Combined role for YAP-TEAD and YAP-RUNX2 signalling in substrate-stiffness regulation of cardiac fibroblast proliferation☆

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ABSTRACT

Cardiac fibrosis is associated with increased stiffness of the myocardial extracellular matrix (ECM) in part mediated by increased cardiac fibroblast proliferation However, our understanding of the mechanisms regulating cardiac fibroblast proliferation are incomplete. Here we characterise a novel mechanism involving a combined activation of Yes-associated protein (YAP) targets RUNX Family Transcription Factor 2 (RUNX2) and TEA Domain Transcription Factor (TEAD).

We demonstrate that cardiac fibroblast proliferation is enhanced by interaction with a stiff ECM compared to a soft ECM. This is associated with activation of the transcriptional co-factor, YAP. We demonstrate that this stiffness induced activation of YAP enhances the transcriptional activity of both TEAD and RUNX2 transcription factors. Inhibition of either TEAD or RUNX2, using gene silencing, expression of dominant-negative mutants or pharmacological inhibition, reduces cardiac fibroblast proliferation. Using mutants of YAP, defective in TEAD or RUNX2 activation ability, we demonstrate a dual role of YAP-mediated activation of TEAD and RUNX2 for substrate stiffness induced cardiac fibroblast proliferation.

Our data highlights a previously unrecognised role of YAP mediated RUNX2 activation for cardiac fibroblast proliferation in response to increased ECM stiffness.

1. Introduction

Cardiac fibrosis is one of the main pathophysiological processes contributing to heart failure, which affects 26 million people worldwide [1]. For some conditions that lead to fibrosis, such as volume or pressure overload, treatments that target risk factors may help limit its progression. However, new therapeutic strategies are urgently needed to directly inhibit the mechanisms that lead to fibrosis, particularly in patients where risk factor management is ineffective.

Cardiac fibroblasts (CF), the main non-myocyte cell type resident in the myocardial interstitium [2], perform several important functions that maintain normal myocardial homeostasis, including regulation of myocardial extracellular matrix (ECM) and myocardial integrity, as well as functioning as mechano-electric transducers [3]. In the healthy heart, CF are quiescent, maintaining a slow baseline turnover of ECM proteins

[4,5]. However, in response to a range of signals, including angiotensin II [6,7], TGF-β [8] and mechanical cues, CF trans-differentiate into a myofibroblast phenotype, characterised by increases in the cytoskeletal protein smooth muscle alpha-actin (SMA) expression, increased extracellular matrix synthesis and increased proliferation [9–12]. Myofibroblast phenotypic modulation is essential for myocardial healing after cardiac injury. For example, increased collagen synthesis by CF after myocardial infarction is essential for scar formation and structural stabilisation of the infarcted area. However, aberrant, or excessive ECM deposition and myofibroblast proliferation has been implicated in the development of maladaptive fibrosis, which alters the mechanical properties of the myocardium (increased stiffness), impairs cardiac function and promotes the development of heart failure [13–16]. Increased proliferation of resident cardiac fibroblasts is believed to be an important contributary factor [17–19], as expansion of the cardiac

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fibroblast population increases the number of ECM producing cells, thus exacerbating fibrosis.

Multiple signals have been implicated in the regulation of cardiac fibroblast proliferation, including TGF-β [8,20], angiotensin-II [6,7], endothelin-1 [21,22], bFGF [8] and PDGF [23]. In addition to these soluble mediators, the composition [24,25] and mechanical properties of the local ECM also play an important role. For example, when fibroblasts are cultured on rigid plastic plates in vitro, they spontaneously trans-differentiate into myofibroblasts with an increased proliferation rate [26–28]. Studies culturing fibroblasts on hydrogels of tuneable stiffness also demonstrate a mechanistic link between increased ECM-stiffness and myofibroblast differentiation [29,30]. Several biomechanical signalling mechanisms have been implicated in the regulation of cardiac fibroblast activation and proliferation. Mechanical sensing depends on the development of intracellular force in the actin stress fibres. As a result, inhibition of pathways that regulate actin polymerisation and tension, such as the RhoA-ROCK pathway, blocks myofibroblast activation [31–33]. Thus, mechanical signals are transmitted, via actin cytoskeleton remodelling, to actin sensitive transcription factors that control myofibroblast phenotype.

Here, we investigated the role of the transcriptional co-factors, Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ/WWTR1) in the biomechanical regulation of cardiac fibroblast proliferation. YAP and TAZ are downstream targets of the Hippo pathway and play a critical role in regulation of organ size and cell proliferation [34–36]. Several lines of evidence suggest that YAP and TAZ play a central role in regulating myofibroblast phenotype and cardiac fibrosis. For example, expression of the YAP/TAZ target gene, connective tissue growth factor (CTGF), is associated with a myofibroblast phenotype [37] and upregulated in response to increased ECM stiffness [30]. YAP/TAZ activity has been shown to positively regulate myofibroblast activation from various cell types [38–43] and cardiac fibroblast specific deletion of YAP in mice attenuated fibrosis after myocardial infarction [38].

A large body of literature documents the role of TEAD family transcription factors (TEAD1-4) as the major downstream effectors of YAP an TAZ in many different cell types [44–49]. For example, cellular phenotypes induced by overexpression of constitutively active mutants of YAP and TAZ are prevented by point mutations to block interaction with TEAD proteins [48,50,51]. Furthermore, deletion or silencing of TEADs results in phenotypes resembling those caused by loss or inhibition of YAP [52–54]. The activity of YAP-TEAD complexes plays a central role in the regulation of cell proliferation and transformation in many cell types [55,56]. However, YAP and to a lesser extent, TAZ, have also been shown to interact with [49,57–61] and either positively [51,57,59–61] or negatively [62] regulate the transcriptional activity of other, non-TEAD family, transcription factors [57]. RUNX2 is notable amongst these, being the first YAP interaction partner identified [57] and one of only two non-TEAD transcription factors identified in a recent proteomic YAP/TAZ interactome screen [49]. Interestingly, YAP has been reported to act as an activator [51] and a repressor [63] of RUNX2 transcriptional activity. Despite the early research on YAP interaction with RUNX2, relatively little is known about the functional significance of RUNX2 in the regulation cardiac fibroblast proliferation. Studies in endothelial cells, HEK293 cells and NIH3T3 fibroblasts suggest a role of YAP-dependent regulation of RUNX2 transcriptional activity in cellular transformation and growth [51,58]. Here we investigated the functional importance of YAP-mediated regulation of RUNX2 in the biomechanical control of cardiac fibroblast proliferation.

2. Methods

2.1. Reagents

All chemicals were obtained from Sigma unless otherwise stated. Antibodies to total YAP (#140745), YAP phospho-serine-397 (#13619), YAP phospho-serine-127 (#4911) and total TAZ (#4883) were from Cell Signalling Technologies. Antibody to GAPDH (MAB374) was from Merck Millipore.

2.2. Cardiac fibroblast culture

Male Sprague Dawley rats were killed by cervical dislocation in accordance with schedule 1 of the U.K. Animals (Scientific Procedures) Act 1986 and Directive 2010/63/EU of the European Parliament and with the approval of the University of Bristol. Hearts were removed and flushed with PBS before chopping into 2 mm^2 pieces and digestion with 1 mg/ml collagenase (Worthington Biochemical Corporation) overnight. The cell suspension was pelleted and resuspended in Advanced DMEM/F12 supplemented with 10 % foetal bovine serum, 100 U/ml penicillin/streptomycin and 2.5 mM L-glutamine. Cardiac fibroblasts were allowed to adhere to tissue culture plastic for 4 h and non-adherent myocytes washed away.

Where indicated, cells were cultured on collagen coated polyacrylamide hydrogels. These were either purchased from Cell Guidance Systems or were made as previously described [64]. Briefly, gels were cast onto 22mm^2 glass coverslips that had first been activated by incubation in 1 M HCl for 1 h, followed by treatment with (3-aminopropyl) triethoxysilane (APES) for 2 min and finally, incubation in 0.5 % glutaraldehyde for 30 min. Gel stiffness was controlled by varying the percentage of acrylamide and bis-acrylamide (see supplement fig. 1). Following polymerisation, gels were coated with 0.5 mg/ml Sulfo-SANPAH (SIGMA) by exposure to UV light for five minutes. This cross-linking step was repeated before covalent attachment of 0.2 mg/ml type 1 collagen, overnight at 4° C. The next day, gels were extensively washed in PBS before cell seeding.

2.3. Quantitative real time PCR

Quantification of mRNA levels was performed by RT-qPCR as described previously [65]. Total RNA was extracted using Ambion Pure-Link kits (Thermo Fisher) and was reverse transcribed using QuantiNova RT kit (Qiagen) and random primers. Quantitative PCR was performed using KappaFAST SYBR Green using a Qiagen Roto-Gene Q PCR machine (15′@95 °C;15′@62 °C;5′@72 °C). Primers sequences are described in Supplement Fig. 2. Data were normalised to total amount of RNA.

2.4. Western Blotting

Total cell lystes were prepared in $1\times$ reducing Laemmli sample buffer (2 % SDS, 10 glycerol, 50 mM Tris pH 6.8, 2.5 % β-mercaptoethanol, 0.002 % bromophenol blue). Proteins were denatured by heating to 95 °C for 5 min before electrophoresis using Bio-Rad 4–15 % polyacrylamide mini-TGX gels in a Mini-Protean II electrophoresis apparatus. Proteins were transferred to PVDF membrane using a semi-dry Turbo blotter system (Bio-Rad). Membranes were blocked for 1 h at room temperature in 5 % low-fat milk powder in Tris buffered saline (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM KCl) containing 0.2 % Tween20 (1xTBS.T) before incubation with primary antibody overnight at 4 °C. Blots were extensively washed in 1xTBS.T before incubation with HRP-conjugated secondary antibodies (Sigma). Specific proteins were detected using Immobilon ECL reagent and a ChemiDoc-MP digital imaging system (Bio-Rad).

2.5. Plasmids, siRNA and adenoviral vectors

Replication deficient adenoviral vectors expressing YAP$_{S127A}$ and TAZ$_{4SA}$ were generated by cloning cDNAs into the multiple cloning site of the pDC515 adenovirus shuttle vector (Microbix) as previously described [66–68]. YAP mutants of both WW-domains, which disrupts YAP interaction with RUNX2 [51], in the context of the YAP$_{S127A}$

activation mutation were generated by mutating W199A, W202A, W258A and P261A by PCR. YAP mutant containing S89A mutation, which disrupts interaction with TEAD [51], in the context of YAP$_{S127A}$ was also generated by PCR. Resulting mutants were cloned into pDC515 and recombinant adenoviruses generated by co-transfection of HEK293 cells with the pDC515 expression vector and the adenoviral genome vector pBGHfrt, as previously described [69]. Clones of replication deficient adenoviruses were amplified by infection of four 150 cm2 flasks of HEK293 cells and the resulting adenovirus purified using AdEasy adenovirus purification kit (Agilent). Purified adenoviral stocks were titrated by end-point dilution plaque assay in HEK293 cells.

Plasmid TEAD-LUC expressing firefly luciferase under the control of a synthetic promoter containing eight copies of a consensus TEAD binding element was obtained from Addgene (#34615) and has been described previously [66]. Plasmid minP-LUC, containing the Troponin-T minimal promoter was generated by digesting 8xGTIIC-luciferase plasmid (Addgene #34615) with *Kpn1* and *BglII* to remove the TEAD elements, followed by blunt end re-ligation. To construct plasmid pNL3.3-SNUL:RUNX2 (abbreviated to RUNX2-SNLUC herein), a synthetic promoter containing six copies on a consensus RUNT-binding element was synthesised using the Invitrogen GeneArt service. This was sub-cloned into the *Kpn1* and *Nhe1* restriction elements upstream of the minimal promoter of plasmid pNL3.3[secNLUC/minP], which expresses a secreted form of nano-luciferase reporter gene. GAL4-secNLUC nano-luciferase reporter (GAL4-NLUC) was created by subcloning the 5xGAL4 binding elements from plasmid pG5E1b-LUC (a gift from Ugo Moens, University of Tromsø, Norway) into the Nhe1 and Xho1 sites of pNL3.3[*sec-Nluc*/minP] (Promega).

Synthetic Silencer Select siRNAs targeting YAP, TAZ and RUNX2 were purchased from Invitrogen Life Technologies.

2.6. Transient transfection and reporter gene assays

TEAD and RUNX2 reporter gene activity was determined by quantifying the cellular firefly luciferase and secreted nanoluciferase reporter gene activity in cells transiently transfected with TEAD-LUC or RUNX2-secNLUC reporter plasmids. Plasmid transfection was performed by electroporation of 1×10^6 cells with a total of 5 μg of plasmid DNA using an a Nucleofector-1.5 electroporation device (Lonza) set to program A-024. For gene silencing, cells were transfected with 100 pmoles of Silencer Select siRNA (Life Technologies). At the indicated times post transfections, cell lysates were prepared in ice cold Cell Culture Lysis (CLB) buffer (Promega) and assayed for firefly luciferase activity using the luciferase assay system (Promega) and a Glomax Discover luminometer (Promega) according to the manufacturer's instructions. Secreted nano luciferase activity was quantified in the cell conditioned media using the NanoGlo assay system (Promega).

2.7. Cell proliferation assays

Cell proliferation was quantified using Edu incorporation and counting total cell number/mm^2. For Edu labelling assays, cells were treated as indicated and incubated with 10 uM Edu for 4 h. Cells were fixed in 70 % ethanol and Edu incorporation detected using the Edu-CLICK-488 assay kit (SIGMA). Nuclei were counter stained with DAPI and Edu positive and total nuclei numbers manually counted using ImageJ software. For total cell numbers, cells were fixed in 70 % ethanol, -24, -48- and -72-hours post treatment and nuclei stained with DAPI. Nuclei were counted using Cell Profiler software and expressed as cells/mm^2.

2.8. YAP immunostaining

Cells cultured on stiff or soft polyacrylamide hydrogels were fixed in 4 % methanol-free formaldehyde for 10 min at room temperature. Cells were permeabilised in 0.1 % Triton-X-100 for 5 min before blocking in 5 % BSA in PBS. Cells were incubated with 1:100 rabbit anti-YAP antibody (#14074; Cell Signalling Technologies) for 2 h. Following washing in PBS, cells were incubated an Alexa Fluor 488 conjugated anti-rabbit antibody. Nuclei were counter stained with DAPI. Images were captured using an Olympus microscope and a 60× oil immersion lens.

2.9. Cell area and nuclear intensity analysis

Cell area was quantified from phase contrast micrographs of subconfluent cell cultures. Cell area was determined by manually tracing the periphery of individual cells using the freehand selection tool within Fiji Image J software. YAP nuclear intensity was quantified using Cell Profiler software. Briefly, DAPI stained nuclei images were used to identify nuclear regions in the corresponding YAP-stained images, that were quantified.

2.10. Data and statistical analysis

Raw experimental data was collated and graphed using Microsoft Excel, with final figures constructed using Microsoft Powerpoint. Statistical analysis was performed using Graphpad Instat software. Data is presented as means ± standard error of the mean. Data was analysed either using one-way Anova with Student Newman Keuls pos*t*-test, or where appropriate student's t-test, as indicated.

3. Results

3.1. Substrate stiffness regulates cardiac fibroblast proliferation

We initially characterised the role of substrate stiffness on cardiac fibroblast proliferation by culturing cells on collagen bio-functionalised polyacrylamide hydrogels of tuneable stiffness. Proliferation of cardiac fibroblasts cultured on stiff (50 kPa) substrates, detected by incorporation of BrdU, was significantly greater than cells cultured on intermediate (8 kPa) or soft (0.5 kPa) substrates (45.29 ± 2.99 % vs 29.81 ± 2.83 % vs 8.34 ± 2.69 %, $p < 0.0001$ for 50 kPa, 8 kPa and 0.5 kPa respectively. Fig. 1A and Supplement Fig. 3). Total cell number increased significantly between 24 and 48 h ($p < 0.05$) and 48–72 h ($p < 0.001$) post seeding onto stiff substrates (Fig. 1B). Importantly, cell numbers on soft substrates were significantly lower than on stiff substrates on day 2 ($p < 0.05$) and day 3 (Fig. 1B and supplement fig. 4; $p < 0.001$).

3.2. Substrate stiffness regulates cell morphology, actin stress fibre organisation and YAP activation

Changes in actin cytoskeleton organisation have been implicated in sensing changes in local substrate compliance and regulating the activation of YAP [70]. We therefore investigated the effect of substrate stiffness on cardiac fibroblast morphology, actin stress fibre organisation and markers (phosphorylation and cellular localisation) of YAP activation. Cells cultured on a stiff substrate (50 kPa) displayed a typical spindle shaped spread morphology with a mean cell area of 3120 ± 1935 μm^2 (Fig. 2A, B). These cells exhibited pronounced phalloidin stained F-actin stress fibres (Fig. 2A). In contrast, cells cultured on a soft substrates displayed a rounded contracted morphology (Fig. 2A) with a significantly reduced cell area of 1091 ± 768 um^2 (Fig. 2B) and disorganised and less prominent F-actin stress fibres· (Fig. 2A). Cells cultured on a soft substrates also displayed higher levels of YAP phosphorylated on serine 397 (a marker of YAP inactivation; Fig. 2C) although levels of total YAP protein remained unaffected (Fig. 2D). However, levels of nuclear YAP were significantly reduced in cells cultured on a soft substrates compared to those cultured on a stiff substrate (Fig. 2E and F; enlarged copy of 2E as supplement fig. 5). To test the functional importance of actin stress fibres in stiffness dependent YAP activation, we treated cells with the myosin II ATPase inhibitor,
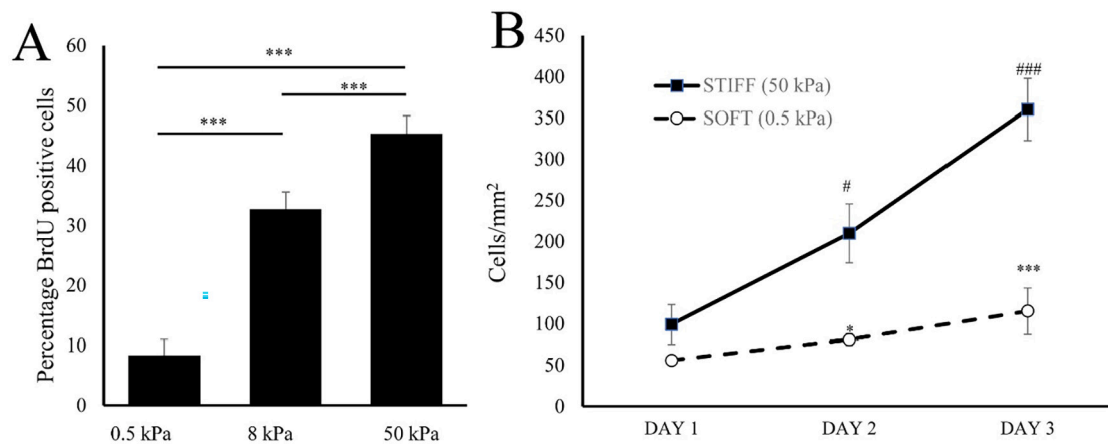
Fig. 1. *Substrate stiffness regulates the proliferation of cardiac fibroblasts.*
Cardiac fibroblasts were cultured on collagen coated polyacrylamide hydrogels of indicated stiffness. The next day cells were incubated with Edu for 4 h (*A*; $n = 9$), or cell number was quantified at day 1, 2 and 3 post seeding (*B*; n = 9). Data are expressed as mean $\pm$ SE and analysed either by paired student *t*-test or ANOVA with Student Newman Keul's post-test. * and # indicate $p < 0.05$, ** indicates $p < 0.01$, *** and ### indicate $p < 0.0001$.
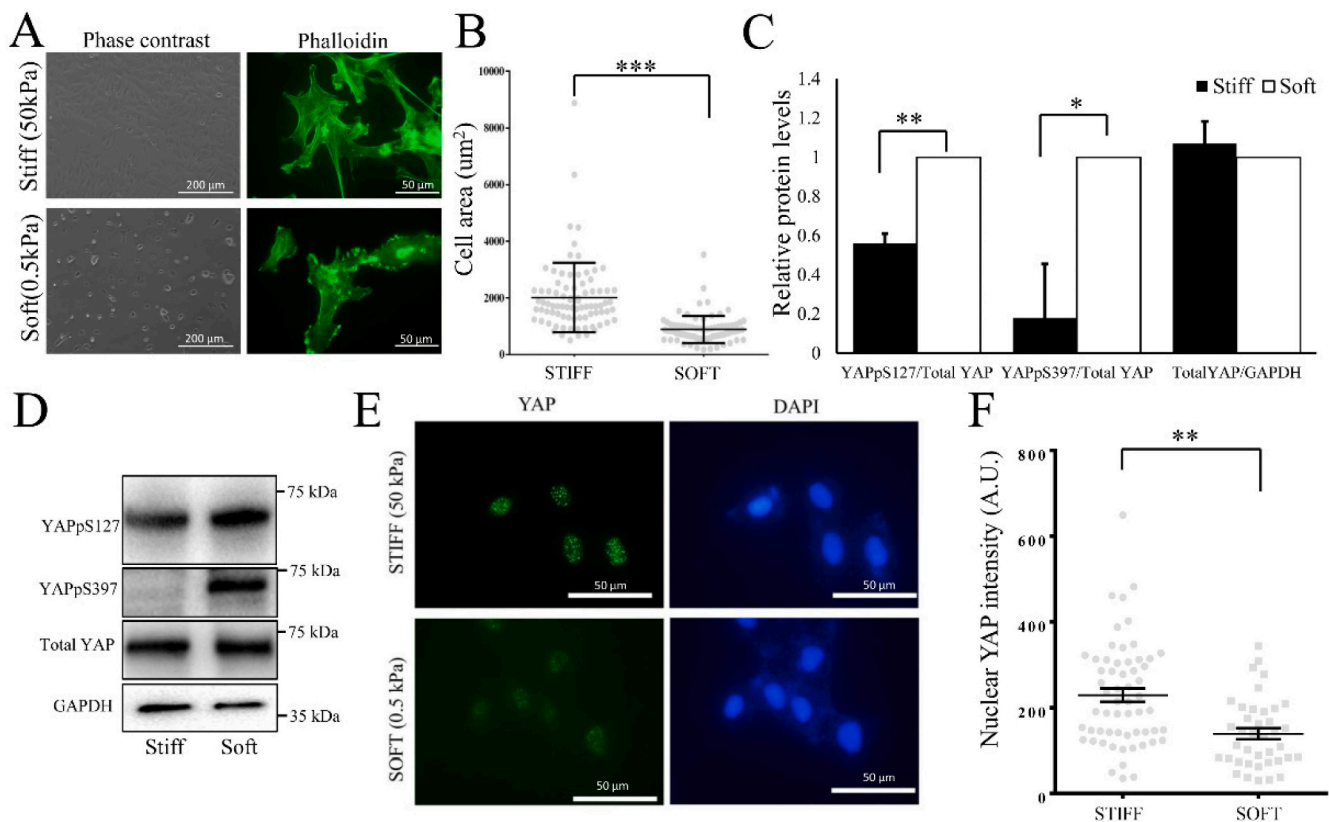

Fig. 2. *Substrate stiffness regulates cell morphology, actin stress fibre organisation, cell area and YAP localisation.*
Cardiac fibroblasts were seeded on stiff (50 kPa) and soft (0.5 kPa) substrates. Cell morphology was analysed by phase contrast microscopy (A and B) and actin stress fibres by phalloidin staining (A). Cells were seeded on stiff or soft matrices and total cellular YAP, phospho YAP$_{S127}$, phospho YAP$_{S397}$ and GAPDH protein levels quantified by western blotting and densitometric analysis (C and D). Cells were cultured on stiff or soft substrates and analysed by immunostaining for YAP cellular localisation (E) with densitometric analysis of nuclear YAP levels (F). Data are expressed as mean $\pm$ and analysed using paired Student *t*-test. * indicates $p < 0.05$, ** indicates p < 0.01 and *** indicates $p < 0.001$.

blebbistatin. In agreement with our earlier data, cells cultured on a stiff substrate displayed a larger area than cells on soft substrates (supplement fig. 6). Treatment of cells on stiff substrates with blebbistatin significantly reduced cell area to levels not significantly different to cells on soft substrates. Furthermore, phosphorylation of YAP, was significantly lower in cells growing on stiff substrates, compared to soft ones. Importantly, blebbistatin treatment resulted in a significant increase in

YAP phosphorylation (supplement fig. 7). Taken together, these data indicate that a stiff ECM promotes cell spreading, actin stress fibre formation, reduced YAP phosphorylation and increased nuclear levels of YAP.

3.3. Cardiac fibroblast proliferation induced by stiff substrates is dependent on YAP and TAZ

We next tested if cardiac fibroblast proliferation on stiff substrates is dependent on YAP and TAZ. Cells were transiently transfected with siRNA targeting YAP and TAZ. Transfection of cells cultured on tissue culture plastic, which has a stiffness in the GPa range, with siRNA targeting YAP and TAZ resulted in a strong reduction in YAP and TAZ protein levels (Fig. 3 A, B and C), without affecting cell morphology (supplement fig. 8) and a significant reduction in Edu incorporation (Fig. 3D). YAP/TAZ silencing also significantly reduced cell numbers 48 h and 72 h post-transfection (Fig. 3E). Importantly, forced expression of a constitutively active YAP mutant (YAP$_{S127A)}$, rescued cell proliferation rates of cells cultured on a soft-substrates to levels similar to those measured in cells cultured on a stiff substrate (Fig. 3F).

3.4. Increased substrate stiffness promotes RUNX2 and TEAD activity and target gene expression in cardiac fibroblasts

Since YAP and TAZ have been reported to be able to physically interact with RUNX2 [57], we tested if RUNX2 activity in cardiac fibroblasts is dependent on substrate stiffness. To specifically quantify RUNX2 activity we transfected cells with a plasmid encoding the GAL4 DNA-binding domain fused to the RUNX2 transcriptional activation domain (GAL4-RUNX2), together with a secreted nano-luciferase reporter gene under the control of a promoter containing five GAL4 binding elements. This demonstrated a significant reduction in GAL4-RUNX2 activity in cells cultured on a soft, compared to a stiff substrate (Fig. 4A). Furthermore, activity of RUNX2-secNLUC, which is responsive to endogenous RUNX2 activity, was also significantly lower in cells interacting with a soft compared to a stiff substrate (Fig. 4B). Moreover, we observed a gradual increase in RUNX2 reporter gene activity as substrate stiffness increased (Fig. 4C). These data suggest that RUNX2 activity is sensitive to substrate stiffness in cardiac fibroblasts. As a positive control, we also measured TEAD activity, which has

previously been shown to have substrate stiffness-dependent transcriptional regulation [70]. As expected, TEAD-dependent reporter activity was also significantly reduced in cells cultured on soft substates (Fig. 4D), whereas a reporter gene under the control of a minimal promoter lacking TEAD or RUNX2 binding elements remained unaffected (Fig. 4E). We also observed a marked and significant increase in TEAD activity above 8 kPa (Fig. 4F). These data suggest that RUNX2 as well as TEAD is sensitive to substrate stiffness in cardiac fibroblasts.

We used RT-qPCR to quantify changes in mRNA levels of RUNX2 target genes, to further test that increased substrate stiffness promotes RUNX2-mediated gene expression. We identified putative RUNX2 target genes (FN1, ITGB1 and COROC1C) from published RUNX2 gene sets (members of either TRANSFAC predicted transcription factor targets or CHEA Transcription Factor Targets Chip targets [71]), which have also been associated with the regulation of cell proliferation [72–74]. We also analysed expression of the well characterised TEAD-target genes CCN1 and CTGF. We first confirmed the RUNX2 dependence of FN1, ITGB1 and CORO1C using siRNA-mediated silencing of RUNX2 (Supplement Fig. 9) or expression of DN-RUNX2 (Fig. 4G). Silencing of RUNX2 (Supplement fig. 9) or expression of DN-RUNX2 (Fig. 4G) significantly inhibited mRNA levels of FN1, ITGB1 and CORO1C, without affecting levels of the housekeeping genes 36B4 or UBC or affecting cell morphology (supplement fig. 8), thus confirming the RUNX2-dependence of these genes. Interestingly, expression of DN-TEAD did not affect mRNA levels of these RUNX2 target genes but did strongly inhibit the classical TEAD targets CCN1 and CTGF (Fig. 4H). CTGF expression was not inhibited by DN-RUNX2, while CCN1 mRNA levels were repressed by both DN-TEAD and DN-RUNX2. This suggests that RUNX2 and TEAD can regulate distinct target genes in cardiac fibroblasts and implies that repression of RUNX2 target gene expression does not occur secondary to inhibition of TEAD. Analysis of mRNA levels of these genes in cells cultured on stiff and soft substates demonstrated a significantly increased expression on stiff compared to soft substrates, consistent with our reporter gene data indicating elevated TEAD and RUNX2 activity in cells cultured on stiff substrates (Fig. 4I).
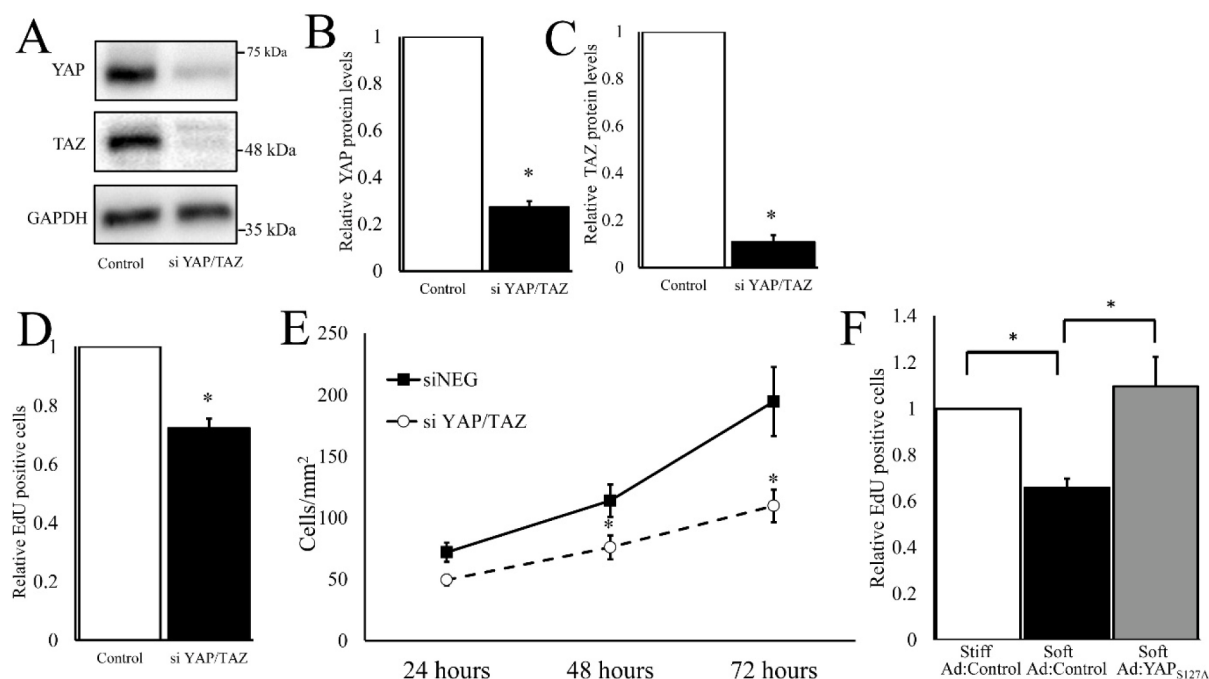
Fig. 3. *Cardiac fibroblast proliferation stimulated by stiff substrates is dependent on YAP and TAZ*
Cardiac fibroblasts were transiently transfected with siRNA targeting YAP and TAZ (si YAP/TAZ) or non-targeting control siRNA (Control). Total cell lysates were analysed for protein levels of YAP, TAZ and GAPDH by western blotting (*A, B and C*; $n = 4$). Cells were also labelled and detected by incorporation of Edu, 24 h post transfection (*D*; $n = 4$). Cell number was quantified after 24-, 48- and 72-h post-transfection (*E*; $n = 7$). Data are expressed as mean $\pm$ and analysed using paired Student t-test or ANOVA with Student Newman Keul's po*st*-test. * indicates $p < 0.05$.

Fig. 4. *Stiff substrates upregulate RUNX2 and TEAD activity in cardiac fibroblasts*
Cardiac fibroblasts were transfected with a plasmid (GAL4-RUNX2) encoding the GAL4 DNA-binding domain fused to the RUNX2 transcriptional activation domain, together with a secreted nano-luciferase reporter gene under the control of a promoter containing five GAL4 binding elements, t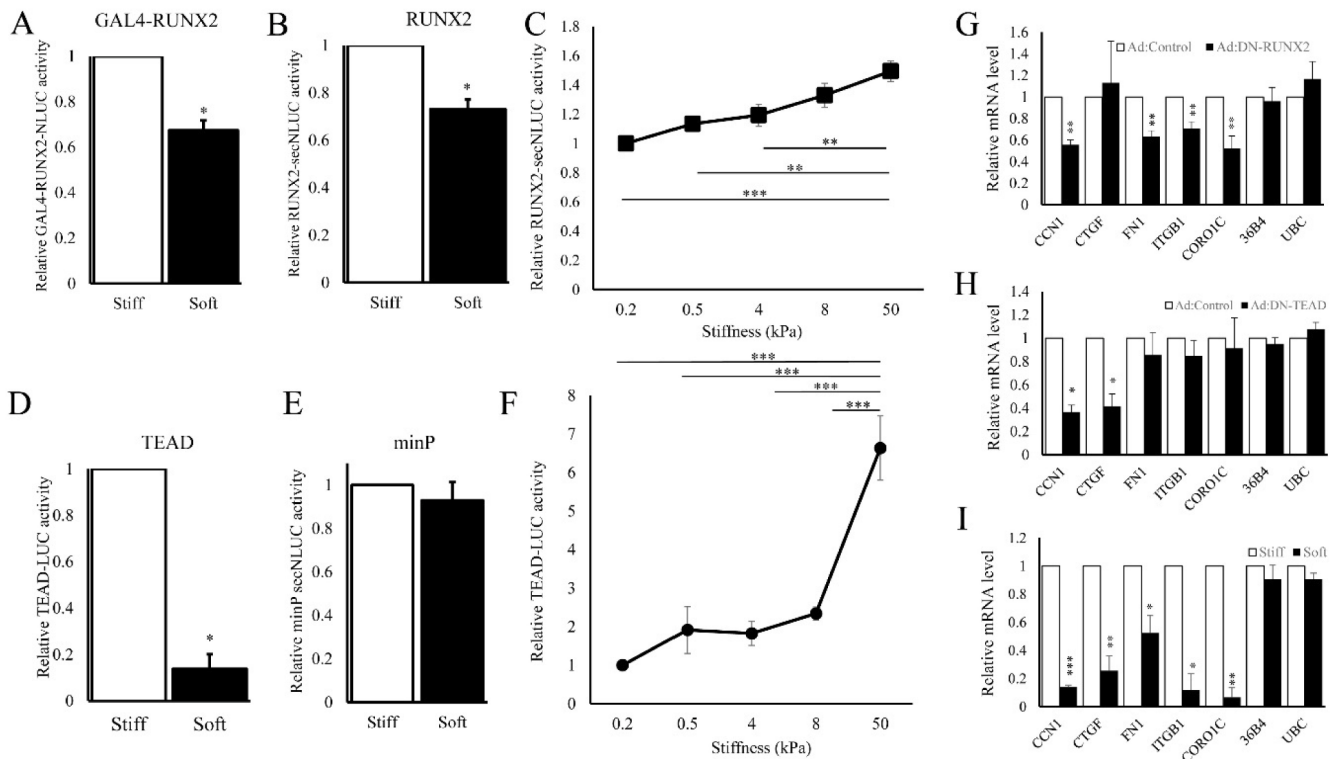o allow specific quantification of RUNX2 activity (*A*, *n* = 8). Cells were transfected with RUNX2-secNLUC reporter plasmids, which is responsive to endogenous RUNX2 activity (*B*; *n* = 3 and *C*; n = 7). Cells were transfected with TEAD-LUC (*D*, n = 3) or a minimal promoter reporter which is lacking TEAD or RUNX2 binding elements (minP; *E*; n = 3). All cells were seeded onto either a stiff (50 kPa) or soft (0.5 kPa) substrate post transfection or on the indicated substrate stiffness (*C* and *F*). Reporter gene activity was quantified 24 h after transfection. Data are expressed as mean ± and analysed using paired Student t-test (A,B, D, E) or ANOVA with student Newman Keuls post-test (C, F, G, H, I). * Indicates $p < 0.05$, ** indicates $P < 0.01$, *** indicates $p < 0.001$.

3.5. YAP and TAZ enhance RUNX2 and TEAD activity in cardiac fibroblasts

To confirm that YAP/TAZ activate RUNX2, we next used siRNA-mediated silencing and adenovirus-mediated expression of constitutively active mutants of YAP (Ad:YAP$_{S127A}$) and TAZ (Ad:TAZ$_{4SA}$) to test if YAP and TAZ affected RUNX2 activity in cardiac fibroblast. Firstly, as a positive control we showed that silencing of YAP and TAZ strongly downregulated (to 0.082 ± 0.039 fold of controls $p < 0.001$) TEAD activity, consistent with the well characterised role of YAP and TAZ as TEAD co-factors (Fig. 5A). Importantly, YAP and TAZ silencing also significantly downregulated (to 0.38 ± 0.11 fold of controls, $p < 0.05$) RUNX2 reporter gene activity (Fig. 5B). Moreover, expression of active YAP or TAZ significantly increased TEAD activity (to 2.27 ± 0.41 fold, p < 0.05 and 3.19 ± 0.85 fold, $p < 0.01$ for YAP and TAZ respectively) and RUNX2 activity (by 5.14 ± 1.85 fold of control, p < 0.05 and 6.45 ± 1.09 fold of control, p < 0.05 for YAP and TAZ respectively) but did not affect the activity of a minimal promoter-dependent reporter (Fig. 5C and supplement fig. 10). Moreover, the reduction in RUNX2 (quantified using GAL4-RUNX2; Fig. 5D) and TEAD activity (Fig. 5E) in cells cultured on a soft substrate was completely reversed by exogenous expression of YAP$_{S127A}$. Hence YAP appears to be the upstream activator of RUNX2 in response to increased stiffness.

3.6. TEAD and RUNX2 activity are both required for cardiac fibroblast proliferation

We used multiple pharmacological and molecular approaches to test the functional importance of TEAD activity for maximal cardiac fibroblast proliferation. We initially treated cells with CPD3.1, a recently identified inhibitor of YAP-TEAD interaction and TEAD activity [56]. Incubation of cells with CPD3.1 resulted in a dose dependent inhibition of proliferation, detected by reduced incorporation of the thymidine analogue, Edu, in cells cultured on plastic (Fig. 6A) and inhibited Edu incorporation induced by a stiff substrate (Fig. 6B). Treatment with CPD3.1 had no effect on cell morphology or phosphorylation of YAP (Supplement Figs. 11 and 12 respectively), as this inhibitor acts by disrupting YAP-TEAD interaction. Consistent with this CPD3.1 also inhibited the total cell number after 72 h of treatment (Fig. 6C). Treatment of cells with Vertaporfin, another well characterised YAP-TEAD inhibitor [75], also significantly inhibited incorporation of Edu (Fig. 6D). Lastly, we used a dominant-negative form of TEAD (DN-TEAD), generated by fusing the TEAD1 DNA-binding domain to the transcriptional repressor domain of the *Drososphila engrailed* (*en*) gene, as previously described [76]. Adenoviral-mediated expression of DN-TEAD, efficiently reduced TEAD reporter gene activity, without affecting the activity of a minimal promoter driven control reporter construct (Fig. 6E). Furthermore, expression of DN-TEAD, inhibited the proliferation of cardiac fibroblasts cultured on a plastic substrate (Fig. 6F). Consistent with this, expression of DN-TEAD also significantly reduced the total cell number after 72 h (Fig. 6G). Taken together, these data demonstrate that TEAD activity is required for maximal cardiac fibroblast proliferation.

We next tested the importance of RUNX2 for cardiac fibroblast proliferation using a similar combination of pharmacological and molecular biology approaches. We initially used the RUNX2 inhibitor CADD522 [77]. Treatment of cells with CADD522 dose dependently inhibited RUNX2 reporter gene activity without significantly affecting
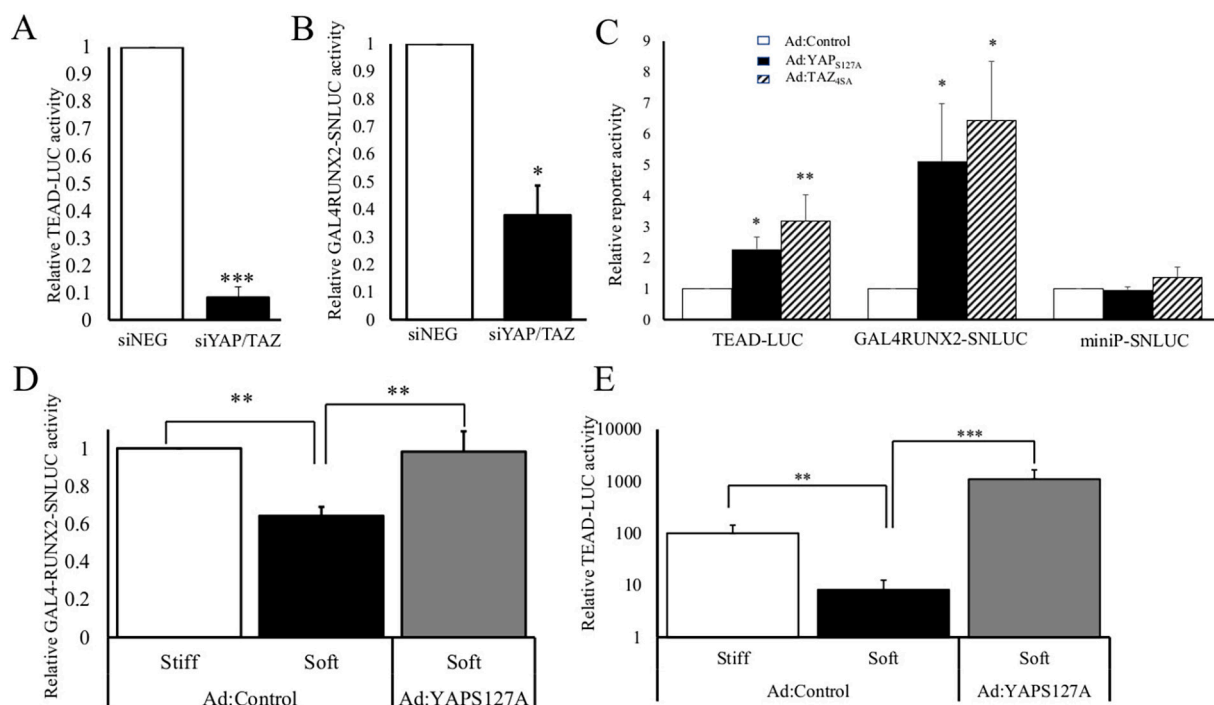
Fig. 5. *YAP and TAZ enhance RUNX2 and TEAD activity in cardiac fibroblasts*
Cardiac fibroblasts were transfected with siRNA targeting YAP and TAZ (siYAP/TAZ) or non-targeting control siRNA (siNEG), together with TEAD-LUC (*A; n = 3*) or GAL4RUNX2-SNLUC (*B, n = 3*). Cells were cultured on cell culture plastic plates and reporter gene activity were then quantified 24 h post transfection. Cells were transfected with TEAD-LUC, RUNX2-SECNLUC or a minimal promoter-SNLUC (miniP-secNLUC), The next day, CFs were infected with either a control adenovirus (Ad:Control) or adenovirus expressing constitutively-active YAP (Ad:YAP$_{S127A}$) or TAZ (Ad:TAZ$_{48A}$). Media was collected and reporter gene activity was quantified 24 h later (*C; n = 6*). CFs were seeded on stiff and soft matrices post transfection with either GAL4RUNX2-SNLUC (*D; n = 5*) or TEAD-LUC (*E; n = 4*) reporter gene plasmids. The next day, cells were infected with either a control adenovirus (Ad:Control) or adenovirus expressing constitutively-active YAP (Ad:YAP$_{S127A}$). 24 h after infection, media was collected, and the activity of the reporter genes were quantified. Data are expressed as mean $\pm$ and analysed using paired Student *t*-test or ANOVA with Student Newman Keul's post-test. * indicates $p < 0.05$, ** indicates $p < 0.01$ and *** indicates $p < 0.001$.

the activity of a control reporter gene driven by a minimal promoter lacking RUNX2 binding elements (Fig. 7A). Treatment of cells with CADD522 significantly inhibited Edu incorporation in cardiac fibroblasts cultured on a plastic substrate (Fig. 7B) and significantly reduced Edu incorporation induced by a stiff (50 kPa) collagen coated hydrogel (Fig. 7C). Consistent with these data, CADD522 treatment also significantly reduced cell number after 72 h (Fig. 7D). To further test the role of RUNX2 in cardiac fibroblast proliferation, we transiently transfected cells with siRNA to silence RUNX2 expression. Transfection with siR-UNX2 strongly repressed expression of RUNX2 protein levels, without affecting levels of the housekeeping protein, GAPDH (Fig. 7E) or cellular morphology (supplement fig. 8), indicating efficient and specific silencing of RUNX2. Consistent with this, RUNX2 silencing significantly repressed RUNX2 reporter gene activity, without affecting activity of a minimal promoter control reporter (Fig. 7F). Importantly, RUNX2 silencing significantly reduced Edu incorporation of cells cultured on a plastic substrate (Fig. 7G) and reduced total cell numbers 48 and 72 h after siRNA transfection (Fig. 7H). Finally, we used a dominant-negative RUNX2, consisting of the RUNX2 DNA-binding domain fused to the engrailed repressor domain. Adenoviral mediated expression of DN-RUNX2 strongly reduced the activity of a RUNX2-dependent reporter gene (to 0.29 ± 0.09 fold of control, $n = 4$, $p = 0.0044$), without affecting the activity of a control reporter gene, lacking RUNX2 binding elements (Fig. 7I). Expression of DN-RUNX2 significantly inhibited Edu incorporation (Fig. 7J) and reduced total cell numbers after 48 and 72 h (Fig. 7K). Taken together, these data demonstrate that RUNX2 activity is essential for maximal cardiac fibroblast proliferation.

3.7. Combined role of YAP-TEAD and YAP-RUNX2 in substrate stiffness-dependent cardiac fibroblast proliferation

Our data demonstrates the proliferation of cardiac fibroblasts is dependent on both TEAD and RUNX2 activity. Our data also demonstrates that substrate stiffness controls YAP activation, which enhances both TEAD and RUNX2 activity. Although our data clearly demonstrates the ability of YAP to activate RUNX2 in these cells, other mechanisms have also been documented to control RUNX2 activity [78]. We therefore sought to test the specific functional importance of YAP-mediated RUNX2 activity in substrate stiffness dependent proliferation in cardiac fibroblasts. To this end, we generated a series of YAP mutants, all based on the S127A mutation, which promotes activation of YAP by enhancing its nuclear localisation [35]. We mutated YAP serine-94 to alanine, which has previously been shown to block YAP interaction with TEAD [45]. To disrupt YAP interaction with RUNX2, we mutated two residues (in each of the two WW-domains W199A and W202A in WW domain 1 and W258A and P261A in WW domain 2; abbreviated to WW1/2 herein), as previously described [45]. Finally, we generated a compound mutant containing both the S94A and all the WW-domain mutation, thus creating a YAP mutation unable to interact with either TEAD or RUNX2 (see schematic Fig. 8A). Adenovirus mediated delivery of these YAP mutants resulted in expression of similar levels of YAP overexpression (Fig. 8B). Expression of YAP$_{S127A}$ and YAP$_{S127A/WW2}$, but not YAP$_{S127A/S94A}$ or YAP$_{S127A/S94A/WW2}$, induced TEAD activity (by 9.7 ± 3.3 fold and 6.4 ± 1.7 fold respectively), consistent with disruption of YAP-TEAD interaction by the S94A but not the WW2 mutation(s) (Fig. 8C). Expression of YAP$_{S127A}$ and YAP$_{S127A/S94A}$, but not YAP$_{S127A/WW2}$ or YAP$_{S127A/S94A/WW2}$ induced RUNX2 activity (by 3.0 ± 0.7 fold and 2.6 ± 0.6 fold, respectively), consistent with disruption of YAP-
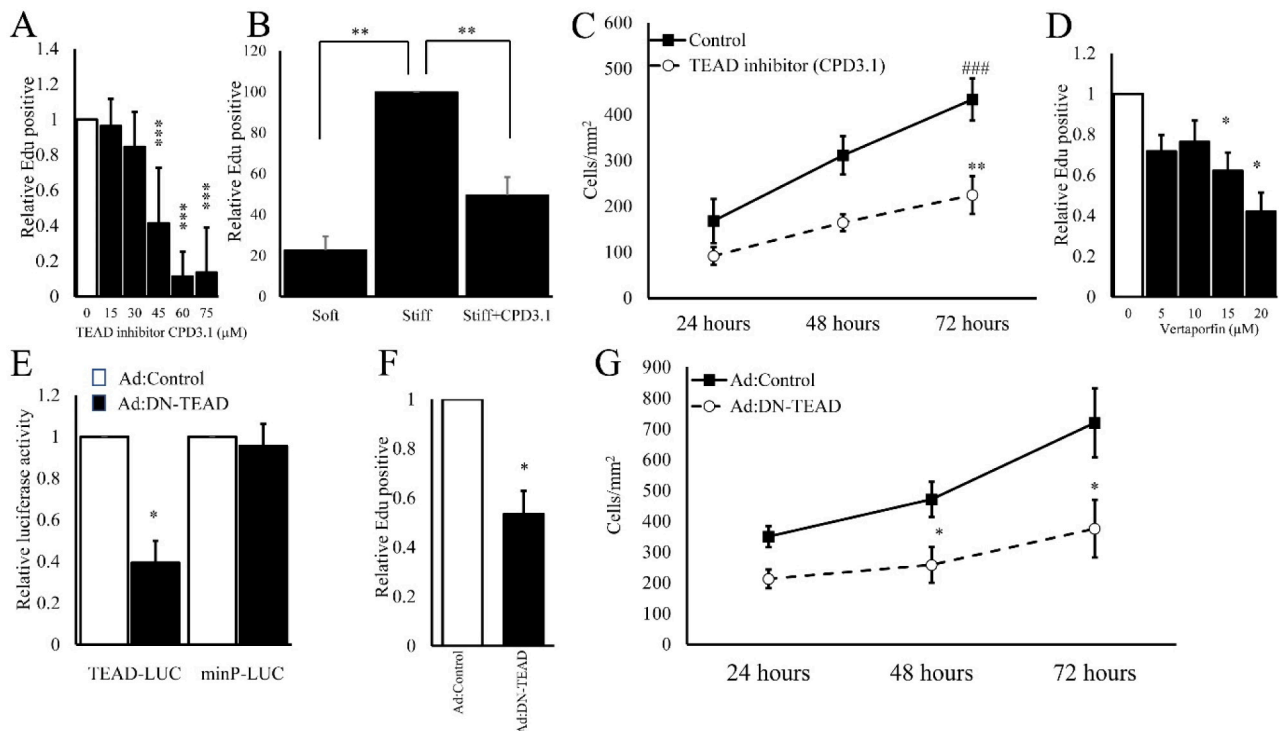
Fig. 6. *Proliferation of cardiac fibroblasts is TEAD dependent*
Cardiac fibroblasts were seeded on plastic cell culture plates (A) or soft (0.5 kPA) and stiff (50 kPA) hydrogels (B). The next day cells were incubated with indicates doses (0–75 uM in A and 50 uM in B) of CPD3.1, a pharmacological TEAD inhibitor. Incorporation of Edu was assessed during a 4-hour labelling window 18 h after addition of CPD3.1(A and B; $n = 3$). Cells were treated with 50 uM CPD3.1 and total cell numbers quantified after 24, 48 and 72 h (C). Cells were seeded on plastic cell culture plates and incubated with indicated doses (0–20 uM) of verteporfin, a pharmacological YAP-TEAD inhibitor. Incorporation of Edu was assessed during a 4-hour labelling window, 18 h after addition of Verteporfin (D; n = 3). Cells were transfected with either TEAD-LUC or minimal promoter as a control (minP-LUC). The next day cells were infected with either a control adenovirus (Ad:Control) or adenovirus overexpressing dominant-negative TEAD (Ad:DN-TEAD). Reporter activity was quantified 24 h post infection (E; n = 4). Edu incorporation was quantified 24 h post infection with Ad:Control or Ad:DN-TEAD (F). Total cell number was quantified 24, 48 and 72 h post infection with Ad:Control or Ad:DN-TEAD (G). Data are expressed as mean ± SEM and analysed either by paired student t-test or ANOVA with Student Newman Keul's post-test. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** and ### indicate $p < 0.001$.

RUNX2 interaction by the WW2 mutations but not the S94A mutation (Fig. 8D). Importantly, the activity of a control minimal promoter lacking TEAD or RUNX2 binding elements was unaffected by ectopic expression of any of the YAP mutants (Fig. 8E). We next tested the ability of these YAP mutants to rescue cardiac fibroblast proliferation on soft substrate, which normally do not support maximal proliferation. Control virus infected cells displayed a high rate of proliferation on a stiff substrate, which was significantly reduced in cells cultured on a soft substrate (Fig. 8F). Expression of YAP$_{S127A}$ increased proliferation on a soft substrate to levels not significantly different to levels on a stiff substrate. Interestingly, expression of YAP$_{S127A/S94A}$, which lacks the ability to activate TEAD, was able to rescue proliferation on a soft substrate to levels similar to YAP$_{S127A}$, implying that YAP-dependent activation of TEAD is not essential for YAP-mediated proliferation in these cells. Expression of YAP$_{S127A/WW2}$, which lacks the ability to activate RUNX2, significantly increased proliferation on a soft substrate compared to control, although this was to lower levels than achieved by YAP$_{S127A}$ and YAP$_{S127A/S94A}$. Importantly, expression of compound mutant, YAP$_{S127A/S94A/WW2}$ was unable to increase proliferation on soft substrates above control levels. Taken together, these data indicate that both YAP-mediated activation of TEAD and RUNX2 are required for maximal proliferation of cardiac fibroblasts.

4. Discussion

YAP and TAZ are candidate oncogenes with well characterised roles in regulating organ size and cell proliferation. A large body of literature documents the role of YAP as a co-factor for TEAD family transcription factors. Here we present evidence supporting a combined role for YAP-TEAD and YAP-RUNX2 signalling in mediating substrate stiffness dependent regulation of cardiac fibroblast proliferation. We show that the substrate stiffness dependent regulation of cardiac fibroblast proliferation is associated with YAP activation and increased activity of both TEAD and RUNX2. Using siRNA-mediated silencing of YAP/TAZ and overexpression of constitutively active YAP mutants, we demonstrate that YAP and TAZ can activate RUNX2 transcriptional activity, in addition to stimulating TEAD activity. Using pharmacological inhibition, gene silencing and expression of dominant-negative mutants, we demonstrate a requirement for both TEAD and RUNX2 activity for maximal cardiac fibroblast proliferation. Using YAP mutants that are defective in their ability activate either TEAD or RUNX2, we functionally link YAP-TEAD and YAP-RUNX2 to stiffness-dependent cardiac fibroblast proliferation. These data implicate for the first time an important role for YAP-RUNX2 signalling in mediating increased cardiac fibroblast proliferation in response to elevated ECM stiffness, a characteristic of tissue fibrosis.

Cardiac fibrosis is associated with increased stiffness of the myocardium [79]. Healthy diastolic human myocardium has been reported to have a stiffness of 1.7 kPa [80], with fibrotic myocardium having stiffnesses in the range of 20–100 kPa. Although the soft substrates (0.5 kPa) used in our experiments are slightly softer than healthy human myocardium, we still observe significant differences in proliferation rate, TEAD and RUNX2 activity when comparing various stiffnesses (0.5, 4 and 8 kPa) with 50 kPa substrates, indicating that the mechanism we described is physiologically relevant. Resident cardiac fibroblasts respond to stiffening in the local ECM by differentiating into
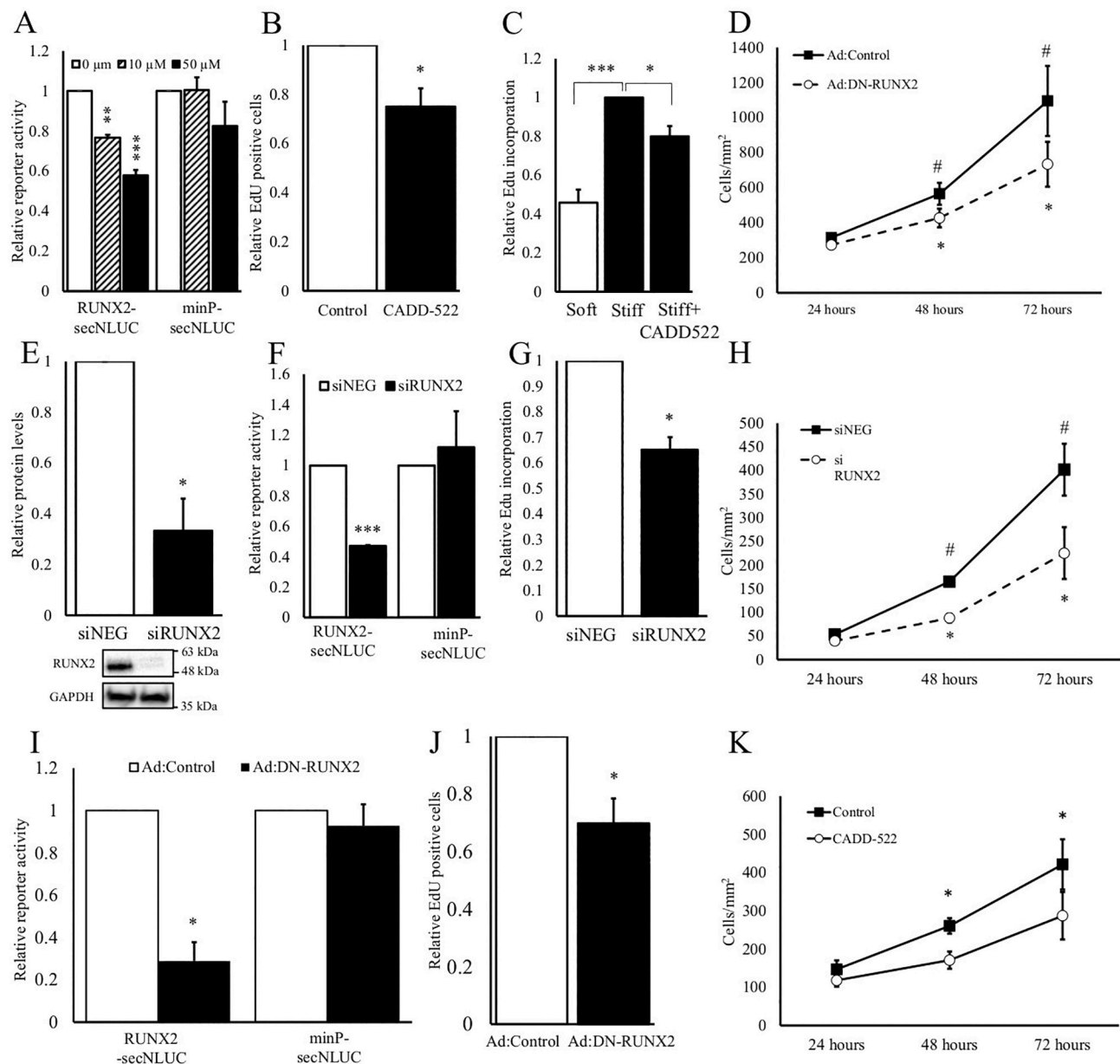
Fig. 7. *Proliferation of cardiac fibroblasts is dependent on RUNX2 activity*
Cardiac fibroblasts were transfected with either RUNX2-secNLUC or minP-secNLUC plasmids. A day later, cells were treated with indicated doses (0–50 μM) of pharmacological RUNX2 inhibitor (CADD-522). Reporter activity was quantified 24 h post stimulation (A; *n = 3*). Cells were stimulated with 50 μM CADD-522, either on plastic (B) or stiff (50 kPa) or soft (0.5 kPa) substrate (C), and labelled with Edu for 4 h, 18 h after addition of CADD-522. Total cell number was quantified 24-, 48- and 72-hour post CADD-522 treatment (D). Cells were transiently transfected with siRNA targeting RUNX2 (siRUNX2) or non-targeting control siRNA (siNEG). Total cell lysates were analysed for protein levels of RUNX2 and GAPDH by western blotting (E; *n = 5*). Cells were co-transfected with siNEG or siRUNX2 together with GAL4-secNLUC or minP-secNLUC and their activities were quantified 24 h post transfection(F, *n = 3*). Edu incorporation was quantified 24 h post siRNA transfection (G; *n = 3*). Total cell number was quantified 24-, 48- and 72-hour post siRNA transfection (H; *n = 6*). Cells were infected with either a control adenovirus (Ad:Control) or adenovirus expressing dominant-negative RUNX2 (Ad:DN-RUNX2) and transfected with GAL4-secNLUC or minP-secNLUC, as indicated (I). Cells were Edu labelled 24 h post adenovirus infection (J). Total cell number was quantified 24, 48 and 72 h post infection with Ad:Control or Ad:DN-RUNX2 (K).

activated myofibroblasts [81], a hallmark of cardiac fibrosis, characterised by elevated expression of α-SMA. Increased ECM stiffness is associated with elevated proliferation rates in many cell types [82–85], although the effects on cardiac fibroblasts has not been extensively characterised. Herum et al. reported increased cardiac fibroblast proliferation in response to mechanical static stretching but not in response to increased ECM stiffness [12]. This contrasts with our data showing a marked increase in new DNA synthesis and total cell number when cells were cultured on stiff, compared to soft, substrates. This difference may reflect the source of the cells used (embryonic heart compared to adult

heart used here) or differences in the compliance of the substrates studied. Nevertheless, cardiac fibroblast proliferation is increased in vivo in regions of fibrosis [86,87], consistent with the paradigm of enhanced proliferation in regions of increased stiffness. This proliferative expansion of the cardiac fibroblast population likely contributes to creation of a positive feedback loop driving fibrosis progression [15,18]. However, the mechanisms involved in transducing tissue stiffness into changes in cardiac fibroblast proliferation are poorly understood.

Substrate stiffness is associated with actin polymerisation, cytoskeleton organisation and cell morphology in many adherent cell types
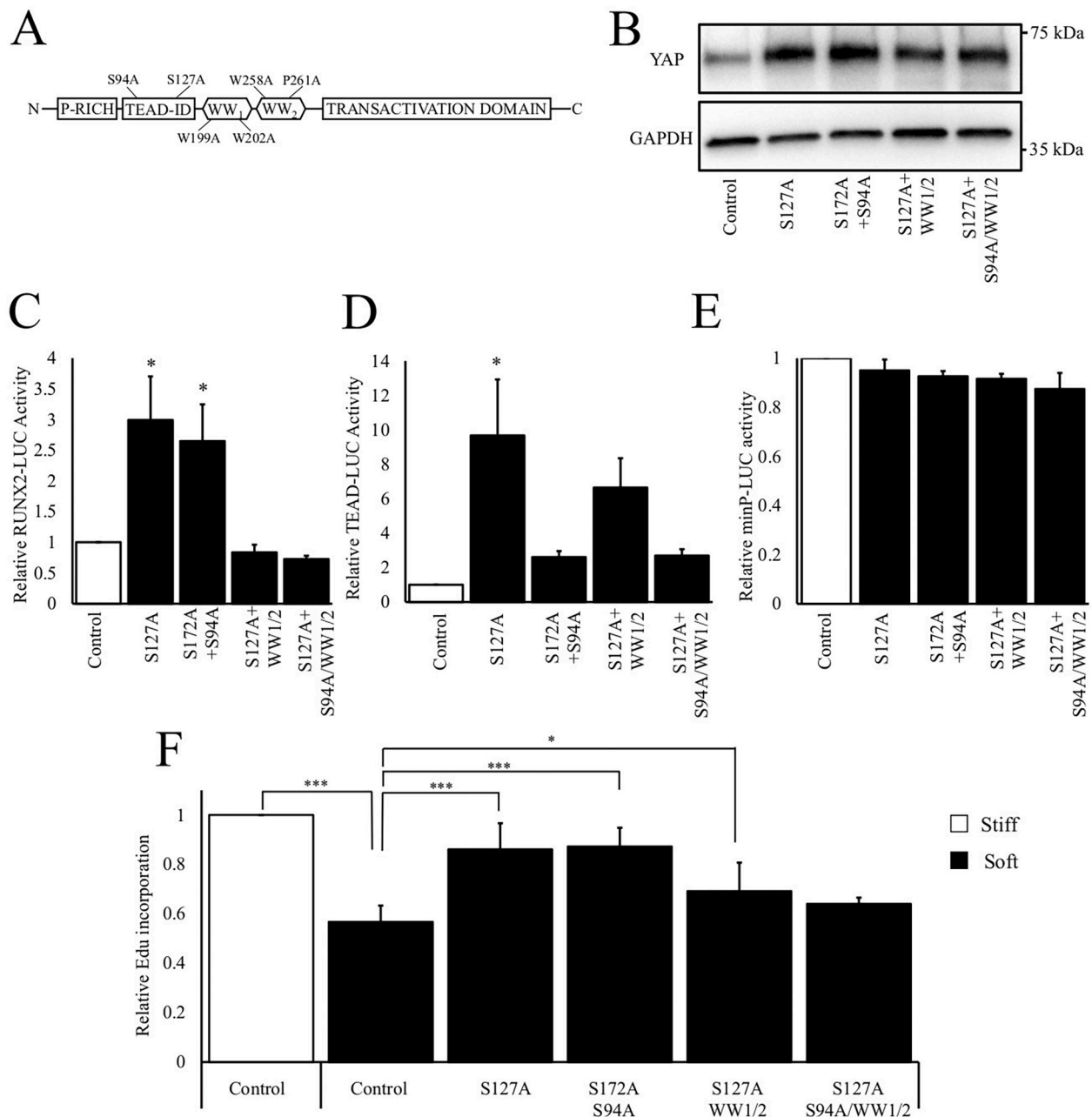
Fig. 8. *Dual requirement of TEAD and RUNX2 interaction domains of YAP for cardiac fibroblast proliferation*
Cardiac fibroblasts were infected with adenoviral vectors expressing indicated YAP mutants (A and B). Total cell lysates were analysed for YAP expression levels by Western blotting (B) Cells were transfected with RUNX2-secNLUC, (C; n-3) TEAD-LUC (D, n = 3) or minP-secNLUC (E, n = 3) and reporter gene activity assays 24 h post virus infection. Cells were infected with either Control adeno virus or adenoviruses expressing indicated YAP mutant were seeded onto stiff (50 kPa) or soft (0.5 kPa) substrates. The next day cells were labelled with Edu for 4 h and Edu incorporation quantified (F; n = 5). Data are expressed as mean ± SEM and analysed by ANOVA with Student Newman Keul's post-test. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicate $p < 0.001$.

[88,89]. Furthermore, cell spreading, and formation of actin stress fibres is tightly coupled to proliferation [90]. Consistent with this, we observed increased cell spreading and formation of prominent actin stress fibres in cardiac fibroblast cultured on stiff compared to soft substrates. This was also associated with an increased proliferation rate of cells interacting with a stiff substrate, consistent with previous observations in other cell types. Stiffness-dependent changes in actin cytoskeleton organisation are transduced into transcriptional responses, and ultimately modulate proliferation, via actin-sensitive transcriptional co-factors and transcription factors. Prominent amongst these is the transcriptional co-factor YAP [70], which stimulates the transcriptional activity of TEAD transcription factors [44]. We detected a

reduction in YAP phosphorylation on serine 397, a post-translational modification associated with YAP inactivation, in cardiac fibroblasts cultured on stiff substrates. We also detected increased nuclear localisation of YAP and increased TEAD-transcriptional activity in cells on stiff substrates, indicating stiffness-dependent activation of YAP-TEAD signalling. YAP has been demonstrated to positively regulate proliferation in many cell types [35,45,66] and our data confirms a similar function in cardiac fibroblasts. Importantly, YAP is activated in cardiac fibroblasts in vivo in response to myocardial infarction, and genetic deletion of YAP in cardiac fibroblasts attenuates fibrosis [38]. Furthermore, selective activation of YAP, either via overexpression of a constitutively active YAP mutant or via genetic deletion of negative

regulators of YAP function (Lats1/2) in vivo, promotes fibrosis and enhances cardiac fibroblast proliferation [91,92]. Taken together, this suggests that fibrosis related stiffening of the myocardium enhances YAP activation, which promotes cardiac fibroblast proliferation and fibrosis.

A large body of evidence suggests that TEAD transcription factors are mediators of YAP function [50,93,94]. YAP has also been shown to interact with several other transcription factors, including RUNX2 [57]. In this study we characterised the importance of TEAD and RUNX2 in mediating the substrate stiffness-dependent effects of YAP on cardiac fibroblast proliferation. We confirmed that increased substrate stiffness-stimulates TEAD activity, consistent with previous reports in other cells [70]. Importantly, our data demonstrates that RUNX2 activity, detected using two different reporter gene systems, is also sensitive to substrate stiffness. To our knowledge, this is the first report of substrate stiffness-dependent regulation of RUNX2 activity. Consistent with this, we detected upregulation of RUNX2 target mRNAs (FN1, ITGB1 and CORO1C) in cells cultured on stiff compared to soft substrates, in addition to the classical TEAD target mRNAs (CCN1 and CTGF). Importantly, all of these genes have been associated with increase cell proliferation and the development of tissue fibrosis [37,95–99]. Furthermore, the RUNX2 regulated target mRNAs that we analysed were sensitive to inhibition by dominant-negative RUNX2 but not by dominant-negative TEAD, implying that effects of substrate stiffness and YAP on RUNX2 are not simply a secondary effect of TEAD inhibition. Instead, our data suggest that YAP-TEAD and YAP-RUNX2 represent parallel pathways that control distinct sets of target genes (e.g. CCN1 via YAP-TEAD and FN, CORO1C and ITGB1 via YAP-RUNX2). This conclusion is further strengthened by our data obtained using the YAP$_{S94A}$ mutant, which retains the ability to activate RUNX2, despite lacking the ability to activate TEAD. Our data indicates that the substrate stiffness dependent regulation of RUNX2 activity is mediated by YAP/TAZ since YAP/TAZ silencing on a stiff substrate significantly reduces RUNX2 activity, whereas expression of constitutively active YAP rescues RUNX2 activity in cells interacting with soft substrates.

Although YAP is known to interact with RUNX2, the functional consequences of this interaction are unclear, with some reports demonstrating YAP-mediated inhibition of RUNX2 activity [63,100] and others reporting activation [51,57]. These conflicting reports may reflect cell type dependent differences or may indicate promoter context dependent effects of YAP on RUNX2. Consistent with this, Zaidi et al. [63] reported that YAP had divergent effects on various RUNX2-dependent promoters, showing repression of some, but not all, by the YAP-RUNX2 complex. Interestingly, Zaidi et al. did not find any regulation of a reporter gene under the control of a synthetic promoter comprised of six multimerised RUNX2 binding elements, in response to YAP overexpression. This contrasts with our data demonstrating a strong induction of a similar RUNX2-dependent reporter gene by YAP. Our data is consistent with YAP acting as a co-activator of RUNX2 and taken together with our other data, indicates that RUNX2 and TEAD are both regulated by YAP/TAZ in a substrate-stiffness dependent manner.

Our data supports a functional role for YAP-TEAD and YAP-RUNX2 signalling in promoting cardiac fibroblast proliferation. Inhibition of YAP-TEAD activity using the YAP-TEAD protein:protein interaction inhibitor, CPD3.1 [56], or expression of dominant-negative TEAD, both significantly inhibited proliferation of cardiac fibroblasts on a stiff substrate. In contrast to YAP and TAZ, there are relatively few studies characterising the function of TEAD family transcription factors on cell proliferation. However, studies that have directly inhibited TEAD activity, either using peptide inhibitors that block YAP interaction, or using active or dominant-negative approaches, have indicated a role for TEADs in the proliferation of keratinocytes [101], NIH3T3 cells [102] and mesothelioma cells [103]. Importantly, overexpression of an active-YAP mutant (YAP$_{S127A/S94A}$) devoid of ability to activate TEAD, was still able to rescue proliferation in cardiac fibroblasts cultured on soft substrates to levels not significantly different from proliferation in cells expressing YAP$_{S127A,}$ which is competent in TEAD activation. This clearly demonstrates that YAP has mitogenic activity in cardiac fibroblasts that is independent of its ability to activate TEAD and implicates a role for additional YAP-activated pathways in substrate stiffness dependent proliferation. This contrasts with the effects of the same YAP mutant in NIH3T3 cells, where YAP$_{S94A}$ mutants unable to activate TEAD completely lost the ability to drive serum independent growth. This absolute dependence on YAP-mediated TEAD activation may reflect the absence of serum mitogens in the NIH3T3 experiments compared to ours that were performed in the presence of 5 % serum, which is likely to activate other mitogenic signalling pathways that permit-YAP induced proliferation in the absence of TEAD activity. Our study demonstrates that activation of RUNX2 is also an important mediator of YAP-dependent proliferation in cardiac fibroblasts and contributes towards the effects of substrate stiffness in these cells. RUNX2 has been reported to have pro-mitogenic and anti-mitogenic effects in various cell types. For example, loss of function of RUNX2 in the mouse causes increased proliferation of skeletal lineage cells in vitro [104,105], whilst over expression of RUNX2 induces growth arrest of osteoblasts [105]. Using pharmacological inhibition, siRNA-mediated silencing, and expression of a dominant-negative form of RUNX2, we demonstrate that RUNX2 activity is essential for maximal proliferation of cardiac fibroblast on stiff substrates. However, expression of a YAP WW-domain, defective in RUNX2 activation, retained some ability to drive cardiac fibroblast proliferation on soft substrates, albeit to a lesser extent that the TEAD-defective mutant. It seems likely that YAP-dependent TEAD activation, which remains functional in this YAP-WW-domain mutant, can compensate for the lack of RUNX2 activation in these cells. Or putting things another way, activation of TEAD becomes essential when RUNX2 activity is suppressed. Consistent with this, we found that a YAP mutant harbouring both the S94A and the WW-domain mutations, which was devoid of TEAD or RUNX2 activation properties, lacked any ability to promote cardiac fibroblast proliferation on soft substrates.

In summary, our data demonstrates that substrate stiffness regulates RUNX2 and TEAD activity in cardiac fibroblasts via YAP and that YAP-RUNX2 and YAP-TEAD signalling act together to promote proliferation of cardiac fibroblasts on stiff matrices. Characterization of the mechanisms underlying the biomechanical regulation of cardiac fibroblast proliferation is important for our understanding of the pathogenesis and prevention of cardiac fibrosis. YAP-RUNX2 signalling may represent a new target for pharmacological inhibition of cardiac fibroblast proliferation and limiting the progression of cardiac fibrosis.

CRediT authorship contribution statement

Reza Ebrahimighaei: Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Graciela B. Sala-Newby:** Resources. **Claire Hudson:** Methodology, Investigation. **Tomomi E. Kimura:** Methodology, Investigation. **Tom Hathway:** Resources. **Joseph Hawkins:** Investigation. **Madeleine C. McNeill:** Investigation. **Andrew C. Newby:** Writing – original draft, Writing – review & editing, Supervision. **Mark Bond:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

None of the authors of this manuscript have any conflicts of interest to declare.

Data availability

Data will be made available on request.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2022.119329.

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