Identification of a common regulatory pathway that determines cell survival and cell cycle progression in cardiac fibroblasts

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Abstract:

Relative resistance to apoptosis and the ability to proliferate and produce a collagen-rich scar determine the critical role of cardiac fibroblasts in wound healing and tissue remodeling following myocardial injury. Identification of cardiac fibroblast-specific factors and mechanisms underlying these aspects of cardiac fibroblast function is therefore of considerable scientific and clinical interest. In the present study, gene knockdown and over-expression approaches, and promoter binding assays, showed that DDR2, a mesenchymal cell-specific collagen receptor tyrosine kinase localized predominantly in fibroblasts in the heart, acts via ERK1/2 MAPK-activated SRF transcription factor to enhance the expression of anti-apoptotic cIAP2 in cardiac fibroblasts, conferring resistance against oxidative injury. Further, DDR2 was found to act via ERK1/2 MAPK-activated SRF to transcriptionally up-regulate Skp2 that in turn facilitated post-translational degradation of p27, the cyclin-dependent kinase

inhibitor that causes cell cycle arrest, to promote G1-S transition, as evidenced by Rb phosphorylation, increased PCNA levels and flow cytometry. DDR2-dependent ERK1/2 MAPK activation also suppressed FoxO3a-mediated transcriptional induction of p27. Notably, DDR2 levels positively correlated with SRF, cIAP2 and PCNA levels in cardiac fibroblasts from Spontaneously Hypertensive Rats. To conclude, DDR2-mediated ERK1/2 MAPK activation facilitates coordinated regulation of cell survival and cell cycle progression in cardiac fibroblasts via SRF.

Keywords: Cardiac fibroblasts, Discoidin Domain Receptor 2 (DDR2), ERK1/2 MAPK, Serum Response Factor (SRF), cIAP2, FoxO3a, Skp2, p27, apoptosis resistance, G_1-S transition

Abbreviations:

DDR2- Discoidin Domain Receptor 2

SRF- Serum Response Factor

ERK1/2 MAPK- Extracellular signal-regulated kinase1/2 Mitogen-activated Protein Kinase

cIAP2- Cellular inhibitors of apoptosis protein 2

FoxO3a-Forkhead box O 3a transcription factor

Skp2- S-Phase Kinase Associated Protein 2

MAPK- Mitogen-Activated Protein Kinase

Introduction:

Cardiac fibroblasts, the principal stromal cells in the myocardium and a major source of matrix proteins, pro-inflammatory cytokines and pro-fibrotic factors, play an important role in wound healing following cardiac muscle damage (Furtado et al., 2016). Unlike cardiac myocytes, cardiac fibroblasts retain their replicative capacity throughout adult life and are relatively resistant to pro-apoptotic signals such as Angiotensin II, TNF-α, hypoxia and oxidative stress that do not favor the survival of cardiac myocytes in the injured myocardium (Humeres and Frangogiannis, 2019). In the altered cytokine milieu of the damaged heart, marked by progressive loss of functional cardiomyocytes, normally quiescent cardiac fibroblasts survive and undergo phenotypic transformation into active myofibroblasts that migrate to the site of injury, proliferate and produce matrix components to replace the lost tissue with a scar that maintains the structural and functional integrity of the heart (Humeres and Frangogiannis, 2019). The relative resistance of cardiac fibroblasts to death signals that prevail in the myocardium *post injury* and their ability to proliferate and produce a collagen-rich scar are key determinants of their central role in acute wound healing.

Surprisingly, however, while there has been a great deal of interest in the regulation of collagen turnover in cardiac fibroblasts (Fan et al., 2012), only a limited number of studies have addressed the molecular mechanisms that determine cell survival and cell cycle progression in cardiac fibroblasts (Gao et al., 2014; Lv et al., 2016; Mayorga et al., 2004; Olsen et al., 2017; Olson et al., 2005; Philip and Shivakumar, 2013; Pramod and Shivakumar, 2014). Moreover, it is of obvious interest to ascertain

whether the cell survival and cell cycle pathways are under distinct regulatory mechanisms or are co-ordinately regulated in cardiac fibroblasts to achieve an efficient coupling of the two processes that are indispensable for optimal myocardial recovery from an acute insult.

We had demonstrated an obligate role for Discoidin Domain Receptor 2 (DDR2), a mesenchymal cell-specific collagen receptor tyrosine kinase localized predominantly in fibroblasts in the heart, in collagen type I expression in cardiac fibroblasts and wound healing in response to Ang II, which points to the centrality of DDR2 in cardiac fibroblast response to injury (George et al., 2016). DDR2 has been implicated in a variety of fundamental cellular processes such as proliferation, survival and differentiation (Leitinger, 2014; Lin et al., 2010; Valiathan et al., 2012). While there are sporadic reports on the link between DDR2 and cell proliferation, demonstrated mostly in DDR2 null mice and in cancer cells (Hammerman et al., 2011; Labrador et al., 2001; Maeyama et al., 2008; Olaso et al., 2001, 2002), the direct involvement of DDR2 in the cell cycle machinery *per se* and the relevant signalling pathways activated by it remain, to the best of our knowledge, poorly defined. Further, the protective role of DDR2 in cells exposed to ambient stress and the relevant effectors and mechanisms involved remain largely obscure.

The present study provides robust evidence for the first time that mitogen-stimulated cardiac fibroblasts exploit a common regulatory mechanism involving collagen receptor (DDR2)-dependent activation of ERK1/2 MAPK and Serum Response Factor (SRF) to achieve coordinated regulation of apoptosis resistance and cell cycle

progression, which would facilitate their survival and function in the injured myocardium.

Results:

DDR2 mediates Ang II-stimulated expression of anti-apoptotic cIAP2 in cardiac fibroblasts

The intra-cardiac generation of Ang II is enhanced following myocardial injury, which exposes the different cell types in the heart to its pleiotropic actions (Sopel et al., 2011). Paradoxically, while Ang II is reported to induce apoptosis in cardiac myocytes (Booz and Baker, 1998; Kajstura et al., 1997), it promotes cardiac fibroblast activation *post injury* and is a potent pro-fibrotic factor with marked stimulatory effect on collagen expression in cardiac fibroblasts (Sopel et al., 2011). These observations prompted us to explore the possible pro-survival role of Ang II in cardiac fibroblasts and delineate the underlying mechanisms, focusing specifically on the role of DDR2.

First, we examined the effect of Ang II on the expression of cellular Inhibitor of Apoptosis, cIAP2, which was earlier shown by us to play a key role in cardiac fibroblast resistance to oxidative stress (Philip and Shivakumar, 2013). Ang II induced a 3-fold increase in cIAP2 mRNA at 6 h (Figure 1A) and a 2-fold increase in cIAP2 protein expression at 12 h post-treatment (Figure 1B), determined by RT-qPCR and western blotting, respectively.

Notably, DDR2 knockdown in cardiac fibroblasts using specific siRNA prevented Ang II-stimulated cIAP2 expression (Figure 1C), showing that DDR2 mediates the stimulatory effect of Ang II on cIAP2 expression. Further, DDR2 over-expression in

un-stimulated cells enhanced cIAP2 expression (Figure 1D), clearly demonstrating its role in cIAP2 regulation.

DDR2-dependent ERK1/2 MAPK activation acts via Serum Response Factor to transcriptionally up-regulate cIAP2 expression in Ang II-stimulated cardiac fibroblasts

Since bioinformatic analysis of cIAP2 promoter region revealed binding sites for Serum Response Factor (SRF), we probed its possible involvement in the regulation of Ang II-dependent cIAP2 expression. SRF knockdown with specific siRNA was found to abolish cIAP2 in Ang II-stimulated cells, indicating its role in cIAP2 expression (Figure 2A). Importantly, while SRF knockdown did not affect DDR2 expression (Figure 2A), knockdown of DDR2 down-regulated SRF levels (Figure 2C), confirming that DDR2 regulates cIAP2 via SRF in Ang II-stimulated cells. Further, chromatin immunoprecipitation assay (ChIP) demonstrated SRF binding to the promoter region of cIAP2 in Ang II-treated cells, which was attenuated in DDR2-silenced cells (Figure 2B).

As previously reported by us, we observed DDR2-dependent activation of ERK1/2 MAPK in Ang II-stimulated cells (Figure 2D). Further, ERK1/2 MAPK knockdown down-regulated Ang II-stimulated SRF expression and cIAP2 expression (Figure 2E and Figure 2F), showing that DDR2 increases cIAP2 levels via ERK1/2 MAPK and SRF in Ang II-stimulated cells.

DDR2-dependent cIAP2 expression protects cardiac fibroblasts against oxidative damage

We had reported earlier that cIAP2, induced in response to H_2O_2, protects cardiac fibroblasts against oxidative damage (Philip and Shivakumar, 2013). Additionally, we

had also shown that H_2O_2 treatment promotes Ang II production in cardiac fibroblasts (Anupama et al., 2016). Pursuing these observations, we found that blocking the Ang II receptor, AT1, with candesartan attenuated cIAP2 expression (Figure 3A.) and promoted apoptosis in H_2O_2-treated cells (Figures 3B and S2A), showing that Ang II produced in response to H_2O_2 is responsible for cIAP2 induction and protection against oxidative damage. Interestingly, H_2O_2 treatment also enhanced DDR2 and SRF expression, which was abolished by candesartan (Figure 3A). Further, we found that DDR2 and SRF knockdown in H_2O_2-treated cardiac fibroblasts reduced cIAP2 expression (Figures 3C and 3D) and promoted cell death (Figures 3E and S2B). Together, these data point to the pro-survival role of Ang II in cardiac fibroblasts under oxidative stress by a mechanism involving DDR2-dependent transcriptional up-regulation of cIAP2 expression by SRF.

An obligate role for DDR2 in G_1-S transition in cardiac fibroblasts via transcriptional and post-translational regulation of p27

To evaluate the role of DDR2 in G_1-S transition, we employed a combination of gene knockdown and over-expression approaches and examined cell cycle status in relation to DDR2 expression. 10% Fetal Calf Serum was used to obtain a robust mitogenic effect, which would facilitate an unequivocal assessment of the regulatory role of DDR2 in the cardiac fibroblast cell cycle.

DDR2 knockdown in mitogen-stimulated cardiac fibroblasts inhibits G1-S transition

Sub-confluent cultures of cardiac fibroblasts were transfected with DDR2 siRNA (or control siRNA) and, following revival for 12 h in 10% serum-supplemented medium, the cells were serum-deprived for synchronization. Post synchronization, cells were

exposed to mitogenic stimulation (10% Fetal Calf Serum) and collected at either 8 h for analysis of various cell cycle regulatory molecules by western blotting or at 14 h for flow cytometric analysis of G1-S transition. Flow cytometric analysis showed that DDR2 knockdown in mitogen-stimulated cells results in cell cycle arrest at the G1 phase (Figure 4A). Further, western blot analysis showed that DDR2 knockdown in mitogen-stimulated cells results in a significant reduction in the levels of Proliferating Cell Nuclear Antigen (PCNA), an S-phase marker (Figure 4B). Skp2 is an E3 ubiquitin ligase that targets various inhibitors of G1-S transition, which includes p27 belonging to the CIP/KIP family of cyclin-dependent kinase (CDK) inhibitors (Chen et al., 2008). We found that DDR2 knockdown attenuates Skp2 levels (Figure 4B), resulting in p27 induction (Figure 4B) and Rb hypophosphorylation (Figure 4C), culminating in G1 arrest of mitogen-stimulated cells. Consistent with G1 phase arrest, cyclin E, an S-Phase cyclin, but not Cyclin D1, a G1-phase cyclin, was significantly reduced in DDR2-silenced, mitogen-stimulated cells (Figure 4D). Together, these data suggest that DDR2 has an obligate role in G1-S transition via positive regulation of Skp2 and negative regulation of p27.

Regulation of Skp2 by DDR2-dependent SRF

Based on bioinformatics analysis, the possible involvement of SRF in DDR2-dependent Skp2 expression was probed next. SRF knockdown was found to down-regulate Skp2 (Figure 5A), showing that SRF is involved in the regulation of Skp2. Following this, it was of obvious interest to examine the link between DDR2 and SRF. DDR2 knockdown in serum-stimulated cells led to down-regulation of SRF (Figure 5B). Moreover, ERK1/2 MAPK knockdown in mitogen-stimulated cells reduced SRF levels (Figure 5C). Since DDR2 promotes ERK1/2 activation (Figure

5D), these data point to DDR2-dependent regulation of SRF via ERK1/2 MAPK. Together, the findings demonstrate a role for DDR2-dependent SRF activation in the regulation of Skp2.

DDR2-dependent binding of SRF to the Skp2 gene promoter was confirmed by ChIP assay (Figure 5E).

Regulation of p27 by DDR2

i) Post-translational regulation via SRF-dependent Skp2

Since Skp2 is known to post-translationally degrade p27 (Chen et al., 2008) and DDR2-dependent SRF activation regulates Skp2 (Figures 5A-E), we examined whether SRF silencing would affect p27 levels. SRF knockdown was found to induce p27 and reduce PCNA expression in mitogen-stimulated cells (Figure 5F) and cause cell cycle arrest at the G1 phase (Figure 5G), suggesting that SRF may transcriptionally regulate Skp2 expression to degrade p27 and promote G1-S transition.

ii) Transcriptional regulation through modulating FoxO3a activity

Since p27 was found to be up-regulated in DDR2-silenced, mitogen-stimulated cells (Figure 4B), we probed its transcriptional regulation by FoxO3a transcription factor whose phosphorylation leads to its inactivation and sequestration in the cytoplasm while its non-phosphorylated form translocates to the nucleus and transcribes the p27 gene to result in G1 arrest (Pramod and Shivakumar, 2014; Santo et al., 2013; Weidinger et al., 2008; Yang and Hung, 2009; Zhang et al., 2013). We found that DDR2 knockdown in mitogen-stimulated cells reduces FoxO3a phosphorylation,

leading to its activation (Figure 6A), which corresponded with enhanced p27 and reduced PCNA levels (Figure 4B).

Consistent with these observations, chromatin immunoprecipitation showed enhanced binding of FoxO3a to the p27 gene promoter in DDR2-silenced, mitogen-stimulated cells (Figure 6B).

Subsequently, we also examined the mechanism by which DDR2 regulates FoxO3a. While DDR2 knockdown inhibited ERK1/2 MAPK in serum-stimulated cells (Figure 5D), ERK1/2 MAPK knockdown in mitogen-stimulated cells reduced FoxO3a phosphorylation (Figure 6C), resulting in enhanced p27 and reduced PCNA (Figure 6D). The data show that DDR2 promotes ERK1/2 MAPK activation to inhibit FoxO3a-dependent transcriptional induction of p27, promoting G1-S transition.

Considered in tandem, the data point to SRF/Skp2-dependent post-translational and FoxO3a-dependent transcriptional regulation of p27 by DDR2.

DDR2 over-expression in mitogen-deprived cardiac fibroblasts facilitates G_1-S transition

Cardiac fibroblasts were transfected with plasmid construct containing DDR2 cDNA driven by CMV promoter and grown under serum-free conditions. Over-expression of DDR2 in mitogen-starved cells promoted G_1-S transition, demonstrated by flow cytometry (Figure 7A). This was accompanied by enhanced Skp2 levels, reduced p27 levels, elevated cyclin D1/E levels, enhanced Rb phosphorylation and enhanced PCNA expression (Figures 7B and C). Further, DDR2 overexpression also led to ERK1/2 MAPK activation (Figure 7D), a significant increase in SRF levels (Figure 7E) and elevated Phospho-FoxO3a (inactivation) levels (Figure 7E). As expected, siRNA-mediated ERK1/2 MAPK inhibition in DDR2-overexpressing cells attenuated

PCNA expression (Figure 7F), showing that ERK1/2 MAPK mediates the DDR2 effects.

Enhanced expression of DDR2 correlates with enhanced levels of SRF, cIAP2 and PCNA in freshly isolated cardiac fibroblasts from Spontaneously Hypertensive Rats

Looking for a possible association of DDR2 with augmented cIAP2 expression and cardiac fibroblast hyperplasia in Spontaneously Hypertensive Rats (SHR), we analysed expression levels of DDR2, SRF, cIAP2 and PCNA in freshly prepared cardiac fibroblasts (following 2.5 h of pre-plating of freshly isolated cells) from 6-month old male SHR. Consistent with the in vitro findings, enhanced levels of DDR2 in cardiac fibroblasts from SHR correlated well with enhanced levels of SRF, cIAP2 and PCNA (Figure 7G). Further, while serum from both SHR and control rats enhanced the levels of DDR2, SRF, cIAP2 and PCNA in cardiac fibroblasts in vitro, serum from SHR had more pronounced effects (Figure 7H), which were reduced upon pre-treatment of the cells with candesartan.

A schematic representation of the plausible molecular events that integrate apoptosis resistance and proliferation under the regulatory control of DDR2 in cardiac fibroblasts is provided in Supplementary Figure S1A.

Discussion

Cardiac fibroblasts, an abundant cell type in the heart, are the only intracardiac source of collagen types I and III. Although their ability to survive and proliferate in the hostile ambience of the injured myocardium and generate a fibrillar collagen-rich scar underlies wound healing in the short-term, their persistence in the infarct scar due to relative resistance to apoptosis promotes adverse myocardial remodeling in

the long-term, which exacerbates disease and contributes to the progression of heart failure. A better understanding of the molecular pathways that regulate apoptosis resistance and cell cycle progression in cardiac fibroblasts can contribute to our ability to limit adverse fibrotic remodeling of the heart.

After examining several apoptotic regulators, a role for constitutively-expressed Bcl2 in protecting cardiac fibroblasts against a variety of pro-apoptotic stimuli has been reported earlier (Mayorga et al., 2004). On the other hand, we had demonstrated that augmented cIAP2 expression protects cardiac fibroblasts against oxidative damage (Philip and Shivakumar, 2013). The present study sought to analyze the role of DDR2 as a possible pro-survival factor in cardiac fibroblasts. Currently, there are only sporadic studies that report the anti-apoptotic role of DDR2 in tumour cell lines and cell types such as pulmonary fibroblast and hepatic stellate cells, with limited mechanistic insights (Duncan et al., 2012; Jia et al., 2018; Li et al., 2012; Luo et al., 2013). We provide robust evidence that Ang II enhances cIAP2 expression via DDR2-dependent activation of ERK1/2 MAPK that in turn leads to SRF activation and binding to the cIAP2 gene promoter (Figures 1 and 2). It is pertinent to point out here that both Ang II and ERK1/2 MAPK have been shown to activate SRF (Esnault et al., 2017; Hautmann Martina B. et al., 1997; Luo et al., 2006; Taurin et al., 2009). Further, SRF has been identified as an important factor in myofibroblast differentiation leading to tissue fibrosis (Chai et al., 2007; Small, 2012; Zhou et al., 2017), and is reported to play a role in cell survival and cell proliferation in other cell types (Schratt et al., 2004; Werth et al., 2010). Exploring the significance of Ang II-dependent increase in cIAP2 expression, we found that exposure of cells to oxidative stress (25uM H2O2) enhances DDR2, SRF and cIAP2 levels while knockdown of DDR2 or SRF abolishes cIAP2 expression and causes cell death under conditions of

oxidative stress (Figure 3 C, D and E). Interestingly, candesartan, the AT1 receptor antagonist, attenuated H2O2-stimulated DDR2, SRF and cIAP2 expression and compromised viability in cardiac fibroblasts (Figure 3 A and B), showing that oxidative stress induces Ang II production that in turn protects the cells against oxidative injury via DDR2-dependent cIAP2 induction. It is also noteworthy that serum from SHR had a stimulatory effect on DDR2, SRF and cIAP2 expression in cardiac fibroblasts isolated from normal rats, which was reduced by candesartan (Figure 7H), implying a role for circulating Ang II in SHR in triggering pro-survival mechanisms in cardiac fibroblasts.

These findings need to be considered in tandem with earlier reports that Ang II induces apoptosis in cardiac myocytes (Booz and Baker, 1998; Kajstura et al., 1997). The pro-apoptotic effect of Ang II on cardiac myocytes, on the one hand, and its anti-apoptotic effect on cardiac fibroblasts, on the other, constitute yet another example of "the apoptotic paradox" that was previously reported in a setting of idiopathic pulmonary fibrosis wherein TGF-β1 exerts pro-apoptotic effects on epithelial cells and anti-apoptotic effects on myofibroblasts in the lung (Thannickal and Horowitz, 2006). The pro-survival role of Ang II alongside its stimulatory effect on collagen expression in cardiac fibroblasts may facilitate fibroblast-mediated wound healing upon injury in the short-term but may also contribute to the persistence of these cells in the scar in an active state long after the termination of the healing process, which ultimately would lead to myocardial stromal expansion and pump dysfunction. Ang II is recognized to be a potent pro-fibrotic factor and its role in cardiac fibroblast function has been viewed mostly in terms of its effects on collagen turnover (Sopel et al., 2011). By focusing on the anti-apoptotic action of Ang II on cardiac fibroblasts, mediated by DDR2 signaling, this study uncovers a less

known facet of the pro-fibrotic action of Ang II and, possibly, offers an additional explanation for the beneficial effects of Ang II inhibitors that are used extensively in the clinical setting and are known to minimize adverse myocardial remodeling post myocardial injury (Hanatani et al., 1995; Oishi et al., 2006; Prescott et al., 1991; Sladek et al., 1996).

Beyond demonstrating the obligate role of DDR2 in cell survival, this study also uncovers a critical role for DDR2 in cell cycle progression in cardiac fibroblasts. While a role for DDR2 in promoting proliferation in hepatic stellate cells, skin fibroblasts, chondrocytes and cancer cell types has been demonstrated (Hammerman et al., 2011; Labrador et al., 2001; Maeyama et al., 2008; Olaso et al., 2001, 2002), an anti-proliferative and lack of proliferative effect of DDR2 have also been reported in other cell types (Kawai et al., 2012; Olaso et al., 2011; Wall et al., 2005). Moreover, these studies did not probe the direct influence of DDR2 on cell cycle regulatory elements. Against this backdrop, the present study provides evidence of the direct involvement of DDR2 in the cell cycle machinery to facilitate G1-S transition in mitogen-stimulated cardiac fibroblasts.

G1-S transition occurs following activation of cyclin-dependent kinases (CDKs) through association with cyclins, and consequent phosphorylation of the retinoblastoma protein (Rb) and E2F-dependent transcription of S-phase genes, including PCNA and Cyclin E. On the other hand, inhibition of CDK activity by cyclin-dependent kinase inhibitors (CDKIs) leads to Rb hypophosphorylation and cell cycle arrest (Henley and Dick, 2012; Malumbres and Barbacid, 2009). The abundance of p27Kip1, an important member of the Cip/Kip family of CDKIs and critical regulator of G1-S transition, is regulated by post-translational and transcriptional mechanisms. S-phase kinase-associated protein 2 (Skp2), an F-box protein of the SCF ubiquitin

ligase complex (Chen et al., 2008; Frescas and Pagano, 2008), facilitates cell cycle progression through the proteasomal degradation of p27. p27 is also regulated transcriptionally (Khattar and Kumar, 2010; Lees et al., 2008). Forkhead box O 3a (FoxO3a), a member of the Forkhead box O (FoxO) family of transcription factors, enhances p27 gene expression, and phosphorylation-dependent inhibition of FoxO3a activity facilitates cell-cycle progression through transcriptional repression of p27 in mitogen-stimulated cells (Pramod and Shivakumar, 2014; Santo et al., 2013; Weidinger et al., 2008; Yang and Hung, 2009; Zhang et al., 2013). This study focused on the regulation of these mediators by DDR2 and sought to delineate the underlying mechanisms.

Flow cytometry demonstrated that DDR2 knockdown in mitogen-stimulated cells causes cell cycle arrest at the G1 phase (Figure 4A), accompanied by a significant reduction in the levels of PCNA, along with reduced Skp2 levels, induction of p27 (Figure 4B), and hypophosphorylation of Rb (Figure 4C). Further, cyclin E but not cyclin D1 was reduced in DDR2-silenced, mitogen-stimulated cells (Figure 4D). Cyclin D1 serves as a key sensor and integrator of extracellular signals of cells in early to mid-G_1 phase whereas cyclin E is synthesized during progression to S phase and it associates with Cdk2 and activates its kinase activity shortly before entry of cells into the S phase (Obaya and Sedivy, 2002)**.** Thus, our data suggest that DDR2 may act at the G1 phase, preventing S phase entry by inhibiting Cyclin D1/CDK activation through p27 induction. Additional evidence of an obligate role for DDR2 in G1-S transition came from experiments showing that over-expression of DDR2 in mitogen-deprived cells increases the expression of cyclin D1, cyclin E and Skp2, reduced p27 and promoted Rb phosphorylation, culminating in enhanced PCNA expression and G1-S transition (Figure 7A-F). It is noteworthy that, while

DDR2 over-expression in mitogen-starved cells induced cyclin D1, DDR2 knockdown in mitogen-stimulated cells did not inhibit cyclin D1 expression, indicating that serum may overcome the effect of DDR2 knockdown, a possibility that warrants further investigation.

Focusing on the mechanisms involved in cell cycle regulation by DDR2, we found that DDR2 facilitates G1-S transition through FoxO3a-mediated transcriptional and SRF/Skp2-mediated post-translational inhibition of p27 expression. Our findings showed that DDR2 knockdown in mitogen-stimulated cells leads to inhibition of ERK1/2 activity (Figure 5D) and consequent FoxO3a hypophosphorylation (activation) (Figure 6 A and C), which in turn transcriptionally enhances p27 expression, as shown by FoxO3a binding to the p27 gene promoter (Figure 6B). Thus, DDR2 acts as a negative regulator of FoxO3a. Further, while DDR2 knockdown in mitogen-stimulated cells resulted in ERK1/2 MAPK inhibition (Figure 5D) and consequent down-regulation of SRF (Figure 5 B and C), SRF knockdown reduced Skp2 levels (Figure 5A), resulting in increased p27, reduced PCNA and G1-S transition (Figure 5 F and G). Together with the observation that over-expression of DDR2 in mitogen-deprived cells increases the expression of SRF and Skp2 (Figure 7B), these data clearly show that DDR2 is a positive regulator of Skp2, as confirmed by SRF binding to the Skp2 gene promoter, which was abolished upon DDR2 silencing (Figure 5E). To the best of our knowledge, there is only a single report showing the link between SRF and Skp2 expression (Werth et al., 2010). Together, the data demonstrate that DDR2-induced ERK1/2 MAPK activation promotes transcriptional repression of p27 through FoxO3a phosphorylation and SRF-mediated post-translational degradation of p27 by Skp2.

It is pertinent to note that beyond demonstrating the obligate role of DDR2 in cell survival and cell cycle progression in cardiac fibroblasts, this study uncovers a common pathway of regulation of these processes wherein the DDR2-ERK1/2 MAPK-SRF signaling pathway regulates apoptosis resistance via cIAP2 and cell cycle progression via Skp2 that promotes proteasomal degradation of p27 to facilitate Rb phosphorylation and G1-S transition.

The role of DDR2 as an important determinant of cardiac size and organ growth was previously demonstrated in DDR2-null mice (Cowling et al., 2014). Investigations on this mouse model had demonstrated, by echocardiography, reduced left ventricular chamber dimensions and reduced cardiomyocyte length, resulting in decreased heart size and weight. Moreover, the study reported a reduction in cardiac interstitial collagen density at baseline and reduced rates of collagen synthesis in cardiac fibroblasts isolated from DDR2-null mice, compared to the wild type littermate fibroblasts. In the present study, the link between DDR2 and factors that mediate apoptosis resistance and G1-transition in an in vivo setting of hypertension-induced myocardial disease underscored the role of DDR2 in regulating cardiac fibroblast growth. We found significantly increased levels of DDR2 that correlated well with increased levels of SRF, cIAP2 and PCNA in cardiac fibroblasts freshly isolated from Spontaneously Hypertensive Rats (Figure 7G), which present a genetic model of hypertensive heart disease with a role for the renin-angiotensin system in mediating the pathological changes in the myocardium (Gouldsborough et al., 2003). Interestingly, serum from SHR induced the expression of DDR2, SRF and PCNA in cardiac fibroblasts from normal rats, which was attenuated by candesartan (Figure 7H), indicating the effect of circulating Ang II on cardiac fibroblasts in SHR rats.

In summary, cardiac fibroblasts are major contributors to tissue repair following acute cardiac injury, and to cardiac fibrogenesis associated with most forms of chronic heart disease. Phenotypic transformation, proliferation, collagen production and persistence *post healing* due to apoptosis resistance are distinct attributes of cardiac fibroblasts that are key to their pivotal role in myocardial pathophysiology. Identification of cardiac fibroblast-specific factors and their downstream effectors that regulate these processes is therefore of considerable scientific interest with obvious clinical relevance. Our earlier studies had demonstrated an obligate role for DDR2 in collagen expression in cardiac fibroblasts (George et al., 2016) and in their phenotypic transformation in response to Ang II (unpublished data). Further, in this communication, we present evidence that DDR2-dependent ERK1/2 MAPK activation facilitates the coordinated regulation of apoptosis resistance and cell cycle progression in cardiac fibroblasts via SRF. Together, the data place these cardinal aspects of cardiac fibroblast function within a single mechanistic framework of molecular pathways under the regulatory control of DDR2, which defines the fibroblast phenotype and acts as a 'master switch' in these cells. The predominant localization of DDR2 in cardiac fibroblasts and its regulatory role in cardiac fibroblast function lend support to the postulation (George et al., 2016) that it is a potential drug target in the control of cardiac fibroblast-mediated adverse tissue remodeling.

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Declaration of interests: None

Figure legends:

Figure 1: DDR2 mediates Ang II-stimulated expression of anti-apoptotic cIAP2 in cardiac fibroblasts. Sub-confluent quiescent cultures of cardiac fibroblasts were stimulated with Angiotensin II (Ang II) (1μM). **(A)** cIAP2 mRNA levels were determined by Taqman Real-time PCR analysis at 6 h of Ang II treatment. β-actin served as the endogenous control. *p<0.05 vs. control. (**B**) Protein was isolated at 12 h of Ang II treatment and subjected to western blot analysis for detection of c-IAP2, with β-actin as loading control. *p< 0.05 vs. control). (**C**) RNAi-mediated silencing of DDR2 confirmed its role in regulating cIAP2 gene expression in Ang II-stimulated cardiac fibroblasts. Cardiac fibroblasts were transiently transfected with DDR2 siRNA (5 pmol) or control (scrambled) siRNA prior to treatment with Ang II for 12 h. cIAP2 protein expression was examined, with β-actin as loading control. Validation of DDR2 silencing is also shown. ** p< 0.01 (comparisons as depicted in the Figure). (**D**) Cardiac fibroblasts were transfected with DDR2 cDNA over-expression plasmid (DDR2OE) (with empty vector control, Control OE), post-revival, the cells were serum-deprived for 24 h. Cells were collected and cIAP2 protein

expression was examined, with β-actin as loading control. ** p< 0.01 (comparisons as depicted in the Figure). Validation of DDR2 over-expression is also shown. Data are representative of 3 independent experiments, n=3. Mean ± SEM (Standard Error of Mean).

Figure 2: DDR2-dependent ERK1/2 MAPK activation acts via SRF to transcriptionally up-regulate cIAP2 expression in Ang II-stimulated cardiac fibroblasts. (**A**) Sub-confluent quiescent cultures of cardiac fibroblasts were transiently transfected with SRF siRNA (10 pmol) or control (scrambled) siRNA prior to treatment with Ang II (1μM) for 12 h. DDR2 and cIAP2 protein expression was examined by western blot analysis, with β-actin as loading control. ** p< 0.01 (comparisons as depicted in the Figure). (**B-D**) Sub-confluent quiescent cultures of cardiac fibroblasts in M199 were transiently transfected with DDR2 siRNA (5pmol) or control (scrambled) siRNA prior to treatment with Ang II (1μM). (**B**) Cells were collected at 3 h post-Ang II treatment and chromatin was immunoprecipitated by anti-SRF antibody followed by PCR amplification and analysed on a 2% agarose gel for presence of 169bp region of cIAP2 gene promoter (**C**) Cells were collected at 12 h post-Ang II treatment and SRF protein expression was examined by western blot analysis, with β-actin as loading control. * p< 0.05 (comparisons as depicted in the Figure). (**D**) Cells were collected at 12 h post-Ang II treatment and phospho-ERK1/2 protein level was examined by western blot analysis, with total ERK1/2 level as loading control.** p< 0.01 (comparisons as depicted in the Figure). (**E-F**) Sub-confluent quiescent cultures of cardiac fibroblasts in M199 were transiently transfected with ERK1/2 siRNA (10pmol) or control (scrambled) siRNA prior to treatment with Ang II (1μM). (**E**) Cells were collected at 12 h post-Ang II treatment

and SRF protein expression was examined by western blot analysis, with β-actin as loading control. * p< 0.05 (comparisons as depicted in the Figure).. ERK1/2 knockdown validation is also shown. (**F**) Cells were collected at 12 h post-Ang II treatment and cIAP2 protein expression was examined by western blot analysis, with β-actin as loading control. * p< 0.05 (comparisons as depicted in the Figure). ERK1/2 knockdown validation is shown. Data are representative of 3 independent experiments, n=3. Mean ± SEM (Standard Error of Mean).

Figure 3: DDR2-dependent cIAP2 expression protects cardiac fibroblasts against oxidative damage. (**A-B**) Effect of AT1 receptor antagonist, Candesartan, on H_2O_2-treated cardiac fibroblasts was analysed. Four sets of sub-confluent quiescent cultures of cardiac fibroblasts were serum-deprived for 24 h: i) no treatment (control), ii) 25μM H_2O_2 iii) pre-incubated with 10uM candesartan (AT1 receptor antagonist) for 1 h and was treated with 25μM H_2O_2 iv) 10uM candesartan alone. (**A**) Cells were collected 12 h post-H_2O_2 addition and analysed by western blot for the expression of cIAP2, DDR2 and SRF, with β-actin as loading control.* p< 0.05 and ** p< 0.01 (comparisons as depicted in the Figure). (**B**) Cells were collected 3 h post-H_2O_2 addition and analysed by flow cytometry for annexin/PI uptake and represented as percentage of cells. Live cells in 25μM H_2O_2 + 10uM candesartan *p<0.05 vs control and candesartan alone. Apoptotic cells (early apoptotic + late apoptotic cells) in 25μM H_2O_2 + 10uM candesartan *p<0.05 vs control and candesartan alone. Necrotic cells were few in number (see also Supplementary Figure S2 A). (C**-E**) Effect of DDR2 or SRF gene silencing on H_2O_2-treated cardiac fibroblasts was analysed. Sub-confluent quiescent cultures of cardiac fibroblasts in M199 were transiently transfected with DDR2 siRNA (5pmol) or SRF siRNA(10pmol)

with respective control (scrambled) siRNA prior to treatment with25μM H_2O_2. (**C and D**) Cells were collected 12 h post-H_2O_2 addition and analysed by western blot for the expression of cIAP2, with β-actin as loading control. ** p< 0.01 (comparisons as depicted in the Figure). (**E**) Cells were collected 3 h post-H_2O_2 addition and analysed by flow cytometry for annexin/PI uptake and represented as percentage of cells. (* vs control + control siRNA, # vs 25μM H_2O_2+ control siRNA) *, # - p <0.05; **, ## - p< 0.01 and ***, ### -p<<0.01(see also Supplementary Figure S2 B). Data are representative of 3 independent experiments, n=3. Mean ± SEM (Standard Error of Mean).

Figure 4: DDR2 knockdown in mitogen-stimulated cardiac fibroblasts inhibits G1-S transition. (A-D) Sub-confluent cultures of cardiac fibroblasts were transfected with DDR2 siRNA (or control siRNA) and, following revival for 12 h in 10% serum-supplemented medium, the cells were serum-deprived for synchronization. Post synchronization, cells were exposed to mitogenic stimulation (10% Fetal Calf Serum). (**A**) Cells were collected at 14 h for flow cytometric analysis of G1-S transition showing distribution of cells in each phase in percentage. ** p<0.01 vs no mitogen + control siRNA, ## p< 0.01 vs mitogen-stimulated + control siRNA. (**B**) Cells were collected at 8 h and analysed by western blotting for expression levels of PCNA, Skp2 and p27, with β-actin as loading control.** p< 0.01 (comparisons as depicted in the Figure). (**C**) Cells were collected at 8 h and analysed by western blotting for expression levels of Phospho Rb, with total Rb as loading control.** p< 0.01 (comparisons as depicted in the Figure). (**D**) Cells were collected at 8 h and analysed by western blotting for expression levels of Cyclin E and Cyclin D1, with β-actin as loading control.** or ## p< 0.01 (comparisons as depicted in the Figure).

Data are representative of 3 independent experiments, n=3. Mean ± SEM (Standard Error of Mean).

Figure 5: Regulation of Skp2 by DDR2-dependent SRF. (**A-G**) Sub-confluent cultures of cardiac fibroblasts were transfected with SRF siRNA (**A, F and G**), ERK1/2 siRNA (**C**) or DDR2 siRNA (**B, D** and **E**) (with control siRNA) and, following revival for 12 h in 10% serum-supplemented medium, the cells were serum-deprived for synchronization. Post synchronization, cells were exposed to mitogenic stimulation (10% Fetal Calf Serum). (**A**) SRF siRNA transfected cells were collected at 8 h and analysed by western blotting for expression levels of Skp2, with β-actin as loading control.* $p<0.05$ and ** $p< 0.01$ (comparisons as depicted in the Figure). Validation of SRF knockdown is also shown. (**B**) DDR2 siRNA transfected cells were collected at 8 h and analysed by western blotting for expression levels of SRF, with β-actin as loading control. ** $p<0.01$(comparisons as depicted in the Figure). Validation of DDR2 knockdown is also shown, (**C**) ERK1/2 MAPK siRNA-transfected cells were collected at 8 h and analysed by western blotting for expression levels of SRF, with β-actin as loading control. * $p<0.05$ (comparisons as depicted in the Figure). Validation of ERK1/2 MAPK knockdown is also shown. (**D**) DDR2 siRNA transfected cells were collected at 8 h and phospho-ERK1/2 protein level was examined by western blot analysis, with total ERK1/2 level as loading control. ** $p< 0.01$ (comparisons as depicted in the Figure). (**E**) Sub-confluent cultures of cardiac fibroblasts were transfected with DDR2 siRNA (or control siRNA) and, following revival for 12 h, cells were synchronized and exposed to mitogenic stimulation (10% Fetal Calf Serum). Cells were collected at 3 h and chromatin was immunoprecipitated using anti-SRF antibody and the image shows PCR amplified 150bp region of the Skp2 gene promoter on 2% agarose gel.

Regulation of p27 by DDR2: i) Post-translational regulation of p27 via SRF-dependent Skp2. (**F**) SRF siRNA transfected cells were collected at 8 h and analysed by western blotting for expression levels of PCNA and p27, with β-actin as loading control. * $p<0.05$, ** $p< 0.01$ for PCNA and ## $p<0.01$ for p27 (comparisons as depicted in the Figure). (**G**) Cells were collected at 14 h for flow cytometric analysis of G1-S transition showing distribution of cells in each phase in percentage. ** $p<0.01$ vs no mitogen + control siRNA, ## $p< 0.01$ vs mitogen-stimulated + control siRNA. Data are representative of 3 independent experiments, n=3. Mean ± SEM (Standard Error of Mean).

Figure 6: Regulation of p27 by DDR2: ii) Transcriptional regulation through modulating FoxO3a activity. (**A-F**) Sub-confluent cultures of cardiac fibroblasts were transfected with DDR2 siRNA (**A** and **B**) or ERK1/2 siRNA (**C and D**) (with control siRNA) and, following revival for 12 h in 10% serum-supplemented medium, the cells were serum-deprived for synchronization. Post synchronization, cells were exposed to mitogenic stimulation (10% Fetal Calf Serum). (**A**) DDR2 siRNA transfected cells were collected at 8 h and analysed by western blotting for expression levels of Phospho-FoxO3a (T32)/total FoxO3a, with β-actin as loading control. ** $p< 0.01$ (comparisons as depicted in the Figure). Validation of DDR2 knockdown is also shown. (**B**) DDR2 siRNA transfected cells were collected at 3 h and chromatin was immunoprecipitated using anti-FoxO3a antibody followed by PCR amplification and analysed on a 2% agarose gel for presence of 280bp region of p27 gene promoter. (**C**) ERK1/2 MAPK siRNA transfected cells were collected at 8 h and analysed by western blotting for expression levels of Phospho-FoxO3a (T32)/total FoxO3a, with β-actin as loading control. ** $p< 0.01$ (comparisons as depicted in the Figure). Validation of ERK1/2 MAPK knockdown is also shown. (**D**) ERK1/2 MAPK

siRNA transfected cells were collected at 8 h and analysed by western blotting for expression levels of p27 and PCNA, with β-actin as loading control. ** p<0.01 for p27 or ## p< 0.01 for PCNA (comparisons as depicted in the Figure). Data are representative of 3 independent experiments, n=3. Mean ± SEM (Standard Error of Mean).

Figure 7: DDR2 over-expression in mitogen-deprived cardiac fibroblasts facilitates G1-S transition. (**A-E**) Cardiac fibroblasts transfected with DDR2 cDNA over-expression plasmid (DDR2OE) or empty vector control (Control OE) were subjected to western blot analysis (with β-actin as loading control) or flow cytometric analysis. Validation of DDR2 over-expression is also shown. (**A**) Flow cytometric profile of G1-S transition showing distribution of cells in each phase, ** p<0.01 vs control OE. (**B**) p27, Skp2 and PCNA protein levels were examined,** p< 0.01vs control OE. (**C**) Cyclin E, Cyclin D1 and P-Rb/Total Rb protein levels were examined by western blot analysis,** p< 0.01vs control OE. (**D**) phospho-ERK1/2 / total ERK1/2 MAPK protein level, * p< 0.05 vs control OE. (**E**) phospho-FoxO3a/Total FoxO3a and SRF protein level, * p< 0.05 and ** p< 0.01 vs control OE. (**F**) Cardiac fibroblasts were co-transfected with DDR2 cDNA over-expression plasmid (DDR2OE) with empty vector control (Control OE) and ERK1/2 MAPK siRNA (with control siRNA), post revival, the cells were serum-deprived for 24 h and collected and subjected to western blot analysis for protein levels of PCNA (with β-actin as loading control).* p<0.05 and ** p< 0.01 (comparisons as depicted in the Figure). DDR2 over-expression validation and validation of ERK1/2 MAPK knockdown is also shown.

Enhanced expression of DDR2 correlates with enhanced levels of SRF, cIAP2 and PCNA in freshly isolated cardiac fibroblasts from Spontaneously Hypertensive Rats.

(**G**) Cardiac fibroblasts freshly isolated cells from 6-month old male SHR and corresponding Wistar rats were analysed by western blotting for levels of DDR2, SRF, cIAP2 and PCNA, with β-actin as loading control. ** p< 0.01 vs Wistar. (**H**) Synchronized sub-confluent cultures of cardiac fibroblasts from Wistar rats were exposed to serum obtained from male SHR or Wistar rats (6-months) with or without pre-incubation with 10μM candesartan. After 12 h incubation, DDR2, SRF, PCNA and cIAP2 protein levels were examined in these cells, with β-actin as loading control. **p< 0.01(comparisons as depicted in the Figure). Data are representative of 3 independent experiments, n=3. Mean ± SEM (Standard Error of Mean).

Materials and Methods:

Materials

Angiotensin II, CCG-1423, Candesartan cilexetil and M199 were obtained from Sigma-Aldrich, (St. Louis, MO, USA). Random primers, reverse transcriptase, RNAase inhibitor, dNTPs and were obtained from Promega (Madison, WI, USA). The PureLink RNA isolation kit and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA, USA). The Low cell# ChIP kit protein A × 48 was from Diagenode (Denville, NJ, USA). TaqMan probes for mRNA expression and Chemiluminescence western blot detection reagent were from Thermo Fisher Scientific (Waltham, MA, USA). DDR2 and control siRNAs were from Ambion (Foster City, CA, USA). SRF siRNAs were custom-designed from Eurogentec (Liege, Belgium). Signal Silence ® P44/42 MAPK (ERK1/2) siRNA was obtained from Cell Signaling Technology (Danvers,MA,USA).The rat DDR2/CD167b Gene ORF cDNA clone expression plasmid was obtained from Sino Biologicals (Beijing, China). Opti-MEM and fetal Calf

serum (FCS) were from GIBCO (Waltham, MA, USA). All cell culture ware was purchased from BD Falcon (Corning, NY USA). Primary antibodies against DDR2, p27KIP1, extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase (MAPK), Total FoxO3a and Phospho-FoxO3a were obtained from Cell Signaling Technology (Danvers, MA, USA). The primary antibodies for cIAP2, Cyclin D1, Cyclin E, Skp2, were from Santa Cruz Biotechnology (Dallas, TX, USA). Rb and Phospho-Rb antibodies were purchased from Elabscience (Houston, TX, USA). Primary antibodyagainst SRF (Serum Response Factor) was obtained fromThermo Fisher Scientific (Waltham, MA, USA). Loading control β-Actin antibody was obtained from Sigma-Aldrich, (St. Louis, MO, USA). All antibodies were used after dilution (1:1000), except SRF (1:50) and FoxO3a (1:50) for chromatin immunoprecipitation (ChIP). XBT X-ray Film was from Carestream (Rochester, NY, USA). The study on rats was approved by the Institutional Animal Ethics Committees of SCTIMST (B form No: SCT/IAEC-233/AUGUST/2017/94 and SCT/IAEC-268/FEBRUARY/2018/95).

METHODS:

Isolation of cardiac fibroblasts

Cardiac fibroblasts were isolated from young adult male Sprague–Dawley rats (2–3 months old) as described earlier (Kumaran and Shivakumar, 2002). Subconfluent cultures of cardiac fibroblasts from passage 2 or 3 were used for the experiments. Cells were serum-deprived for 24 h prior to treatment with 1μM Ang II, 25μM H_2O_2 or 10% Fetal Calf Serum (mitogen). Cells were pre-incubated with 10μM Candesartan (AT1 receptor antagonist) for 1 h before the addition of 25μM H_2O_2 in the appropriate group.

Cardiac fibroblasts were also isolated from 6 month-old male Wistar and Spontaneously Hypertensive Rats (SHR) as described earlier (Kumaran and Shivakumar, 2002). Cells were collected after a brief wash 2.5 h post initial plating to obtain cardiac fibroblasts. These cells were collected and processed further to analyse the expression of various genes and proteins.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis

Subconfluent cultures of cardiac fibroblasts were subjected to the indicated treatments and total RNA was isolated using the PureLink RNA isolation kit (Invitrogen), according to the manufacturer's instructions. Following DNase I treatment, 2µg of total RNA was reverse transcribed to cDNA with random primers and M-MLV reverse transcriptase. TaqMan RT-qPCR analysis was carried out using the ABI prism 7500 Sequence Detection System (Applied Biosystems, CA, USA) with specific FAM-labeled probes for cIAP2(Birc2) (Assay ID: Rn00572734_m1), and VIC-labeled probes for β-actin (Rn00667869_m1). PCR reactions were performed under the following thermal cycling conditions: 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Gene expression was quantified using C_T values. mRNA expression was normalized to that of β-actin. The relative fold-change in target mRNA levels of treated versus control was quantified using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Subconfluent cultures of cardiac fibroblasts in serum-free M199 were treated with Ang II (1µM), or 10% Fetal Calf Serum (mitogen) and relative protein abundance was determined by western blot analysis following standard protocols, with β-actin as

loading control. Enhanced chemiluminescence reagent was used to detect the proteins with X-ray Film.

RNA interference and overexpression

Cardiac fibroblasts at passage 3 were seeded on 60mm dishes at equal density. After 24 h, the cells were incubated in Opti-MEM for 5–6 h with Ambion pre-designed Silencer-Select siRNA, custom-designed siRNA from Eurogentech or scrambled siRNA (control siRNA) at the given concentrations (10nM for DDR2, 20nM for SRF and ERK1/2 MAPK) and Lipofectamine 2000 (8µl).

Constitutive expression of DDR2 was achieved under the control of a CMV promoter. The DDR2 plasmid was verified by restriction mapping. For overexpression, the plasmid vector for DDR2 (1µg/µl) was transfected using Lipofectamine 2000. Following a post-transfection recovery phase in M199 with 10% FCS for 12 h, the cells were serum-deprived for 24 h and then treated with Ang II (1µM) or 10% FCS for the indicated durations. Cell lysates were prepared in Laemmli sample buffer, denatured and used for western blot analysis.

Chromatin Immunoprecipitation (ChIP) assay

The ChIP assay was performed with the Low Cell Number ChIP kit, according to the manufacturer's protocol. Briefly, after treatment of cardiac fibroblasts with 1µM Ang II or 10% FCS for 30 min, the cells were cross-linked with 1% formaldehyde, lysed and sonicated in a Diagenode Bioruptor to generate ~600 bp DNA fragments. The lysates were incubated with anti-SRF or anti-FoxO3a antibody overnight at 4°C with rotation. Immune complexes were precipitated with protein A-coated magnetic beads. After digestion with proteinase K to remove the DNA-protein cross-links from the immune complexes, the DNA was isolated and subjected to PCR using primers

for the specific promoter regions. In samples immunoprecipitated with the SRF antibody, the cIAP2 promoter region was amplified using FP-5'-AAGGGGTAAAAGATTTGAGG-3' and RP-5'-CTATCAACATTGGAGACCAAG-3' and Skp2 promoter region was amplified using FP- 5' AGGACAGCCAAGACTACAAAG 3' and RP- 5' ATAACAGGCAAATGACCCTTC 3'. In samples immunoprecipitated with the FoxO3a antibody, p27 promoter region was amplified with FP-5' GAGACGTGGGGCGTAGAATAC 3' and RP- 5' ATTCGGGGAACCGTCTGAAAC 3'. DNA isolated from an aliquot of the total sheared chromatin was used as loading control for PCR (input control). ChIP with a non-specific antibody (normal rabbit IgG) served as negative control. The PCR products were subjected to electrophoresis on a 2% agarose gel.

Effect of serum from SHR on normal cardiac fibroblasts

Preparation of serum: Blood was collected from the descending aorta of anesthetized rats, and serum was separated by centrifugation. For experiments with cardiac fibroblasts, serum was filtered through 0.22µm membrane and used fresh. Cardiac fibroblasts from Wistar rats were exposed to serum from Wistar rats and SHR to achieve a final serum concentration of 10% in Medium M199.

Flow cytometry

Annexin V- PI staining

Cells were trypsinized, washed twice with cold PBS and re-suspended in 1X Binding Buffer at a concentration of 1 x 10^6 cells/ml. 5 µl of FITC Annexin V and 5 µl PI were added to 1 x 10^5 cells/100ul in a 5 ml culture tube, gently vortexed and incubated for 15 min at RT (25°C) in the dark. Cells were analyzed by flow cytometry after adding

400 µl of 1X Binding Buffer to each tube. Unstained cells, cells stained with only FITC Annexin V and cells stained with only PI were used as controls to set up compensation and quadrants. Flow cytometry was perfomed using BD FACSJazz™ Cell Sorter.

Cell cycle analysis

Cells were trypsinized, washed twice with cold PBS, re-suspended in 500ul PBS, fixed with an equal volume of 70% ethanol and stored at 4^0C overnight. The cells were pelleted, washed twice in 1xPBS, re-suspended in 500ul 1xPBS and incubated at room temperature for 30 minutes with 50ug of RNase A. Following incubation with 200ul of Propidium Iodide in the dark for 15 min, the cells were analyzed by flow cytometry on BD FACSJazz™ Cell Sorter.

Statistical analysis

Data are expressed as Mean±SE. Statistical analysis was performed using Student's *t* test for comparisons involving 2 groups. For comparisons involving more than 2 groups, the data were analyzed by one-way ANOVA. $p \leq 0.05$ was considered significant. The in vitro data presented are representative of 3 independent experiments (n=3). 3 age-matched (6 months) male SHR and Wistar rats were used in the in vivo experiments (n=3).

Supplementary Information:

***Supplementary Figure S1: A schematic representation of the plausible molecular events that integrate apoptosis resistance and proliferation under the regulatory control of DDR2 in cardiac fibroblasts*.**

A) In response to a stimulus (Ang II or 10% FCS), DDR2-dependent activation of ERK1/2 MAPK activates SRF to: i) transcriptionally increase cIAP2, conferring apoptosis resistance, ii) increase Skp2 and promote Skp2-mediated *post-translational* degradation of p27, and iii) inactivate FoxO3a through phosphorylation to transcriptionally repress p27. Transcriptional and post-translational inhibition of p27 results in CyclinD1/$CDK_{4/6}$ complex-dependent phosphorylation of Rb protein, facilitating the transcription of S-phase genes and G1-S transition.

Supplementary Figure S2: Representative scatter plot images of annexin-PI by flow cytometry. (**A**) Effect of AT1 receptor antagonist, Candesartan, on H_2O_2-treated cardiac fibroblasts was analysed. Four sets of sub-confluent, quiescent cultures of cardiac fibroblasts were serum-deprived for 24 h: i) no treatment (control), ii) 25μM H_2O_2 iii) pre-incubated with 10uM Candesartan for 1 h and treated with 25μM H_2O_2 iv) 10uM Candesartan. Cells were collected 3 h post-H_2O_2 addition and analysed by flow cytometry for annexin/PI uptake. The images are representative scatter plots for **Figure 3C**. (**B**) Effect of DDR2 or SRF gene silencing on H_2O_2-treated cardiac fibroblasts was analysed. Sub-confluent quiescent cultures of cardiac fibroblasts in M199 were transiently transfected with DDR2 siRNA (5pmol) or SRF siRNA(10pmol) with respective control (scrambled) siRNA prior to treatment with 25μM H_2O_2. Cells were collected 3 h post-H_2O_2 addition and analysed by flow cytometry for annexin/PI uptake. The images are representative scatter plots for **Figure 3E.**

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Figure 1: DDR2 mediates Ang II-stimulated expression of anti-apoptotic cIAP2 in cardiac fibroblasts

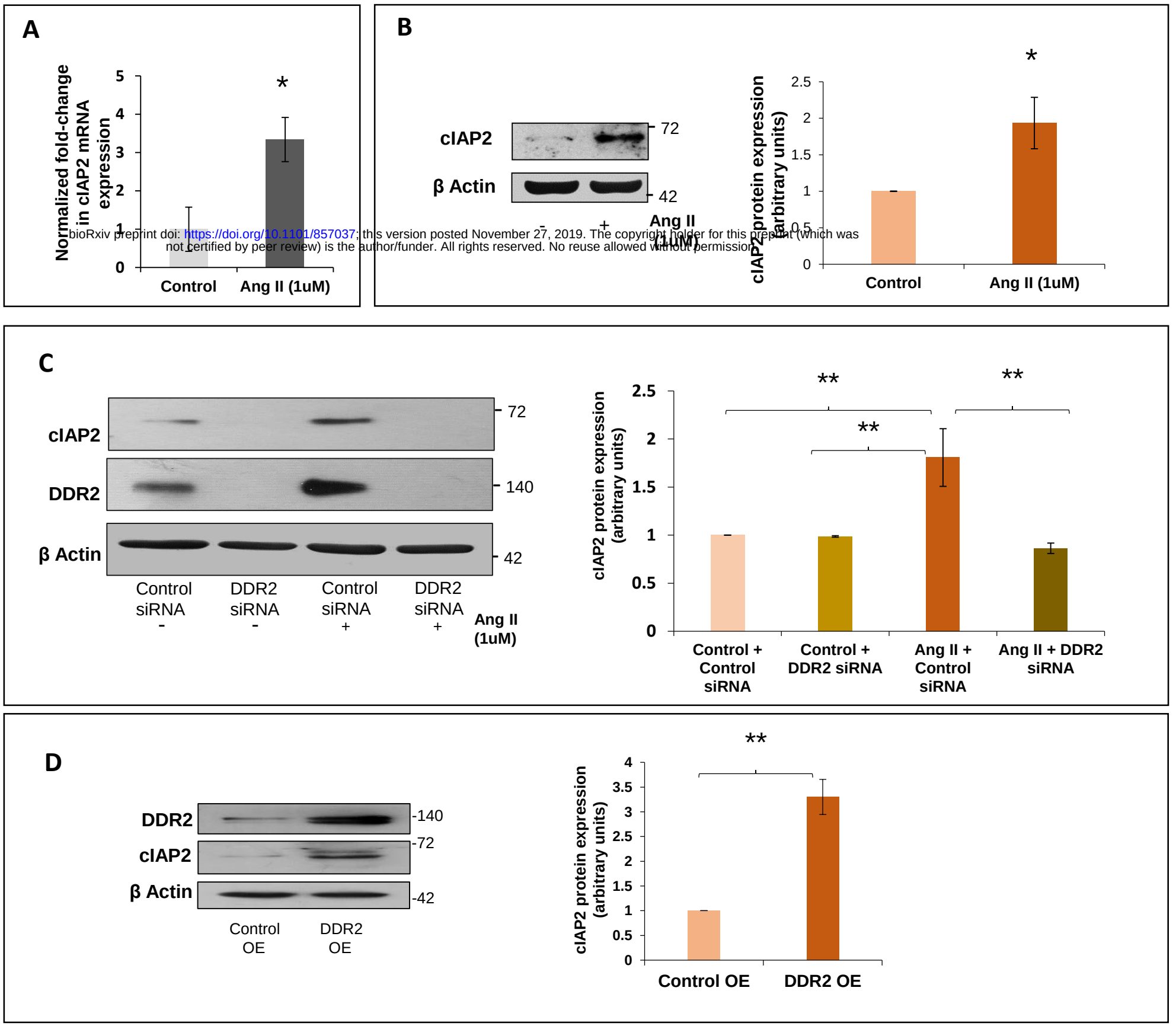
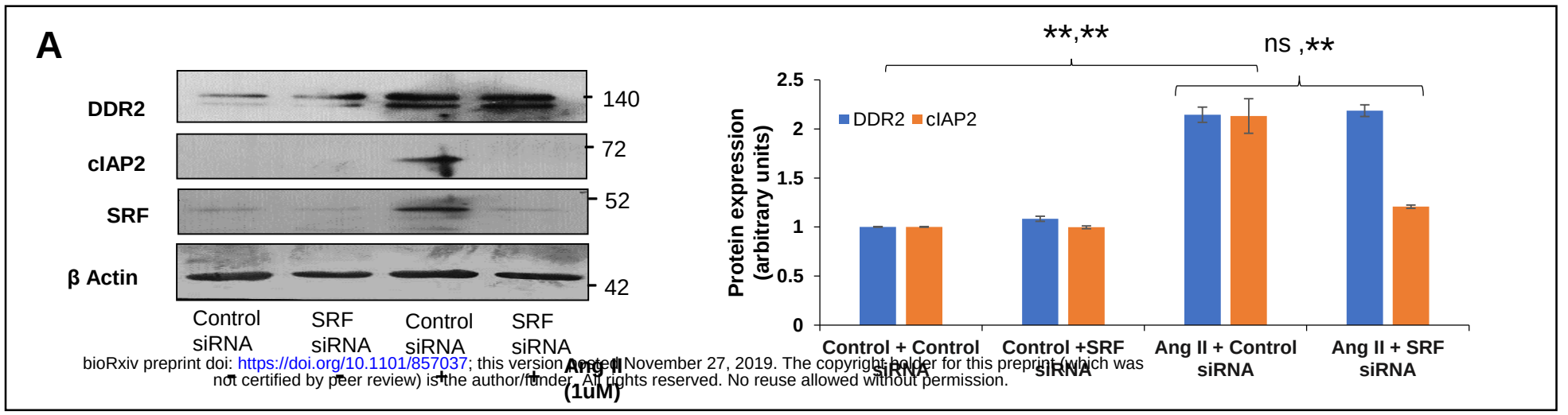
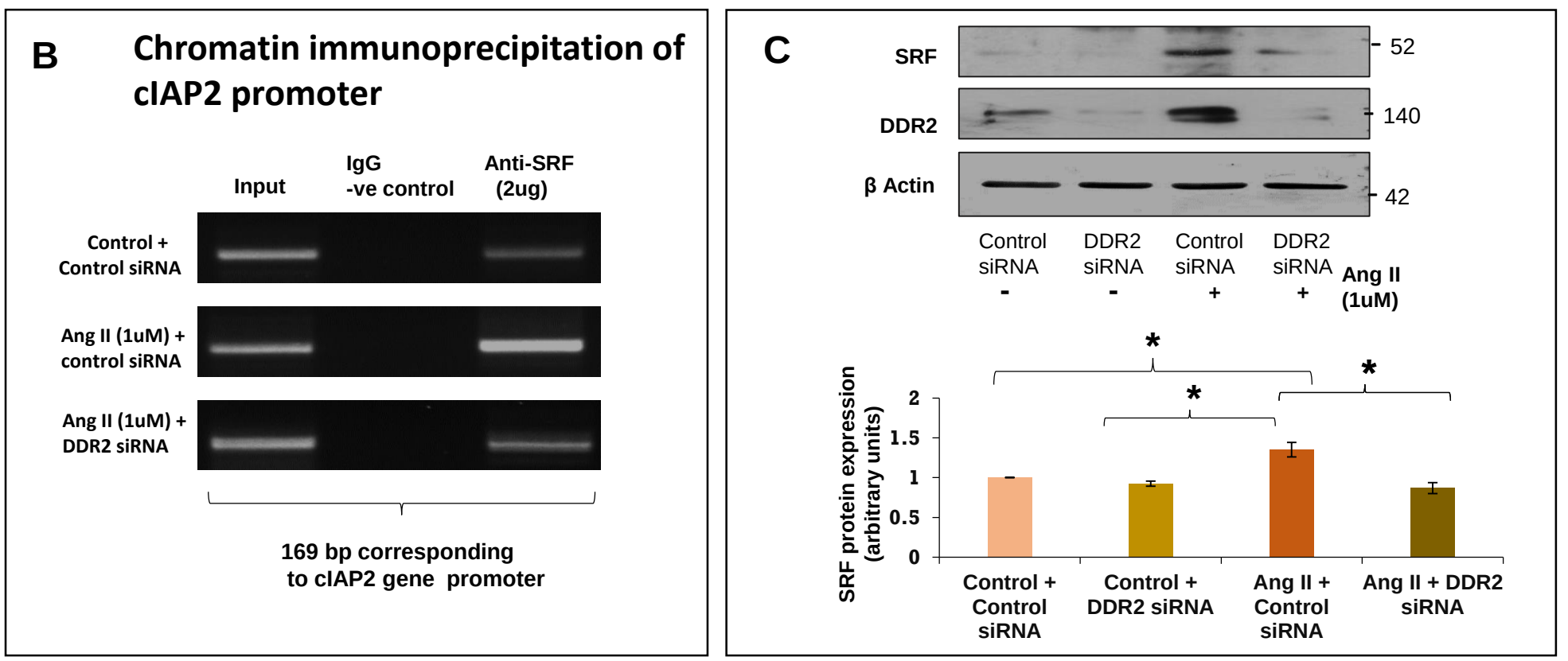
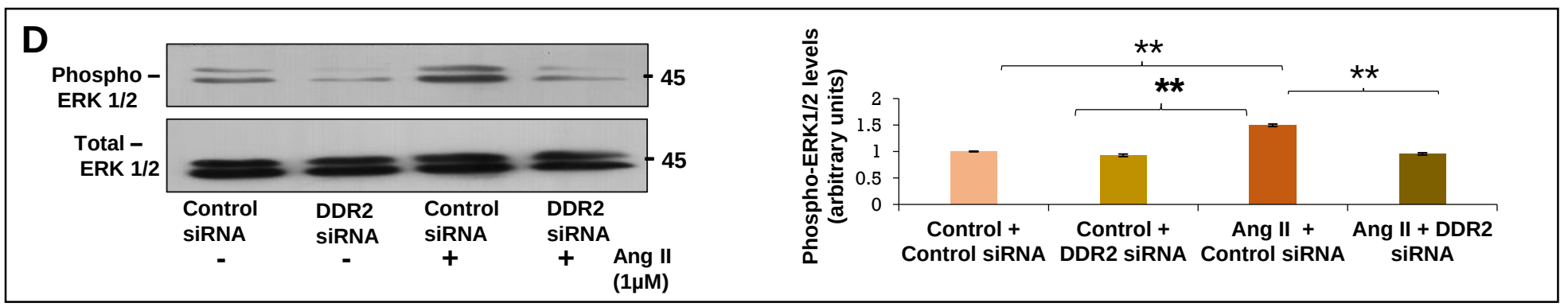
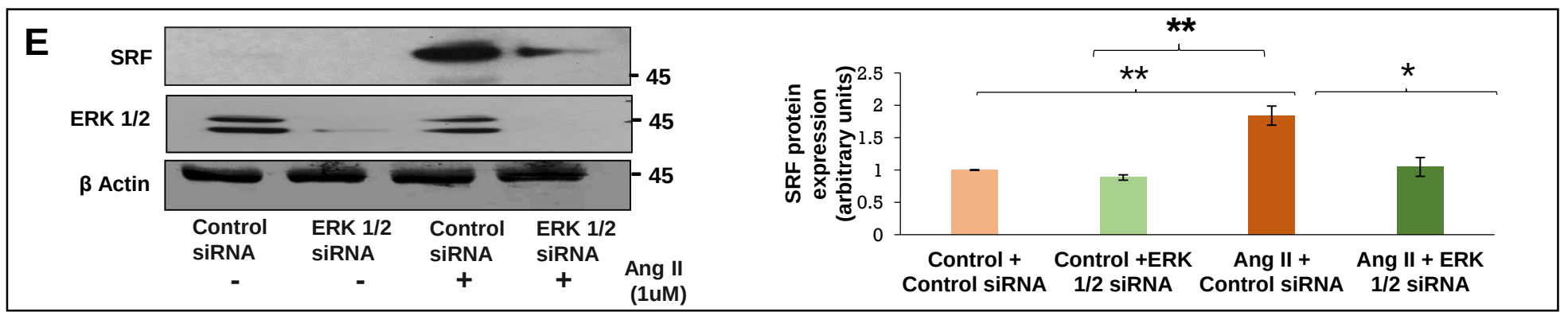


Figure 2: *DDR2-dependent ERK1/2 MAPK activation acts via SRF to transcriptionally up-regulate cIAP2 expression in Ang II-stimulated cardiac fibroblasts*









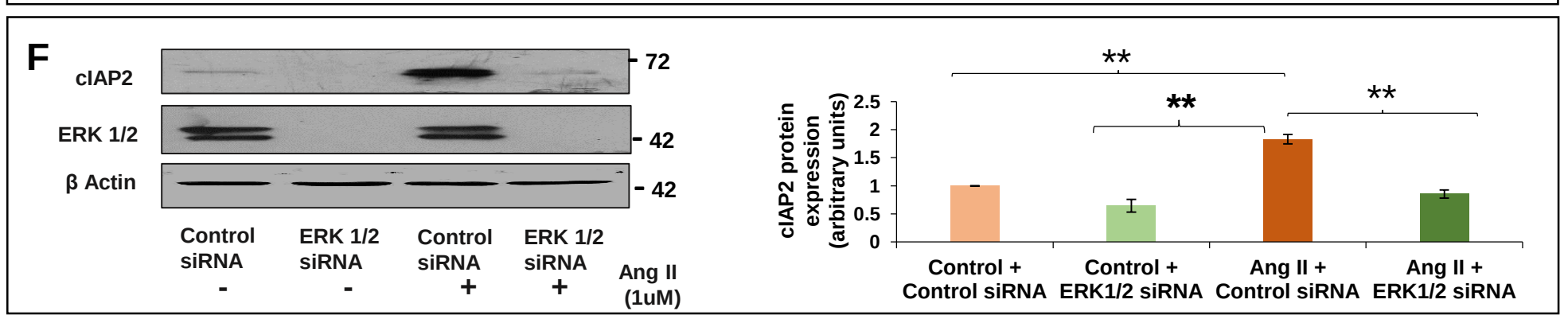
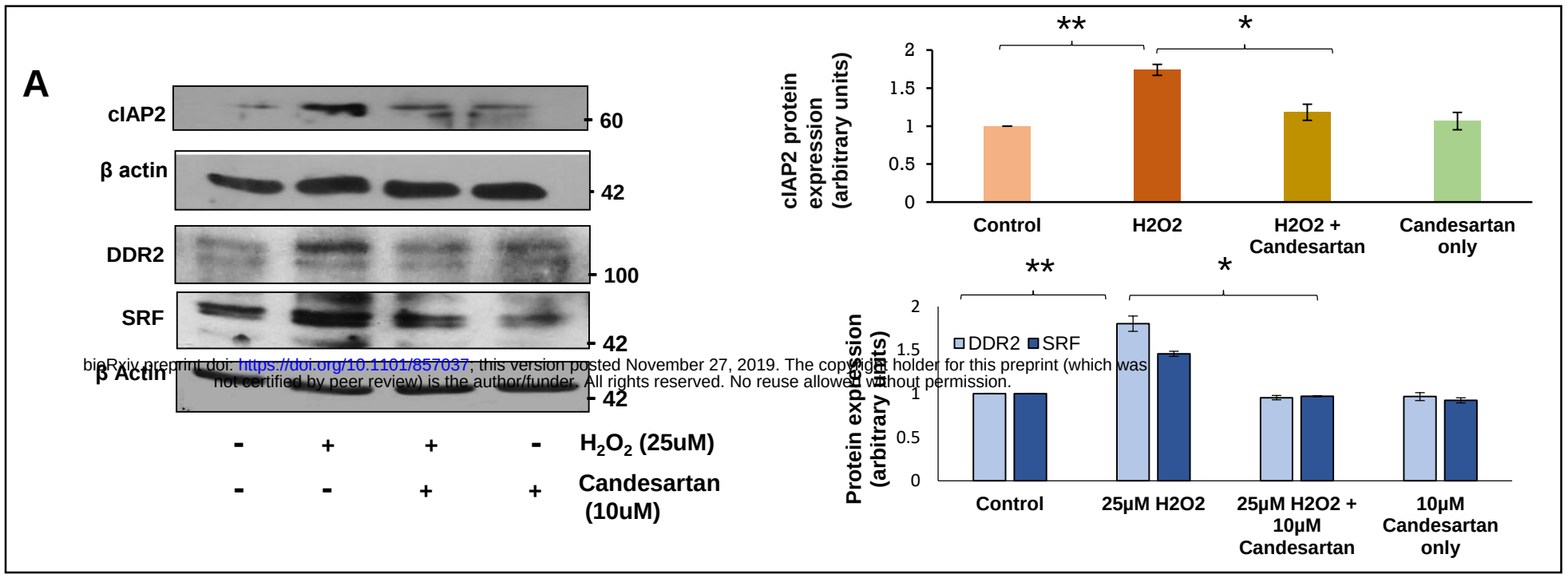
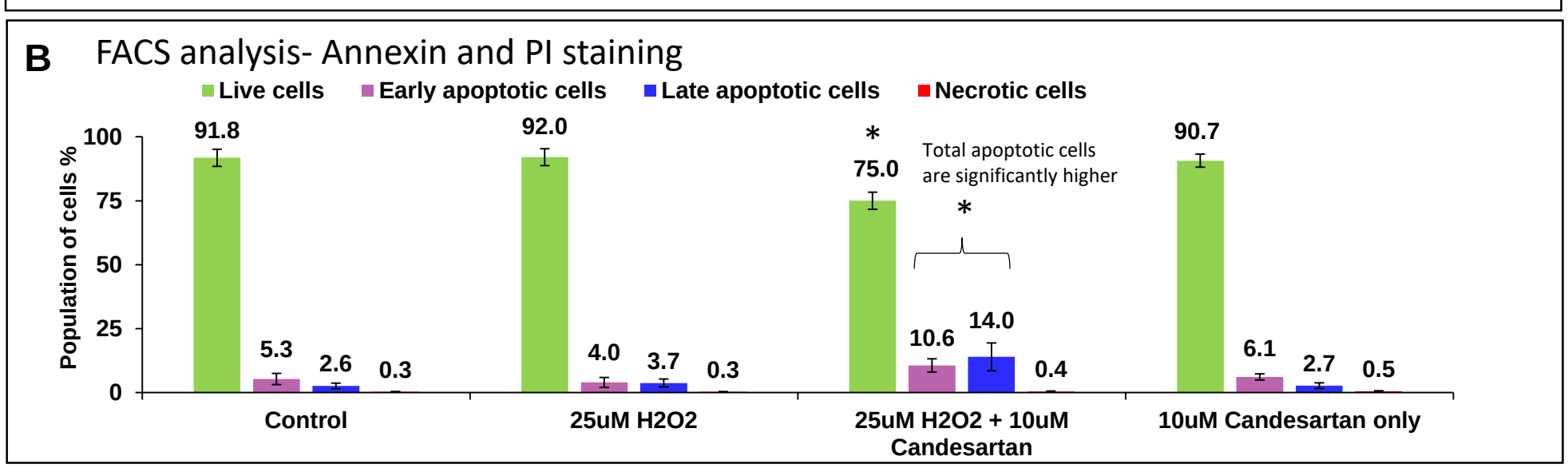
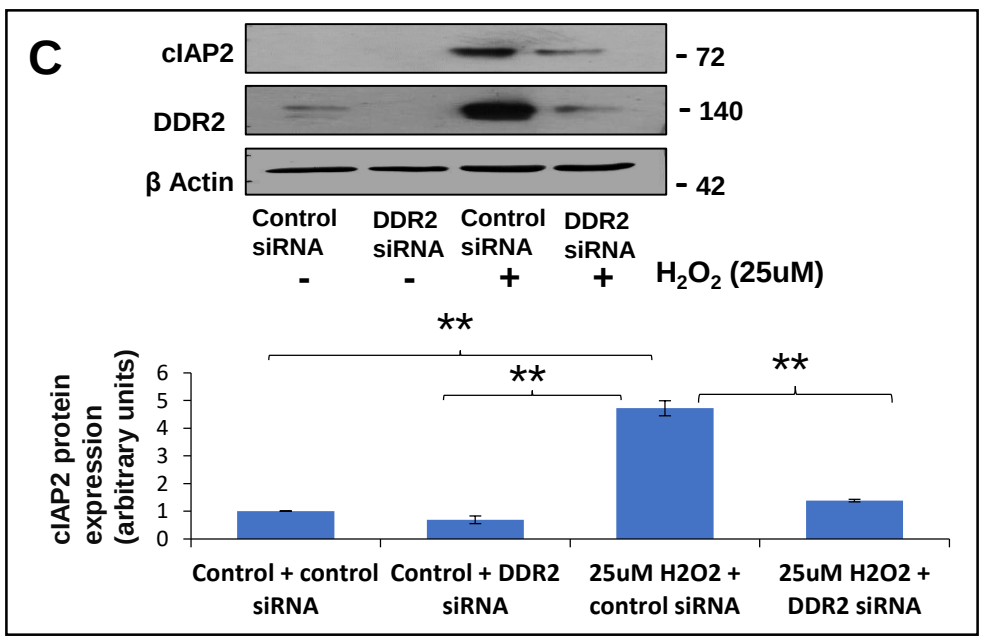
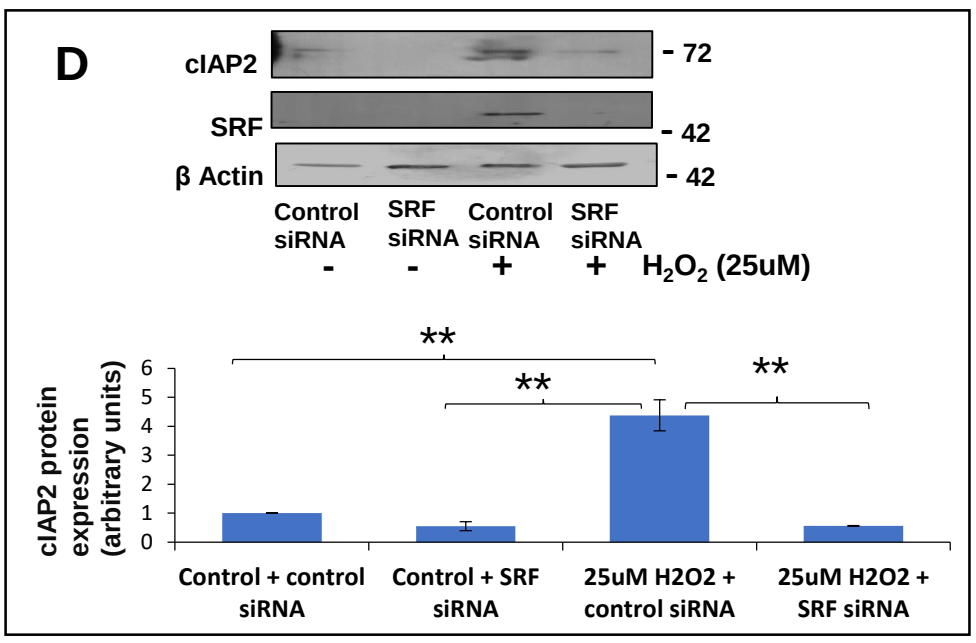


Figure 3: *DDR2-dependent cIAP2 expression protects cardiac fibroblasts against oxidative damage*









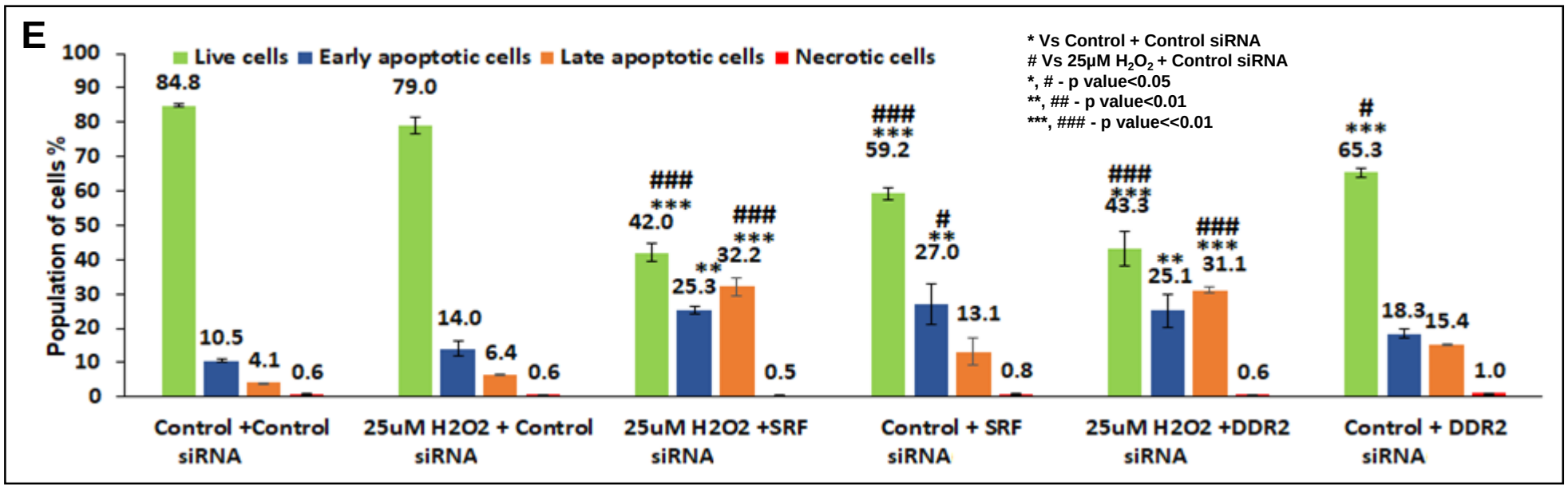
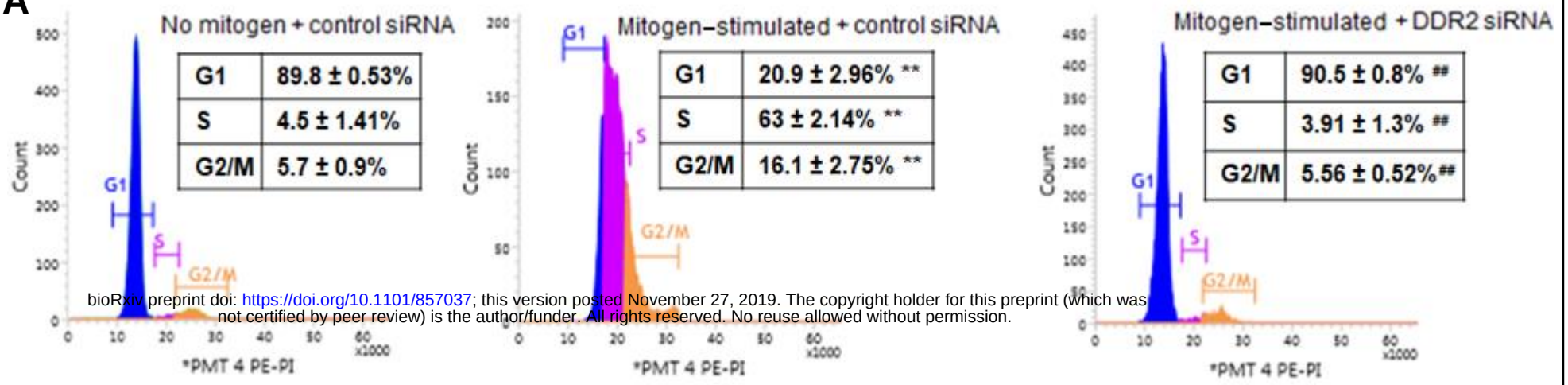


Figure 4: DDR2 knockdown in mitogen-stimulated cardiac fibroblasts inhibits G1-S transition

A



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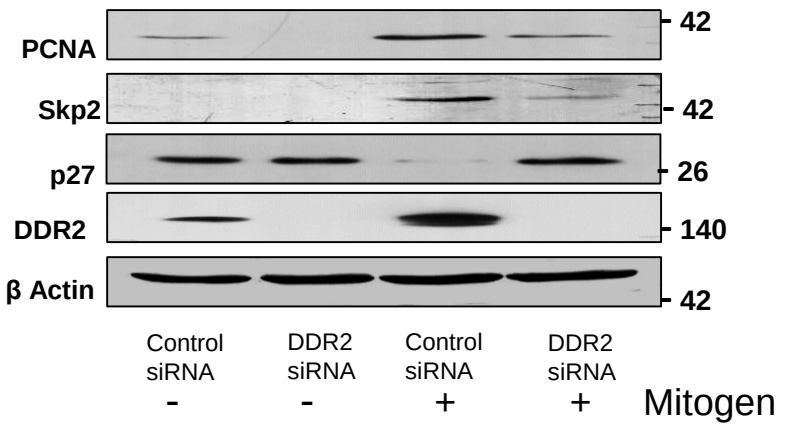

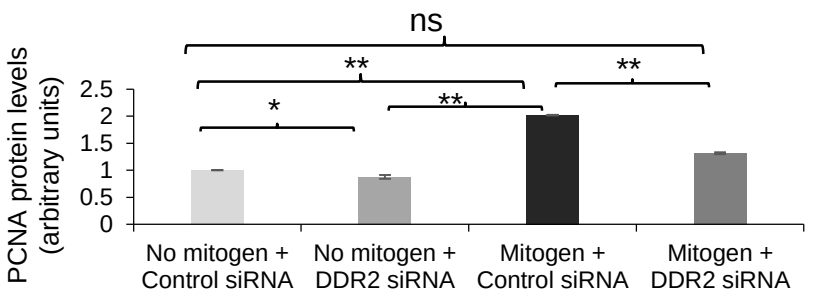

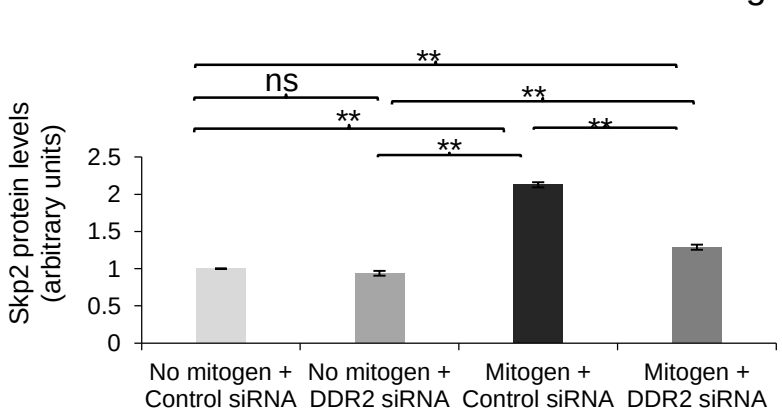

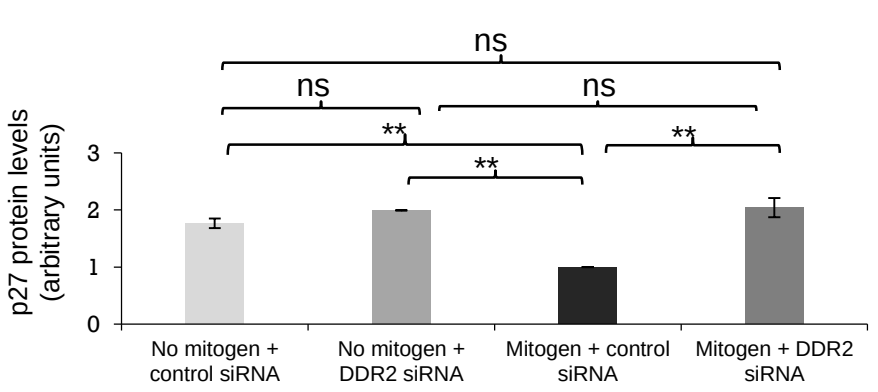

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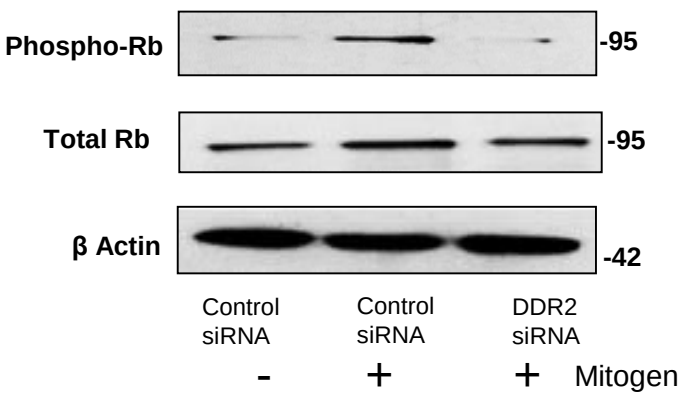

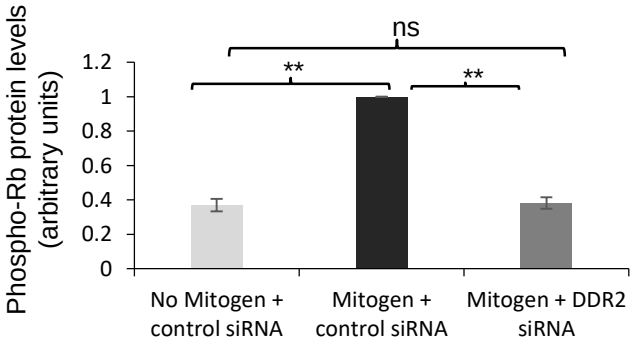

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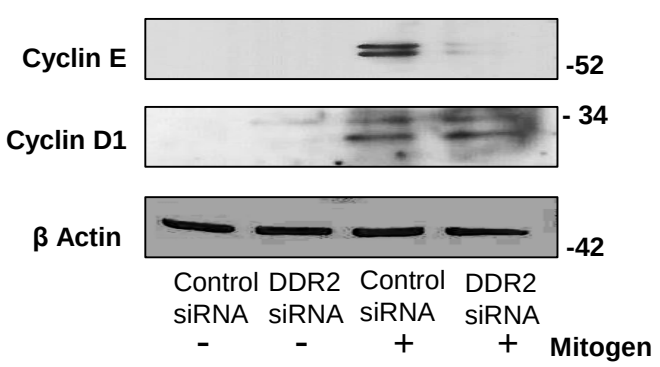


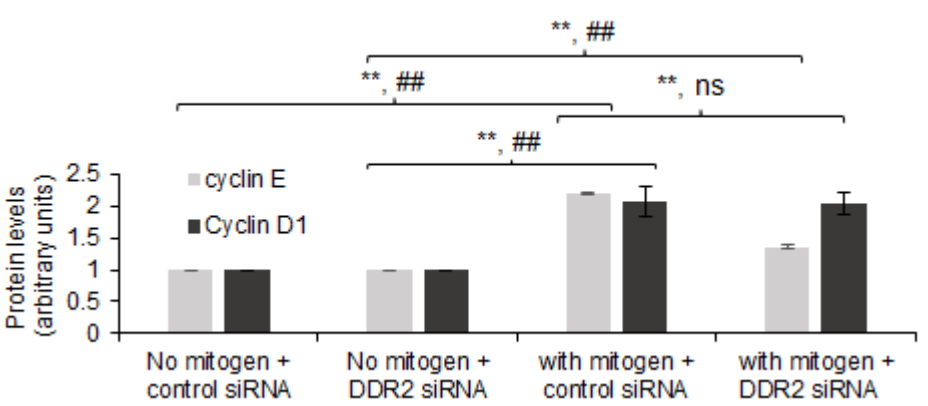
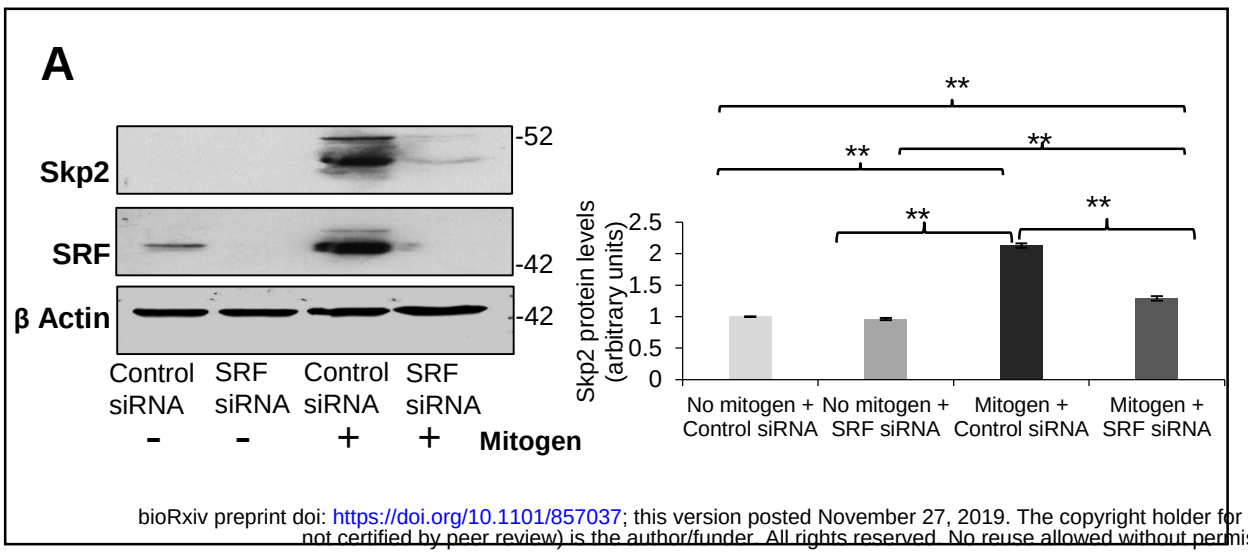
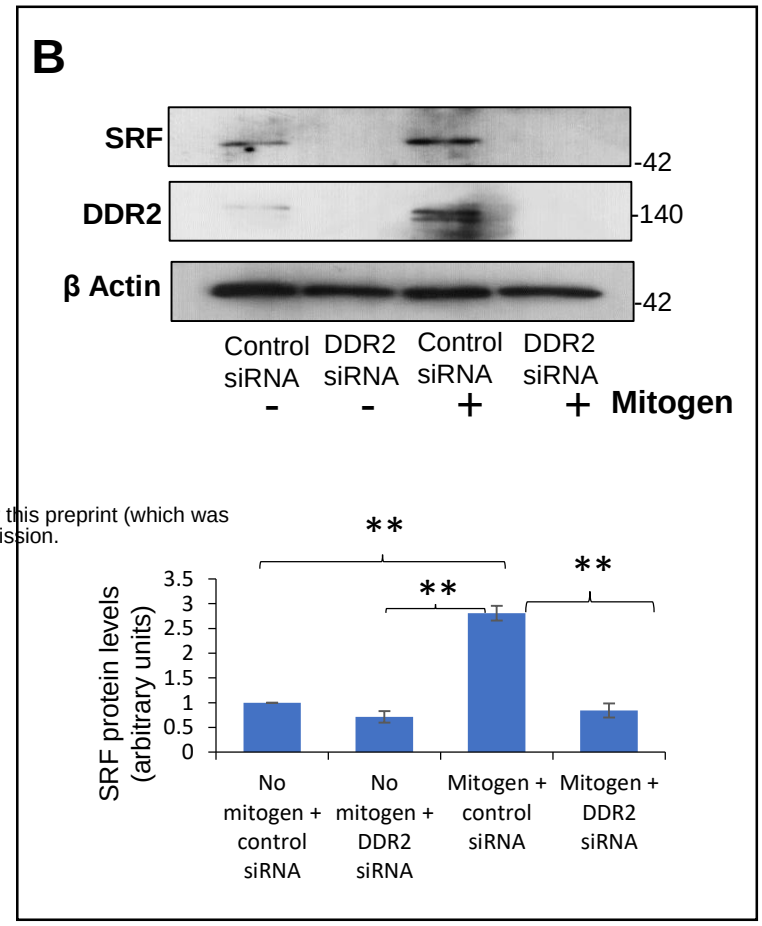
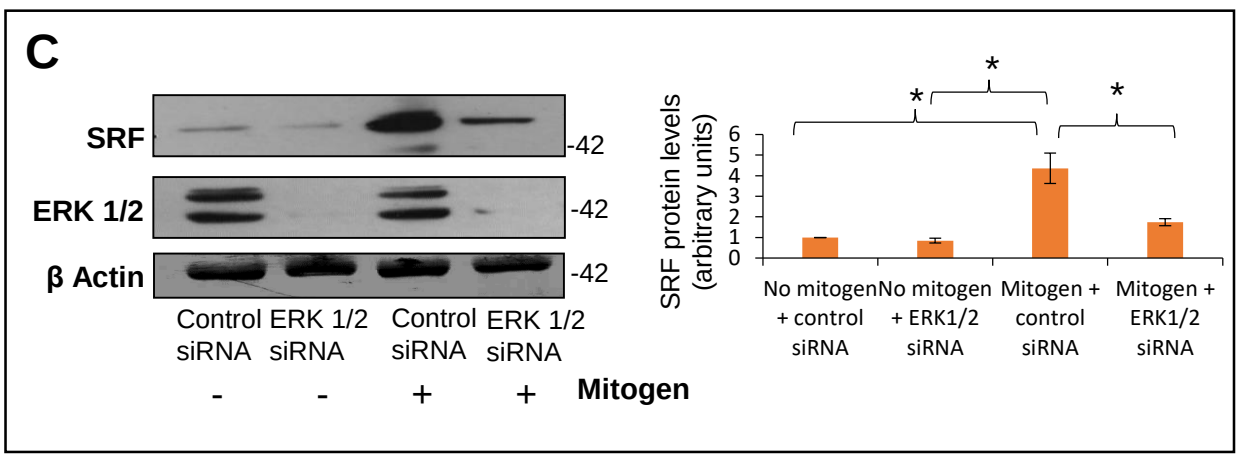
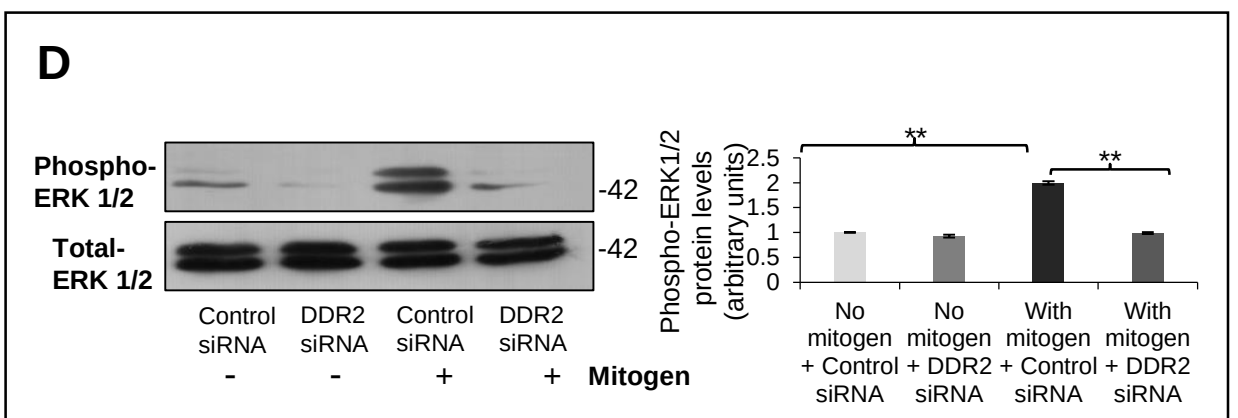


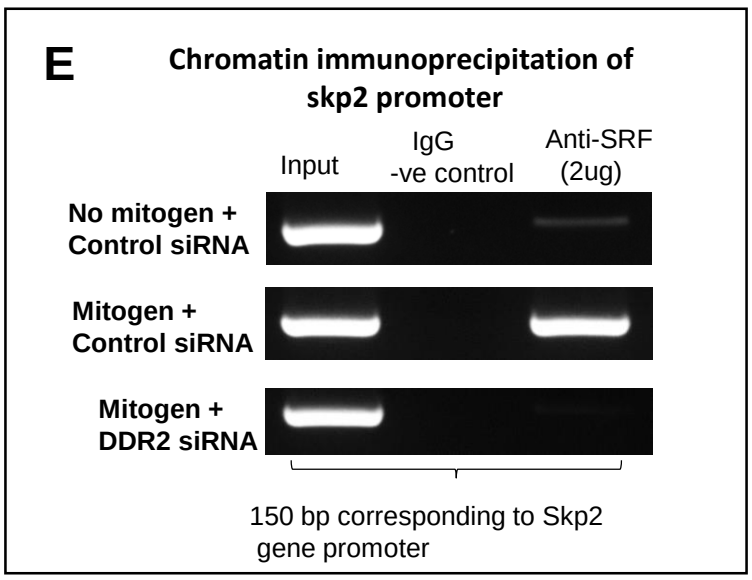
Figure 5: Regulation of Skp2 by DDR2-dependent SRF



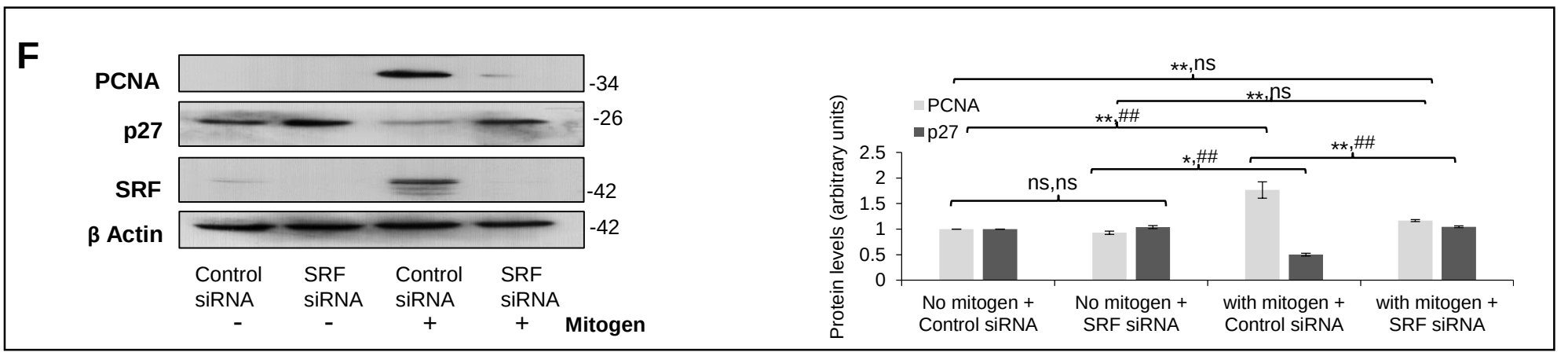








Regulation of p27 by DDR2: i) Post-translational regulation via SRF-dependent Skp2



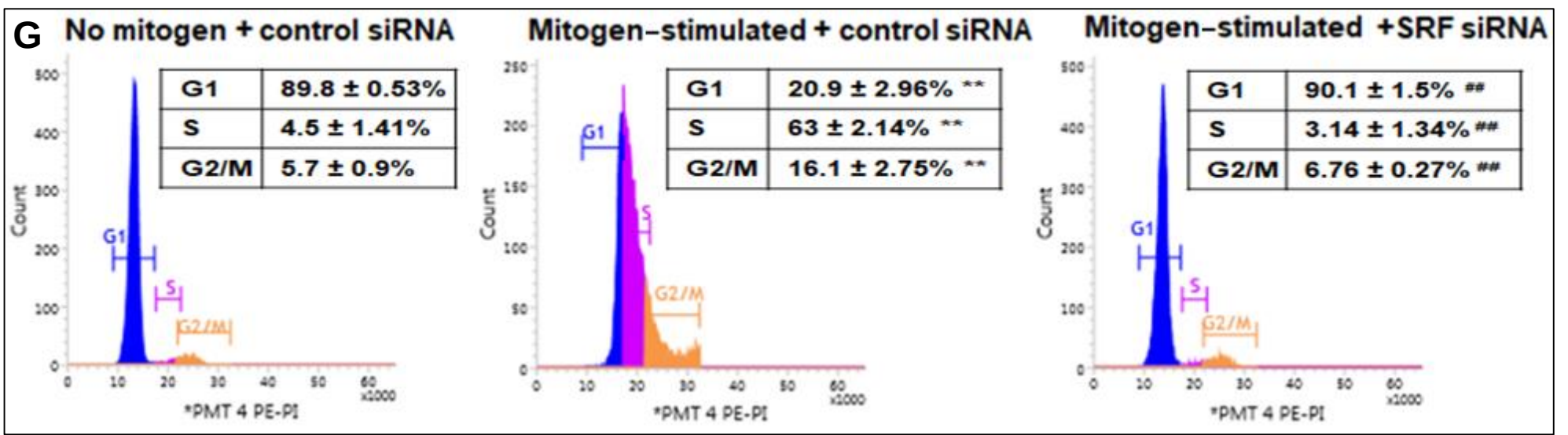
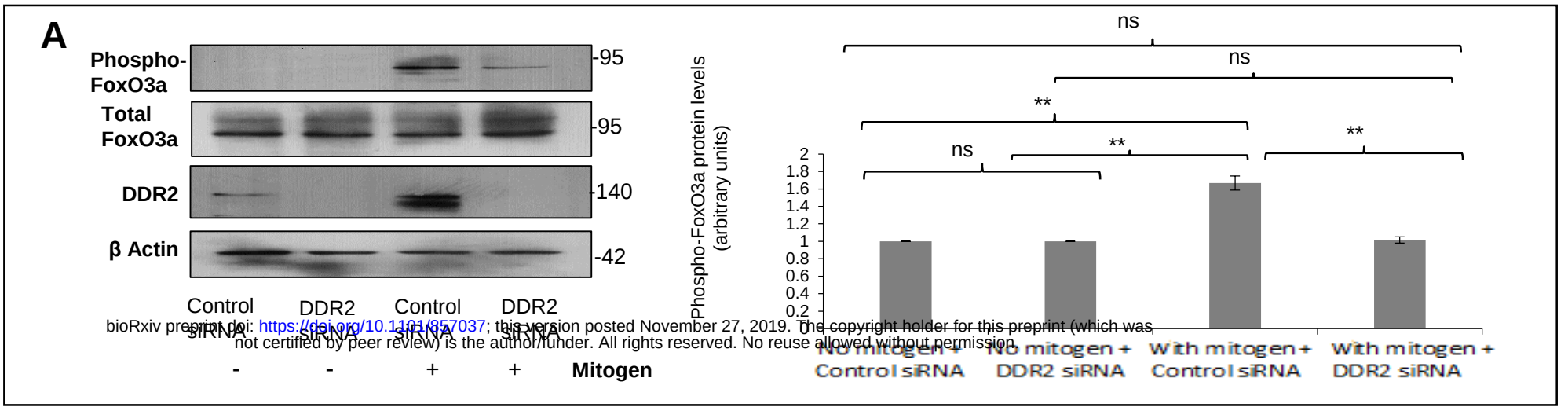
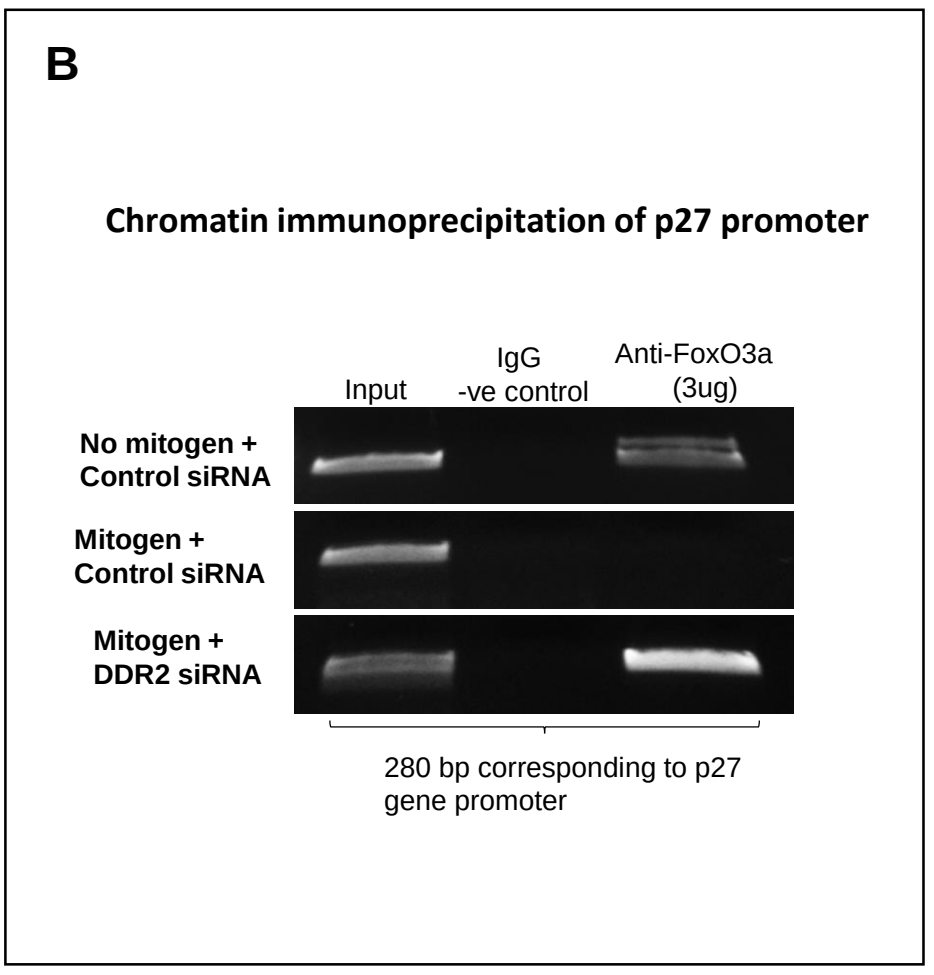
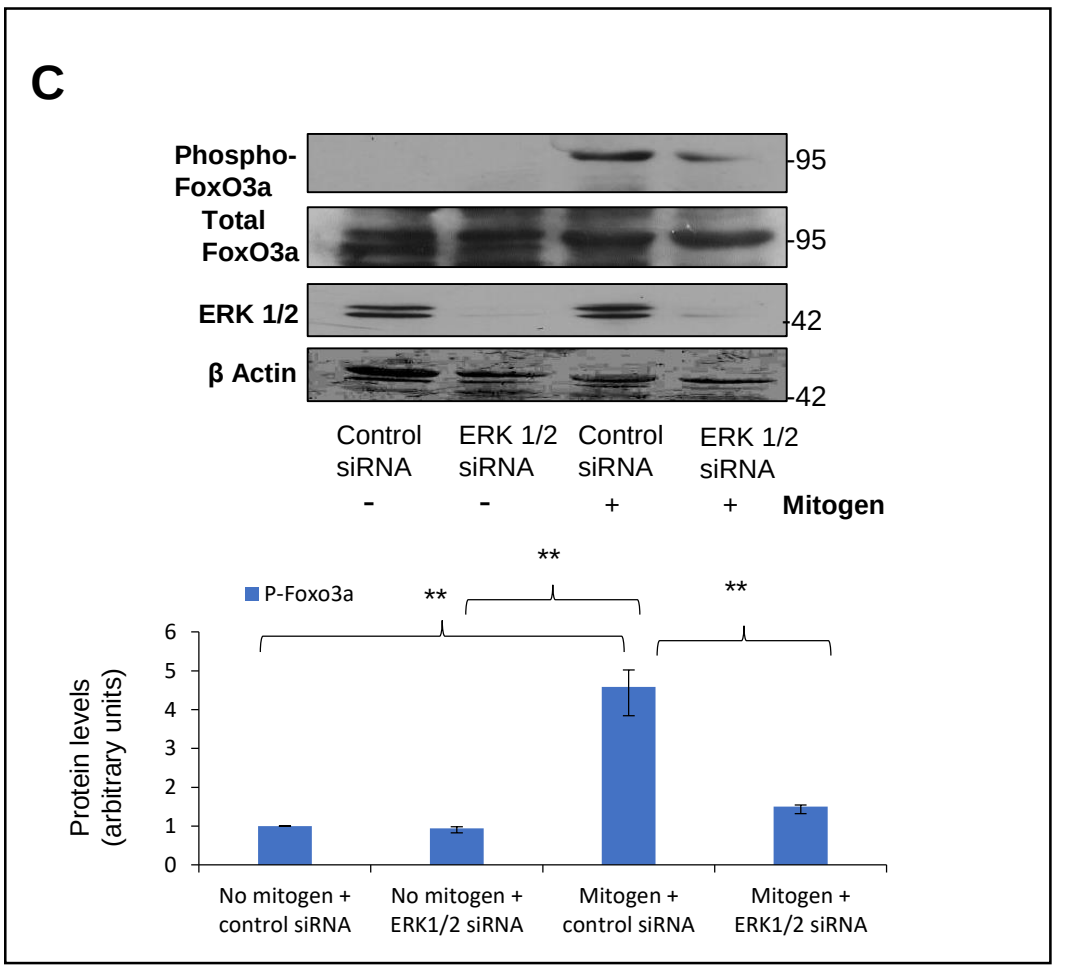


Figure 6: Regulation of p27 by DDR2: ii) Transcriptional regulation through modulating FoxO3a activity







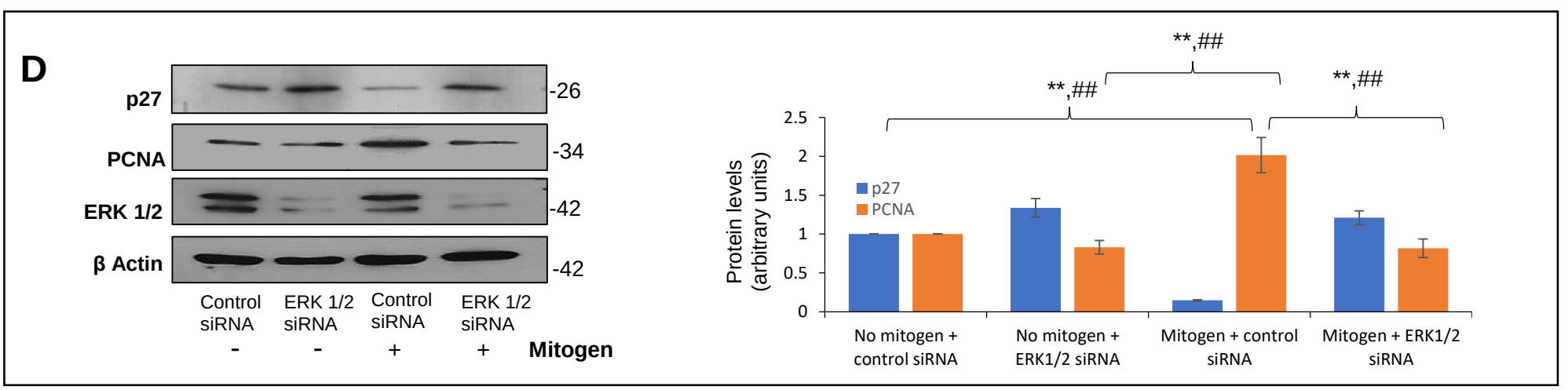
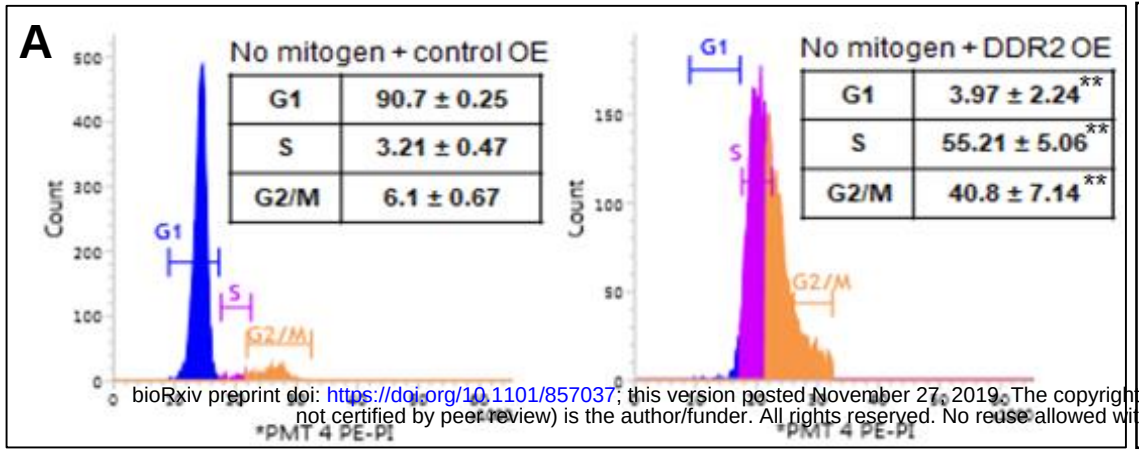
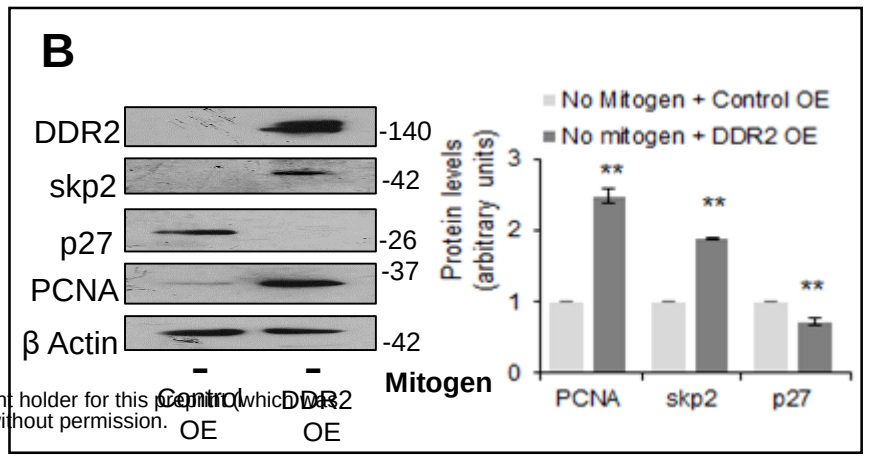
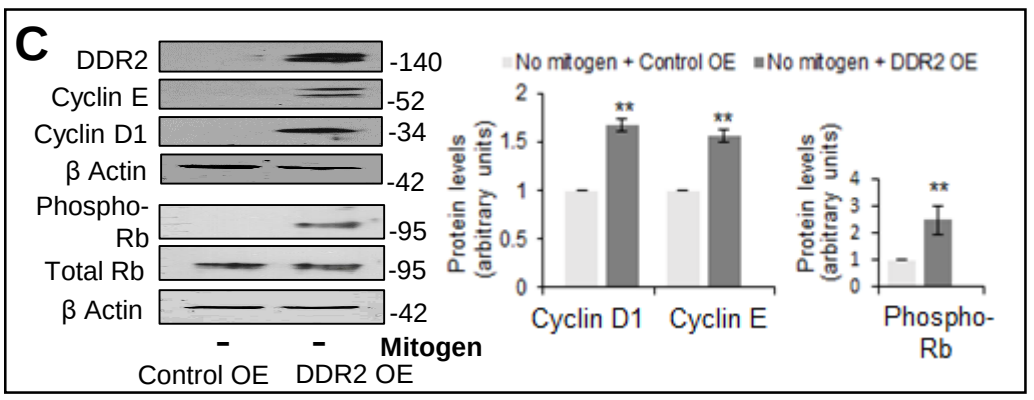
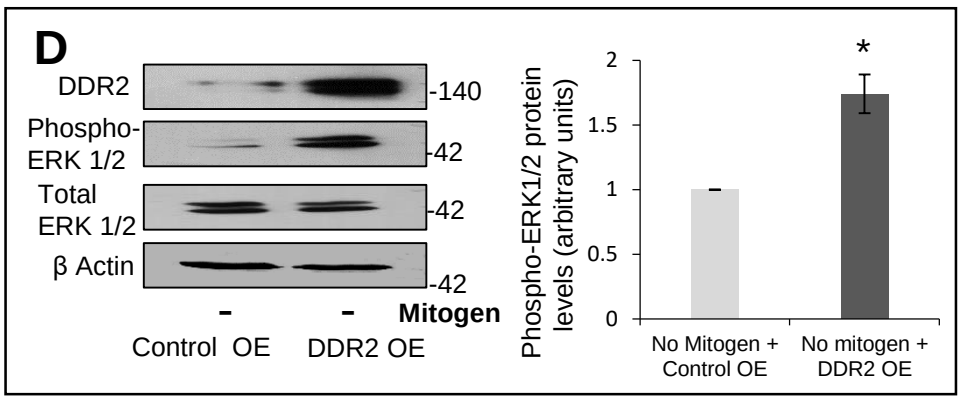


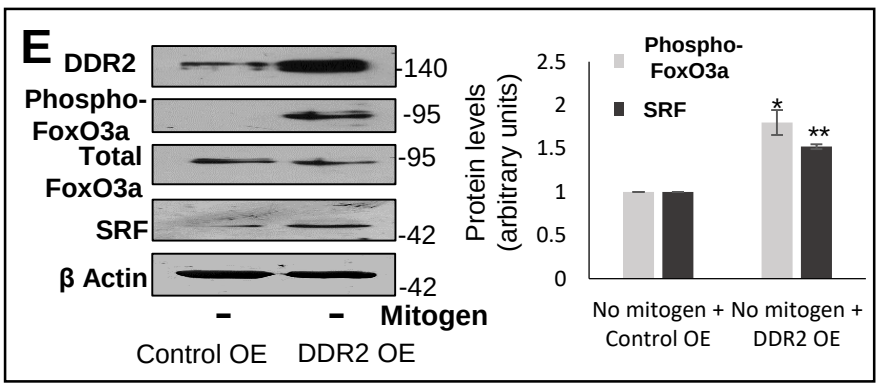
Figure 7: DDR2 over-expression in mitogen-deprived cardiac fibroblasts facilitates G1-S transition

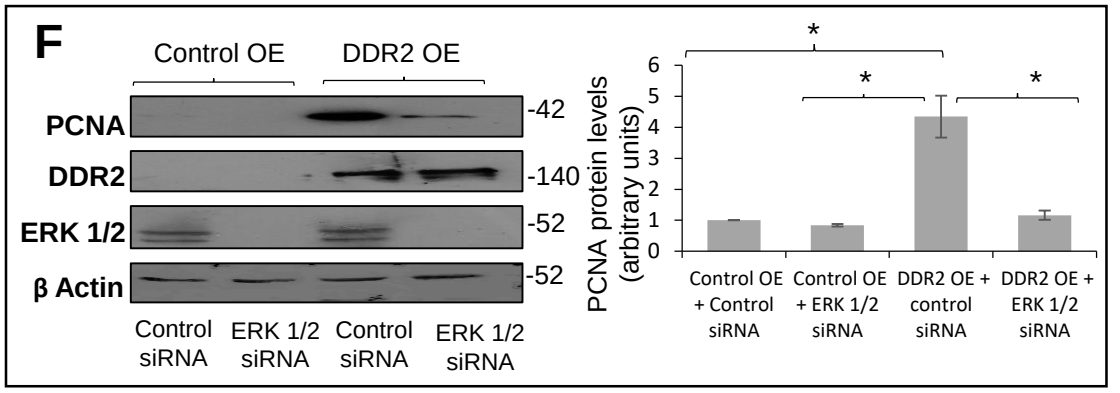












Enhanced expression of DDR2 correlates with enhanced levels of SRF, cIAP2 and PCNA in cardiac fibroblasts freshly isolated from Spontaneously Hypertensive Rats