefore, we measured the rate constant of decay of the caffeine-induced calcium transient (which includes sarcolemmal efflux but not SR calcium uptake) and subtracted this value from that of the electrically stimulated calcium transient (which includes both SR calcium uptake and sarcolemmal efflux)⁴,¹⁵. The K_{SERCA} of the Runx1Δ/Δ mice was 148% and 160% of control Runx1fl/fl and Runx1wt/wt mice (14.4±1.4 vs. 9.7±1.8 vs. 9.0±1.2s⁻¹; P<0.05; Figure 4J). To corroborate that the increased calcium transient amplitude of Runx1Δ/Δ mice resulted in increased cell shortening, we performed edge-detection shortening measurements (Figure 4C). Cardiomyocyte shortening in Runx1Δ/Δ mice 2 wk post-MI was 156%
and 203% of the control Runx1^{fl/fl} and Runx1^{wt/wt} mice (7.5±0.9 vs. 4.8±0.6 vs. 3.7±0.4% of diastolic length; \( P<0.05 \); Figure 4C and K).

**Effect of overexpressing Runx1 on calcium transient amplitude and SR calcium content in normal cardiomyocytes**

To further support the novel link between Runx1 and calcium handling in isolated cardiomyocytes, we performed a gain-of-function study by overexpressing Runx1 via adenoviral-mediated gene transfer (Ad-Runx1) in isolated adult cardiomyocytes from normal hearts. The calcium transient amplitude in Ad-Runx1-transduced cardiomyocytes was 53% of cardiomyocytes transduced with the control adenoviral vector expressing green fluorescent protein (Ad-GFP) (70.5±8.3 vs. 133.4±30.7nM [Ca^{2+}]; \( P<0.05 \); Figure 4L and M). SR calcium content in cardiomyocytes overexpressing Runx1 was 60% of the control cardiomyocytes (388.8±60.3 vs. 651.8±84.4nM [Ca^{2+}]; \( P<0.05 \); Figure 4N).

**Expression of calcium handling proteins in Runx1^{-/-} mice 2 wk post-MI**

To investigate the mechanism by which SERCA activity is increased, we quantified the expression and phosphorylation levels of key calcium handling proteins involved in the control of SERCA-mediated calcium uptake in isolated cardiomyocytes 2 wk post-MI.

Levels of phospholamban (PLB), an inhibitory protein that regulates SERCA activity, were not significantly altered in the Runx1^{-/-} mice (Figure 5A and B). By contrast, phosphorylation of PLB (which relieves SERCA inhibition and improves cardiac contractility) at the PKA-target residue Ser16 [P-PLB (Ser16)] was 331% of the control mice (Runx1^{wt/wt} and Runx1^{fl/fl}) (331.2±94.5 vs. 100±35.6% change; \( P<0.05 \); Figure 5A and C).

Decreased levels of PKC indirectly enable enhanced phosphorylation of PLB and increase cardiac contractility\(^{16}\); however, no between-group differences were detected in the
levels of PKC (Figure 5D and E). Phosphorylation of PLB at the CaMKII-target residue threonine-17 ([P-PLB (Thr17)] in Runx1\textsuperscript{Δ/Δ} mice was 175% of the control mice (Runx1\textsuperscript{wt/wt} and Runx1\textsuperscript{β/β}) (175.0±22.9 vs. 100±8.2% change; \(P<0.05\); Figure 5D and F). A possible regulator of phosphorylation of PLB is protein phosphatase 1 (PP1), which dephosphorylates PLB\textsuperscript{16}. We found that expression of PP1 in Runx1\textsuperscript{Δ/Δ} mice was decreased to 28% of the control mice (Runx1\textsuperscript{wt/wt} and Runx1\textsuperscript{β/β}) post-MI (27.6±19.4 vs. 100±21.3% change; \(P<0.05\); Figure 5G and H).

To confirm that the increased SERCA activity, SR calcium content and calcium transient amplitude observed in Runx1\textsuperscript{Δ/Δ} mice 2 wk post-MI (Figure 4) were PKA-mediated; we investigated the effect of the PKA inhibitor (H89) on calcium handling. Addition of H89 completely blocked enhancement of all three parameters in the Runx1\textsuperscript{Δ/Δ} mice relative to the control Runx1\textsuperscript{wt/wt} and Runx1\textsuperscript{β/β} mice (Figure 5I–K).

**Cardiac contractility in Runx1\textsuperscript{Δ/Δ} mice after ischemia with reperfusion**

Reperfusion of a blocked coronary artery limits cell death following MI; this effect can be achieved clinically via percutaneous coronary intervention. We therefore tested whether Runx1\textsuperscript{Δ/Δ} mice also maintain a preserved LV contractile function in an additional clinically relevant model of ischemia with reperfusion (I/R).

The left anterior descending coronary artery was temporarily ligated *in vivo* for 45min followed by reperfusion and the Runx1\textsuperscript{Δ/Δ} mice recovered for 8 wk. Fractional shortening was assessed using echocardiography before MI and weekly after the induction of MI with reperfusion. As expected, fractional shortening decreased in control Runx1\textsuperscript{β/β} mice after reperfusion (Figure 6A and B). By contrast, Runx1\textsuperscript{Δ/Δ} mice demonstrated markedly preserved fractional shortening, which was 154% of control Runx1\textsuperscript{β/β} mice at 8 wk post-reperfusion (42.7±1.5 vs. 27.7±2.13%; \(P<0.05\); Figure 6A and B). As with the animal model of permanent
coronary artery ligation, infarct size at 8 wks post-reperfusion (and the earlier time point of 24 h post-reperfusion) was not different in Runx1^{Δ/Δ} versus control mice (Figure 6C and Supplemental Material Figure 8C and D).

**Discussion**

Runx1 has been most intensively studied in the hematopoietic system because its function is frequently corrupted in different subtypes of leukemia. Although it is known to have a role in lineage differentiation and tissue function in a range of other systems there is almost no information relating to its role in adult cardiomyocytes other than the observation that it can be reactivated following myocardial insult\(^5,7\). Our novel study addressed a vital question, namely, is increased expression of RUNX1 post-MI merely a marker of ischemic damage or does it play a functional role in adult cardiomyocytes following MI? We provide new evidence that Runx1 has an important role in cardiomyocytes following MI. Reducing Runx1 function preserved cardiac contractility and prevented adverse cardiac remodeling, which suggests that targeting the actions of this gene could have important implications for patient survival post-MI. Importantly, this research transcends discipline boundaries as it not only widens the importance of Runx1 to other fields of medicine but also describes a novel function for this gene.

Our results provide the first detailed quantification of regional Runx1 expression in mouse heart tissue after myocardial infarction. At 4 wk post-MI, Runx1 mRNA was increased within the BZ myocardium and INF region which was sustained until at least 8 wk post-MI, at which time point Runx1 expression also increased within the remote LV myocardium. This is important given that changes in Runx1 expression at the mRNA and protein level are not restricted to rodent MI models but also occur in patients with MI\(^5,7\). In separate experiments, we
were able to demonstrate that Runx1 expression is increased within the BZ and INF region post-MI within the contractile elements of the heart i.e. the cardiomyocytes, as early as 1 to 14 days post-MI (Figure 1).

To determine the specific contribution of Runx1 in cardiomyocytes to reduced cardiac contractility, we generated a new tamoxifen-inducible cardiomyocyte-specific Runx1 deficient mouse with the hypothesis that these mice would demonstrate improved cardiac function. Induction of MI in control transgenic mice led to the expected LV wall thinning, cardiac dilation and reduced contractility 8 wk post-MI. However, all of these adverse cardiac remodeling parameters were absent or reduced in Runx1\(^{-/-}\) mice at this time point. One possible explanation for the observed preservation of systolic function could have been a reduction in the infarct size of Runx1\(^{-/-}\) mice given that infarct size correlates with systolic function\(^1\). However, Runx1\(^{-/-}\) mice exhibited preservation of geometric shape and contractility post-MI, with no difference in infarct size (at both early and late time points) or fibrosis versus the control mice.

To establish the mechanism underlying these notable findings, we first investigated cardiomyocyte size. Control mice demonstrated the expected cardiomyocyte lengthening and thinning (eccentric hypertrophy) at 8 wk post-MI\(^1\). However, these changes were absent in Runx1\(^{-/-}\) mice. Such protection against eccentric hypertrophy at 8 wk post-MI seems highly likely to have afforded Runx1\(^{-/-}\) mice protection from ventricular dilation and thinning, ultimately leading to preserved contractility. Nevertheless, at 2 wk post-MI, the wall thickness in Runx1\(^{-/-}\) mice was comparable to that of control mice (as wall thinning in control mice had not yet begun) but contractile function was still dramatically improved. This finding indicated that prevention of wall thinning and dilation could not fully explain the preserved contractile function observed at 2 wk post-MI.
The above dichotomy led us to investigate calcium handling in cardiomyocytes isolated from Runx1<sup>Δ/Δ</sup> mice 2 wk post-MI, in particular electrically stimulated calcium release (the calcium transient) from the intra-cardiomyocyte calcium store (the SR) and subsequent cell shortening. Patients and animal models with MI typically exhibit calcium transients with lower amplitude and a slower rate of decline than control/healthy cardiomyocytes, an observation largely attributed to reduced SR-mediated calcium uptake via SERCA<sup>15</sup>. Runx1<sup>Δ/Δ</sup> mice exhibited increased calcium transient amplitude and reduced time constant of decline post-MI compared to control mice post-MI, resulting in an increase in cell shortening. The accompanying higher SR calcium content observed in Runx1<sup>Δ/Δ</sup> mice post-MI can explain the enhanced calcium transient amplitude<sup>18</sup> since equalizing the SR calcium content with H89 resulted in a calcium transient equivalent to control.

Analysis of the caffeine-induced calcium transient found no detectable change in NCX activity in Runx1<sup>Δ/Δ</sup> mice post MI as compared to control mice post-MI. However, enhanced SR-mediated calcium uptake via SERCA was observed in the Runx1<sup>Δ/Δ</sup> mice. SERCA activity is a major determinant of the SR calcium content. Furthermore, this pump is regulated predominately by the inhibitory protein phospholamban (PLB). Although expression of PLB was not altered in Runx1<sup>Δ/Δ</sup> mice post-MI, we explored some of the proteins that regulate PLB activity<sup>16</sup>.

PLB-mediated inhibition of SERCA is balanced by phosphorylation by PKA and CaMKII (which relieves SERCA inhibition) and dephosphorylation by PP1 (which returns PLB to its inhibitory state<sup>16</sup>). We found that ventricular cardiomyocytes from Runx1<sup>Δ/Δ</sup> mice exhibited increased PKA-mediated phosphorylation of PLB possibly as a result of reduced levels of PP1. These mechanistic data suggest that PLB phosphorylation stimulates SERCA activity in Runx1<sup>Δ/Δ</sup> mice post-MI, and leads to an increased SR calcium content, which in turn increases electrically...
induced SR-mediated calcium release and doubles cardiomyocyte contraction. Our proposed mechanism was supported by complete blockage of the enhanced calcium transient in cardiomyocytes from Runx1\(^{\Delta/\Delta}\) mice 2 wk post-MI by inhibition of PKA. The enhanced rate of removal of calcium from the cytosol following increased SERCA activity is sufficient to reduce the end diastolic [Ca\(^{2+}\)]\(_{i}\), which not only improves whole heart relaxation but may also limit the stimulation of hypertrophic factors\(^1\).

Previous studies strongly support our proposed mechanism that the effect of Runx1 on SR function is a major contributor to the beneficial effects observed in Runx1\(^{\Delta/\Delta}\) mice post-MI. Decreased SR function has been demonstrated among patients with HF\(^{15}\) and enhanced SR-mediated calcium cycling markedly preserved contractility, reduced adverse cardiac remodeling and delayed progression to heart failure at levels not dissimilar from those observed in the current study\(^{16,19}\).

The key findings of our study will likely initiate further research into the beneficial effects of decreasing Runx1 expression in alternative animal models of cardiac disease. As testament to this goal, we found that Runx1\(^{\Delta/\Delta}\) mice are protected from adverse cardiac remodeling in a separate clinically relevant surgical model. In this model, the blocked coronary artery was subsequently unblocked after a period of ischemia, as would be the case for patients undergoing percutaneous coronary intervention. These additional data further support our study and the translational potential of this new target.

Although our strategy did not result in inactivation of the Runx1 gene in all cardiomyocytes, we postulate that cardiac function improves when only a subset of cardiomyocytes benefits from better Ca\(^{2+}\) handling after inactivation of Runx1. Since it is more feasible in a clinical setting to suppress genes in a subset of cardiomyocytes rather than in all
cells, we think Runx1, or targets showing a similar potency, are particularly attractive for therapeutic interventions.

In conclusion, we have demonstrated for the first time that Runx1 modulates cardiac SR calcium uptake and contractile function. Reducing Runx1 function drives increased contractility post-MI, thereby preserving LV systolic function and preventing adverse cardiac remodeling. Clinical studies clearly demonstrate that preserving cardiac contractility and protecting against adverse cardiac remodeling are key factors to limiting progression from MI to heart failure. Identification of a new therapeutic target that achieves this objective is urgently required. To this end, we envisage that Runx1 will be exploited in future basic and translational studies to limit progression of patients with MI to heart failure, thereby improving survival rates and quality of life.

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Disclosures

None

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References


Figure Legends

Figure 1. Runx1 expression in WT C57BL/6 mice post-MI. (A) Typical images of regional heart sections with RNA in situ hybridization (using RNAscope®). Regions examined were right ventricle (RV), left ventricle (LV), border zone (BZ) and infarct (INF) in 1 day (middle; n=3) and 14 day (bottom; n=3) post-MI and equivalent regions in sham hearts (top; n=5). Probes for Runx1 (pink) and PCM-1 (cardiomyocyte specific; brown) were used; colored punctate dots represent positive staining (arrows). Scale bar (10\(\mu\)m); magnified insert image (5\(\mu\)m). (B) Mean quantification of cardiomyocytes (PCM-1+) and (C) non-cardiomyocytes PCM-1-) with Runx1 positive staining as a % of the total number of cardiomyocytes or non-cardiomyocytes respectively (*\(P<0.05\), 1 day post-MI vs. sham; #\(P<0.05\), 14 day post-MI vs. sham - Student’s t-test). (D) Runx1 expression as measured by rtPCR in cardiomyocytes isolated from whole sham (n=17) and 1 day (n=8), 7 day (n=6) and 14 day (n=3) post-MI hearts (ANOVA). Stock (C57BL/6J; n=4) mice were included to show that there was no detectable difference with sham hearts (ANOVA).

Figure 2. Cardiac function in Runx1\(^{\Delta/\Delta}\) mice. (A) Echocardiography (scale:x=0.1s;y=2mm). (B) 8 wk echocardiographic data for fractional shortening (FS) and (C) LV internal diameter (LVID) at systole (LVIDs), (D) LVID at diastole (LVIDd), (E) LV posterior wall thickness at systole (LVPWs) and (F) LVPW thickness at diastole (LVPWd). (Runx1\(^{\Delta/\Delta}\) MI and Runx1\(^{\text{wt/wt}}\) MI combined [n=11], Runx1\(^{\Delta/\Delta}\) MI [n=9] and Runx1\(^{\Delta/\Delta}\) sham [n=5]), (ANOVA). #\(P<0.05\)=Runx1\(^{\Delta/\Delta}\) MI mice versus Runx1\(^{\Delta/\Delta}\) sham mice. *\(P<0.05\)=Runx1\(^{\Delta/\Delta}\) MI mice versus control Runx1\(^{\text{fl/fl}}\) MI and Runx1\(^{\text{wt/wt}}\) MI mice combined. (G) Pressure–volume (PV) loops of Runx1\(^{\text{wt/wt}}\) and Runx1\(^{\Delta/\Delta}\) 2
wk post-MI. (H) Mean PV data 2 wk post-MI End diastolic volume (EDV), (I) End systolic volume (ESV) and (J) Ejection fraction (EF) (Runx1<sup>β/β</sup> MI and Runx1<sup>wt/wt</sup> MI combined [n=11], Runx1<sup>Δ/Δ</sup> MI [n=8]), *P<0.05 Student’s t-test.

Figure 3. Runx1<sup>Δ/Δ</sup> mice cardiac structure 8 wk post-MI. (A) Picrosirius red stained hearts (BZ,border zone; LV,left ventricle; INF,infarct; RV,right ventricle; SEP,septum; scale: 1mm). Mean (B) area of whole heart (all regions), (C) LV, (D) RV and (E) LV wall thickness at BZ region, (F) septum wall thickness at BZ region level, (G) heart weight to body weight ratio, (H) infarct thickness, (I) LV fibrosis and (J) infarct size (Runx1<sup>β/β</sup> MI and Runx1<sup>wt/wt</sup> MI combined [n=12], Runx1<sup>Δ/Δ</sup> MI [n=5]; *P<0.05), Student’s t-test. (K) Wheat germ agglutinin (WGA) staining of LV cardiomyocytes (longitudinal) of Runx1<sup>β/β</sup> MI (top) and Runx1<sup>Δ/Δ</sup> (bottom) post-MI (scale bar:25mm). (L) Mean LV cardiomyocyte length (Runx1<sup>Δ/Δ</sup> sham [n=55 cardiomyocytes, n=3 hearts], Runx1<sup>β/β</sup> MI [n=122 cardiomyocytes, n=6 hearts], Runx1<sup>wt/wt</sup> MI [n=109 cardiomyocytes from n=6 hearts], Runx1<sup>Δ/Δ</sup> MI [n=102 cardiomyocytes, n=6 hearts]), *P<0.05 linear mixed modelling. (M) Mean SEP cardiomyocyte length (Runx1<sup>Δ/Δ</sup> sham [n=29 cardiomyocytes, n=3 hearts], Runx1<sup>β/β</sup> MI [n=82 cardiomyocytes, n=6 hearts], Runx1<sup>wt/wt</sup> MI [n=84 cardiomyocytes, n=6 hearts], Runx1<sup>Δ/Δ</sup> MI [n=64 cardiomyocytes, n=6 hearts]. (N) WGA staining of LV cardiomyocytes (transverse) of Runx1<sup>wt/wt</sup> MI (Left) and Runx1<sup>Δ/Δ</sup> MI (Right) (scale bar:25mm). (O) Mean LV cardiomyocyte diameter (Runx1<sup>Δ/Δ</sup> sham [n=449 cardiomyocytes, n=3 hearts), Runx1<sup>β/β</sup> MI [n=811 cardiomyocytes, n=6 hearts], Runx1<sup>wt/wt</sup> MI [n=897 cardiomyocytes, n=6 hearts], Runx1<sup>Δ/Δ</sup> MI [n=878 cardiomyocytes, n=6 hearts]). (P) Mean LV cardiomyocyte cross sectional area (Runx1<sup>Δ/Δ</sup> sham [n=403 cardiomyocytes, n=3 hearts), Runx1<sup>β/β</sup> MI [n=714 cardiomyocytes, n=6 hearts], Runx1<sup>wt/wt</sup> MI [n=785 cardiomyocytes, n=6
hearts], Runx1<sup>A/d</sup> MI [n=699 cardiomyocytes, n=6 hearts]. *(Q) Mean SEP cardiomyocyte diameter (Runx1<sup>A/d</sup> sham [n=238 cardiomyocytes, n=3 hearts], Runx1<sup>B/b</sup> MI [n=465 cardiomyocytes, n=6 hearts], Runx1<sup>wt/wt</sup> MI [n=454 cardiomyocytes, n=6 hearts], Runx1<sup>A/d</sup> MI [n=452 cardiomyocytes, n=6 hearts]), *P<0.05 linear mixed modelling.

**Figure 4. Excitation–contraction coupling in Runx1<sup>A/d</sup> mice 2 wk post-MI.** (A) Protocol (B) Typical calcium (Ca<sup>2+</sup>) transients and (C) cell shortening. Mean Ca<sup>2+</sup> transient: (D) peak, (E) minimum and (F) amplitude (Runx1<sup>B/b</sup> MI [n=25 cardiomyocytes, n=3 hearts], Runx1<sup>wt/wt</sup> MI [n=28 cardiomyocytes, n=4 hearts], Runx1<sup>A/d</sup> MI [n=28 cardiomyocytes, n=3 hearts], *P<0.05 Runx1<sup>A/d</sup> MI vs. Runx1<sup>B/b</sup> MI and Runx1<sup>wt/wt</sup> MI combined, linear mixed modelling). (G) Mean time constant of Ca<sup>2+</sup> transient decay. (H) Mean caffeine-induced Ca<sup>2+</sup> transient amplitude. (I) Mean time constant of decay for caffeine-induced Ca<sup>2+</sup> transient amplitude. (J) Mean sarco–endoplasmic reticulum calcium transport ATPase (SERCA) activity. (K) Mean fractional shortening (Runx1<sup>B/b</sup> MI [n=27 cardiomyocytes, n=3 hearts], Runx1<sup>wt/wt</sup> MI [n=27 cardiomyocytes, n=4 hearts], Runx1<sup>A/d</sup> MI [n=23 cardiomyocytes, n=3 hearts]). (L) Ca<sup>2+</sup> transients from cardiomyocytes transduced with Ad-GFP or Ad-Runx1. Mean: (M) Ca<sup>2+</sup> transient peak; Ad-GFP (n=16 cardiomyocytes, n=6 hearts) and Ad-Runx1 (n=22 cardiomyocytes, n=6 hearts), *P<0.05 linear mixed modelling (N) Caffeine-induced Ca<sup>2+</sup> transient amplitude; Ad-GFP (n=13 cardiomyocytes, n=6 hearts) and Ad-Runx1 (n=19 cardiomyocytes, n=6 hearts), *P<0.05 linear mixed modelling.

**Figure 5. Phospholamban regulation in isolated cardiomyocytes from Runx1<sup>A/d</sup> mice 2 wk post-MI.** (A) Western blot of phospholamban (PLB), phosphorylation of PLB at serine-16 [P-
PLB (Ser16)] and pan-actin loading control. Percentage change in protein for (B) PLB and (C) P-PLB (Ser16/total PLB) (Runx1Δ/Δ MI [n=5 hearts] versus Runx1wt/wt and Runx1fl/fl MI [n=5 hearts]), *P<0.05 Student’s t-test. (D) Western blot of PKC, PLB, and PLB phosphorylation at threonine-17 (P-PLB (Thr17)). Percentage change in protein for (E) PKC and (F) P-PLB (Thr17/total PLB) (Runx1Δ/Δ MI [n=5 hearts] versus Runx1wt/wt MI and Runx1fl/fl MI [n=9 hearts]), *P<0.05 Student’s t-test. (G) Western blot of protein phosphatase 1 (PP1). (H) Percentage change in PP1 (Runx1Δ/Δ MI [n=5 hearts] versus Runx1wt/wt MI and Runx1fl/fl MI [n=9 hearts]). (I, J, K) Mean SERCA activity, caffeine–induced calcium transient amplitude and calcium transient amplitude data from Figure 4 compared to mean data obtained from 2 wk post-MI isolated cardiomyocytes with H89 (hatched white column; Runx1wt/wt MI and Runx1fl/fl MI+H89 [n=16 cardiomyocytes, 3 hearts] and hatched red column; Runx1Δ/Δ MI+H89 [n=15 cardiomyocytes, 3 hearts]), *P<0.05 Student’s t-test.

**Figure 6.** Runx1Δ/Δ mice after ischemia with reperfusion (I/R). (A) Echocardiographic images (scale:x=0.1s;y=2mm). (B) Mean echocardiographic fractional shortening data of Runx1fl/fl I/R (n=9) and Runx1Δ/Δ I/R (n=8) [*P<0.05], Student’s t-test. (C) Mean infarct size (Runx1fl/fl I/R [n=9] and Runx1Δ/Δ I/R [n=7]), Student’s t-test.
Figure 1: Heart Morphology and Function in Runx1 knockout Mice

A. Representative images of heart sections from Runx1 Δ/Δ MI, Runx1 wt/wt MI, and Runx1 Δ/Δ MI. RV = right ventricle, LV = left ventricle, SEP = septum, INF = infarcted area.

B. Area (mm²) comparison between groups: Runx1 Δ/Δ MI and Runx1 wt/wt MI.

C. Area (mm²) of LV and RV: Runx1 Δ/Δ MI and Runx1 wt/wt MI.

D. LV wall thickness comparison: Runx1 Δ/Δ MI and Runx1 wt/wt MI.

E. LV transverse section: Runx1 Δ/Δ MI.

F. SEP wall thickness comparison: Runx1 Δ/Δ MI and Runx1 wt/wt MI.

G. Heart weight/body weight ratio comparison: Runx1 Δ/Δ MI and Runx1 wt/wt MI.

H. Infarct thickness and fibrosis comparison: Runx1 Δ/Δ MI and Runx1 wt/wt MI.

I. Infarct size (% of LV) comparison: Runx1 Δ/Δ MI and Runx1 wt/wt MI.

J. LV transverse section: Runx1 Δ/Δ MI.

K. LV longitudinal section Runx1 Δ/Δ MI

L. LV cardiomyocyte length comparison: Runx1 Δ/Δ MI and Runx1 wt/wt MI.

M. SEP cardiomyocyte diameter comparison: Runx1 Δ/Δ MI and Runx1 wt/wt MI.

N. SEP cardiomyocyte cross-sectional area comparison: Runx1 Δ/Δ MI and Runx1 wt/wt MI.
Runx1 Deficiency Protects Against Adverse Cardiac Remodeling Following Myocardial Infarction


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**Coronary artery ligation**

Mice aged 10–12 wk (25–30 g) were anesthetized by inhalation of 4% isofluorane gas (Isoflo, Abbott Laboratories, USA) delivered in O₂ at 1.5 L/min. Animals were given preoperative analgesia of 5 mg/kg carprofen (Rimadyl, Pfizer Animal Health, U.) and 0.1 mg/kg buprenorphine (Vetergesic, Reckitt Benckiser Healthcare Ltd, UK) i.p. delivered in 0.4 mL of sterile saline. Mice were endotracheally intubated and maintained with artificial ventilation at 120 breaths/min with a tidal volume of 120 µL on 1% isofluorane in 0.5 L/min O₂. Mice were positioned in a left lateral oblique position. The skin was incised perpendicular to the sternum in parallel with the ribs 5 mm proximal to the xyphoid process. Underlying muscles were carefully retracted to expose the fifth intercostal space beneath. The intercostal muscle was cut using an electrocautery pen and the ribs retracted to expose the heart. The pericardial sac was opened and removed to provide access to the left anterior descending (LAD) coronary artery which was then ligated with 0.3 metric nylon (W2829 Ethilon, Johnson & Johnson, UK) 1.5 mm distal to the left atrial appendage. Three sutures were evenly pre-placed along the ribs using 0.7 metric non-absorbable prolene (W8711, Johnson & Johnson, UK). The lungs were reinflated until the pre-placed sutures were tied. The thoracic muscles were returned to their original position and skin closed with 0.7 metric absorbable vicryl (W9575, Johnson & Johnson, UK). Animals that underwent the sham procedure had a thoracotomy without LAD ligation. Temporary LAD ligation (I/R model) was achieved by using a piece of polyethylene tubing (PE-10; outer diameter 0.61 mm) against which the coronary artery was tied temporarily (45 min) and then subsequently released by removal of the tubing. Post-operatively, animals were given buprenorphine orally for 3 days.
**RNA isolation, cDNA synthesis and real-time qPCR analysis**

Total RNA was extracted from heart tissue using the miRNeasy Mini Kit (Qiagen, UK) with DNase I treatment (Qiagen, UK). 1 µg of RNA was then reverse transcribed to cDNA with Omniscript reverse transcriptase, dNTPs, RNase inhibitors and oligo dT primers (Omniscript Reverse Transcription kit, Qiagen, UK). Real-time quantitative PCR (qPCR) reactions were run using cDNA with a Runx1 quantitect primer assay (Amplicon length-120bp, Mm_Runx1_1_SG QuantiTect Primer Assay [QT00100380], Qiagen, UK) and SYBR green mastermix (Applied Biosystems, UK) or with a Runx1 Taqman Gene Expression Assay (Amplicon length 81bp, Mm01213404_m1) and Taqman Universal Mastermix Mix II, no UNG (both ThermoFisher Scientific, UK) in a 20 µL final volume or 10 µL final volume respectively with the following cycling conditions: hold for 2 min at 50°C followed by 10 min at 95°C to heat-start the Taq polymerase enzyme, then 40 cycles of 95°C for 10 min, 60°C for 1 min. Relative mRNA levels were analysed using comparative Ct calculations; either $2^{-\Delta\Delta Ct}$ (regional comparisons relative to RV region) or $2^{-\Delta Ct}$ (whole heart or cardiomyocytes) normalised to Gapdh (whole heart; Eurofins MWG Operon, Germany), or Gapdh Taqman Gene Expression Assays (Amplicon length 109 Mm99999915_g1, ThermoFisher Scientific, UK) or Peptidylprolyl Isomerase B; PPIB (isolated cardiomyocytes).

**Generation of mice with conditional cardiomyocyte-specific gene excision of Runx1**

We generated cardiomyocyte-specific Runx1-deficient mice using standard Cre-LoxP-based gene targeting strategies (Supplemental Material Figure 5). This approach utilized mice with LoxP sites flanking exon 4 of the Runx1 gene ($Runx1^{fl/fl}$) and mice with the cardiac-specific alpha-myosin heavy chain (MHC) promoter directing expression of a tamoxifen-inducible Cre recombinase (MerCreMer) to adult cardiomyocytes\(^1\). Cardiomyocyte-specific excision of Runx1 was induced in adult mice by a single i.p. injection of tamoxifen (40 mg/Kg) to prevent cardiac dysfunction arising from repeated injections\(^2\).
Genomic PCR was performed to determine the specificity of the Runx1 gene disruption 7 d after injection with tamoxifen (Supplemental Material Figure 5A–C). Excision of exon 4 (denoted by the PCR product presenting at approximately 310 bp following gel electrophoresis) was clearly present in all male and female mice. However, this size PCR product was absent in control Runx1fl/fl mice, which instead produced only a 275 bp PCR product, denoting insertion of the LoxP sites. Control Runx1wt/wt mice exhibited only a 203 bp PCR product representing an unaltered Runx1 gene as expected. To determine the extent to which the Runx1 gene had been excised from cardiomyocytes, PCR of genomic DNA was performed on both: (i) whole hearts and (ii) cardiomyocytes that had been separated from other cell types by plating and short term culture or by FACS (Supplemental Material Figure 5C). Isolated cardiomyocytes exhibited an increased ratio of the excised PCR product (310 bp) to the combined excised (310 bp) and LoxP product (275 bp) indicating approximately ~50% Runx1 gene excision (Supplemental Material Figure 5C).

Whilst the mRNA Runx1 levels were significantly reduced in whole heart tissue taken from the right (RV) and left ventricle (LV) of Runx1Δ/Δ mice compared to control mice (Runx1wt/wt and Runx1fl/fl) 4 wk post-MI, this was not the case in the border zone (BZ) and infarct (INF) regions reflecting the increased number of non-cardiomyocytes in these regions post-MI (Supplemental Material Figure 5D).

At 2 wk post-MI, mRNA Runx1 levels (relative to the house keeper gene Peptidylprolyl Isomerase B; PPIB) in cardiomyocytes isolated from Runx1Δ/Δ mice were 61% of the control mice (Runx1fl/fl) (0.055±0.006 vs. 0.090±0.012 (2−ΔΔct); P<0.05; Supplemental Material Figure 5E) and Runx1 protein levels in cardiomyocytes isolated from Runx1Δ/Δ mice were 48% of the control mice (Runx1wt/wt and Runx1fl/fl) (47.6±9.6 vs. 100±3.84% change; P<0.05; Supplemental Material Figure 5F) indicating that protein and mRNA expression correlates with the level of gene excision in these cells.
PCR of genomic DNA

Mice were killed by the schedule one procedure. Hearts were removed and perfused via the aorta with saline solution to wash out the blood before being snap frozen in liquid N$_2$ and stored at –80°C. The tissue was homogenized with the TissueLyser system (QIAGEN) using a stainless steel bead in each sample. Tissue was disrupted mechanically in proprietary lysis buffer (Illustra Nucelon Genomic DNA Extraction kit, GE Healthcare) until no visible pieces of tissue remained (~2 min at 25 oscillations/s). Lysates were incubated for at least 3 h with proteinase K at 50°C. DNA was extracted according to the manufacturer’s instructions. Primers used were as described by Chen et al. Runx1 forward primer WT and floxed alleles (Ex4Int – F563) 5’- CCC ACT GTG TGC ATT CCA GAT TGG -3’; Runx1 reverse primer for WT and floxed alleles (Ex4 – R837) 5’- GAC GGT GAT GGT CAG AGT GAA GC -3’; and Runx1 reverse primer for deleted floxed allele (Int3-2) 5’- CAC CAT AGC TTC TGG GTG CAG -3’. Herculase II Fusion DNA polymerase (Agilent) was used under the following stepwise cycling conditions:

1. 95°C 1 min
2. 95°C 20 s
3. 58°C 20 s
4. 68°C 1 min
5. Repeat steps 2–4, 30 times
6. 68°C 4 min
7. 12°C hold

RNAscope® assay

RNA in situ hybridization was performed using the RNAscope® duplex kit (Red/Brown) (Advanced Cell Diagnostics) on the Leica Bond Rx Autostainer to detect Runx1 and PCM-1 using mouse-specific probes (Advanced Cell Diagnostics) on formalin fixed paraffin embedded (FFPE) mouse hearts. PCM1 has been shown to be 95-99.9% specific to cardiomyocytes furthermore, cardiomyocytes could be identified morphometrically by their
elongated nucleus and/or the presence of surrounding muscle striations\textsuperscript{4-6}. Adult mouse hearts at 1 and 14 day post-MI and SHAM FFPE were cut at 4 μm and placed in an oven 60°C for 1 h. The duplex staining protocol was performed following the manufacturer's (Advanced Cell Diagnostics) strict guidelines. For each heart, positive (PPIB and POLR2A) and negative controls (bacterial dapB) were run (Supplemental Material Figure 1). The images were visualized on a brightfield Evos Cell Imaging microscope. Cells were considered to be positive if at least one brown (PCM-1) or red (Runx1) punctate dot was present within the nuclei - a single punctate dot representing a single mRNA transcript\textsuperscript{7}. Cells were manually counted for quantification as previously described\textsuperscript{7} taking three representative images per region of the heart on mid-heart sections where the aorta is confluent with the LV chamber.

**Pressure–volume loop measurements**

Pressure–volume (PV) loop measurements were recorded using the Transonic ADV500 small animal model PV measurement system or a Scisense PV loop system and a 1.2 F 4.5 mm spaced PV admittance catheter (FTH-1212B-4517, Scisense, UK) as previously described\textsuperscript{8}. Mice were anesthetized in a pre-filled induction chamber with 4% isofluorane gas (Isoflo, Abbott Laboratories, USA) delivered in O\textsubscript{2} at 1.5 L/min. Mice were endotracheally intubated and maintained with artificial ventilation at 120 breaths/min with a tidal volume of 120 µL. Isofluorane concentration was gradually reduced to 1.5% during the procedure and maintained at this level for the duration of the measurements. The temperature was maintained at 37°C by a homeothermic monitoring system (Harvard Apparatus, UK). A longitudinal incision was made in the center of the neck and the right carotid artery dissected free of the surrounding tissue. A small incision was made in the vessel and the catheter inserted into the vessel and advanced into the left ventricle of the heart. Steady state readings were then taken for 15 min. Data were recorded in the Labscribe software \textit{via} a four-channel analog-to-digital converter. Data were analyzed offline by averaging the final 2 min of steady state recording.
Echocardiography
Mice were anesthetized as for pressure–volume loop measurements and maintained via facemask on 0.5-1% isofluorane in 1.0 L/min O₂.

Histology
Hearts from killed animals were fixed for a minimum of 24 h in 10% neutral buffered formalin (CellPath, UK) after which time they were embedded into a wax block for sectioning. Each heart was sliced parallel to the long axis every 200 µm to produce 4 µm serial sections. At every 200-µm interval, picrosirius red was used to stain collagen until the mid-point depth of the heart (defined by the largest ventricular cavity size and confluence of the LV chamber with the aorta). Quantification of regional areas and infarct size was performed on each section using Image J and Adobe Photoshop CC 2015 and mean data per heart were generated as described in previous studies⁹. The point at which the infarct region became 50% of muscle and 50% of collagen (as determined using picrosirius red staining) was denoted as the BZ in histological sections. Infarct thickness was defined and measured as the distance between the endocardium and epicardium of the infarcted myocardium. This was performed using three lines drawn perpendicular to the curvature of the ventricular wall and the distance measured with ImageJ on each histological section. Cardiomyocyte size was assessed by staining adjacent sections at the level of the middle of the heart with AlexaFluor-594 conjugated wheat germ agglutinin (WGA; Invitrogen, Paisley, UK). Briefly, de-waxed and rehydrated sections were boiled in sodium citrate buffer for 10 min, followed by blocking in 1% BSA/PBS with 5% goat serum for 1 h. Sections were then incubated with 10 µg/mL WGA for 1 hr at room temperature in the dark. Sections were mounted in ProLong Gold with DAPI (Invitrogen, Paisley, UK). Confocal imaging was used to produce an image of the LV and septum from each heart. A digital grid was placed on the image using ImageJ and grid intersections determined which cardiomyocytes were then chosen for measurement of cell length, diameter (perpendicular to centre of longest axis) and cross-sectional area (thus avoiding cell selection bias).
Infarct size at 24 h post-MI and I/R injury.

Infarct size in the mouse MI model was determined using triphenyltetrazolium chloride (TTC) (dissolved at 1% in a phosphate buffer for 15 min [37°C]) to detect viable tissue in x5 transversely cut myocardial slices per heart. In the mouse I/R model, the area at risk (AAR) and infarct size was measured using previously published protocols\textsuperscript{10} whereby 1% Evans blue delineated the area not at risk, TTC the viable tissue and infarct region white. Heart slices were then placed into 10% neutral buffered formalin for 20 min at room temperature. Digital photographs of the myocardial sections from each heart were laid onto a white background and whole photograph white balanced against the background using Adobe photoshop. The number of pixels of each region was counted using Image J (National Institute of Health, Maryland, USA) and expressed as relative %.

Adult cardiomyocyte isolation

Hearts were removed and the coronary arteries perfused via the aorta at 4.0 mL/min (37°C) with a Modified Isolation Krebs–Henseleit (MIKH) solution for 4 min. The composition of MIKH (in mmol/L) was: NaCl (120.00), KCl (5.40), HEPES (20.00), NaH\textsubscript{2}PO\textsubscript{4} (0.52), MgCl\textsubscript{2}6H\textsubscript{2}O (3.50), taurine (20.00), creatine (10.00), glucose (11.10). The pH was adjusted to 7.4 with NaOH. Perfusion of hearts with MIKH was followed by perfusion with MIKH containing 1.0 mg/mL of type I collagenase (Worthington Biochemical) and 0.1 mg/mL of type XIV protease (Sigma-Aldrich). After ~6 min, the enzymes were removed and the heart perfused with MIKH containing 0.7% BSA (Sigma-Aldrich) but no enzymes for a further 4 min. The left ventricular free wall was then cut into strips and mixed to yield a single-cell suspension in MIKH containing 0.7% BSA. The calcium concentration in solution ([Ca\textsuperscript{2+}]\textsubscript{o}) was raised in this suspension via stepwise increments until 1.0 mmol/L was reached.

Immunoblotting

Isolated cardiomyocytes were lysed in radioimmunoprecipitation assay (RIPA) buffer. The composition of the RIPA buffer (in mmol/L) was: Tris (20), NaCl (150), EDTA (5), EGTA (5),
DTT (1) plus 1.0% Triton X-100 and 0.5% deoxycholate. MI tissue samples in Supplemental Material Figure 2 were disrupted in extraction buffer (0.1 M Tris–HCl, 0.01 M EDTA, 0.04 M DTT, 10% SDS, pH 8.0) using an ultrasonic device. Protease inhibitors added (in mmol/L) were: Na pyrophosphate (2.5), β-glycerophosphate (1.0), Na$_3$VO$_4$ (1.0), PMSF (1.0), NaF (2.0), plus 10 µg leupeptin, one tablet of a protease inhibitor cocktail (Complete Mini, Roche, Germany) and one tablet of phosphatase inhibitor (Phos-stop, Roche, Germany) per 10 mL buffer. The buffer was adjusted to pH 7.4. Lysates were assayed for protein concentration using the bicinchoninic acid assay. BSA was used to produce a standard curve. Samples were mixed with β-mercaptoethanol as the reducing agent and a loading dye before heating at 99˚C for 90 s. The lysates were loaded at a concentration of 10 µg per well into 4–12% Bis-tris gels (NuPAGE, Life Technologies, UK) with 4 µg of lysed thymus tissue as a positive control. Electrophoresis conditions were 75 V for 10 min followed by 165 V for 60–90 min. Protein was transferred to 0.45 µm pore size nitrocellulose membranes (Life technologies, UK).

Membranes were incubated overnight at 4˚C with primary antibodies against: PLB A2 (1:1000; MA3-922, Pierce), Runx1 (1:500; ab35962, AbCam), p-PLB Ser16 and p-PLB Thr17 (1:1000; A010-12 and A010-13, Badrilla), PKC α (1:200; sc-8393, Santa Cruz), PP1 (1:200; sc-7482, Santa Cruz). Pan-actin (1:1000; 4968s, Cell Signalling) or Red Alert Western blot stain (Merck Millipore) were used as loading controls. Secondary antibodies were donkey anti-mouse IRDye 800 CW and donkey anti-rabbit IRDye 680RD (1:10000; 926-32212 and 926-68073, Li-Cor). Western blots were visualized and quantified using a LI-COR fluorescence imager and LI-COR or Quantity One analysis software. Figure 5A, 5D and 5G are separate blots but have been cut and probed separately for each different target. Same lanes were used for Runx1$^{WT}$ MI mice in Figure 5D hence the actin signal is common to both PKC and PLB.
Epifluorescence measurements of field stimulated calcium transients

Isolated cardiomyocytes (1.8 mmol/L [Ca^{2+}]_{o}) were loaded with calcium–sensitive fluorophore (5.0 µmol/L Fura-4F AM, Invitrogen). Cardiomyocytes were incubated in MIKH for 30 min for de-esterification in a cell bath (Cell Microcontrols) followed by superfusion with MIKH at 37°C and field–stimulation (1.0 Hz, 2.0 ms duration, stimulation voltage set to 1.5 x threshold). Caffeine (10 mmol/L, 20 s; without field stimulation) was applied before the protocol. Field-stimulation was started after 10 s of perfusion with MIKH and sustained for 120 s before application of a second caffeine bolus at the end of the protocol. The Fura-4F fluorescence ratio (340/380nm excitation; R_{340/380nm}) was measured using a spinning wheel spectrophotometer (Cairn Research Ltd.; sampling rate of 5.0 kHz) to measure the intracardiomcyte [Ca^{2+}]. Cell-edge detection (IonOptix) was used to measure cell length. Data were analyzed offline. The mean Fura-4F fluorescence ratio was obtained by averaging 12 steady state transients (Origin) and converted to [Ca^{2+}] as previously described^{11}. Particular experiments utilized pretreated (30 min) and perfusion with the PKA inhibitor H89 (1 µmol/L; Tocris Biosciences, Bristol UK) as previously described^{12}.

Determination of the in vivo electrocardiogram (ECG) in Runx1^{Δ/Δ} and control mice 2 wk-post-MI.

Mice were anaesthetised and maintained at 1 % isoflurane in 1 L/min O₂. ECG was recorded with an IX-228/S data acquisition unit and LabScribe2 software (iWorx) using 3 electrodes, placed subcutaneously in the right forelimb, left forelimb and right hindlimb. The ECG was recorded for 5 min, traces analysed with Labscribe3 software (iWorx) and the last 20 s of the traces were used to determine the PR and QT intervals, averaging every 30 beats. The QT interval was corrected for heart rate (QT_{c}), calculated using the QT and RR intervals with the Bazett’s formula, adjusted for mice: QT_{c} = QT/(RR/100)^{0.5}. The QT interval (which coincides well with the action potential duration; APD^{13}) increased between sham and MI mice in both groups as expected^{13}, but there was no significant
Determination of the action potential duration in cardiomyocytes isolated from Runx1Δ/Δ and control hearts 2 wk-post-MI.

Voltage recordings were made on isolated cardiomyocytes using the CellOPTIQ™ electrophysiology platform (Clyde Bioscience Ltd., Glasgow, UK). Cells were loaded with 8 µmol/L Di-4 ANEPPS, and illuminated using a 470nm OptoLED (Cairn Research; Faversham, UK). The APD was measured at a range of lengths (APD20, 40, 60 and 80) and demonstrated no significant difference between control and Runx1Δ/Δ mice post-MI. Together with the ECG measurements above, the experiments demonstrated that an increased APD cannot account for the changes in Ca²⁺ handling between control and Runx1Δ/Δ mice post-MI (Supplemental Material Figure 9C&D).

Determination of calcium entry in cardiomyocytes isolated from Runx1Δ/Δ and control hearts 2 wk-post-MI.

Fura-4F (8 µmol/L)-loaded cardiomyocytes were alternately excited between 360 and 380nm with emission collected at 510nm and electrically paced using field stimulation (2ms, 40V, 1Hz). Two previously published protocols were used to measure the amplitude of the Ca²⁺ transient in the absence of SR Ca²⁺ release (an index of Ca²⁺ influx via the L-type Ca²⁺ channel). The first protocol measured the amplitude of the first stimulated Ca²⁺ transient immediately after application of 10mM caffeine (to empty Ca²⁺ from the sarcoplasmic reticulum; SR)¹⁴, ¹⁵ (Supplemental Material Figure 9E&F) and in separate experiments, the second protocol measured the amplitude of the stimulated Ca²⁺ transient during inhibition of the SR with thapsigargin (1µmol/L for 30 min; Supplemental Material Figure 9G)¹⁶. Both protocols confirmed that there was no change in the amplitude of Ca²⁺ influx between control and Runx1Δ/Δ mice post-MI. Collectively, these experiments...
demonstrated that an increased Ca\(^{2+}\) entry cannot account for the changes in Ca\(^{2+}\) handling between control and Runx1\(^{ΔA}\) mice post-MI.

**Viral overexpression of Runx1 in culture**

Adult rabbit cardiomyocytes were isolated as described above but in sterile filtered isolation MIKH. The [Ca\(^{2+}\)] in solution ([Ca\(^{2+}\)]\(_{o}\)) was raised in this suspension via stepwise increments until 1.0 mmol/L was reached. Cardiomyocytes were gently centrifuged in a hand-operated centrifuge and resuspended in prewarmed minimal essential media (MEM; Life Technologies) supplemented with L-glutamine and penicillin–streptomycin plus 10% fetal calf serum (FCS). The cells were added to wells of a 6-well tissue culture plate at a density of 1 x 10\(^5\) rods per well and incubated at 37°C in 5% CO\(_2\) for 1–2 h to allow the cardiomyocytes to adhere to the base of the well. The media was carefully aspirated under sterile conditions. Freshly warmed MEM without FCS was added to each well. Adenovirus was previously prepared with cloned genes for GFP (control Ad-GFP) or Runx1 with GFP (experimental Ad-Runx1) using standard protocols\(^{17, 18}\). Aliquots of each virus were thawed and diluted to 1:100 with warmed MEM. The PFU of each virus was 2.54 x 10\(^{10}\) for Ad-Runx1 and 1.10 x 10\(^{11}\) for Ad-GFP. The volume of the virus required for infection was calculated based on the number of rod-shaped cardiomyocytes and the PFU of the virus. Cultures were maintained at 37°C in 5% CO\(_2\) for 24 h.

**REFERENCES**


**Supplemental Material Figure 1. Control RNAscope probes.** Typical control heart section images of (A) negative dapB and (B) positive PPIB and POLR2 probes taken from the right ventricle (RV), left ventricle (LV), border zone (BZ) and infarct (INF) regions in MI hearts 1 day post-MI or the equivalent regions in sham hearts.

**Supplemental Material Figure 2. Runx1 expression in WT C57BL/6 mice post-MI. (A)** Typical PV loop traces at 4 weeks post-MI and mean data (n=10 sham, n=13 MI; *P*<0.05,
Student’s t-test) for; (B) Left ventricular (LV) End-systolic pressure, (C) maximum rate of contraction, (D) LV end-diastolic pressure, (E) maximum rate of relaxation, (F) LV end-systolic volume, (G) LV end-diastolic volume and (H) Ejection fraction. (I) Whole heart Runx1 mRNA (n=6 sham, n=7 MI; *P<0.05, Student’s t-test). (J) MI heart (BZ,border zone; LV, left ventricle; INF, infarct; RV, right ventricle; Scale=1mm). (K) Regional Runx1 mRNA 4 wk post-MI (n=4 sham, n=8 MI). #P<0.05 relative to RV and *P<0.05 sham versus MI, Student’s t-test. (RQ, relative quantification). (L) Regional Runx1 protein 3 wk post-MI (n=5), with (M) mean data (ANOVA).

**Supplemental Material Figure 3. Regional Runx1 mRNA 8 wk post-MI**

Runx1 mRNA in different regions (INF, infarct; BZ, border zone and LV, left ventricle) of 8 wk post-MI hearts (n=4 sham, n=6 MI; *P<0.05 Student’s t-test).

**Supplemental Material Figure 4. Echocardiography study – cardiac contractile function in C57Bl/6J mice.** 1 wk post-MI echocardiography study measuring fractional shortening in WT C57Bl/6J sham (n=3-5) and MI (n=3-6) mice; *P<0.05 Student’s t-test.

**Supplemental Material Figure 5. Generation of Runx1Δ/Δ mice.** (A) The RUNT domain and locations of the three primers (colored arrows) used to detect the Runx1wt/wt, Runx1fl/fl and excised Runx1Δ/Δ alleles. LoxP sites are indicated by blue triangles. (B) PCR of genomic DNA; whole hearts from male (M) and female (F) mice (Runx1Δ/Δ n=9 for M and n=4 for F; Runx1fl/fl n=8 for M and n=6 for F; Runx1wt/wt n=6 for M and n=6 for F). A WT and three-band control where used to delineate the appropriate sized bands (bands 1 and 2). (C) PCR of genomic DNA; WT and three-band controls (bands 1 and 2), whole heart preparations (bands 3–5), cardiomyocytes (CMs) from Runx1Δ/Δ mice that had been separated from other cell types by plating and short term culture (band 6) or by FACS (band 7). Mean ± SEM of Runx1 excision as denoted by the ratio of the excised band (310 bp) to the combined excised (310 bp) and LoxP band (275 bp) (band 5 [n=3], band [n=4]) and band 7 (n=2),
*P<0.05 ANOVA. (D) Runx1 mRNA levels (relative to PPIB; 2−ΔCt) in whole heart tissue from the right ventricle (RV), left ventricle (LV), border zone (BZ) and infarct (INF) regions of 4 wk post-MI hearts (Runx1Δ/Δ MI [n=5 hearts] as % of Runx1wt/wt and Runx1fl/fl MI [n=9 hearts] *P<0.05 Student’s t-test). (E) Runx1 mRNA levels (relative to PPIB; 2−ΔCt) in cardiomyocytes separated from other cell types by plating and short term culture from 2 wk post-MI hearts (Runx1Δ/Δ MI [n=7 hearts] as % of Runx1fl/fl MI [n=8 hearts], *P<0.05 Student’s t-test). (F; Left) Western blot of Runx1 in isolated cardiomyocytes from 2 wk post-MI hearts with pan-actin loading control (performed on same blot as Figure 6A) and thymus (Thy) positive control for Runx1.  (F; Right) Percentage change in Runx1 protein in isolated cardiomyocytes from 2 wk post-MI hearts (Runx1Δ/Δ MI [n=5 hearts] as % of Runx1wt/wt and Runx1fl/fl MI [n=5 hearts], *P<0.05 Student’s t-test).

Supplemental Material Figure 6. Blinded study – cardiac function assessed by echocardiography in Runx1Δ/Δ mice. 2 wk post-MI blinded study (control Runx1fl/fl MI (n=8), Runx1wt/wt MI (n=11) and Runx1Δ/Δ MI (n=7), *P<0.05 ANOVA) for the following functional parameters: (A) Fractional shortening (FS), (B) Left ventricular internal diameter at systole (LVIDs), (C) LVID at diastole (LVIDd), (D) LV posterior wall thickness at systole (LVPWs), (E) LVPW thickness at diastole (LVPWd).

Supplemental Material Figure 7. Echocardiography study – cardiac contractile function in Runx1Δ/Δ mice. 1 wk post-MI echocardiography study measuring fractional shortening in Runx1Δ/Δ MI (n=5-6) and control Runx1fl/fl MI and Runx1wt/wt MI mice combined (n=5-10); *P<0.05 Student’s t-test.

Supplemental Material Figure 8. Infarct size at 24 h post-myocardial infarction (MI) and post-ischemia reperfusion (I/R). (A) Typical heart section images of MI using TTC staining. (B) Mean infarct area (IA) as % of left ventricle (LV) [control MI (n=8) and Runx1Δ/Δ
Supplemental Material Figure 9. Isolated cardiomyocyte electrical activity in Runx1\(^{\Delta/\Delta}\) mice. (A) Typical electrocardiogram (ECG) traces from control and Runx1\(^{\Delta/\Delta}\) and mice 2 wk-post-MI with (B) mean QT and QT\(_c\) (corrected) intervals of control Runx1\(^{fl/fl}\) and Runx1\(^{wt/wt}\) mice combined (n=4 sham; n=10 MI) and Runx1\(^{\Delta/\Delta}\) (n=4 sham; n=6 MI), *P<0.05 Student’s t-test. (C) Typical voltage recordings on isolated cardiomyocytes from control and Runx1\(^{\Delta/\Delta}\) and mice 2 wk-post-MI with (D) mean action potential duration (APD) at a range of lengths (APD\(_{20}\), 40, 60 and 80) of control Runx1\(^{fl/fl}\) MI and Runx1\(^{wt/wt}\) MI mice combined (n=28 cardiomyocytes, 6 hearts) and Runx1\(^{\Delta/\Delta}\) MI (n=18 cardiomyocytes, 5 hearts). (E) Typical calcium measurements of the Ca\(^{2+}\) transient in the absence of SR Ca\(^{2+}\) release in isolated cardiomyocytes from control and Runx1\(^{\Delta/\Delta}\) and mice 2 wk-post-MI with (F) mean amplitude of the first stimulated Ca\(^{2+}\) transient immediately after application of 10mM caffeine of control Runx1\(^{fl/fl}\) MI and Runx1\(^{wt/wt}\) MI mice combined (n=31 cardiomyocytes, 7 hearts) and Runx1\(^{\Delta/\Delta}\) MI (n=11 cardiomyocytes, 3 hearts). (G) Mean amplitude of the stimulated Ca\(^{2+}\) transient during thapsigargin-mediated SR inhibition of control Runx1\(^{fl/fl}\) MI and Runx1\(^{wt/wt}\) MI mice combined (n=6 cells; 2 hearts) and Runx1\(^{\Delta/\Delta}\) MI (n=5 cells; 2 hearts).
SUPPLEMENTAL MATERIAL FIGURE 3

Runx1 mRNA relative to RV (RQ)

- INF
- BZ
- LV

RV

- Sham
- MI

* #
SUPPLEMENTAL MATERIAL FIGURE 4

Fractional Shortening (FS) (%)

Time (days post-MI)

- Sham
- MI

* * *
SUPPLEMENTAL MATERIAL FIGURE 5

A

Runx1<sup>wt/wt</sup>

5′ ATG 203 bp STOP 3′

Runx1<sup>Δ/Δ</sup>

5′ ATG 275 bp Neo STOP 3′

Runx1<sup>fl/fl</sup>

5′ ATG 310 bp STOP 3′

Primer 1 (Runx WT Forward)

Primer 2 (Runx WT Reverse)

Primer 3 (Runx Excision Reverse)

Neomycin Resistance Gene

B

1. WT control
2. 3 Band Control
3. Runx1<sup>wt/wt</sup> (whole heart)
4. Runx1<sup>fl/fl</sup> (whole heart)
5. Runx1<sup>Δ/Δ</sup> (whole heart)
6. Runx1<sup>fl/fl</sup> (CMs plated)
7. Runx1<sup>Δ/Δ</sup> (CMs FACS)

C

1 2 3 4 5 6 7

% of Runx1<sup>Δ/Δ</sup> band fluorescence/combined Runx1<sup>fl/fl</sup> and Runx1<sup>Δ/Δ</sup> band

D

Runx1 mRNA in whole heart regions (% of control)

E

Runx1 mRNA in CMs (% of control)

F

Runx1 protein in CMs (% of control)
SUPPLEMENTAL MATERIAL FIGURE 6

A  B  C  D  E

- Runx1 f/f MI
- Runx1 w/wt MI
- Runx1 Δ/Δ MI

A: FS (%)
B: LVtDs (mm)
C: LVtDd (mm)
D: LVPtWs (mm)
E: LVPtWd (mm)
SUPPLEMENTAL MATERIAL FIGURE 7

Time (days post-MI)

Fractional Shortening (FS) (%)

- Runx1^{+/+} MI
- Runx1^{+/-} MI and Runx1^{-/-} MI