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RESEARCH ARTICLE

# Cardiomyocyte intercellular signalling increases oxidative stress and reprograms the global- and phospho-proteome of cardiac fibroblasts

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## Abstract

Pathological reprogramming of cardiomyocyte and fibroblast proteome landscapes drive the initiation and progression of cardiac fibrosis. Although the secretome of dysfunctional cardiomyocytes is emerging as an important driver of pathological fibroblast reprogramming, our understanding of the downstream molecular players remains limited. Here, we show that cardiac fibroblast activation ($\alpha SMA^+$) and oxidative stress mediated by the secretome of TGF$\beta$-stimulated cardiomyocytes is associated with a profound reprogramming of their proteome and phosphoproteome landscape. Within the fibroblast global proteome there was a striking dysregulation of proteins implicated in extracellular matrix, protein localisation/metabolism, KEAP1-NFE2L2 pathway, lysosomes, carbohydrate metabolism, and transcriptional regulation. Kinase substrate enrichment analysis of phosphopeptides revealed potential role of kinases (CK2, CDK2, PKC, GSK3B) during this remodelling. We verified upregulated activity of casein kinase 2 (CK2) in secretome-treated fibroblasts, and pharmacological CK2 inhibitor TBB (4,5,6,7-Tetrabromobenzotriazole) significantly abrogated fibroblast activation and oxidative stress. Our data provides molecular insights into cardiomyocyte to cardiac fibroblast crosstalk, and the potential role of CK2 in regulating cardiac fibroblast activation and oxidative stress.

KEYWORDS
cardiac fibroblasts, cardiomyocytes, intercellular signalling, proteomics, reactive oxygen species

# 1 | INTRODUCTION

Cardiac fibrosis, the deposition of excess extracellular matrix (ECM) proteins by cardiac fibroblasts, is a hallmark feature of pathological myocardial remodelling, driving contractile dysfunction, distortion of cardiac architecture, and heart failure (Azevedo et al., 2016; Frangogiannis, 2021; Maruyama & Imanaka-Yoshida, 2022). Cardiac fibrosis often occurs concurrently with oxidative stress [excessive reactive oxygen species (ROS)], inflammation, and hypertrophy (Aimo et al., 2020; Aragno et al., 2008; Kai et al., 2006; Raman et al., 2019; Shah et al., 2021; Smolgovsky et al., 2021; van der Pol et al., 2019; Wei, 2011), with the scar tissue impairing mechanical and biochemical functions (Frangogiannis, 2021; Li et al., 2018). Several key fibrotic drivers have been identified, including transforming growth factor beta (TGF$\beta$), angiotensin II, endothelin 1, and platelet-derived growth factor, which exert their profibrotic functions by reprogramming the fibroblast proteome and phosphoproteome landscape (Sygitowicz et al., 2021; Travers et al., 2016). Moreover, accruing evidence indicates these molecules also dysregulate physiological paracrine signals between cardiac cells, further promoting pathological remodelling (Basma et al., 2019; Kuo et al., 2019; Lyu et al., 2015).

Physiological crosstalk between atrial cardiomyocytes and fibroblasts is important in regulating fibroblast proliferation and ECM secretion; disruption of this interaction results in atrial fibrosis and fibrillation (Moreira et al., 2020). Dysregulated signalling between cardiomyocytes, cardiac fibroblasts, endothelial cells, and immune cells contributes to the development of hypertrophy (Bang et al., 2014; Lyu et al., 2015; Tian et al., 2020), endothelial dysfunction (Ottaviani et al., 2022; Wang et al., 2014), and fibrosis (Kumar et al., 2019; Ramanujam et al., 2021; Yang et al., 2018). Intercellular communications can be mediated by the cell secretome (secreted factors) comprised of soluble (endothelins, cytokines/chemokines, and natriuretic peptides (Fountoulaki et al., 2015; Martins-Marques, 2021)) and vesicular components (Datta et al., 2017; Kuo et al., 2019; Nie et al., 2018; Ontoria-Oviedo et al., 2018; Wang et al., 2017; Yang et al., 2018). Cardiomyocytes constitute the cardiac majority, 80% by volume (Dämmrich & Pfeifer, 1983), and significantly contribute to the intercellular signalling central to fibrotic development (Cartledge et al., 2015; Dolmatova et al., 2012; Kumar et al., 2019; Tsoporis et al., 2012). Indeed, cardiomyocyte-derived soluble factors influence cellular proliferation (Cartledge et al., 2015; Kumar et al., 2019) and death (Cartledge et al., 2015), collagen expression (Kumar et al., 2019), migration (Kumar et al., 2019), and activation (Dolmatova et al., 2012; Kumar et al., 2019; Tsoporis et al., 2012). Key fibrotic drivers alter cardiomyocyte secreted factors, with angiotensin II causing secretome-mediated promotion of migration and activation in cardiac fibroblasts (Kuo et al., 2019), and TGF$\beta$ altering their composition (Gennebäck et al., 2013). TGF$\beta$ signalling in cardiomyocytes is central to the development of cardiac fibrosis during pressure overload, with knockdown of TGF$\beta$ receptors in cardiomyocytes reducing pathological remodelling and interstitial fibrosis (Koitabashi et al., 2011). While TGF$\beta$ modulates myocardial disease and maladaptive remodelling (Frangogiannis, 2022), its influence on cardiomyocyte secretome-mediated reprogramming of cardiac fibroblast proteome and function remains poorly understood. Such insights into this downstream signalling will potentially uncover therapeutic leads for cardiac fibrosis.

Here, cardiomyocytes derived from human induced pluripotent stem cells (iPSCs) were stimulated with TGF$\beta$, resulting in maladaptive contractile function and proteome alterations. The signalling and remodelling capacity of these cardiomyocytes' secretome was studied in primary human cardiac fibroblasts. Functional and proteomic analysis revealed a pro-fibrotic phenotype, and combined phosphopeptide kinase substrate enrichment analysis and upstream regulatory network prediction identified casein kinase 2 (CK2) as a potential regulator of cardiac fibrosis through oxidative stress and myofibroblast activation.

# 2 | EXPERIMENTAL PROCEDURES

## 2.1 | Generation of iPSC-derived cardiomyocytes

iPSC-cardiomyocytes were generated and cultured as previously described (Lozano et al., 2020, 2022). Briefly, human iPS-Foreskin-2 cell line (Yu et al., 2007) were cultured in TeSR-E8 medium (Stem Cell Technologies, 05990) on vitronectin-coated plates. For directed cardiac differentiation, iPSCs were seeded onto Matrigel (Corning) coated plates at $1\times10^5$ cells/cm$^2$ and cultured in TeSR-E8 medium supplemented with 10 μM Y-27632 (Tocris Bioscience). After 2 days (100% confluency, referred to as day 0), medium was replaced with RPMI-1640 basal medium supplemented with B-27 without insulin supplement (Thermo Fisher Scientific), growth factor reduced Matrigel (1:60 dilution) and 10 μM CHIR99021 (Cayman Chemical). After 24 h, medium was replaced with RPMI-1640 basal medium containing B-27 without insulin supplement for 24 h. On day 2, the medium was changed to RPMI-1640 basal medium containing B-27 without insulin supplement and 5 μM IWP2 (Tocris Bioscience) for 72 h. From day 5, cells were cultured in RPMI-1640 basal medium containing B-27 supplement (Thermo Fisher Scientific) and 200 μg/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich). On day 12, cardiomyocytes were re-plated onto Matrigel-coated plates at $1.5\times10^5$ cells/cm$^2$ in DMEM/F-12 GlutaMAX medium supplemented with 20% foetal bovine serum (Sigma-Aldrich), 0.1 mM 2-mercaptoethanol, 0.1 mM non-essential amino acids, 50 U/mL penicillin/streptomycin and 10 μM Y-27632. From days 14–19, cardiomyocytes were enriched by culturing the cells in glucose-free DMEM medium containing 4 mM lactate (Sigma-Aldrich). Enriched cardiomyocytes (>90% cardiac troponin T positive) were maintained

in RPMI-1640 basal medium containing B-27 supplement and 200 μg/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate.

## 2.2 | Cardiomyocyte cell culture and secretome isolation

Cardiomyocytes were cultured in RPMI-1640 basal medium containing B-27 supplement and 200 μg/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate at 37°C, 5% $CO_2$. TGF$\beta$ (or equal volumes of PBS vehicle control) was added to culture media for a final concentration of 5 ng/mL TGF$\beta$. Cardiomyocytes were cultured under stimuli for 144 h, with media and treatment replaced after 72 h for functional analyses. Secretome of cardiomyocytes was harvested by collecting the conditioned media after both 72-h periods, which was centrifuged at 500 *g* for 5 min at 4°C, followed by 2000 *g* for 10 min at 4°C to remove cell debris prior to storage at −20°C in aliquots.

## 2.3 | Cardiac fibroblast cell culture

Human primary cardiac fibroblasts were obtained from Sigma-Aldrich (306-5a, lot: 3357; atrial, single donor: male, 26 years old) and cultured on flasks (coated with 1% gelatin (bovine skin Type B, Sigma-Aldrich, 9000-70-8) overnight) in media consisting of 50% Cardiac Fibroblast Growth Medium (Cell Applications, 316–500), 40% DMEM/F12 (Gibco, Invitrogen, 11320033), 10% 0.22 μm filtered foetal calf serum (Gibco, Invitrogen, 10099141), and Penicillin-Streptomycin (Gibco, Invitrogen, 15140122) at 37°C, 5% $CO_2$. Cells were passaged 1:3 by surface area and used at passages 6–8. For analysis, fibroblasts were plated (30,000 cells/$cm^2$) on plastic plates coated with 1% gelatin and left to attach overnight, after which cells were serum starved for 24 h in DMEM/F12 medium containing 1x Insulin-Transferring-Selenium-Sodium Pyruvate (Gibco, Invitrogen, 51300044) and Penicillin-Streptomycin. Starve media was removed and cardiomyocyte secretome was added for 48 h, with secretome replaced after 24 h. For TGF$\beta$ control conditions (oxidative stress assay), starved cardiac fibroblasts were cultured with 10 ng/mL TGF$\beta$ (or equal volumes of PBS vehicle) in DMEM/F12 medium containing 1x Insulin-Transferring-Selenium-Sodium Pyruvate and Penicillin-Streptomycin for 48 h, with treatment replaced after 24 h. For CK2 inhibition experiments, 5 μM TBB (4,5,6,7-Tetrabromobenzotriazole, MedChem Express, HY-14394) or equal concentrations of DMSO vehicle control were added concurrently with secretome treatments.

## 2.4 | Cardiomyocyte contraction analysis

Videos of contracting cardiomyocytes were captured using the Olympus IX-71 microscope and CellSens software in biological triplicate and technical duplicate. Videos were analysed using MuscleMotion macro for ImageJ (Sala et al., 2018). More than 20 cells per biological replicate were analysed (values averaged for data presentation) using settings as follows: frames/second (15), speedWindow (2), decrease noise for output (Yes), detect reference frame (Yes). Statistical analysis was performed in GraphPad Prism (v9.4.1) using unpaired *t*-tests.

## 2.5 | Protein quantification

Protein quantification was performed using the microBCA™ Protein Assay Kit (Thermo Fisher Scientific, 23235) as per manufacturer's instructions.

## 2.6 | Sample preparation for mass spectrometry

Sample preparation (5–10 μg/sample) using a sera-bead workflow (Hughes et al., 2019) was performed as previously described (Lozano et al., 2022; Rai et al., 2021). Cell lysates were collected with 2% v/v sodium dodecyl sulphate (SDS), incubated for 5 min at 95°C, quantified using microBCA (Thermo Fisher Scientific, 23235) and normalised to 10 μg/50 μL in 1% SDS, 50 mM 4-(2-hydroxyethyl)−1-piperazineethanesulfonic acid (HEPES), pH 8.0. Samples were reduced (10 mM Dithiothreitol (DTT, Thermo Fisher Scientific) for 1 h, agitated at 350 rpm at room temperature), alkylated with 20 mM iodoacetamide (IAA, Sigma-Aldrich) for 20 min at room temperature protected from light, and finally reaction quenched using 10 mM DTT. A Sera-Mag SpeedBead carboxylate-modified magnetic particle mixture (hydrophobic and hydrophilic, 1:1 mix, Cytiva 65152105050250, 45152105050250) was then added to samples and bound using 50% ethanol (10 min, 1000 rpm, room temperature). Magnetic racks were used to sediment beads for washing with 80% ethanol (three times, 200 μL/wash). Beads were resuspended for overnight digestion (100 μL

50 mM triethylammonium bicarbonate (TEAB), pH 8.0, 1:50 trypsin:protein (Promega, V5111), 37°C, 1000 rpm). Samples centrifuged at 20,000 *g* for 1 min and placed on a magnetic rack to sediment beads for peptide removal. Peptides in the supernatant were collected, acidified (final concentration of 1.5% formic acid), frozen (−80°C for 30 min), and dried using vacuum centrifugation (~1 h). Peptides were then resuspended in 0.07% trifluoroacetic acid (TFA), quantified by Fluorometric Peptide Assay (Thermo Fisher Scientific, 23290) as per manufacturer's instructions, and normalised with 0.07% TFA.

## 2.7 | Phosphopeptide preparation and enrichment

Cardiac fibroblasts were plated (30,000 cells/cm$^2$) on plastic plates coated with 1% gelatin and left to attach overnight, after which cells were serum starved for 24 h in DMEM/F12 medium containing 1x Insulin-Transferring-Selenium-Sodium Pyruvate (Gibco, Invitrogen, 51300044) and Penicillin-Streptomycin. Starve media was removed and cells were stimulated with cardiomyocyte secretome for 15 min, and cells washed 2× in ice-cold PBS and lysed [1% (v/v) SDS, 1:50 HALT protease phosphatase inhibitor cocktail (Thermo Fisher Scientific, 78,442), 50 mM HEPES pH 8] on ice for 5 min, and heat treated at 95°C for 5 min. 100 µg of cell lysates were reduced, alkylated, quenched and digested as described above. Peptides were acidified to a final concentration of 2% formic acid (FA), centrifuged at 20,000 g for 1 min, and lyophilised by vacuum centrifugation. Peptides were reconstituted in Binding/Equilibration Buffer for phosphopeptide enrichment, using the High-Select™ TiO2 Phosphopeptide Enrichment kit, as per manufacturer's instructions (Thermo Fisher Scientific, A32993). Peptides were transferred to $TiO_2$ spin tip and centrifuged twice at 1000 *g* for 5 min. The column was washed twice with binding/equilibration buffer and wash buffer at 3000 *g* for 2 min, followed with MS-grade water wash at 3000 *g* for 2 min. Phosphopeptides were eluted in 100 µL phosphopeptide elution buffer by centrifugation at 1000 *g* for 5 min, dried by vacuum centrifugation and reconstituted in 0.07% TFA and quantified by Fluorometric Peptide Assay (Thermo Fisher Scientific, 23290) as per manufacturer's instructions, and normalised with 0.07% TFA.

## 2.8 | Peptide analysis

Peptides were analysed on a Dionex UltiMate NCS-3500RS nanoUHPLC coupled to a Q-Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer equipped with nanospray ion source in positive, data-dependent acquisition mode as described (Lozano et al., 2022; Rai et al., 2021). Peptides were loaded (Acclaim PepMap100 C18 5 µm beads with 100 Å pore-size, Thermo Fisher Scientific) and separated (1.9 µm particle size C18, 0.075 × 250 mm, Nikkyo Technos Co. Ltd) over 2%−28% acetonitrile containing 0.1% formic acid for 110 min runtime at 300 nL/min at 55°C (butterfly portfolio heater, Phoenix S&T). MS1 scans acquired from 300−1650 m/z (60,000 resolution, $3 \times 10^6$ automatic gain control (AGC), 128 ms injection time) followed by MS/MS data-dependent acquisition (top 25) with collision-induced dissociation and detection in the ion trap (30,000 resolution, $1 \times 10^5$ AGC, 60 ms injection time, 28% normalised collision energy, 1.3 m/z quadrupole isolation width). Precursor Ions with unassigned, 1, and 6–8 charge states, and slightly charged species, were rejected and peptide match disabled. Selected sequenced ions were dynamically excluded for 30 s. Data was acquired using Xcalibur software v4.0 (Thermo Fisher Scientific).

## 2.9 | Database searching and protein/modified peptide identification

Identification and quantification of peptides was performed using MaxQuant (v1.6.14.0) (Tyanova et al., 2016) and Andromeda (Cox et al., 2011) as previously described (Claridge et al., 2021). Tandem mass spectra obtained from cardiomyocytes and remodelled fibroblasts (global and phospho analysis) were analysed as three separate runs; spectra were run against Homo sapiens (human) reference proteome (77,027, Jan 2021 (cardiomyocytes), 81,837, Mar 2023 (cardiac fibroblasts—global remodelled and phosphopeptides)) supplemented with common contaminants. Search parameters for whole cell samples and secretome were as follows: carbamidomethylated cysteine as fixed modification, oxidation of methionine and N-terminal protein acetylation as variable modifications, trypsin/P as proteolytic enzyme with ≤2 missed cleavage sites, search tolerance 7 ppm, fragment ion mass tolerance 0.5 Da, <1% false discovery rate on peptide spectrum match with target-decoy approach at peptide and protein levels, match between runs (MBR) selected and label free quantification (LFQ) algorithm employed. Search parameters for phosphopeptides were as described with modifications: no LFQ and Phospho(STY) included as a variable modification.

## 2.10 | Proteome data analysis and informatics

Statistical analysis and summary values were generated in Perseus (Tyanova et al., 2016) (v1.6.15.0) for proteins with ≥2 razor/unique peptides. Principal component analysis (proteins present in ≥66% of samples within one group) and heatmap visualization (hierarchical clustering, Euclidian distance, average linkage clustering, significant ($p < 0.05$) and unique proteins

(≥50% in one group, 0% in other)) was performed in Perseus. Principal component analysis of cardiomyocytes was performed with missing values imputed from normal distribution for presentation purposes only, imputed values were not used for further analysis. Reprogrammed cardiac fibroblasts were processed in two batches ($n = 12$ total); transformed LFQ samples were filtered to proteins present in ≥4/6 in ≥1/2 treatment and imputed with QRILC prior to Limma batch correction in Perseus (Ritchie et al., 2015; Yu et al., 2020). Student's *T*-test was performed to determine significance ($p < .05$). Venn diagram values were generated using the Bioinformatics Evolutionary Genomics web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). Graphs generated using GraphPad Prism (v8.1.2). GO and functional enrichment annotations were retrieved by submitting protein IDs to g:Profiler (Raudvere et al., 2019) and Reactome (Fabregat et al., 2017). Upregulated proteins in remodelled (48 h) cardiac fibroblasts treated with TGF$\beta$-cardiomyocyte secretome were uploaded to X2K (Clarke et al., 2018) for kinase prediction.

## 2.11 | Analysis of phosphopeptides

Downstream analysis was restricted to peptides with localization scores >0.7. Heatmap visualization (hierarchical clustering, Euclidian distance, average linkage clustering, significant ($p < 0.05$) and unique peptides (≥50% in one group, 0% in other)) was performed in Perseus. Phosphoproteomic analysis was performed using KSEA (Wiredja et al., 2017). All significant and unique (imputed unique *p* value as 0.01 and fold change as 10 (unique to pTGF$\beta$-CM-CF) or 0.1 (unique to pCtrl-CM-CF)) phosphosites uploaded to KSEA for analysis with NetworKIN (cutoff 2).

## 2.12 | Data availability

A list of samples and RAW/processed data (cardiac fibroblast and cardiomyocytes, and phospho-peptides of cardiac fibroblasts) have been uploaded to the MassIVE Knowledge Base (MassIVE-KB) # MSV000092293.

## 2.13 | Reactive oxygen species detection assay

Reactive oxygen species detection assay (Abcam, ab113851) was performed according to manufacturer's instructions, where cardiac fibroblasts were plated at confluency, serum starved for 24 h, and incubated with cardiomyocyte secretome or TGF$\beta$/vehicle as described above. Cells were washed three times with dPBS before incubation with 50 μM DCFDA and 5% FCS (Gibco, Invitrogen, 10099141) in phenol red-free DMEM/F12 medium (Gibco, Invitrogen, 21041025) for 45 min. Cells were washed three times with dPBS and fresh phenol red-free DMEM/F12 medium was added. Fluorescence was measured (excitation/emission 485 nm/535 nm) for 3–4 biological replicates (≥4 technical replicates/biological). Statistical analysis was performed in GraphPad Prism (v9.4.1) using unpaired t-tests.

## 2.14 | Cardiac fibroblast activation imaging

Cells were washed in dPBS, fixed using 4% formaldehyde for 5 min, and washed thrice with dPBS. Cells were permeabilised for 5 min with 0.2% Triton X100 and blocked for 30 min with blocking buffer (3% BSA in 0.2% Triton X100) at 25°C. Cells were stained with anti-alpha smooth muscle actin ($\alpha$SMA) antibody [1A4] (Abcam, ab7817; 1:50 dilution in blocking buffer) for 1 h at 25°C. Cells were washed and incubated with Alexa fluor secondary antibody (Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488, Thermo Fisher Scientific, A-11001; 1:200 dilution) for 20 min. Nuclei were stained with 5 μg/mL Hoescht 33342 (Thermo Fischer Scientific, H1399) as per manufacturer's instructions. Cells were imaged using Olympus IX71 microscope and CellSens software; four fields of view were captured per well. Cells positive for $\alpha$SMA were counted in each capture. Statistical analysis was performed in GraphPad Prism (v9.4.1) using unpaired *t*-tests.

## 2.15 | Western blotting

Samples were lysed in SDS sample buffer (4% w/v SDS, 20% v/v glycerol, 0.01% v/v bromophenol blue, 0.125 M Tris-hydrochloride (Tris-HCl), pH 6.8) with 100 mM DTT), and separated on Norvex 4%–12% Bis–Tris NuPAGE gels with MES running buffer at 150 V for 1 h. Proteins on the gel were electrotransferred onto nitrocellulose membranes using iBlot Dry 2.0 blotting system (Life Technologies). The membranes were blocked with 5% w/v skim milk powder in PBS-Tween (PBST) (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na2HPO4, 0.0018 M KH2PO4, 0.05% w/v Tween 20) for 30 min at room temperature. The membranes were washed and probed with primary antibodies (1:1000 dilution) for 24 h at 4°C in PBST. Membranes were washed and then

probed with secondary antibodies (1:20,000 dilution) for 1 h at 25°C in PBST. Membranes were imaged on a LICOR Odyssey 9120 with Image Studio Software 5.2.5. Primary antibodies used were: GAPDH (D4C6R) Mouse mAb (Cell Signalling Technology 97166), Phospho-CK2 Substrate [(pS/pT)DXE] MultiMab™ Rabbit mAb mix (Cell Signalling Technology, 8738), Recombinant Anti-Casein Kinase 2 beta antibody [EP1995Y] (Abcam, ab76025), Anti-CSNK2A2 antibody (Abcam, ab10474), Anti-CSNK2A1 antibody [8E5] (Abcam, ab70774). Secondary antibodies used were: IRDye 800 goat anti-mouse IgG (#926-32210) or IRDye 680 goat anti-rabbit IgG (#926-68071) (1:15000, LI-COR Biosciences). Densitometry-based quantification of western blots was performed with ImageJ software (Schneider et al., 2012). Statistical analysis was performed in GraphPad Prism (v9.4.1) using unpaired *t*-tests.

## 3 | RESULTS

### 3.1 | TGFβ induces pathological dysfunction and proteome remodelling in cardiomyocytes

TGFβ is a driver of maladaptive cardiomyocyte biology, promoting contractile dysfunction (Li et al., 2008) and driving their profibrotic influence on cardiac fibroblasts (Koitabashi et al., 2011). We induced a pathological phenotype in iPSC-derived cardiomyocytes with TGFβ (TGFβ-CM) or vehicle (Ctrl-CM) for 6 days, and then assessed contractile function using MuscleMotion software (Sala et al., 2018). Using contraction profiles (Figure 1a,b), contraction time (time to peak, Figure 1c), relaxation time (time from peak, Figure 1d), and total contraction duration (Figure 1e) were determined. We found that TGFβ-CM, compared to Ctrl-CM, showed aberrant contractility with significantly slowed contraction ($p < 0.05$) and relaxation ($p < 0.0001$), resulting in an overall increase of total contraction duration ($p < 0.01$), as previously reported in dysfunctional cardiomyocytes (Li et al., 2008).

We investigated whether changes in the proteome landscape supported the contractile dysfunction phenotype of cardiomyocytes (Parreira et al., 2020; Vigil-Garcia et al., 2021). Proteomic profiling identified 3173 and 3304 proteins in Ctrl-CM and TGFβ-CM, respectively (Figure 1f, Table S1). Principal component analysis revealed that the proteomes were distinct (Figure 1g). Pairwise comparative analysis revealed 279 significantly dysregulated proteins (Student's *T*-test, $p < 0.05$, 146 upregulated, 133 downregulated) in response to TGFβ, including NACA (Schroeder et al., 2022), MYLK3 (Williams et al., 2020), KRT19 (Stone et al., 2005), RRAD (Li et al., 2020), NEBL (Maiellaro-Rafferty et al., 2013), PDLIM5 (Verdonschot et al., 2020), and GPC6 (Melleby et al., 2016), proteins associated with contraction and cardiac pathologies (Figure 1h). In TGFβ-CM, Gene Ontology analysis (g:Profiler for components/processes/functions (Figure 1i) and Reactome for pathways (Figure 1j)) of dysregulated proteins (*t*-test $p < 0.05$ or uniquely identified) revealed an upregulation of proteins associated with myofibril (TNNI1/T1, PDLIM4, NEXN), mRNA processing (RNMT, NCBP1, XRN2) and splicing (SF3A2, SRSF10, U2AF1), and a striking downregulation of mitochondrion components including mitochondrial envelope (ACSL3, ACSL4, HK2), inner membrane (TMEM70, CHCHD3, MICOS13), and matrix (HSPD1, AK4, TEFM) (Table S2). Lower levels of both mitochondria (Lopaschuk et al., 2021; Nomura et al., 2018) and alternative mRNA splicing (Beqqali, 2018; Hasimbegovic et al., 2021; Zhu et al., 2017) have been associated with pathological cardiac remodelling and heart failure. Thus, our functional and proteomic data support a model of dysfunctional cardiomyocytes representative of those observed in pathology (Chen et al., 2020; Lopaschuk et al., 2021; Nomura et al., 2018).

### 3.2 | TGFβ-cardiomyocyte secretome reprograms the proteomic landscape of cardiac fibroblasts

To investigate the role of cardiomyocyte secretome signalling in cardiac fibrosis, Ctrl-CM and TGFβ-CM secretomes were obtained and their effect on cardiac fibroblast biology assessed. Cardiac fibroblasts were cultured on 1% gelatin and serum starved overnight prior to a 48-h secretome exposure (Figure 2a); we refer to these secretome-remodelled fibroblasts as 'Ctrl-CM-CF or TGFβ-CM-CF' hereafter. We performed proteomic analysis of Ctrl-CM-CF and TGFβ-CM-CF to interrogate the reprogramming capacity of the cardiomyocyte secretomes in fibroblasts (Figure 2b). A total of 3583 and 3776 proteins were identified in Ctrl-CM-CF and TGFβ-CM-CF (Figure 2c, razor-unique peptides ≥ 2, Tables S3, S4, and Figure S1). Principal component analysis revealed distinct clustering of Ctrl-CM-CF and TGFβ-CM-CF (Figure 2d). Pairwise comparative analysis revealed significant dysregulation (Student's *T*-test, $p < 0.05$) of fibrosis associated proteins including COL6A2 (Williams et al., 2022), AGTRAP (Subbaiah et al., 2022), LOXL4 (González-Santamaría et al., 2016), and PIEZO1 (Braidotti et al., 2022) in TGFβ-CM-CF (Figure 2e, Table S4).

Analysis of significantly dysregulated components (Student's *T*-test, $p < 0.05$) using Gene Ontology enrichment (g:Profiler and Reactome (Figure 2f)) revealed significant alteration of protein metabolism, signalling, lysosome, carbohydrate metabolism, and translational regulation. TGFβ-CM-CF had increased expression of proteins associated with assembly of collagen fibrils and other multimeric structures (COL6A2, LOXL4), focal adhesion (AFAP1, AHNAK, MARCKS, NEXN), endomembrane system (ABCA8, OPTN, POFUT2, VPS26B), protein localization (DYNLL1, RANGAP1, SNX2, TNPO3), and glycogen metabolism (PGM1, PYGL) (Figure 2f, Table S5). Downregulated ontologies included KEAP1-NFE2L2 pathway (CSNK2A1, MAP1LC3B,

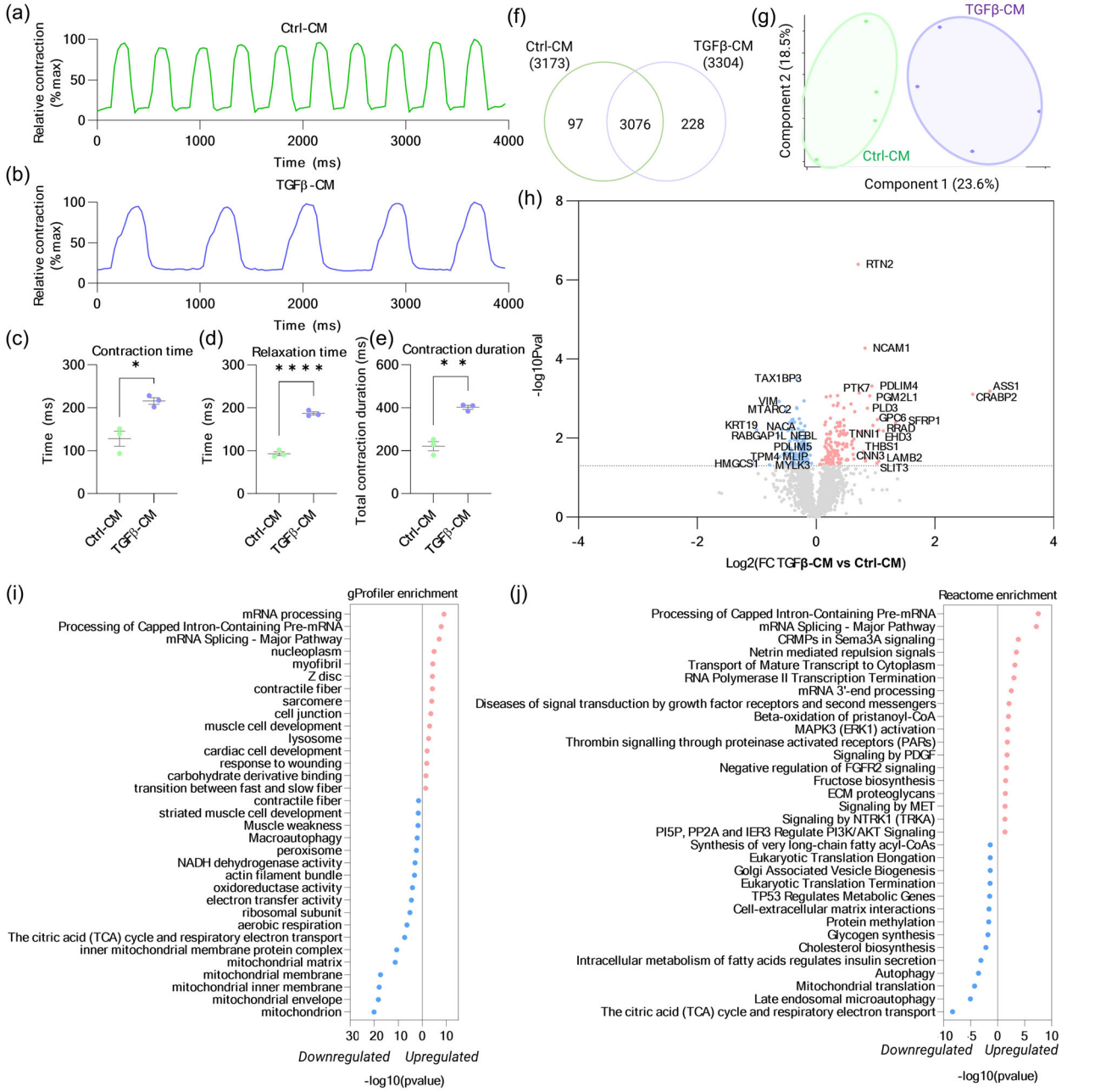


**FIGURE 1** TGF$\beta$ induces contractile dysfunction and changes to global proteome in human iPSC-derived cardiomyocytes. Representative contraction profiles of iPSC-derived cardiomyocytes (CM) treated with (a) vehicle control (Ctrl-CM) or (b) 5 ng/mL TGF$\beta$ (TGF$\beta$-CM). MuscleMotion™ analysis of (c) contraction time, (d) relaxation time, and (e) total contraction duration ($n = 3$, Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$). (f) Venn diagram of proteins quantified in Ctrl-CM and TGF$\beta$-CM using LC-MS/MS. (g) Principal component analysis of Ctrl-CM and TGF$\beta$-CM. (h) Volcano plot of proteins identified (blue are downregulated proteins and pink are upregulated proteins in TGF$\beta$-CM). Gene enrichment analysis of TGF$\beta$-CM versus Ctrl-CM showing significantly dysregulated ($t$-test $p < 0.05$) and unique proteins using (i) g:Profiler and (j) Reactome.

PSMD1/9, TKT), primary lysosome (ARSA, DNAJC3/5, MAN2B1, NPC2), eiF3 complex (EIF3D, EIF3I, EIF3K), and mRNA binding (AQR, CSTF2T, HNRNPL, TAF15) (Figure 2f, Table S5). Notably, various kinases (ADK, ARAF, CSNK1D, CSNK2A1) and phosphatases (PPP3CA, PPP5C) were dysregulated in remodelled fibroblasts, suggesting alterations to phosphorylation-mediated signalling. Furthermore, regulators of pathways involved in fibrosis, including angiotensin II (Cao et al., 2018) (AGTRAP), insulin like growth factor (Garrett et al., 2019) (IGF2), and prostaglandin (Li et al., 2021) (PTGFRN) were dysregulated. A range of transcriptional factors and regulators exhibited altered expression (CHCHD2, HDAC2, HMGA2, CTBP2, DDX54), as did carbohydrate metabolism related proteins (MAN2B1, PGM1, PYGL, TPI1, PKM) including fucosyltransferase POFUT2 which is essential for fucosylation, a post translational modification associated with fibrosis (Li et al., 2018).

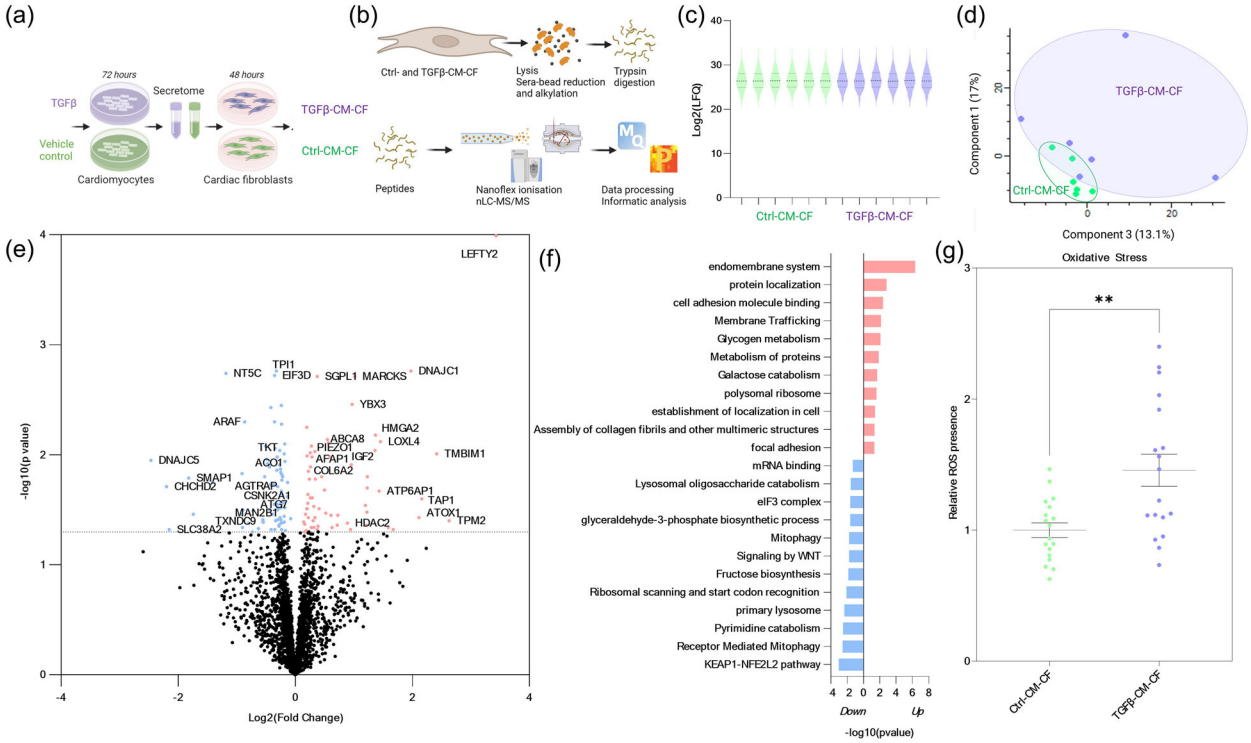


**FIGURE 2** TGF$\beta$-cardiomyocyte derived secretome induces global proteome remodelling and oxidative stress in cardiac fibroblasts. (a) Workflow for treatment of cardiac fibroblasts (CF) with secretomes from TGF$\beta$-CM (TGF$\beta$-CM-CF) and Ctrl-CM (Ctrl-CM-CF) ($n = 6$/treatment). (b) Mass spectrometry-based proteomic profiling workflow, including sera-bead sample preparation with nano-liquid chromatography tandem mass spectrometry and data processing/informatics. (c) Protein LFQ (normalised) intensity of Ctrl-CM-CF and TGF$\beta$-CM-CF. (d) Principal component analysis of Ctrl-CM-CF and TGF$\beta$-CM-CF. (e) Volcano plot of proteins identified (blue—downregulated and pink—upregulated in TGF$\beta$-CM-CF). (f) g:Profiler and Reactome gene enrichment analysis of analysis of TGF$\beta$-CM-CF versus Ctrl-CM-CF significantly dysregulated ($t$-test $p < 0.05$) using g:Profiler and Reactome. (g) Reactive oxygen species levels relative to Ctrl-CM-CF measured using DCFDA (mean ± SEM, $n = 18$ over three biological replicates, ** $p < 0.01$).

Significant downregulation of stress response related KEAP1-NFE2L2 pathway, and altered expression of antioxidant and ROS related proteins TKT (Xu et al., 2016), TXNDC9 (Zhou et al., 2020), MARCKS (Huber et al., 2022), and ATOX1 (Chen et al., 2015) suggested a disruption to the oxidative state of TGF$\beta$-CM-CF. To examine whether these changes were associated with increased oxidative stress, ROS levels were measured in treated fibroblasts using a DCFDA (2′,7′-dichlorofluorescin diacetate)-based ROS detection assay. This revealed a significant increase of oxidative stress in cardiac fibroblasts remodelled with secretome from dysfunctional cardiomyocytes ($p < 0.01$) (Figure 2g), an increase not observed with treatment of TGF$\beta$ alone on fibroblasts (Figure S2).

## 3.3 | TGF$\beta$-cardiomyocyte secretome reprograms phosphoproteomic landscape in cardiac fibroblasts

Phosphorylation-based signalling (i.e., kinase cascades) (Kuzmanov et al., 2020; Maruyama & Imanaka-Yoshida, 2022; Thanigaimani et al., 2017) is integral to the cardiac fibrotic response. To better understand the effect cardiomyocyte secretome has on the cardiac fibroblast phosphoproteome landscape, cardiac fibroblasts were treated with cardiomyocyte secretome for 15 min prior to phosphopeptide enrichment and mass spectrometry-based proteomic analysis (Figure 3a). A total of 1611 phosphopeptides with localization probability >0.7 were detected with 89% of sites occurring on serine (Figure 3b, Table S6). Intensity values were used for analysis (Figure S3). A total of 1471 phosphopeptides were identified in phospho-Ctrl-CM-CF (pCtrl-CM-CF) and 1498 in phospho-TGF$\beta$-CM-CF (pTGF$\beta$-CM-CF) (Figure 3c, Table S6). Gene Ontology enrichment of significantly dysregulated ($t$-test, $p < 0.05$, Table S6) or uniquely identified phosphoproteins revealed enrichment of cytoskeleton organization (MAP1B, NEXN, SIPA1L1), MTOR signalling (PRKAA1, TSC2, EIF4B), signalling by interleukins (ANXA2, JUN, HNRNPA2B1), mRNA 3′-end processing (SRSF6, RBM8A, FYTTD1), and mRNA splicing (POLR2A, SNRNP70, SRSF6) (Figure 3d, Table S7).

To identify putative kinases in fibroblasts involved downstream of secretome-mediated signalling, we performed kinase substrate enrichment analysis (KSEA (Wiredja et al., 2017)) on phosphopeptide data (significantly dysregulated or unique) and upstream regulatory network analysis (X2K (Clarke et al., 2018)) on remodelled fibroblast data (significantly increased) to

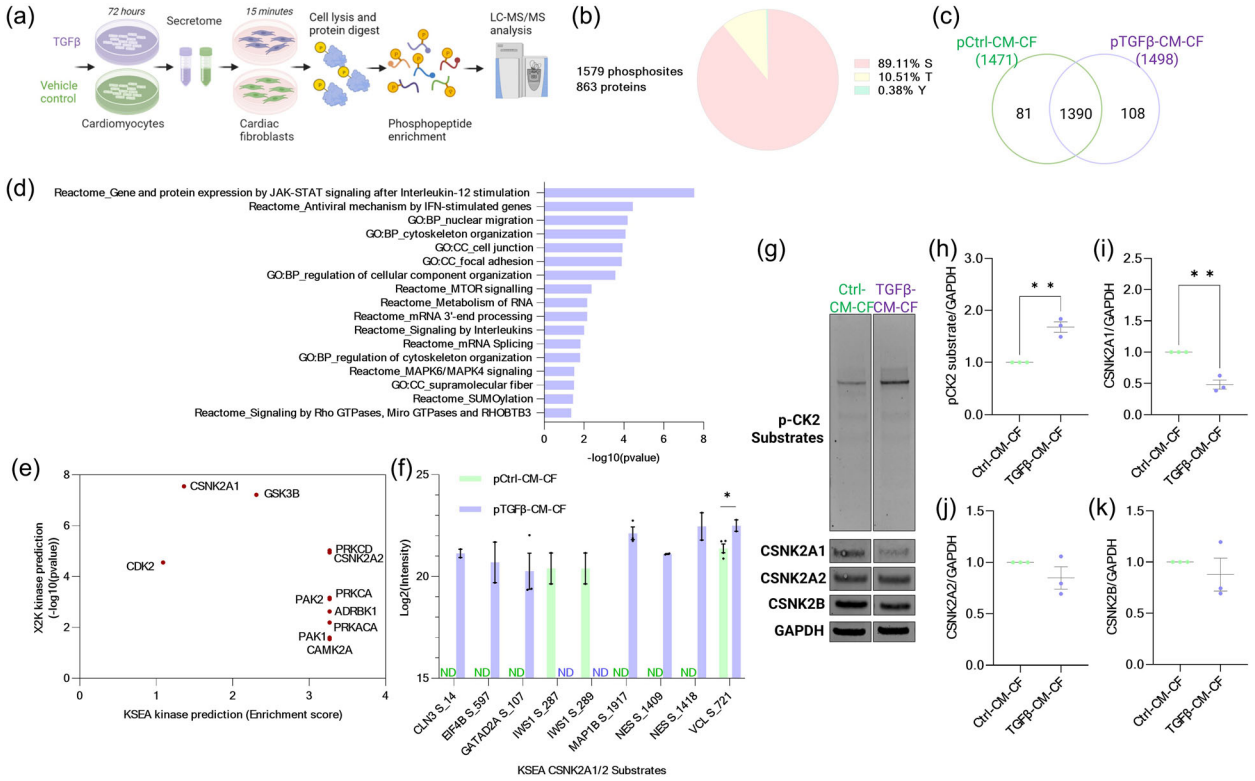


**FIGURE 3** TGF$\beta$-cardiomyocyte derived secretome regulates CK2 activity in cardiac fibroblasts. (a) Phosphoproteomics workflow for the analysis of Ctrl-CM and TGF$\beta$-CM secretome signalling in cardiac fibroblasts ($n = 4$/treatment). (b) Number of proteins, and number and site distribution of phosphosites (localization score > 0.7) detected. (c) Venn diagram of phosphopeptides identified in pCtrl-CM-CF and pTGF$\beta$-CM-CF. (d) Reactome and g:Profiler gene enrichment of unique/differentially phosphorylated proteins ($p < 0.05$). (e) Kinase activity prediction with Kinase Substrate Enrichment Analysis (KSEA) analysis of significant and unique phosphopeptides and X2K analysis of significant and unique remodelled proteins. Kinases with a KSEA enrichment score > 0 and X2K prediction $p$ value < 0.05 are presented. (f) Intensity levels of CK2 substrates identified by KSEA analysis. (g) Immunoblot and densitometry analysis of Ctrl-CM-CF and TGF$\beta$-CM-CF for phosphorylated (h) CK2 substrates, (i) CSNK2A1, (j) CSNK2A2, and (k) CSNK2B relative to GAPDH (20 µg protein/lane, $n = 3$). ** $p < 0.01$. All three biological replicates and full uncropped blots available in Figure S4.

predict kinases with increased activity. These orthogonal approaches allowed direct (from phosphopeptides, KSEA) and indirect (from downstream remodelling effects, X2K) prediction of kinase activity. Phosphopeptide analysis revealed 17 kinases with increased activity (enrichment score > 0, Table S8) and upstream regulatory analysis predicted 106 kinases with significantly increased activity ($p < 0.05$, Table S9). The 11 kinases predicted by both tools were shortlisted for further investigation. These included known cardiac fibrosis regulators CDK2 (Qi et al., 2017), PRKACA (Lv et al., 2016; Wang et al., 2017), PKC (PRKCA, PRKCD) (Song et al., 2015), GSK3B (Guo et al., 2017), and casein kinase 2 (CK2) subunits CSNK2A1 and CSNK2A2 (Figure 3e). CK2 is a constitutively active, laterally acting kinase involved in essential physiological processes, development, and disease (Bian et al., 2013; Borgo et al., 2021a, 2021b). As CK2 has, to the best of our knowledge, not been previously associated with cardiac fibrosis, we selected it for further investigation.

Mapping of KSEA CSNK2A1/CSNK2A2 kinase-substrate links revealed six peptides uniquely identified in pTGF$\beta$-CM-CF, two uniquely identified in pCtrl-CM-CF, and one significantly upregulated in pTGF$\beta$-CM-CF (Figure 3f, Table S6). Immunoblot analysis of Ctrl-CM-CF and TGF$\beta$-CM-CF (Figure 3g, Figure S4) validated significantly increased levels of phosphorylated CK2 substrates (Figure 3h), the significant decrease of CSNK2A1 subunit detected by mass spectrometry (Figure 3i), and no significant observed differences in CK2 subunits CSNK2A2 (Figure 3j) and CSNK2B (Figure 3k).

## 3.4 | Pharmacological inhibition of CK2 abrogates levels of ROS and stress fibre formation in TGF$\beta$-CM-CF

To investigate the involvement of CK2 in cardiomyocyte-fibroblast pathological crosstalk, cardiac fibroblasts were challenged (as previously reported in dermal fibroblasts (Zhang et al., 2015)) with 5 µM TBB, a pharmacological inhibitor of CK2, during their treatment with cardiomyocyte secretome for 48 h. TBB significantly abrogated TGF$\beta$-CM-driven increased ROS levels in cardiac fibroblasts ($p < 0.05$, Figure 4a). Furthermore, due to the involvement of oxidative stress and observed proteome

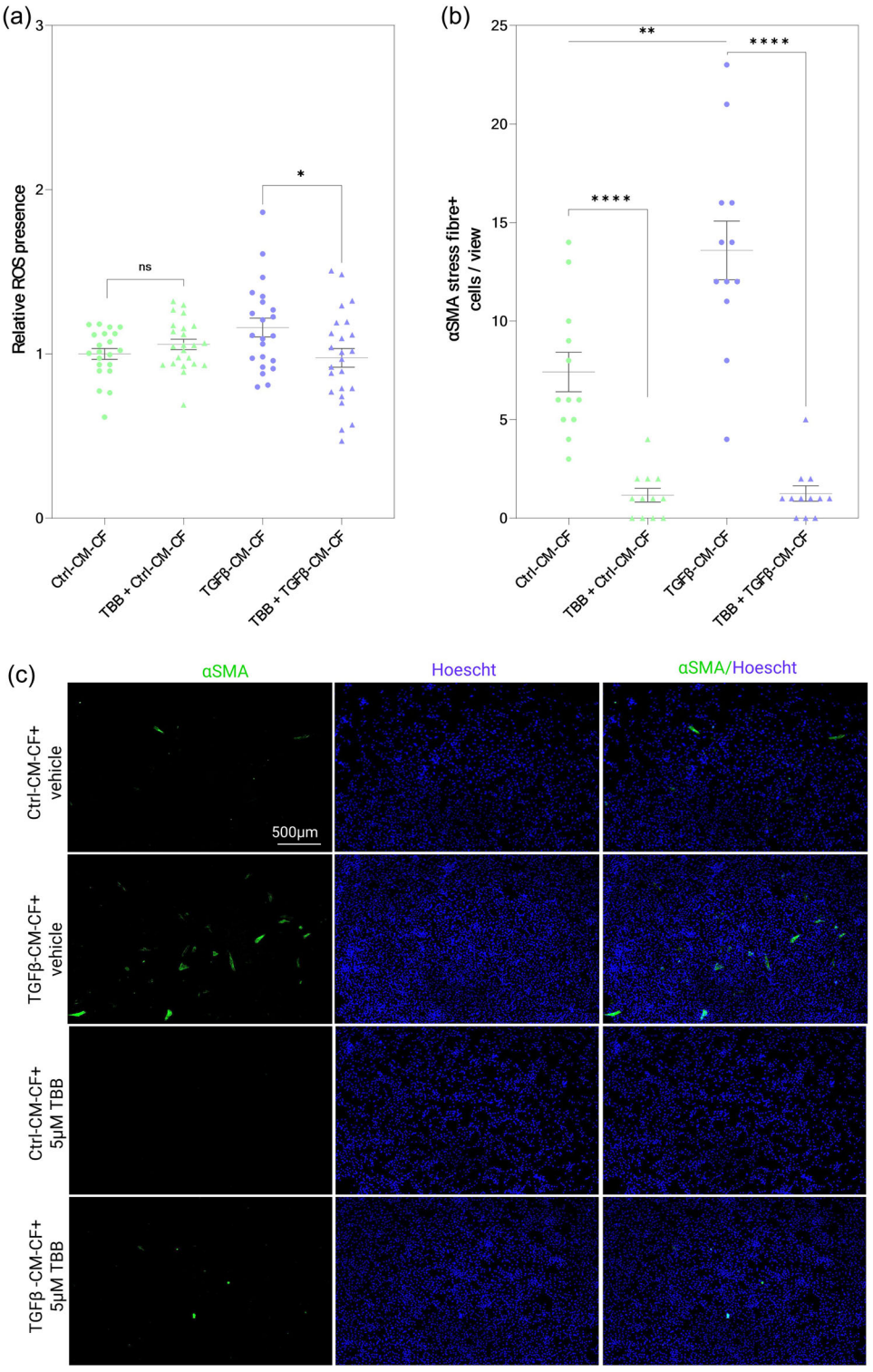


**FIGURE 4** CK2 inhibition abrogates oxidative stress and activation in TGF$\beta$-CM-CF. Cotreatment of cardiac fibroblasts with secretome and 5 μM TBB or DMSO vehicle. (a) Reactive oxygen species levels relative to Ctrl-CM-CF measured using DCFDA ($n = 21–24$ over four biological replicates, Mean ± SEM, * $p < 0.05$). (b) αSMA+ cells per view ($n = 12$ over three biological replicates, Mean ± SEM. **** $p < 0.0001$). (c) Representative immunofluorescence microscopy images of remodelled and TBB- or vehicle-treated cardiac fibroblasts stained with anti-αSMA antibody and Hoechst (nuclei), scale bar = 500 μm.

changes in fibroblast activation, fluorescence microscopy was used to quantify the number of $\alpha$SMA$^+$ cells. TGF$\beta$-CM significantly increased cardiac fibroblast activation ($p < 0.01$, Figure 4b,c), and TBB significantly downregulated this TGF$\beta$-CM mediated fibroblast activation ($p < 0.0001$, Figure 4b,c). Additionally, TBB significantly reduced the number of $\alpha$SMA$^+$ cells in Ctrl-CM-CF ($p < 0.0001$).

## 4 | DISCUSSION

Cardiomyocyte-fibroblast crosstalk plays a significant role in both physiological and pathological regulation of cardiac biology (Cartledge et al., 2015; Dolmatova et al., 2012; Kumar et al., 2019; Moreira et al., 2020; Tsoporis et al., 2012), and insights into these interactions allow for the discovery of new disease mechanisms and targets. Here, we examined the effect of dysfunctional-cardiomyocyte derived secretome on cardiac fibroblast global and phospho-proteome, and function. Analysis of remodelled fibroblasts provided insights into pathological associated processes including transcriptional regulation, carbohydrate metabolism, kinase signalling, and oxidative stress.

Intercellular signalling by the secretome is mediated by a cumulation of soluble factors, extracellular vesicle, and extracellular particles, and includes their collective effects on phosphorylation driven signalling and cellular remodelling (Gärtner et al., 2018; Zhang et al., 2019). EVs can drive phosphorylation signalling and surface receptor changes in recipient cells (Fatmous et al., 2022) and act synergistically with soluble fractions to alter cell behaviour (Gomes et al., 2022). Indeed, dysfunctional cardiomyocytes exhibit altered secretion of signalling proteins and changing extracellular vesicle composition which can affect cardiac fibroblast function (Kuhn et al., 2020; Ontoria-Oviedo et al., 2018). We speculate that broad changes in these secreted factors (soluble, vesicular, and particular) during cardiomyocyte dysfunction cooperate to drive the observed altered functions and phosphorylation/global proteomic landscape remodelling of cardiac fibroblasts.

With the emergence of epigenetic and transcriptional therapeutic strategies for cardiac remodelling (McKinsey et al., 2023), understanding transcriptional regulation and its role in cardiac fibroblast pathology (Lighthouse & Small, 2016) is critical. Amongst the dysregulated proteome of remodelled cardiac fibroblasts were various transcription factors and regulators, including HDAC2, CTBP2, CHCHD2, and AQR. Histone deacetylases (HDAC) are associated with cardiac remodelling and fibrosis (Nural-Guvener et al., 2014) and HDAC2 is implicated in cardiac hypertrophy (Eom et al., 2011). C-terminal-binding protein 2 (CTBP2) orchestrates epithelial-mesenchymal transition (Ma et al., 2020), a process associated with endothelial cellular transformation to profibrotic phenotypes (Li et al., 2016), and its variants are associated with congenital heart disease (Gordon et al., 2022; Jin et al., 2017). CHCHD2 is an important regulator of mitochondrial dynamics (Ruan et al., 2022), with mitochondrial dysfunction associated with fibrotic diseases (Li et al., 2020) and cardiac fibroblast activation (Garvin & Hale, 2022). Furthermore, dysregulation of RNA binding protein AQR is associated with changes to glucose metabolism and protein ubiquitination (Song et al., 2018), both of which are processes altered during cardiac remodelling (Spänig et al., 2019; Tran & Wang, 2019).

Metabolic proteins with altered expression in remodelled fibroblasts were primarily involved in carbohydrate metabolism. Upregulation of glycogen phosphorylase (PYGL) and phosphoglucomutase 1 (PGM1) suggest changes to glycogen breakdown and altered metabolite input for glycogen storage, glycosylation, glycolysis, and the pentose phosphate pathway (Backe et al., 2020; Tegtmeyer et al., 2014). Key enzymes involved in glycosylation (POFUT2), glycolysis (TPI1, PKM), and the pentose phosphate pathway (TKT) are also dysregulated in TGF$\beta$-CM-CF. Metabolic shifts in diseased cardiac fibroblasts are not well characterised but changes to rates of glycolysis have been reported (Lombardi et al., 2019; Tian et al., 2020). As alterations to glycosylation (Haukedal & Freude, 2021; Peixoto et al., 2019) and the pentose phosphate pathway (Jin & Zhou, 2019; Tu et al., 2019) are implicated in other tissue remodelling associated diseases (e.g., cancer, neurodegeneration), investigation into cardiac fibrosis-related changes may provide further insight into pathogenesis.

Here, we present evidence that dysfunctional cardiomyocyte secretome promotes ROS levels in cardiac fibroblasts. Oxidative stress is a central aspect of not only cardiac fibrosis, but broader cardiac remodelling (Aimo et al., 2020; D'Oria et al., 2020; Shah et al., 2021; van der Pol et al., 2019; Zhang et al., 2021). Significantly upregulated by TGF$\beta$-CM secretome were copper chaperone and transcription factor ATOX1 and MARCKS (myrisotylated alanine-rich C-kinase substrate), two proteins whose expression levels are associated with ROS production (Chen et al., 2015; Huber et al., 2022) and may contribute to observed oxidative stress. Further, lysosomal protein downregulation may promote ROS levels as lowered lysosomal activity is associated with disease and cardiac fibrosis, partially due to its impact on oxidative stress (Parenti et al., 2021; Stypmann et al., 2002; van de Vlekkert et al., 2019).

Other potential contributing factors include the downregulation of proteins involved in counteraction of oxidative stress; CHCHD2 (Aras et al., 2015), TKT (Xu et al., 2016) and TXNDC9 (Zhou et al., 2020), and oxidative stress response KEAP1-NFE2L2 pathway (Cuadrado et al., 2019; Thanas et al., 2020) associated proteins; NFE2L2 signalling product TKT (Mitsuishi et al., 2012), and MAP1LC3B (Jiang et al., 2015) which is associated with KEAP1 degradation (KEAP1 a negative regulator of NFE2L2 signalling (Baird & Yamamoto, 2020)).

Phosphorylation-based signalling is the driving force of fibrotic development (Kuzmanov et al., 2020; Maruyama & Imanaka-Yoshida, 2022; Thanigaimani et al., 2017), and successful identification and inhibition of central regulators may offer new approaches to treat cardiac remodelling. TGF$\beta$-CM-CF displayed altered expression of kinases and phosphatases including ADK, ARAF, PPP3CA/calcineurin A, and PPP5C. ADK and ARAF kinases were downregulated in cardiac fibroblasts by dysfunctional cardiomyocyte secretome. Loss of ADK worsens cardiac dilation but not fibrosis during pressure overload (Fassett et al., 2019), and is associated with altered ERK/mTORC signalling (Fassett et al., 2019) and DNA methylation (Murugan et al., 2021). Downregulated in the hearts of patients with dilated cardiomyopathy (Clerk et al., 2022), ARAF inhibits Nodal signalling (Liu et al., 2013), which contributes to fibroblast reprogramming (Li et al., 2019) and may contribute to the significantly increased expression of LEFTY2 (a ligand upregulated by Nodal signalling (Liu et al., 2022)) in TGF$\beta$-CM-CF. Additionally, phosphatases dysregulated included PPP3CA/Calcineurin A (upregulated) and PPP5C (downregulated). PPP3CA is upregulated in spontaneously hypertensive rat hearts (Bogdanova et al., 2021) and is important for profibrotic signalling in cardiac fibroblasts (White et al., 2012). PPP5C not only carries phosphatase activity, but acts as a co-chaperone, and abnormal expression levels are associated with various cancers and neurodegenerative diseases (Zhang et al., 2023).

Further dysregulation of kinase activity was predicted with analysis of phosphoproteomic data. KSEA and X2K analysis of phosphopeptides and global remodelled data, respectively, implicated various kinases in the response of cardiac fibroblasts to secretome from dysfunctional cardiomyocytes. Amongst those predicted by one or both tools were kinases previously associated with cardiac fibrosis; CDK2 (Qi et al., 2017), ADRBK1/GRK2 (Li et al., 2022; Tanaka et al., 2020), PRKACA (Lv et al., 2016; Wang et al., 2017), PKC (PRKCA, PRKCD) (Song et al., 2015), GSK3B (Guo et al., 2017), PAK1 (Zhou et al., 2021) and ROCK1 (Rikitake et al., 2005). Other kinases included CK2, RPS6KA2 and CSNK1G1, further investigation of which may provide insights into pathological signalling. Application of kinase inhibitors have proven effective for oncological, neurodegenerative, dermatological, and inflammatory conditions (Cohen et al., 2021), but are yet to be successfully applied to cardiac diseases. Identification of effective targets and the development of inhibitors with minimal cardiotoxicity will be essential for the application of phosphorylation-based therapeutics.

Phospho- and global proteome remodelling of cardiac fibroblasts indicated CK2 dysregulation, validation of which revealed CSNK2A1 subunit downregulation and CK2 activity upregulation. The contradictory increased activity and decreased catalytic subunit expression has been reported previously for CK2 (Borgo et al., 2021; Piazza et al., 2006; Roelants et al., 2015; Roffey & Litchfield, 2021), but the intricacies of CK2 regulation remain poorly understood (Borgo et al., 2021). However, CK2 is constitutively active and acts laterally to phosphorylate >530 different proteins in humans (Bian et al., 2013; Borgo et al., 2021a, 2021b), including key fibrotic signalling pathways; PI3K-Akt, IKK/NFkB, Wnt/b-catenin and TNF-$\alpha$ (Borgo et al., 2021b; Ma et al., 2018). Here, KSEA and NetworKin identified CK2 substrates involved in cytoskeleton dynamics (NES (Bernal & Arranz, 2018), MAP1B (Ulloa et al., 1993)), endomembrane (CLN3 (Mirza et al., 2019)), focal adhesions (VCL (Carisey & Ballestrem, 2011)) and transcriptional regulation (EIF4B (Shahbazian et al., 2010), IWS1 (Cermakova et al., 2021)), supporting widespread effects of CK2 disruption to cardiac fibroblasts.

TBB, an inhibitor of CK2, significantly reduced activation and oxidative stress in TGF$\beta$-CM-CF. CK2 has previously been associated with endothelial cell stress fibre formation in vitro (Kramerov et al., 2011), and its inhibition with TBB has proven antifibrotic in vitro and in vivo, reducing dermal fibrosis in mice (Zhang et al., 2015). Mechanisms for CK2 modulation of ROS levels remain unclear, with conflicting reports of CK2 promoting (Gagnon et al., 2021; Ka et al., 2015). suppressing (Jang et al., 2020; Schaefer & Guerra, 2017; Schneider et al., 2009), and having no effect (Costa et al., 2022; Hansen et al., 2011; Schneider et al., 2009) on oxidative stress. Differing biological settings of these studies suggest a complex and contextually dependent network of CK2 mediated oxidative state regulation. Supported by the remodelled proteome, interactions with KEAP1-NF2EL2 signalling may contribute as NFE2L2 is phosphorylated by CK2 (Apopa et al., 2008), and CK2-KEAP1-NFE2L2 dysregulation has been associated with increased ROS (Song et al., 2020). Moreover, CK2 is involved in cardiac remodelling and cardiac hypertrophy (Eom et al., 2011; Kuzmanov et al., 2020) and is a therapeutic target for various pathologies (Borgo et al., 2021), with inhibitors in clinical trials for cancers and severe coronavirus-19 (NCT03897036, NCT03904862, NCT03571438, NCT04668209, NCT04663737). While kinase inhibitors often exhibit severe cardiotoxicity (Grela-Wojewoda et al., 2022), CK2 inhibitors show promising efficacy and safety profiles (Marschke et al., 2011; Padgett et al., 2010) and are approved for cholangiocarcinoma treatment (CX-4945 Granted Orphan Drug Designation, 2017). To the best of our knowledge, no animal or human studies have reported cardiotoxicity associated with CK2 inhibition, indicating potential for its successful therapeutic application in cardiac fibrosis. However, further investigation into CK2 is required to validate its involvement in cardiac fibrosis and suitability as a therapeutic target.

In this study, we have identified in pathologically remodelled cardiac fibroblasts the dysregulation of components associated with transcription, carbohydrate metabolism, oxidative stress, and phosphorylation-based signalling, and report the abrogation of increased oxidative stress and activation by CK2 inhibitor, TBB. These findings provide insights into secretome mediated intercellular signalling which promotes pathological remodelling, highlighting the role of cardiomyocyte-driven oxidative stress and reprogramming in fibroblasts. While these findings suggest the involvement of CK2 in this pathological crosstalk, further studies to validate its involvement in cardiac fibroblast remodelling and to delineate causation between CK2 activity and observed oxidative stress and activation are needed. Future studies into the effects of cardiomyocyte-cardiac fibroblast crosstalk will advance our understanding of pathological remodelling mechanisms and identify new therapeutic targets.

## AUTHOR CONTRIBUTIONS

**Bethany Claridge**: Conceptualization; formal analysis; investigation; methodology; visualization; writing—original draft. **Alin Rai**: Conceptualization; investigation; supervision; writing—review & editing; **Jarmon G. Lees**: Methodology; resources; **Haoyun Fang**: Methodology; resources; **Shiang Y. Lim**: Methodology; resources; **David W. Greening**: Conceptualization; funding acquisition; investigation; resources; supervision; writing—review & editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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