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TRANSLATIONAL SCIENCE
Heart failure and cardiomyopathies

Enhanced Parkin-mediated mitophagy mitigates adverse left ventricular remodelling after myocardial infarction: role of PR-364

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Abstract

Background and Aims Almost 30% of survivors of myocardial infarction (MI) develop heart failure (HF), in part due to damage caused by the accumulation of dysfunctional mitochondria. Organelle quality control through Parkin-mediated mitochondrial autophagy (mitophagy) is known to play a role in mediating protection against HF damage post-ischaemic injury and remodelling of the subsequent deteriorated myocardium.

Methods This study has shown that a single i.p. dose (2 h post-MI) of the selective small molecule Parkin activator PR-364 reduced mortality, preserved cardiac ejection fraction, and mitigated the progression of HF. To reveal the mechanism of PR-364, a multi-omic strategy was deployed in combination with classical functional assays using *in vivo* MI and *in vitro* cardiomyocyte models.

Results *In vitro* cell data indicated that Parkin activation by PR-364 increased mitophagy and mitochondrial biogenesis, enhanced adenosine triphosphate production via improved citric acid cycle, altered accumulation of calcium localization to the mitochondria, and initiated translational reprogramming with increased expression of mitochondrial translational proteins. In mice, PR-364 administered post-MI resulted in widespread proteome changes, indicating an up-regulation of mitochondrial metabolism and mitochondrial translation in the surviving myocardium.

Conclusions This study demonstrates the therapeutic potential of targeting Parkin-mediated mitophagy using PR-364 to protect surviving cardiac tissue post-MI from progression to HF.

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Structured Graphical Abstract

Key Question

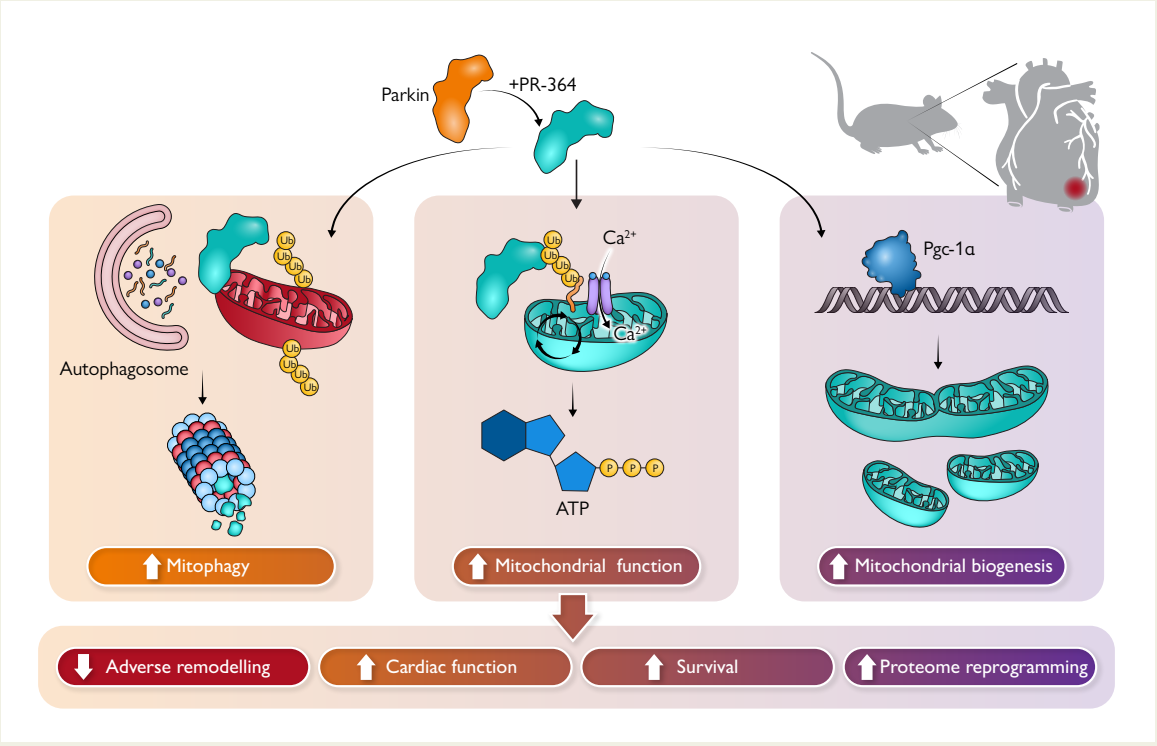
Does pharmacologically-induced Parkin-dependent mitophagy improve cardiac function following acute myocardial infarction (MI)?

Key Finding

In vitro cell data indicated that Parkin activation by PR-364 increased mitophagy and mitochondrial biogenesis. In mice, administration of Parkin activator PR-364 at 2 hours post-MI preserved cardiac function and reduced adverse ventricular remodelling by initiating translational reprogramming and increasing mitochondrial function in the heart.

Take Home Message

Parkin activation through PR-364 provides promising therapeutic potential to preserve cardiac function after acute MI.



Parkin activator proposed therapeutic mechanisms. Inactive Parkin (red) is activated by PR-364; activated Parkin (green) promotes enhanced mitophagy and mito-biogenesis, improves mitochondrial function via increased mitochondrial calcium level and biogenesis, and ultimately leads to limited post-myocardial infarction cardiac remodelling and heart failure. MI, myocardial infarction.

Keywords Myocardial infarction • Proteomics • Multi-omics • Heart failure • Translational reprogramming • Mitochondrial function • Parkin-dependent mitophagy

Translational perspective

Heart disease is the leading cause of death worldwide, and developing novel therapeutic approaches remains a top healthcare priority. This work presents a novel small molecule compound developed to selectively increase the activity of Parkin ubiquitin ligase. This study showed the significant therapeutic potential of PR-364 for the prevention of heart failure progression and in cardiac protection for patients after experiencing a myocardial infarction.

Introduction

Despite progress in diagnosis and treatment of myocardial infarction (MI), 13% of survivors develop heart failure (HF) at 30 days and 20–30% do so at 1 year post-discharge.[1] As the myocardium has high energy demands, mitochondria are required to generate 6 kg/day of adenosine triphosphate (ATP) to maintain normal cardiac contractile function,[2] making the organelle critical in maintaining optimal cardiac performance. It is unsurprising then that damage and dysfunction of

mitochondria play key roles in pathogenesis in cardiovascular diseases.[3,4] Preventing accumulation of damaged mitochondria after an MI is an important therapeutic strategy for reducing subsequent adverse myocardial remodelling, and one of the main mechanisms of this quality control process is Parkin-mediated mitophagy.

Mitophagy is an autophagic response that specifically targets and removes dysfunctional mitochondria that are damaged in response to stress conditions, including hypoxia and increased cytosolic calcium (Ca^{2+}) overload.[5] Mitophagy is critical for cell survival under stress conditions such as MI,[6,7] and the process is mediated by a variety of response factors, foremost among which is the PINK1/Parkin pathway.[8] Parkin, a cytosolic E3 ubiquitin ligase, is a central factor mediating selective mitophagy of damaged mitochondria for mitochondrial quality control.[9,10] Parkin's ubiquitin ligase activity occurs largely at damaged or depolarized mitochondria and is dependent on the activity of the PTEN-induced putative kinase 1 (PINK1).[11] Upon mitochondrial depolarization, PINK1 accumulates on the mitochondrial outer membrane,[11,12] and once stabilized, phosphorylates ubiquitin as well as directly on Parkin ubiquitin-like (Ubl) domain.[13] These phosphorylation events recruit and activate Parkin, which in turn ubiquitinates outer mitochondrial membrane proteins, resulting in recruitment of the autophagy adapter protein p62[14] and leading to the elimination of the damaged mitochondria via mitophagy.[15,16] The removal of damaged mitochondria via mitophagy is essential for maintaining cellular fitness,[17] and studies designed to increase autophagic flux and mitophagy have previously demonstrated beneficial cardioprotective effects.[18,19]

Balanced mitochondrial homeostasis requires both clearance of damaged mitochondria (mitophagy) and the generation of new functional mitochondria (biogenesis). Mitochondrial biogenesis requires the synthesis of new proteins encoded by mitochondrial and nuclear DNA. Mitochondrial biogenesis acts to preserve mitochondrial function and cellular homeostasis, and the process is governed by a key transcription factor, peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α).[20] Canonically, PGC-1α regulates expression of nuclear-encoded mitochondrial genes through induction of nuclear respiratory factors (NRFs), mitochondrial transcription factor A (TFAM), downstream transcriptional targets of PGC-1α, and oestrogen-related receptor alpha. Parkin-interacting substrate (PARIS) is a transcriptional repressor that binds to the PGC-1α promoter to suppress mitobiogenesis.[21,22] The use of targeting pathways that increase mitophagy represents a promising therapeutic direction, but in spite of extensive studies, currently there are no pharmaceuticals available to modulate mitophagy and mitochondrial biogenesis for clinical management of HF.[23]

There is an exponential increase in biological complexity once initial gene transcripts are spliced, translated into amino acid sequences, and post-translationally modified.[24] High-throughput proteomics technologies combined with advanced bioinformatics are extensively used to identify molecular signatures of diseases based on protein pathways and signalling cascades.[25] Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) technology are powerful tools to detect protein changes and low-abundance protein alterations in injured tissue samples and experimental disease models.[26,27] At the same time, analysis of heart tissues remains challenging, as the sarcomeric proteins that comprise the contractile myofilament apparatus dominate the cardiac proteome.[28,29] Optimization of sample preparation with in-depth LC–MS/MS approaches has significantly increased the detection sensitivity and coverage of the low-abundance signalling proteins in the cardiac tissues,[27] which has expanded our understanding of complex networks during cardiac diseases.[30] Growing numbers of studies using proteomics have improved our understanding of the cellular system and biological insights of MI pathophysiology.[31–33]

In this work, we present a novel small molecule, PR-364 (Progenra), developed to selectively activate Parkin. We assessed PR-364 therapeutic benefit and cardiac outcomes in a mouse MI model with clinically relevant experimental paradigms and studied its potential cellular mechanisms using *in vitro* cell models. Utilizing LC–MS/MS techniques combined with bioinformatic pathway analysis tools, we investigated PR-364 therapeutic potential of enhancing the Parkin-dependent mitophagy for prevention of HF progression post-MI.

Methods

Detailed methods are available in Supplementary data online, *Methods*.

Results

PR-364 promotes selective Parkin-dependent mitophagy and mitochondrial biogenesis in differentiated H9c2 cells

PR-364 was discovered using a TR-FRET-based assay in a high-throughput screening for activators of Parkin from a diversity-based small molecule library. PR-364 exhibited potent and selective activation of Parkin ($EC_{50} = 2.60 \pm 0.23$ μM, *Figure 1A*) with half-life ($t_{1/2}$) of 3.5 ± 0.37 h (see Supplementary data online, *Figure S1*). In contrast, PR-364 did not affect 14 other E3 ligases including HHARI which is closely related to Parkin ($EC_{50} > 30$ μM; *Figure 1B*). Most importantly, Parkin activation by PR-364 *in vitro* was confirmed using the gold standard gel-based autoubiquitination assay (*Figure 1C*). Treatment of Parkin with PR-364 resulted in dose-dependent increase in ubiquitination by Parkin (*Figure 1D*). Proteomics analysis also indicates Parkin-specific protein changes, including decreased protein levels in CDGSH iron sulphur domain 2 and ras homolog family member T2 (Rhot2), which have been suggested to be down-regulated with activation of Parkin-mediated mitophagy[34–37] (see Supplementary data online, *Figure S2*). Collectively, these data strongly suggest that PR-364 is a selective and potent Parkin activator.

To evaluate PR-364 selective activation to Parkin-dependent mitophagy, we differentiated the H9c2 rat cell line[38] towards a cardiac phenotype[39] and then transfected the cells with control (siControl) or Parkin (siParkin) siRNA for 72 h prior to PR-364 or vehicle treatment for 6 h (*Figure 1E and F*). The level of p62 (sequestosome1), a mitophagy adaptor that is crucial for damaged mitochondrial clearance,[14] was measured using western blots (*Figure 1G*), demonstrating significant *P*-value for interactions between the PR-364 and Parkin silencing, which suggested that the effect of PR-364 in siControl and siParkin cells is significantly different.

Alternative mitophagy pathways, regulated by Fundc1 or Bnip3, were also measured with PR-364 treatment and no significant activation was observed (see Supplementary data online, *Figure S3*). We next assessed the translocation of p62 to the mitochondria by treating the H9c2 cells with PR-364 or vehicle for 6 h followed by addition of bafilomycin to block autophagic flux (3 h before harvesting).[40,41] We isolated the heavy membrane fraction enriched for mitochondria,[42] hereafter referred to as the 'mitochondrial fraction', and probed for markers of mitophagy. PR-364 increased the translocation of p62 into the mitochondria in the H9c2 cells (*Figure 1H and I*). Additional fluorescent microscopy measurement using mitophagy dye staining H9c2 cells with 6 h

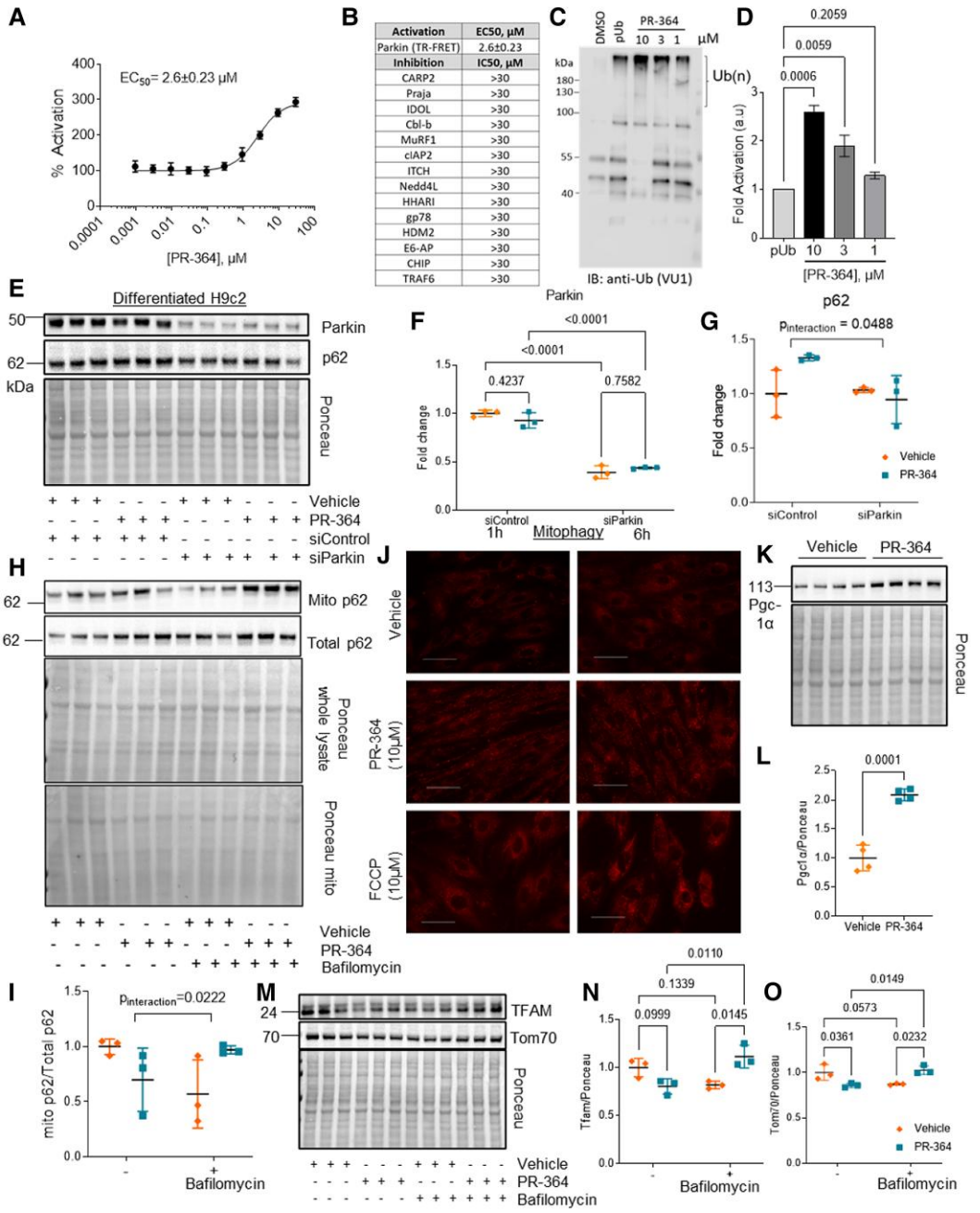


Figure 1 PR-364 selectively activates Parkin and promotes Parkin-dependent mitophagy *in vitro*. (*A*) Recombinant His6-SUMO-Parkin (50 nM) was incubated with DMSO or PR-364 at indicated concentrations in the presence of MBP-TcPINK1 (2 nM) and ubiquitination reaction was carried out for 90 min at room temperature. EC_{50} value is reported as mean ± standard deviation ($n = 3$). (*B*) Per cent activation for Parkin and per cent inhibition for other E3 ligases was plotted and EC_{50} was determined ($n = 3$). (*C*) Western blot probed with anti-Ubiquitin antibody (VU-1) after PR-364. (*D*) PhosphoSer65ubiquitin (50 nM) was used as a Parkin activation control in the western blot. The signal intensities of PR-364 doses relative to pUb signal were shown on the graph, by one-way analysis of variance. (*E*) Western blot analyses of Parkin and p62 to measure mitophagy activation ($n = 3$/group) in H9c2 cells. (*F* and *G*) Western blot quantifications of Parkin and p62. Two-way analysis of variance with Šidák correction for multiple analyses was used to demonstrate Parkin levels after silencing. p62 up-regulation was significant by PR-364 in siControl cells but not in siParkin cells. (*H*) Western blot analysis of vehicle- vs. PR-364-treated cells with the addition of bafilomycin at 3 h before harvesting ($n = 3$/group). (*I*) Quantification of p62 in of mitochondrial p62 vs. total p62. Error bars represent mean ± standard deviation. Two-way analysis of variance with interaction was used to demonstrate the effects of PR-364 on p62 protein levels with or without bafilomycin (50 nmoles), which inhibits mitophagy flux. (*J*) 60× images of live H9c2 cells with PR-364 treatment and stained with mitophagy dye, to demonstrate mitophagy. Scale bars represent 50 μm. (*K*) Cells were treated with either vehicle (DMSO) or PR-364 for 6 h, with or without addition of bafilomycin (50 nmoles) 3 h before harvesting and being analysed by western blot for protein expression. (*L*) Western blot quantification of Pgc-1α protein level ($n = 3$/group), by *t*-test. (*M*–*O*) Western blot and quantification of Tfam and Tom70 ($n = 3$/group), by multiple comparisons post two-way analysis of variance

treatment of vehicle, PR-364, or FCCP (positive control)—confirmed an increase in mitophagy in PR3-64-treated cells (*Figure 1J*).

It has been previously reported that mitophagy and mitochondrial biogenesis were concurrent in mouse hearts after MI through Parkin activation.[43] To determine whether PR-364 also triggers mitochondrial biogenesis *in vitro*, we monitored PGC-1α protein expression, the major regulator of mitochondrial biogenesis,[44,45] following PR-364 treatment. The results showed a 2-fold increase in Pgc-1α protein (*Figure 1K and L*). As concurrent mitophagy can be a confounding factor for the analysis of mitochondrial biogenesis markers and mitochondrial protein content,[46,47] to control for this, we treated cells with bafilomycin and compared the results following PR-364 treatment. The data showed an increase in the accumulation of both TFAM and the mitochondrial outer membrane protein TOM70, biogenesis markers downstream targets of Pgc-1α[48,49] (*Figure 1M–O*), in PR-364-treated cells.

PR-364 increases mitochondrial function in human AC16 cardiomyocytes

To focus on the effects of PR-364 in a model of human cardiomyocytes, proliferating AC16 adult human ventricular cardiomyocytes were used to study the functional changes *in vitro*.[50] AC16 cardiomyocytes (passage between 4 and 10) were incubated with 3 μM PR-364 or volume equivalent vehicle for 6 h (*Figure 2A*). Comprehensive functional assays and omics experiments were performed to understand the mitochondrial functional changes induced by PR-364 *in vitro*, including real-time Seahorse mito-stress assay, flow cytometry, and western blots, discovery proteomics and targeted metabolomics (*Figure 2A*). We measured 218 metabolites in the central carbon chain,[51] and identified 4734 proteins from discovery proteomics (*Figure 2B*). Statistical analysis of the multi-omics data identified 39 metabolites and 188 proteins changed by PR-364 in the AC16 cells (*Figure 2C*). ClueGO pathway analysis of the metabolomics data showed five metabolites (malic acid, ATP, aconitic acid, citric acid, and phosphoenolpyruvate) from the citric acid (TCA) cycle were all increased in abundance (*Figure 2D*). Interestingly, ATP was >6-fold increased by PR-364 after 6 h treatment in the metabolomic data, reflecting increased mitochondrial function. Concurrently, adenosine 5-diphosphate and adenosine 5-monophosphate metabolite abundances in PR-364-treated AC16 cells were also increased (see Supplementary data online, *Figure S4*), demonstrating increased ATP production and mitochondrial activity following PR-364 treatment. Real-time live-cell Seahorse Mito-stress assay measuring mitochondrial activities confirmed that PR-364 increased AC16 cardiomyocyte basal respiration, maximum respiration, and ATP production, indicative of increased mitochondrial function (*Figure 2E*).

ClueGO pathway analysis using the discovery proteomics data identified increased quantity of five proteins (voltage-dependent anion channel 1, voltage-dependent anion channel 2, peptidase, mitochondrial processing subunit alpha, prohibitin 2, and YME1-like 1 ATPase), indicating an alteration in mitochondrial calcium (Ca^{2+}) transport pathway, essential for cardiac contractility[52–54] (*Figure 2F*), as well as six proteins [mitochondrial ribosomal protein S9 (MRPS9), mitochondrial ribosomal protein L37 (MRPL37), mitochondrial ribosomal protein L43 (MRPL43), mitochondrial ribosomal protein L10 (MRPL10), mitochondrial ribosomal protein S18a (MRPS18a), and mitochondrial ribosomal protein S25 (MRPS25)] in mitochondrial translation (*Figure 2G*). To determine the functional outcome of the proteomic increase in mitochondria in the proteins comprising the calcium ion import pathway, we performed fluorescent microscopy and flow cytometry. To measure mitochondrial Ca^{2+}, we used RhoD2AM, a fluorescent mitochondria-specific Ca^{2+} probe[55] together with MitoTracker Green FM (mitochondrial labelling dye; see Supplementary data online, *Figure S5A*). To quantitatively assess whether PR-364 increased mitochondrial Ca^{2+} level, we used flow cytometry to compare the fluorescent intensity ratio of RhoD2AM to MitoTracker Green FM in vehicle- and PR-364-treated cells ($n = 8915–9416$ cells each group). The results showed PR-364 increased the RhoD2AM:MitoTracker Green FM ratio at 6 h (see Supplementary data online, *Figure S5B*), indicating an increase in Ca^{2+} level. Protein expression of mitochondrial calcium uptake 1 (MICU1), a negative regulator of mitochondrial Ca^{2+} uniporter (MCU)-mediated mitochondrial calcium uptake[56,57] and a target of Parkin-mediated degradation,[58] was significantly lower in cells treated with PR-364 (see Supplementary data online, *Figure S5C*). Interestingly, no significant changes in the levels of expression of other members of the calcium uniporter machinery (MCU, MICU2, or MICU3; Supplementary data online, *Figures S5D*) were detected, suggesting that the accumulation of Ca^{2+} following PR-364 treatment may be mediated directly by Parkin-targeted degradation of MICU1.

PR-364 promotes proteomic changes *in vivo*

To interrogate the *in vivo* effects of PR-364, we administered PR-364 to wild-type (WT) adult male mice for 6 h, along with an inhibitor of autophagic flux[59] chloroquine. PR-364-treated mice demonstrated an increase in autophagy markers, including mitochondria-localized Parkin, increased LC3-II/I ratio, and mitochondrial mass (represented by increased protein abundance of matrix protein Cox4[60]), indicating that PR364 was able to activate mitophagy (see Supplementary data online, *Figure S6*). To confirm that PR-364 can induce mitophagy following MI, we treated male mice 2 h post-permanent coronary artery ligation (post-PCAL) with PR-364, and left ventricle (LV) tissue was collected 6 h post-PCAL ($n = 3$, *Figure 3A*). As expected, PR-364 was able to induce elevated levels of the autophagic marker LC3-II in total lysate and mitochondrial fraction in the hearts of PR-364-treated mice following MI (*Figure 3B and C*).

To further characterize the global proteome changes *in vivo* at baseline condition without perturbation, PR-364 or vehicle was administered to healthy adult male mice ($n = 4$) at 0 h, and the non-infarcted LV was collected at 6 h (*Figure 3E*). Total proteins were extracted and prepared for discovery proteomics analysis using LC–MS/MS. Twenty-five up-regulated proteins and 40 down-regulated proteins were significantly altered by PR-364 after 6 h compared with vehicle mice. Interestingly, in all proteins that were statistically changed by 6 h of PR-364 treatment, mitochondrial ribosomal protein S14 (MRPS14), involved in mitochondrial translation, was the top altered protein in expression with more than 6-fold up-regulation compared with vehicle control group ($n = 4$, $P < .0001$ by *t*-test, *Figure 3F*). PR-364 altered the proteome profiles and showed distinct separation from the vehicle control-treated state based on principal component analysis (PCA; *Figure 3G*). Gene Set Enrichment Analysis[61,62] results showed biological pathways enriched in the top or bottom of the ranked list, with the 'na_pos' and 'na_neg' phenotypes corresponding to enrichment in up-regulated or down-regulated proteins. The positive enrichment score reflects the up-regulation of mitochondrial translation by PR-364 (*Figure 3H*). Later proteomics analysis in a separate MI cohort showed the same pathway up-regulation in mice after PR-364 treatment (*Figure 5*). Further, MitoPLEX,[51] a targeted proteomic assay, designed to quantify select mitochondrial proteins (OXPHOS, TCA, and other mitochondrial functions), confirmed that PR-364 increased

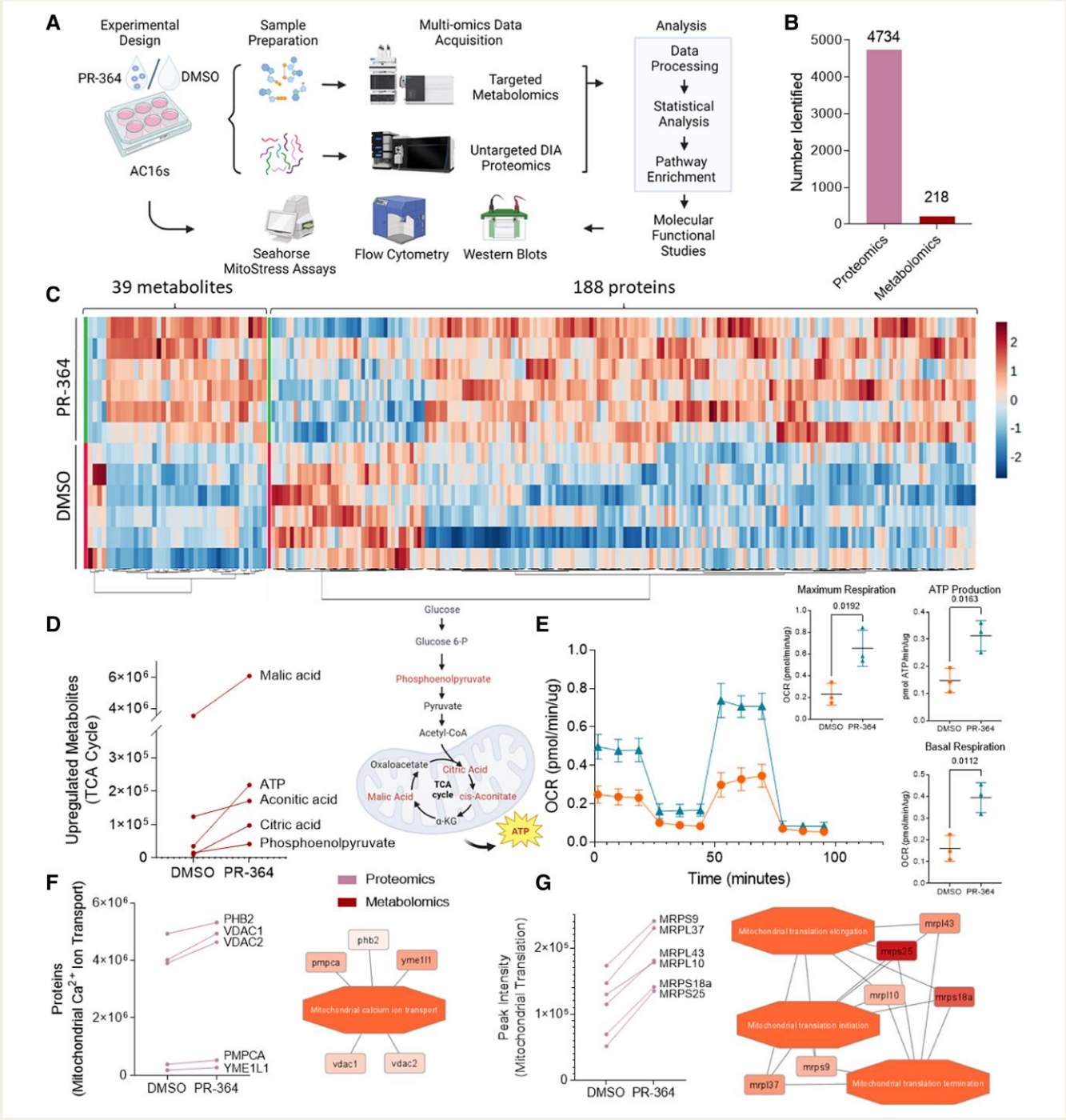


Figure 2 PR-364 up-regulates metabolite and protein expression, improves mitochondrial respiration and calcium ion uptake in AC16 cardiomyocytes. (*A*) Experimental design for *in vitro* AC16 studies (graph created using biorender.com). (*B*) Numbers of proteins were identified and metabolites that were measured using mass spectrometry–based untargeted proteomics or targeted metabolomics. (*C*) Heat map of 39 metabolites and 188 proteins that were significantly different between PR-364 and vehicle AC16 cells. The scale bar indicates *z*-scores of protein intensity values, with highly abundant proteins depicted in dark red and lower abundance proteins depicted in dark blue. $n = 6$, $P < .05$ by unpaired *t*-test for each metabolite measured or protein identified. (*D*) Pathway analysis of the 39 metabolites revealed enrichment of the TCA cycle by PR-364. Metabolites showing mean values at each dot; $n = 6$, by *t*-test for each metabolite. Specifically, mean mass spectrometry peak intensity $ATP_{vehicle} = 34\,660$, mean mass spectrometry peak intensity $ATP_{PR\text{-}364} = 218\,512$; $n = 6$, $P < .0001$ by *t*-test, statistics not shown in the figure (TCA cycle illustration was created using Biorender.com). (*E*) Seahorse mito-stress assay reveals increased ATP production and maximal respiration after PR-364 administration; mean $ATP_{vehicle} = 0.148$ pmol/min/μg, mean $ATP_{PR\text{-}364} = 0.312$ pmol/min/μg; $n = 3$; $P < .05$ by *t*-test. (*F*) Proteomics pathway analysis showed PR-364 increased expression of some proteins involved in mitochondrial calcium ion transport. Proteins showing mean values at each dot; $n = 6$; $P < .05$ by *t*-test for each protein, statistics not shown on graph. (*G*) Proteomics pathway analysis showed PR-364 increased expression of proteins involved in mitochondrial translation. Proteins showing mean values at each dot; $n = 6$; $P < .05$ by *t*-test for each protein, statistics not shown on graph

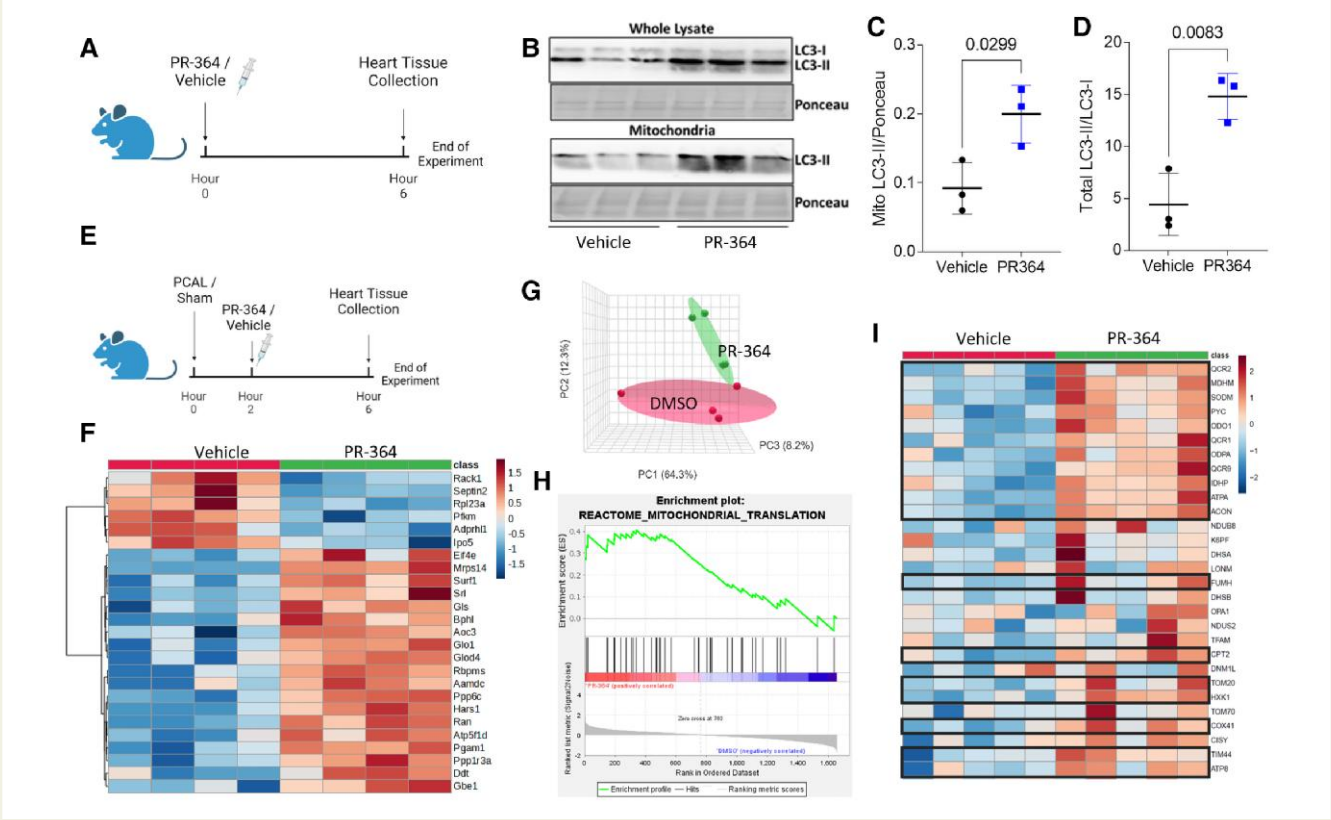


Figure 3 PR-364 increases mitochondrial translation and mitophagy in 6 h. (*A*) Experimental design of the 6 h mouse cohort with myocardial infarction at 2 h to study the proteomic effect of PR-364 post-myocardial infarction *in vivo* (graph created using biorender.com). (*B*) Western blot image of LC3-I and LC3-II detection in total left ventricle lysate (top) and mitochondrial enriched fraction lysate (bottom). (*C* and *D*) Quantifications of mito LC3-II to mito ponceau stain and LC3-II to LC3-I ratio in the total left ventricle lysate ($n = 3$, mean ± standard deviation, by *t*-test). (*E*) Experimental design of the 6 h control mouse cohort to study the proteomic effect of PR-364 to healthy heart *in vivo* (graph created using biorender.com). (*F*) Heat map of the top 25 proteins that are significantly altered by PR-364 (the scale bar indicates *z*-scores of protein intensity values; $n = 4$, $P < .05$ each protein by *t*-test). (*G*) Principal component analysis plot of the proteomic profiles between PR-364 mice and vehicle mice. (*H*) Gene set enrichment analysis of the proteomic data showed enriched mitochondrial translation by PR-364. (*I*) MitoPLEX measurement of the heart tissue samples (the scale bar indicates *z*-scores of protein intensity values; $n = 5$, proteins changed with $P < .005$ are boxed in black, by unpaired *t*-test)

the abundance of a number of mitochondrial proteins, consistent with increased mitochondrial mass (*Figure 3I*).

PR-364 preserves cardiac function and reduces mortality post-myocardial infarction

To study the effects of PR-364 on the heart following ischaemia, either a single dose of 1 mg/kg PR-364 or vehicle (Dimethylsulfoxide, DMSO) was injected i.p. 2 h post-MI in a clinically relevant *in vivo* MI paradigm where adult WT and Parkin knockout (PKO) male mice were subjected to MI by PCAL.[63,64] Echocardiograms were performed at baseline before MI, Day 14, and Day 28 post-MI to track cardiac functional changes (*Figure 4A*). Mice were monitored for a total of 28 days prior to euthanasia and tissue collection. PR-364-injected WT mice had a 75% survival rate ($n = 20$ at baseline, with 15 surviving by collection time on Day 28) compared with 64% in vehicle WT mice post-MI ($n = 25$ at baseline, with 16 surviving by collection time on Day 28; *Figure 4B*). Importantly, PR-364 was not able to replicate the same trend of increased survival in PKO mice (see Supplementary data online, *Figure S7A*). While two groups of WT mice maintained similar body weights at the end of the 28-day protocol (*Figure 4C*), the heart weight to tibia length ratio (HW/TL) indicated greater ventricular enlargement in the vehicle group compared with the PR-364 group (*Figure 4D*). This significant difference in the HW/TL ratio was not observed in the PKO mice (*Figure 4D*). Additional images of whole hearts on Day 28 demonstrated myocardial enlargement in the vehicle WT group, while the hearts from PR-364 WT mice maintained sizes similar to the vehicle condition (*Figure 4E*). Ejection fraction (EF) and fractional shortening (FS) from M-mode echocardiograms showed significant preservation of cardiac function in the PR-364 group at both time points post-MI compared with the vehicle group in the WT mice (*Figure 4F*). However, this preservation was not observed in PKO mice (*Figure 4F*, Supplementary data online, *Figure S7B*). Additionally, the EF and FS in the WT PR-364 group were significantly higher compared with the PKO PR-364 group at Day 14, with this trend continuing at Day 28 (*Figure 4F*, Supplementary data online, *Figure S7B*). These results indicate that PR-364 was not able to rescue cardiac function post-MI in PKO mice without Parkin.

Masson's trichome staining for myocardial injury showed a larger scar area in the vehicle WT group compared with the PR-364 WT group post-MI, indicating PR-364 attenuated pathological cardiac remodelling (*Figure 4G*). PR-364 was also not able to prevent scar tissue

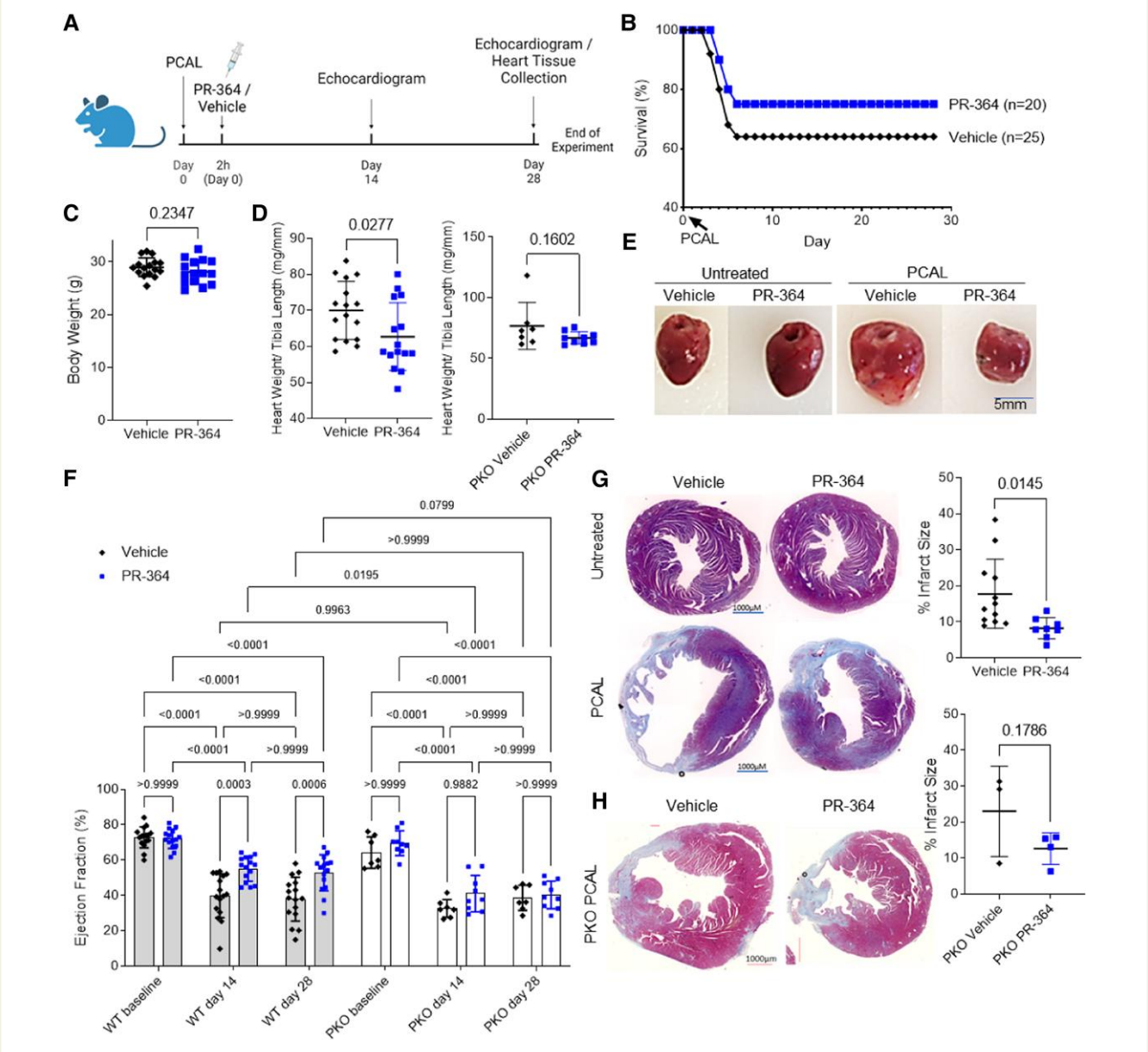


Figure 4 PR-364 protects myocardium by maintaining cardiac function, preventing cardiac remodelling, and reducing mortality post-myocardial infarction. (*A*) Twenty-eight-day *in vivo* paradigm (graph created using biorender.com). (*B*) Survival curve of the PR-364- or vehicle-treated wild-type mice post-permanent coronary artery ligation. (*C*) Measurement of body weight of each wild-type mouse in both vehicle and PR-364 groups, not significant by *t*-test. (*D*) Heart weight to tibia length ratios in vehicle vs. PR-364-treated wild-type and Parkin knockout mice, respectively, mean ± standard deviation, by unpaired *t*-test. (*E*) Representative pictures of hearts from untreated (no permanent coronary artery ligation) and permanent coronary artery ligation wild-type mice in both vehicle- and PR-364-treated groups. (*F*) Echocardiogram recordings of the wild-type and Parkin knockout mice on baseline (before permanent coronary artery ligation), Day 14, and Day 28. Ejection fractions were calculated based on the M-mode echo at each time point, by two-way analysis of variance with Šídák's multiple comparisons test. (*G*) 60× representative heart sections after Masson Trichrome staining to examine infarct size in hearts of untreated and permanent coronary artery ligation wild-type mice after vehicle vs. PR-364 treatment, mean ± standard deviation, by unpaired *t*-test. (*H*) Representative Parkin knockout heart sections after Masson Trichrome staining to examine cardiac infarct size with vehicle vs. PR-364 treatment post-permanent coronary artery ligation. Mean ± standard deviation, by unpaired *t*-test

development in the PKO mice post-MI (*Figures 4H*, Supplementary data online, *Figure S7C*). These data suggest PR-364 leads to Parkin-specific cardiac protection by maintaining high EF and FS, preserving cardiac physiology, and reducing mortality post-MI. Vehicle-treated WT mice showed a significant increase in LV end-diastolic diameter (LVEDD) starting on Day 14, whereas PR-364 WT group preserved LVEDDs until Day 28, indicating PR-364 was able to delay the ventricular remodelling post-MI (see Supplementary data online, *Figure S8A*). Septum thickness (IVSd) was unchanged between PR-364 and vehicle groups (see Supplementary data online, *Figure S8B*). Interestingly, the MI model by PCAL had limited or no effects on female mice mortality, and additional PR-364 treatment in female mice showed no significant

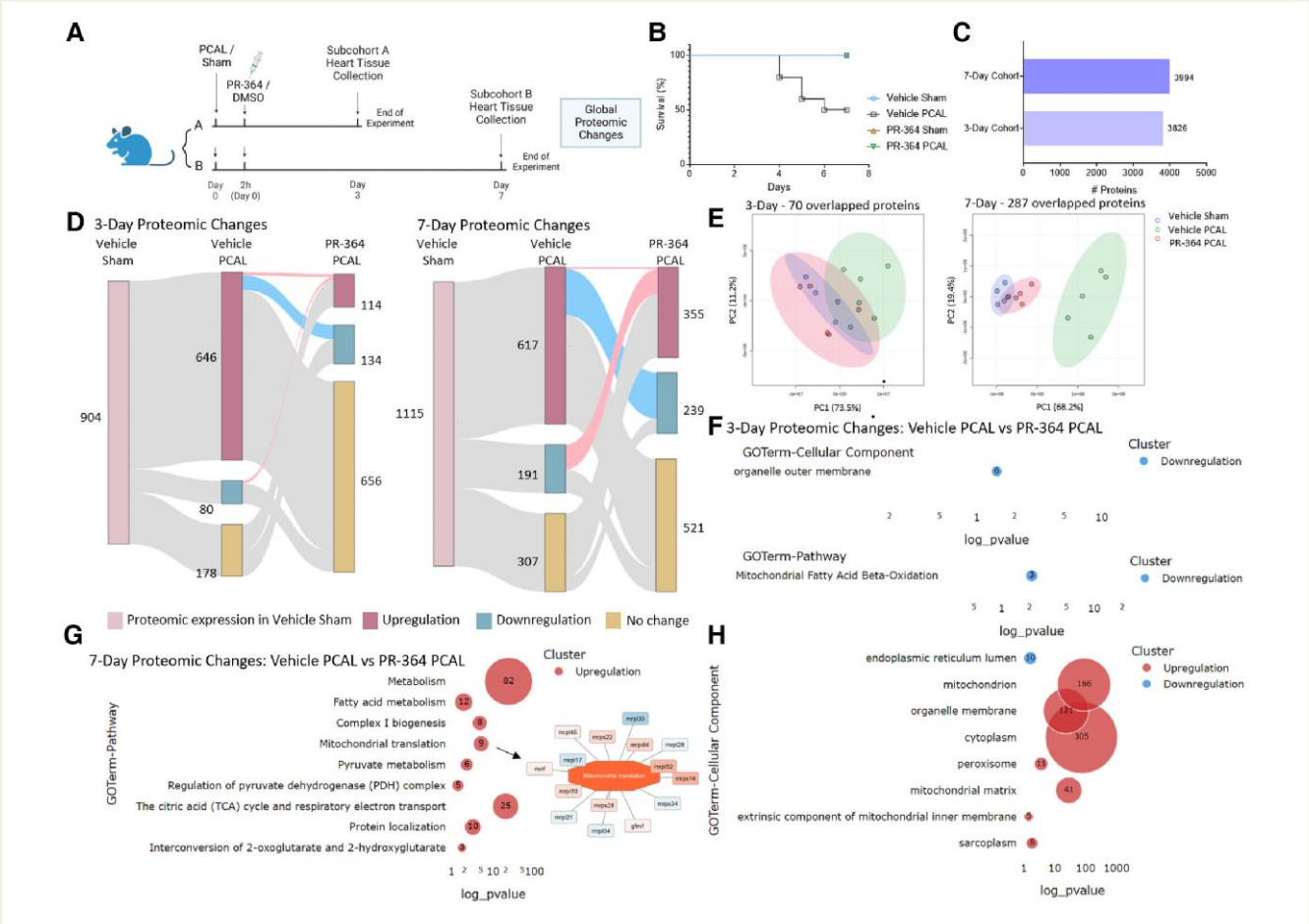


Figure 5 PR-364 reduces mortality and leads to translational reprogramming of the heart in mice post-permanent coronary artery ligation. (*A*) Experimental schematic of the 3-day (*A*) and 7-day (*B*) mouse cohorts (graph created using biorender.com). (*B*) Seven-day cohort survival curve. (*C*) The numbers of total proteins detected by mass spectrometry in the two cohorts. (*D*) Sankey diagrams showing the proteomic changes in two cohorts. Left: From the 3826 identified proteins in the Day 3 mice, 904 proteins (~24% of total detected proteome) had significant changes either between vehicle sham control group vs. vehicle permanent coronary artery ligation group or between vehicle permanent coronary artery ligation group vs. PR-364 permanent coronary artery ligation group (cut-off $P < .05$ by *t*-test). Seventy proteins (646 up + 80 down—656 no change) showed significant changes in both comparisons. Right: from the 3994 identified proteins in the Day 7 mice, 1115 proteins (~28% of total detected proteome) showed significant changes either between vehicle sham control group vs. vehicle permanent coronary artery ligation group or between vehicle permanent coronary artery ligation group vs. PR-364 permanent coronary artery ligation group (cut-off $P < .05$ by *t*-test). Two hundred and eighty-seven proteins (617 up + 191 down—521 no change) showed significant changes in both comparisons. (*E*) Principal component analysis plots of the 70 proteins from 3 days (left) and 287 proteins from 7 days (right), indicating PR-364 translationally reprogrammed a subgroup of proteome in the permanent coronary artery ligation hearts back to the normal healthy state (vehicle sham). (*F*) Bubble plots of GO network analysis of the 3-day vehicle permanent coronary artery ligation and PR-364 permanent coronary artery ligation group (114 up + 134 down proteins). (*G* and *H*) Bubble plots of GO network analysis of the 7-day vehicle permanent coronary artery ligation and PR-364 permanent coronary artery ligation group (355 up + 239 down proteins)

changes in cardiac function or heart size (see Supplementary data online, *Figure S9*).

PR-364 leads to early translational reprogramming of the heart and prevents pathological proteomic changes post-myocardial infarction

In the 28-day cohort, mouse mortality generally peaked between 3 and 7 days after the MI (*Figure 4B*). Thus, we were interested in understanding the effects of PR-364 on the cardiac proteome profiles in treated mice on Day 3 (before the mortality starts) and Day 7 (in the surviving mice). The previously described *in vivo* experimental paradigm was repeated with the hearts harvested earlier: either on Day 3 (sub-cohort A) or on Day 7 (sub-cohort B; *Figure 5A*). Non-ischaemic regions of LVs were collected for total protein extraction and global proteomic analysis via LC–MS/MS. In the 7-day cohort, PR-364-treated mice had 100% survival post-MI, whereas the mice treated with vehicle had 50% mortality [vehicle PCAL group $n = 10$ at baseline, and $n = 5$ by the end of the 7-day protocol, $n = 5$ all other groups, $P = .0019$ by log-rank (Mantel–Cox) test, *Figure 5B*]. A total of 3994 proteins were identified in the 7-day cohort and 3826 proteins in the 3-day cohort, separately ($n = 5$ each group, *Figure 5C*). From the 3826 identified proteins in the 3-day mice, 904 proteins (~24% of total detected

proteome) had significant changes either between vehicle sham control group vs. vehicle PCAL group or between vehicle PCAL group vs. PR-364 PCAL group (cut-off $P < .05$ by *t*-test, *Figure 5D*). From the 3994 identified proteins in the 7-day mice, 1115 proteins (~28% of total quantified proteome) showed significant changes either between vehicle sham control group vs. vehicle PCAL group or between vehicle PCAL group vs. PR-364 PCAL group (cut-off $P < .05$ by *t*-test, *Figure 5D*).

We further investigated the number of proteins that were differently expressed (DEPs) after an MI but were reversed to sham levels by the single dose of PR-364 (highlighted in *Figure 5D*). From the DEPs, 70 (646 up + 80 down and 656 proteins with no change) were identified for the 3-day cohort and 287 proteins (617 up + 191 down and 521 proteins with no change) for the 7-day cohort. The PCA plots were used to visualize groups of protein similarity by sample clustering (*Figure 5E*, Supplementary data online, *Figure S10*). We observed PR-364 PCAL cluster closely resembled the vehicle sham group in the PCA plot for both 3 and 7 days, while the vehicle PCAL cluster separated from the vehicle sham cluster by 3 days and further away by 7 days on the PCA plot. The close resemblance of the PR-364 PCAL group to vehicle sham group on the PCA plot suggested similar proteomes and protective amelioration of protein changes by PR-364 post-MI. Pairwise comparison of the reverted proteins by PR-364 post-MI by analysing the pathway and cellular component changes between PR-364 PCAL and vehicle PCAL groups using ClueGO,[65] revealed limited pathway alteration in the 3-day groups, potentially due to proteome changes dominated by the acute MI effects (*Figure 5F*). However, by Day 7, multiple cellular pathways were shown to be up-regulated by PR-364, including 82 proteins involved in metabolism and mitochondrial mass (*Figure 5G* and *H*). These results indicated PR-364 was able to prevent large-scale proteomic changes in the surviving LV tissue, with a cardiac LV proteome that resembles a healthy sham heart.

Discussion

Heart disease remains a leading cause of death worldwide, and developing novel therapeutic approaches remains a top healthcare priority. This work presents a novel small-molecule compound developed to selectively increase the activity of Parkin ubiquitin ligase. We hypothesize that PR-364 benefits the heart post-MI by inducing Parkin-dependent mitophagic clearance of damaged mitochondria, promoting mitochondrial biogenesis through PGC-1α pathway, and improving the function of the remaining mitochondrial network with increased Ca^{2+} levels and protein translational reprogramming in the post-ischaemic myocardium; PR-364 thus results in preserved cardiac function and reduced adverse cardiac remodelling. PR-364 enhances these effects even when administered after the injury with a single dose required. Our biochemical, *in vitro*, and *in vivo* data strongly suggest that PR-364 specifically activates Parkin and its downstream effects (*Structured Graphical Abstract*). However, the possibility of non-specific binding to non-E3 ubiquitin ligase proteins cannot be ruled out. Further investigation into direct binding partners and effects on non-cardiac tissues is ongoing but beyond the current scope of this work.

In this study, we first investigated the effects on survival and cardiac function of PR-364 post-MI. We adopted a previously published mouse MI model[64] and modified it to a clinically relevant experimental paradigm, where adult male mice received MI via PCAL followed by a single dose of PR-364 or vehicle at 2 h. The timing of PR-364 administration post-MI mimics the actionable intervention timing post-MI found in the clinical setting. The paradigm was also designed to assess whether a single dose would be sufficient as a therapeutic strategy to benefit patients for long term, as most of the current FDA-approved treatments for MI require continued therapy and long-term management.[66,67] Increased mitophagy has been suggested to alleviate ischaemia/reperfusion cardiac injury as well as myocardial injury;[68–70] therefore, we believe a temporary PR-364-mediated increase in mitophagy reduces the number of damaged mitochondria that would otherwise release proapoptotic mediators (e.g. ROS, cytochrome *c*), inflammatory mediators (e.g. mtDNA, hsp60), attenuating the early cascade of injury signalling.

The PCAL-induced MI mortality in mice[64] peaked in both cohorts during the first week post-MI in either 7-day cohort or 28-day cohort. The reasonable cause for this is either LV rupture, arrythmia, or both.[71] This correlates to the high mortality observed during the first 30 days after MI in patients.[72] Although PR-364-treated mice did not exhibit a statistically significant reduction in mortality immediately following the infarct, it is nonetheless remarkable that the drug was able to preserve cardiac function in the surviving mice.

PR-364 also preserved cardiac function long-term post-MI, with higher EF and FS by both 14 and 28 days, with smaller infarct size observed in the treated heart. The risk of developing HF is proportional to the magnitude of the infarct and the degree of no-reflow.[73,74] Accordingly, with no-reflow for the PCAL procedure, we were able to focus our further proteomic analysis on the surviving tissue (border zone and remote myocardium). Ejection fraction links highly to the clinical outcomes as well as the quality of life in patients with heart diseases,[75,76] and our data demonstrate the impressive therapeutic potential of PR-364 in preserving cardiac function post-MI.

The targeting of PR-364 for Parkin is supported by the fact that Parkin-deficient mice experienced no benefits from PR-364 treatment post-MI, supporting our hypothesis that cardiac protection conferred by PR-364 is Parkin mediated. Further, the Parkin silencing in the *in vitro* data also excluded the activation of alternative mitophagy pathways such as the one directed by Fundc1[68,77] or Bnip3.[78] Our H9c2 cell data suggested that PR-364 enhanced Parkin-mediated mitophagy through mitophagy and increased mitochondrial biogenesis. In the heart, mitophagy functions as an adaptive response to stress and plays an important role in mitochondrial quality control. While it has been argued that Parkin is present in the heart at low levels and is therefore biologically unimportant,[79] it is activated in response to ischaemic injury;[80] and, in mice, Parkin deficiency leads to aggravated post-MI injury and loss of pharmacological cardioprotection.[80–82] The importance of Parkin for cardiac protection was also confirmed in our recent study of the glucagon-like peptide-1 receptor (GLP1R) allosteric agonist, 6,7-dichloro-2-methylsulfonyl-3-*N*-tert-butylaminoquinoxaline.[43] In concert with mitophagy, cardiomyocytes increase their mitochondrial mass to meet the needs of increased energy demand through mitobiogenesis. Thus, our data support increased mitophagy and mitobiogenesis by PR-364 interaction with Parkin resulting in accelerated mitochondrial turnover in cardiac protection. Previous studies have noted the sex differences in outcomes and disease progression, often with conflicting results; in general, women have a lower incidence of major adverse cardiac events compared with men, and animal studies show that mitophagy and mitochondrial function are sexually dimorphic.[83–85] A study using the UK Biobank showed that the incidence rates of MI per 10 000 person-years were 7.76 (95% confidence interval 7.37–8.16) in women and 24.35 (23.57–25.16) in men.[86] In our study, MI was achieved in the mouse model via PCAL, which mimics that of men with obstructive coronary artery disease, while relatively more women suffer from microvascular coronary dysfunction.[87,88] Intriguingly, this cardioprotective effect of PR-364 was exclusively observed in male mice, whose female counterparts ($n = 8$ female

animals/group) neither succumbed to post-MI death nor suffered significant reductions in EF despite their equivalent MI sizes. Other studies have established that female heart mitochondria, while lower in number per cardiomyocyte than in males, have higher mitochondrial efficiency while producing less reactive oxygen species; interestingly, they also retain Ca^{2+} better than male mitochondria.[89] Furthermore, our data imply that by directly activating Parkin, PR-364 increases mitophagy and mitochondrial function in male hearts to match basal levels of mitochondrial function in female hearts, preserving cardiomyocytes and improving survival.

In the heart, Ca^{2+} plays an important role in contraction and relaxation of cardiomyocytes. Ca^{2+} flux across the inner mitochondrial membrane regulates cell bioenergetics, cytoplasmic Ca^{2+} signals, and activation of cell death pathways.[56] We found altered proteins that were enriched in the pathway of mitochondrial Ca^{2+} transport in PR364-treated AC16 cardiomyocytes which were supported by the direct detection of the increased mitochondrial Ca^{2+} level using RhoD2AM. In addition, the increase in mito-Ca^{2+} levels due to PR-364 may explain the altered mitochondrial function using Seahorse assay, and the up-regulated TCA cycle metabolites (including ATP). Our mouse data showed significant improvement in cardiac function by PR-364 (*Figure 4*) and up-regulated metabolic pathways and mitochondrial proteins (*Figure 5*) post-MI, which all closely associates to Ca^{2+} regulation.[90] Further studies are required, but we propose that PR-364-mediated Parkin activation improves cardiac function by clearing dysfunctional mitochondria, initiating mitochondrial biogenesis (newly synthesized mitochondria better able to produce and maintain ATP levels) and improved function of the remaining mitochondrial networks by increasing mitochondrial calcium levels through discrete targeting of negative regulators of the mitochondrial calcium importer.

The metabolic switch from efficient mitochondrial oxidative metabolism to less-efficient glycolysis is one of the factors contributing to HF in ischaemic patients.[91] By 7-day post-MI, the mice that received a dose of PR-364 at 2 h compared with vehicle had increased pathways in metabolism (including fatty acid metabolism and pyruvate metabolism), mitochondrial biogenesis and translation, and TCA cycle, supporting better energy supply in the PR-364-treated heart to supplement cardiac demand. It was interesting that there were limited pathway alterations by PR-364 on Day 3, likely due to the overwhelming proteomic changes caused by MI. The advancement of pathways and cellular components activated on Day 7 compared with Day 3 suggested that by Day 7, the single PR-364 dose at 2 h post-MI was able to continue either up-regulating protective mechanisms or suppressing the progression of pathological pathways to preserve cardiac functions, while the hearts in the vehicle mice kept deteriorating. Studying the proteomic consequences on Day 28 post-MI in the heart in the surviving mice is one of our future steps, and we expect to see more progressive divergence in proteomic profiles between the PR-364-treated mice and the vehicle-treated mice.

Post-translational modifications (PTMs) have enormous impact on the stability and activity of proteins, and it is important to consider the effects of various potential PTMs after Parkin activation by PR-364 on cardiac protection as well as the PTM status of Parkin itself. One possibility is that PR-364 causes structural change in the Parkin protein that enhances its activity. We are currently performing studies to determine what if any effect PR-364 has on the phosphorylation of Parkin by PINK1.

An alternate hypothesis that could explain the benefit of PR-364 is inhibition of inflammation and apoptosis. Post-MI remodelling is associated with interleukin-1β (IL-1β)-driven inflammation[92] and apoptosis,[93] which is driven by the activation of the NLRP3 inflammasome, essential for proteolytic maturation of pro-IL-1β. The inflammasome forms upon assembly of NLRP3, Caspase 1, ASC, and pro-IL-1β on the mitochondrial outer membrane under conditions where oxidized mitochondrial DNA and/or cardiolipin are accessible to the complex.[94,95] Activated Parkin is an inhibitor of NLRP3, and IL-1β promotes activation of Parkin by increasing pUb and NEDD8, suggesting feedback inhibition of inflammation and an alternate pathway of cardioprotection provided by PR-364.[96,97] Parkin activation by PR-364 may therefore inhibit IL-1β-driven inflammation and apoptosis post-MI in the heart, affecting multiple immune cells. The work on these hypotheses is ongoing and is outside the scope of this study.

Conclusions

In this study, we demonstrated the cardioprotective benefit of using the small-molecule compound PR-364 to preserve cardiac function and physiology post-MI in male mice. Mechanistically, we observed that PR-364 activated Parkin which induced Parkin-dependent mitophagy and mitochondrial biogenesis, initiated translational reprogramming, increased mitochondrial Ca^{2+} level, and increased ATP production. Overall, this study showed the significant therapeutic potential of PR-364 for prevention of HF progression and in cardiac protection for patients after experiencing an MI.

Supplementary data

Supplementary data are available at *European Heart Journal* online.

Declarations

Disclosure of Interest

The authors declare no conflicts of interest. The Parkin activating compound PR-364 was provided by Progenra, Inc., which did not interfere with the results and conclusions presented here. Progenra, Inc. plans to file patents on PR-364.

Data Availability

The MS proteomics, metabolomics, and MitoPLEX data have been deposited to the ProteomeXchange Consortium via the Mass Spectrometry Interactive Virtual Environment (MassIVE) partner repository with the dataset identifier: MSV000092828. Raw data files for peptides and metabolites are available upon request from the corresponding author. To view the dataset's files, log into the MassIVE FTP server with this URL: https://massive.ucsd.edu/. Username for web access: MSV000092828_reviewer and reviewer password: JVE12345!

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Ethical Approval

All animal experiments were designed and performed in compliance with the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Cedars-Sinai Medical Center under the protocol IACUC-008858.

Pre-registered Clinical Trial Number

Not applicable.

References

1. Jenča D, Melenovský V, Stehlik J, Staněk V, Kettner J, Kautzner J, *et al.* Heart failure after myocardial infarction: incidence and predictors. *ESC Heart Fail* 2021;**8**:222–37. https://doi.org/10.1002/ehf2.13144
2. Neubauer S. The failing heart—an engine out of fuel. *N Engl J Med* 2007;**356**:1140–51. https://doi.org/10.1056/NEJMra063052
3. Knowlton AA, Chen L, Malik ZA. Heart failure and mitochondrial dysfunction: the role of mitochondrial fission/fusion abnormalities and new therapeutic strategies. *J Cardiovasc Pharmacol* 2014;**63**:196–206. https://doi.org/10.1097/01.fjc.0000432861.55968.a6
4. Poznyak AV, Ivanova EA, Sobenin IA, Yet S-F, Orekhov AN. The role of mitochondria in cardiovascular diseases. *Biology (Basel)* 2020;**9**:137. https://doi.org/10.3390/biology9060137
5. Zhang R, Krigman J, Luo H, Ozgen S, Yang M, Sun N. Mitophagy in cardiovascular homeostasis. *Mech Ageing Dev* 2020;**188**:111245. https://doi.org/10.1016/j.mad.2020.111245
6. Marek-Iannucci S, Ozdemir AB, Moreira D, Gomez AC, Lane M, Porritt RA, *et al.* Autophagy-mitophagy induction attenuates cardiovascular inflammation in a murine model of Kawasaki disease vasculitis. *JCI Insight* 2021;**6**:e151981. https://doi.org/10.1172/jci.insight.151981
7. Li Y, Liang P, Jiang B, Tang Y, Liu X, Liu M, *et al.* CARD9 promotes autophagy in cardiomyocytes in myocardial ischemia/reperfusion injury via interacting with Rubicon directly. *Basic Res Cardiol* 2020;**115**:29. https://doi.org/10.1007/s00395-020-0790-6
8. MacVicar TDB, Mannack LVJC, Lees RM, Lane JD. Targeted siRNA screens identify ER-to-mitochondrial calcium exchange in autophagy and mitophagy responses in RPE1 cells. *Int J Mol Sci* 2015;**16**:13356–80. https://doi.org/10.3390/ijms160613356
9. Dorn GW II. Central Parkin: the evolving role of Parkin in the heart. *Biochim Biophys Acta* 2016;**1857**:1307–12. https://doi.org/10.1016/j.bbabio.2016.03.014
10. Vives-Bauza C, Zhou C, Huang Y, Cui M, de Vries RLA, Kim J, *et al.* PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. *Proc Natl Acad Sci U S A* 2010;**107**:378–83. https://doi.org/10.1073/pnas.0911187107
11. Narendra DP, Jin SM, Tanaka A, Suen D-F, Gautier CA, Shen J, *et al.* PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. *PLoS Biol* 2010;**8**:e1000298. https://doi.org/10.1371/journal.pbio.1000298
12. Jin SM, Youle RJ. The accumulation of misfolded proteins in the mitochondrial matrix is sensed by PINK1 to induce PARK2/Parkin-mediated mitophagy of polarized mitochondria. *Autophagy* 2013;**9**:1750–7. https://doi.org/10.4161/auto.26122
13. Ge P, Dawson VL, Dawson TM. PINK1 and Parkin mitochondrial quality control: a source of regional vulnerability in Parkinson's disease. *Mol Neurodegener* 2020;**15**:20. https://doi.org/10.1186/s13024-020-00367-7
14. Geisler S, Holmström KM, Skujat D, Fiesel FC, Rothfuss OC, Kahle PJ, *et al.* PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* 2010;**12**:119–31. https://doi.org/10.1038/ncb2012
15. Chen Y, Dorn GW II. PINK1-phosphorylated mitofusin 2 is a Parkin receptor for culling damaged mitochondria. *Science* 2013;**340**:471–5. https://doi.org/10.1126/science.1231031
16. Wauer T, Simicek M, Schubert A, Komander D. Mechanism of phospho-ubiquitin-induced PARKIN activation. *Nature* 2015;**524**:370–4. https://doi.org/10.1038/nature14879
17. Ma K, Chen G, Li W, Kepp O, Zhu Y, Chen Q. Mitophagy, mitochondrial homeostasis, and cell fate. *Front Cell Dev Biol* 2020;**8**:467. https://doi.org/10.3389/fcell.2020.00467
18. Godar RJ, Ma X, Liu H, Murphy JT, Weinheimer CJ, Kovacs A, *et al.* Repetitive stimulation of autophagy-lysosome machinery by intermittent fasting preconditions the myocardium to ischemia-reperfusion injury. *Autophagy* 2015;**11**:1537–60. https://doi.org/10.1080/15548627.2015.1063768
19. Marek-Iannucci S, Thomas A, Hou J, Crupi A, Sin J, Taylor DJ, *et al.* Myocardial hypothermia increases autophagic flux, mitochondrial mass and myocardial function after ischemia-reperfusion injury. *Sci Rep* 2019;**9**:10001. https://doi.org/10.1038/s41598-019-46452-w
20. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* 2007;**450**:736–40. https://doi.org/10.1038/nature06322
21. Stevens DA, Lee Y, Kang HC, Lee BD, Lee YI, Bower A, *et al.* Parkin loss leads to PARIS-dependent declines in mitochondrial mass and respiration. *Proc Natl Acad Sci U S A* 2015;**112**:11696–701. https://doi.org/10.1073/pnas.1500624112
22. Shin J-H, Ko HS, Kang H, Lee Y, Lee Y-I, Pletinkova O, *et al.* PARIS (ZNF746) repression of PGC-1alpha contributes to neurodegeneration in Parkinson's disease. *Cell* 2011;**144**:689–702. https://doi.org/10.1016/j.cell.2011.02.010
23. Bonora M, Wieckowski MR, Sinclair DA, Kroemer G, Pinton P, Galluzzi L. Targeting mitochondria for cardiovascular disorders: therapeutic potential and obstacles. *Nat Rev Cardiol* 2019;**16**:33–55. https://doi.org/10.1038/s41569-018-0074-0
24. Fert-Bober J, Murray CI, Parker SJ, Van Eyk JE. Precision profiling of the cardiovascular post-translationally modified proteome: where there is a will, there is a way. *Circ Res* 2018;**122**:1221–37. https://doi.org/10.1161/CIRCRESAHA.118.310966
25. Cho WCS. Proteomics technologies and challenges. *Genomics Proteomics Bioinformatics* 2007;**5**:77–85. https://doi.org/10.1016/S1672-0229(07)60018-7
26. Chen A, Chen Z, Xia Y, Lu D, Jia J, Hu K, *et al.* Proteomics analysis of myocardial tissues in a mouse model of coronary microembolization. *Front Physiol* 2018;**9**:1318. https://doi.org/10.3389/fphys.2018.01318
27. Ai L, Binek A, Kreimer S, Ayres M, Stotland A, Van Eyk JE. High-field asymmetric waveform Ion mobility spectrometry: practical alternative for cardiac proteome sample processing. *J Proteome Res* 2023;**22**:2124–30. https://doi.org/10.1021/acs.jproteome.3c00027
28. Doll S, Dreßen M, Geyer PE, Itzhak DN, Braun C, Doppler SA, *et al.* Region and cell-type resolved quantitative proteomic map of the human heart. *Nat Commun* 2017;**8**:1469. https://doi.org/10.1038/s41467-017-01747-2
29. Kane LA, Neverova I, Van Eyk JE. Subfractionation of heart tissue. In: Vivanco F (ed.), *Cardiovascular Proteomics: Methods and Protocols*. Totowa, NJ: Humana Press, 2007, 87–90.
30. Lindsey ML, Mayr M, Gomes AV, Delles C, Arrell DK, Murphy AM, *et al.* Transformative impact of proteomics on cardiovascular health and disease. *Circulation* 2015;**132**:852–72. https://doi.org/10.1161/CIR.0000000000000226
31. Binek A, Fernández-Jiménez R, Jorge I, Camafeita E, López JA, Bagwan N, *et al.* Proteomic footprint of myocardial ischemia/reperfusion injury: longitudinal study of the at-risk and remote regions in the pig model. *Sci Rep* 2017;**7**:12343. https://doi.org/10.1038/s41598-017-11985-5
32. Bai H, Sun K, Wu JH, Zhong ZH, Xu SL, Zhang HR, *et al.* Proteomic and metabolomic characterization of cardiac tissue in acute myocardial ischemia injury rats. *PLoS One* 2020;**15**:e0231797. https://doi.org/10.1371/journal.pone.0231797
33. Wang X, Shen X, Weil BR, Young RF, Canty JM, Qu J. Quantitative proteomic and phosphoproteomic profiling of ischemic myocardial stunning in swine. *Am J Physiol Heart Circ Physiol* 2020;**318**:H1256–71. https://doi.org/10.1152/ajpheart.00713.2019
34. Wu HH, Zhu Q, Liang N, Xiang Y, Xu TY, Huang ZC, *et al.* CISD2 regulates oxidative stress and mitophagy to maintain the balance of the follicular microenvironment in PCOS. *Redox Rep* 2024;**29**:2377870. https://doi.org/10.1080/13510002.2024.2377870
35. Shlevkov E, Kramer T, Schapansky J, LaVoie MJ, Schwarz TL. Miro phosphorylation sites regulate Parkin recruitment and mitochondrial motility. *Proc Natl Acad Sci U S A* 2016;**113**:E6097–106. https://doi.org/10.1073/pnas.1612283113
36. Wang H, Ni H-M, Chao X, Ma X, Rodriguez YA, Chavan H, *et al.* Double deletion of PINK1 and Parkin impairs hepatic mitophagy and exacerbates acetaminophen-induced liver injury in mice. *Redox Biol* 2019;**22**:101148. https://doi.org/10.1016/j.redox.2019.101148
37. Martinez A, Sanchez-Martinez A, Pickering JT, Twyning MJ, Terriente-Felix A, Chen PL, *et al.* Mitochondrial CISD1/Cisd accumulation blocks mitophagy and genetic or pharmacological inhibition rescues neurodegenerative phenotypes in Pink1/Parkin models. *Mol Neurodegener* 2024;**19**:12. https://doi.org/10.1186/s13024-024-00701-3
38. Hescheler J, Meyer R, Plant S, Krautwurst D, Rosenthal W, Schultz G. Morphological, biochemical, and electrophysiological characterization of a clonal cell (H9c2) line from rat heart. *Circ Res* 1991;**69**:1476–86. https://doi.org/10.1161/01.RES.69.6.1476
39. Branco AF, Pereira SP, Gonzalez S, Gusev O, Rizvanov AA, Oliveira PJ. Gene expression profiling of H9c2 myoblast differentiation towards a cardiac-like phenotype. *PLoS One* 2015;**10**:e0129303. https://doi.org/10.1371/journal.pone.0129303
40. Mauvezin C, Neufeld TP. Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome-lysosome fusion. *Autophagy* 2015;**11**:1437–8. https://doi.org/10.1080/15548627.2015.1066957
41. Redmann M, Benavides GA, Berryhill TF, Wani WY, Ouyang X, Johnson MS, *et al.* Inhibition of autophagy with bafilomycin and chloroquine decreases mitochondrial quality and bioenergetic function in primary neurons. *Redox Biol* 2017;**11**:73–81. https://doi.org/10.1016/j.redox.2016.11.004
42. Gottlieb RA, Adachi S. Nitrogen cavitation for cell disruption to obtain mitochondria from cultured cells. *Methods Enzymol* 2000;**322**:213–21. https://doi.org/10.1016/S0076-6879(00)22022-3
43. Germano JF, Huang C, Sin J, Song Y, Tucker KC, Taylor DJR, *et al.* Intermittent use of a short-course glucagon-like peptide-1 receptor agonist therapy limits adverse cardiac remodeling via Parkin-dependent mitochondrial turnover. *Sci Rep* 2020;**10**:8284. https://doi.org/10.1038/s41598-020-64924-2

44. LeBleu VS, O'Connell JT, Gonzalez Herrera KN, Wikman H, Pantel K, Haigis MC, *et al.* PGC-1α mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. *Nat Cell Biol* 2014;**16**:992–1003, 1–15. https://doi.org/10.1038/ncb3039
45. Chen L, Qin Y, Liu B, Gao M, Li A, Li X, *et al.* PGC-1α-mediated mitochondrial quality control: molecular mechanisms and implications for heart failure. *Front Cell Dev Biol* 2022;**10**:871357. https://doi.org/10.3389/fcell.2022.871357
46. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy. *Autophagy* 2012;**8**:445–544. https://doi.org/10.4161/auto.19496
47. Gottlieb RA, Andres AM, Sin J, Taylor DP. Untangling autophagy measurements: all fluxed up. *Circ Res* 2015;**116**:504–14. https://doi.org/10.1161/CIRCRESAHA.116.303787
48. Zheng L, Bernard-Marissal N, Moullan N, D'Amico D, Auwerx J, Moore DJ, *et al.* Parkin functionally interacts with PGC-1α to preserve mitochondria and protect dopaminergic neurons. *Hum Mol Genet* 2017;**26**:582–98. https://doi.org/10.1093/hmg/ddw418
49. Kelly DP, Scarpulla RC. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev* 2004;**18**:357–68. https://doi.org/10.1101/gad.1177604
50. Davidson MM, Nesti C, Palenzuela L, Walker WF, Hernandez E, Protas L, *et al.* Novel cell lines derived from adult human ventricular cardiomyocytes. *J Mol Cell Cardiol* 2005;**39**:133–47. https://doi.org/10.1016/j.yjmcc.2005.03.003
51. Stotland AB, Spivia W, Orosco A, Andres AM, Gottlieb RA, Van Eyk JE, *et al.* MitoPlex: a targeted multiple reaction monitoring assay for quantification of a curated set of mitochondrial proteins. *J Mol Cell Cardiol* 2020;**142**:1–13. https://doi.org/10.1016/j.yjmcc.2020.03.011
52. Singh H. Mitochondrial ion channels in cardiac function. *Am J Physiol Cell Physiol* 2021;**321**:C812–25. https://doi.org/10.1152/ajpcell.00246.2021
53. Kwong JQ. The mitochondrial calcium uniporter in the heart: energetics and beyond. *J Physiol* 2017;**595**:3743–51. https://doi.org/10.1113/JP273059
54. Garbincius JF, Elrod JW. Is the failing heart starved of mitochondrial calcium? *Circ Res* 2021;**128**:1205–7. https://doi.org/10.1161/CIRCRESAHA.121.319030
55. Krstic AM, Power AS, Ward M-L. Visualization of dynamic mitochondrial calcium fluxes in isolated cardiomyocytes. *Front Physiol* 2022;**12**:808798. https://doi.org/10.3389/fphys.2021.808798
56. Mallilankaraman K, Doonan P, Cárdenas C, Chandramoorthy HC, Müller M, Miller R, *et al.* MICU1 is an essential gatekeeper for MCU-mediated mitochondrial Ca(2+) uptake that regulates cell survival. *Cell* 2012;**151**:630–44. https://doi.org/10.1016/j.cell.2012.10.011
57. Liu JC, Liu J, Holmström KM, Menazza S, Parks RJ, Fergusson MM, *et al.* MICU1 serves as a molecular gatekeeper to prevent in vivo mitochondrial calcium overload. *Cell Rep* 2016;**16**:1561–73. https://doi.org/10.1016/j.celrep.2016.07.011
58. Matteucci A, Patron M, Vecellio Reane D, Gastaldello S, Amoroso S, Rizzuto R, *et al.* Parkin-dependent regulation of the MCU complex component MICU1. *Sci Rep* 2018;**8**:14199. https://doi.org/10.1038/s41598-018-32551-7
59. Mauthe M, Orhon I, Rocchi C, Zhou X, Luhr M, Hijlkema KJ, *et al.* Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy* 2018;**14**:1435–55. https://doi.org/10.1080/15548627.2018.1474314
60. Böttinger L, Guiard B, Oeljeklaus S, Kulawiak B, Zufall N, Wiedemann N, *et al.* A complex of Cox4 and mitochondrial Hsp70 plays an important role in the assembly of the cytochrome c oxidase. *Mol Biol Cell* 2013;**24**:2609–19. https://doi.org/10.1091/mbc.e13-02-0106
61. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005;**102**:15545–50. https://doi.org/10.1073/pnas.0506580102
62. Reimand J, Isserlin R, Voisin V, Kucera M, Tannus-Lopes C, Rostamianfar A, *et al.* Pathway enrichment analysis and visualization of omics data using g:Profiler, GSEA, Cytoscape and EnrichmentMap. *Nat Protoc* 2019;**14**:482–517. https://doi.org/10.1038/s41596-018-0103-9
63. Muthuramu I, Lox M, Jacobs F, De Geest B. Permanent ligation of the left anterior descending coronary artery in mice: a model of post-myocardial infarction remodelling and heart failure. *J Vis Exp* 2014;**94**:52206. https://doi.org/10.3791/52206
64. Lindsey ML, Bolli R, Canty JM, Du X-J, Frangogiannis NG, Frantz S, *et al.* Guidelines for experimental models of myocardial ischemia and infarction. *Am J Physiol Heart Circ Physiol* 2018;**314**:H812–38. https://doi.org/10.1152/ajpheart.00335.2017
65. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, *et al.* ClueGO: a cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* 2009;**25**:1091–3. https://doi.org/10.1093/bioinformatics/btp101
66. Ryan TJ, Anderson JL, Antman EM, Braniff BA, Brooks NH, Califf RM, *et al.* ACC/AHA guidelines for the management of patients with acute myocardial infarction:executive summary. *Circulation* 1996;**94**:2341–50. https://doi.org/10.1161/01.CIR.94.9.2341
67. Ibanez B, James S, Agewall S, Antunes MJ, Bucciarelli-Ducci C, Bueno H, *et al.* 2017 ESC guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: the Task Force for the management of acute myocardial infarction in patients presenting with ST-segment elevation of the European Society of Cardiology (ESC). *Eur Heart J* 2017;**39**:119–77. https://doi.org/10.1093/eurheartj/ehx393
68. Chen M, Chen Z, Wang Y, Tan Z, Zhu C, Li Y, *et al.* Mitophagy receptor FUNDC1 regulates mitochondrial dynamics and mitophagy. *Autophagy* 2016;**12**:689–702. https://doi.org/10.1080/15548627.2016.1151580
69. Zhang W, Siraj S, Zhang R, Chen Q. Mitophagy receptor FUNDC1 regulates mitochondrial homeostasis and protects the heart from I/R injury. *Autophagy* 2017;**13**:1080–1. https://doi.org/10.1080/15548627.2017.1300224
70. Bravo-San Pedro JM, Kroemer G, Galluzzi L. Autophagy and mitophagy in cardiovascular disease. *Circ Res* 2017;**120**:1812–24. https://doi.org/10.1161/CIRCRESAHA.117.311082
71. Pouleur A-C, Barkoudah E, Uno H, Skali H, Finn PV, Zelenkofske SL, *et al.* Pathogenesis of sudden unexpected death in a clinical trial of patients with myocardial infarction and left ventricular dysfunction, heart failure, or both. *Circulation* 2010;**122**:597–602. https://doi.org/10.1161/CIRCULATIONAHA.110.940619
72. Ye Q, Zhang J, Ma L. Predictors of all-cause 1-year mortality in myocardial infarction patients. *Medicine (Baltimore)* 2020;**99**:e21288. https://doi.org/10.1097/MD.0000000000021288
73. Ndrepepa G, Tiroch K, Keta D, Fusaro M, Seyfarth M, Pache J, *et al.* Predictive factors and impact of no reflow after primary percutaneous coronary intervention in patients with acute myocardial infarction. *Circ Cardiovasc Interv* 2010;**3**:27–33. https://doi.org/10.1161/CIRCINTERVENTIONS.109.896225
74. Stone GW, Dixon SR, Grines CL, Cox DA, Webb JG, Brodie BR, *et al.* Predictors of infarct size after primary coronary angioplasty in acute myocardial infarction from pooled analysis from four contemporary trials. *Am J Cardiol* 2007;**100**:1370–5. https://doi.org/10.1016/j.amjcard.2007.06.027
75. Wohlfahrt P, Nativi-Nicolau J, Zhang M, Selzman CH, Greene T, Conte J, *et al.* Quality of life in patients with heart failure with recovered ejection fraction. *JAMA Cardiol* 2021;**6**:957–62. https://doi.org/10.1001/jamacardio.2021.0939
76. Chen X, Xin Y, Hu W, Zhao Y, Zhang Z, Zhou Y. Quality of life and outcomes in heart failure patients with ejection fractions in different ranges. *PLoS One* 2019;**14**:e0218983. https://doi.org/10.1371/journal.pone.0218983
77. Liu L, Feng D, Chen G, Chen M, Zheng Q, Song P, *et al.* Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-induced mitophagy in mammalian cells. *Nat Cell Biol* 2012;**14**:177–85. https://doi.org/10.1038/ncb2422
78. Gao A, Jiang J, Xie F, Chen L. Bnip3 in mitophagy: novel insights and potential therapeutic target for diseases of secondary mitochondrial dysfunction. *Clin Chim Acta* 2020;**506**:72–83. https://doi.org/10.1016/j.cca.2020.02.024
79. Dorn GW II. Parkin-dependent mitophagy in the heart. *J Mol Cell Cardiol* 2016;**95**:42–9. https://doi.org/10.1016/j.yjmcc.2015.11.023
80. Andres AM, Tucker KC, Thomas A, Taylor D, Sengstock J, Jahania D, *et al.* Mitophagy and mitochondrial biogenesis in atrial tissue of patients undergoing heart surgery with cardiopulmonary bypass. *JCI Insight* 2017;**2**:e89303. https://doi.org/10.1172/jci.insight.89303
81. Andres AM, Lee P, Hernandez G, Huang C, Ratliff EP, Thornton CA, *et al.* Abstract 314: infarct size reduction by statins requires parkin and mitophagy. *Circ Res* 2012;**111**:A314. https://doi.org/10.1161/res.111.suppl_1.A314
82. Andres AM, Hernandez G, Lee P, Huang C, Ratliff EP, Sin J, *et al.* Mitophagy is required for acute cardioprotection by simvastatin. *Antioxid Redox Signal* 2014;**21**:1960–73. https://doi.org/10.1089/ars.2013.5416
83. Colom B, Oliver J, Garcia-Palmer FJ. Sexual dimorphism in the alterations of cardiac muscle mitochondrial bioenergetics associated to the ageing process. *J Gerontol A Biol Sci Med Sci* 2015;**70**:1360–9. https://doi.org/10.1093/gerona/glu014
84. Gottlieb RA, Thomas A. Mitophagy and mitochondrial quality control mechanisms in the heart. *Curr Pathobiol Rep* 2017;**5**:161–9. https://doi.org/10.1007/s40139-017-0133-y
85. Kerola AM, Palomaki A, Rautava P, Nuotio M, Kyto V. Sex differences in cardiovascular outcomes of older adults after myocardial infarction. *J Am Heart Assoc* 2021;**10**:e022883. https://doi.org/10.1161/JAHA.121.022883
86. Millett ERC, Peters SAE, Woodward M. Sex differences in risk factors for myocardial infarction: cohort study of UK Biobank participants. *BMJ* 2018;**363**:k4247. https://doi.org/10.1136/bmj.k4247
87. Merz CNB. The Yentl syndrome is alive and well. *Eur Heart J* 2011;**32**:1313–5. https://doi.org/10.1093/eurheartj/ehr083
88. Bairey Merz CN, Shaw LJ, Reis SE, Bittner V, Kelsey SF, Olson M, *et al.* Insights from the NHLBI-Sponsored Women's Ischemia Syndrome Evaluation (WISE) study: part II: gender differences in presentation, diagnosis, and outcome with regard to gender-based pathophysiology of atherosclerosis and macrovascular and microvascular coronary disease. *J Am Coll Cardiol* 2006;**47**:S21–9. https://doi.org/10.1016/j.jacc.2004.12.084
89. Ventura-Clapier R, Moulin M, Piquereau J, Lemaire C, Mericskay M, Veksler V, *et al.* Mitochondria: a central target for sex differences in pathologies. *Clin Sci (Lond)* 2017;**131**:803–22. https://doi.org/10.1042/CS20160485
90. Mammucari C, Gherardi G, Rizzuto R. Structure, activity regulation, and role of the mitochondrial calcium uniporter in health and disease. *Front Oncol* 2017;**7**:139. https://doi.org/10.3389/fonc.2017.00139
91. Lopaschuk GD, Karwi QG, Tian R, Wende AR, Abel ED. Cardiac energy metabolism in heart failure. *Circ Res* 2021;**128**:1487–513. https://doi.org/10.1161/CIRCRESAHA.121.318241

92. Toldo S, Mauro AG, Cutter Z, Abbate A. Inflammasome, pyroptosis, and cytokines in myocardial ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 2018;**315**: H1553–68. https://doi.org/10.1152/ajpheart.00158.2018
93. Wang P, Qian H, Xiao M, Lv J. Role of signal transduction pathways in IL-1β-induced apoptosis: pathological and therapeutic aspects. *Immun Inflamm Dis* 2023;**11**:e762. https://doi.org/10.1002/iid3.762
94. Elliott EI, Miller AN, Banoth B, Iyer SS, Stotland A, Weiss JP, *et al.* Cutting edge: mitochondrial assembly of the NLRP3 inflammasome complex is initiated at priming. *J Immunol* 2018;**200**:3047–52. https://doi.org/10.4049/jimmunol.1701723
95. Shimada K, Crother TR, Karlin J, Dagvadorj J, Chiba N, Chen S, *et al.* Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 2012;**36**: 401–14. https://doi.org/10.1016/j.immuni.2012.01.009
96. Balasubramaniam M, Parcon PA, Bose C, Liu L, Jones RA, Farlow MR, *et al.* Interleukin-1β drives NEDD8 nuclear-to-cytoplasmic translocation, fostering parkin activation via NEDD8 binding to the P-ubiquitin activating site. *J Neuroinflammation* 2019; **16**:275. https://doi.org/10.1186/s12974-019-1669-z
97. Panicker N, Kam TI, Wang H, Neifert S, Chou SC, Kumar M, *et al.* Neuronal NLRP3 is a parkin substrate that drives neurodegeneration in Parkinson's disease. *Neuron* 2022; **110**:2422–37.e9. https://doi.org/10.1016/j.neuron.2022.05.009