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Specific Regulation of Non-canonical p38α Activation by Hsp90-Cdc37 Chaperone Complex in Cardiomyocyte

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Abstract

Rationale—p38 is an important stress activated protein kinase involved in gene regulation, proliferation, differentiation and cell death regulation in heart. p38 kinase activity can be induced through canonical pathway via upstream kinases or by non-canonical autophosphorylation. The intracellular p38 kinase activity is tightly regulated and maintained at low level under basal condition. The underlying regulatory mechanism for canonical p38 kinase activation is well studied, but the regulation of non-canonical p38 autophosphorylation remains poorly understood.

Object—We investigate the molecular basis for the regulation of non-canonical p38 autophosphorylation and its potential functional impact in cardiomyocytes.

Methods and Results—Using both proteomic and biochemical tools, we establish that Hsp90-Cdc37 chaperones are part of the p38α signaling complex in mammalian cells both *in vitro* and *in vivo*. The Hsp90-Cdc37 chaperone complex interacts with p38 via direct binding between p38 and Cdc37. Cdc37 expression is both sufficient and necessary to suppress non-canonical p38 activation via autophosphorylation at either basal state or under TAB1 induction. In contrast, Cdc37 expression has no impact on p38 activation by canonical upstream kinase MKK3 or oxidative stress. Furthermore, Hsp90 inhibition results in p38 activation via autophosphorylation, and p38 activity contribute to apoptotic cell death induced by Hsp90 inhibition.

Conclusion—Our study has revealed a so far uncharacterized function of Hsp90-Cdc37 as an endogenous regulator of non-canonical p38 activity.

Keywords

p38 MAP kinase; Non-canonical pathway; Hsp90; Cdc37; Autophosphorylation

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Introduction

Oxidative stress, pro-inflammatory cytokines and many other extracellular stimuli activate intracellular stress responses through stress signaling pathways, including stress activated protein kinase (SAPK) p38 as a member of the mitogen activated protein kinase (MAPK) superfamily [1-5]. Although the role of p38 in heart remains controversial, activation of p38 in response to ischemia-reperfusion has been demonstrated repetitively. Several evidences suggest p38α contributes to myocyte cell death by promoting production of pro-inflammatory cytokines including TNFα, IL-1, and IL-8. This is also supported by the protective effect of p38-specific inhibitor SB203580 in ischemia. On the other hand, protective effect of p38 through activation of Hsp27 has also been suggested. There are two modes of p38 activation in ischemia. One is the canonical p38 pathway, which is activated by a cascade of phosphorylation through upstreamMEKKs such as Ask1[6], and MKK3, 4, or 6, leading to phosphorylation of the TGY motif within the p38 activation loop[3, 4, 7]. In addition, non-canonical MKK-independent activation of p38 has been demonstrated through intrinsic autophosphorylation [8, 9] that can be facilitated by direct interaction with TAB1 or ZAP-70. It has been speculated that these two different pathways are activated by different stimuli, and contribute differently to regulate p38 functions in response to ischemia-reperfusion.

The canonical p38 activation is a highly regulated process involving well studied upstream kinases (MKKs) and protein phosphatases (MKPs, PP2Cα/β) as its positive and negative regulators respectively [10-12]. In contrast, the regulatory mechanism for non-canonical p38 pathway remains poorly understood. Autophosphorylation is an intrinsic property of p38 kinase [8, 9], and although TAB1 and ZAP-70 are reported to function as positive inducers of p38 autophosphorylation[13-16], the molecular mechanism in its negative regulation remains unclear. It is important to note that p38 activity is maintained at low level in cells in the absence of stress stimulus, suggesting that autophosphorylation activity of p38 is normally suppressed at the basal state.

In this study, we identified Hsp90-Cdc37 chaperone complex as a specific component of p38 kinase signaling complex. Hsp90 constitutes 1-2% of cellular proteins[17] to regulate the stability and the activity of its client proteins[18], including protein kinases [19-23]. In most cases, Hsp90 functions to stabilize its client protein kinases and promotes kinase activation. The loss of pro-survival kinases at both protein and activity levels are implicated as a major mechanism underlying the anti-cancer effects of Hsp90 inhibitors. Based on the evidence that p38 protein level is not affected by Hsp90 inhibition, earlier reports have classified p38 as a non-client protein of Hsp90[24-26].

Using biochemical and molecular approaches, we have shown both *in vitro* and *in vivo* that p38 specifically interacts with Hsp90 chaperone through direct binding to its co-chaperone Cdc37. Recombinant Hsp90-Cdc37 is sufficient to attenuate autophosphorylation of recombinant p38 kinase in an Hsp90 activity dependent manner. Furthermore, Cdc37 is both sufficient and necessary for suppressing basal p38 activity, and exogenous Cdc37 expression suppresses non-canonical p38 activation via TAB1, but has no impact on canonical p38 activation via MKK3. In cultured cardiomyocytes, inhibition of Hsp90 leads to rapid p38 autophosphorylation, which contributes to apoptotic cell death resulted from Hsp90 inhibition. In conclusion, we for the first time establish that p38 is a *bona fide* client protein of Hsp90-Cdc37 chaperone complex. Hsp90-Cdc37 is essential to specifically suppress non-canonical autophosphorylation of p38 while has no impact on canonical p38 activation. Therefore, Hsp90-Cdc37 represents the first identified non-canonical pathway-specific negative regulator for p38. This finding is a potentially important addition to the mechanistic repertoire for the diverse function of Hsp90 as a master signaling modulator.

Materials and methods

Immunoprecipitation

Recombinant proteins were cloned from ventricular cDNA library prepared using SSII RT, and oligo dT primer following the company's protocol (Invitrogen). 0.1 - 0.3 μg of precipitated proteins were mixed into binding buffer, and incubated at 4 °C in the presence of anti-Cdc37 (Abcam) and Protein A (GE Healthcare) for 3 hours. Anti-FLAG M2 affinity gel (Sigma) was used for FLAG co-IP, and the experiment was conducted following the company's protocol.

Kinase Assay

Kinase assay was carried out following a protocol provided by Cell Signaling. Briefly, 0.3 μg of GST recombinant proteins were mixed in kinase assay buffer, with or without 5 μmol/L SB203580 or 10 μmol/L GA. For a detection of *de novo* phosphorylation, 5 μCi of γ-P32-ATP was included. Mixtures were incubated for 15 minutes at 30 °C, and iced immediately. Phosphorylation was detected by autoradiography.

shRNA-Mediated Cdc37 Knockdown

shCdc37 was cloned into pSuper. Its efficacy was verified, and pSuper-shCdc37 was digested with PacI and cloned into pAdEasy through homologous recombination to produce Adv-shCdc37.

TUNEL staining

Cells were grown on laminin coated glass coverslips, fixed in 2% paraformaldehyde, and permeabilized in 1% Triton X-100, 1% sodium citrate solution. TUNEL staining was carried out using ApopTag Peroxidase in situ Apoptosis Detection Kit (Chemicon International) following company's protocol. Apoptotic occurrence was determined by counting TUNEL positive cells which co-localize with DAPI staining, and dividing by the total number of nucleus. Statistical significance was determined by student's t-test.

Detailed information for material and method is provided in On-line Supplemental Information.

Results

Identification of HSP90-Cdc37 in cardiac p38α complex

We and others have reported that p38 activation in heart has a number of pathological consequences[1, 14, 27-29]. In order to study the molecular mechanisms of p38 regulation and function in heart, p38α protein complex was isolated through co-immunoprecipitation (co-IP) from transgenic mouse heart expressing N-terminally FLAG-tagged dominant negative p38α (FLAG-DNp38α). We used DNp38α instead of WTp38α since p38 phosphorylation, as wells as the interaction between WTp38 and many of its binding partners are relatively transient[13]. The molecular identities of the p38 signaling complex was established by LC-MS/MS analysis with >95% confidence and are summarized in Online Table I. In addition to known p38 interacting proteins, Hsp90α, Hsp90β, and co-chaperone Cdc37 were identified with significant peptide coverage (Figure 1A). The presence of Hsp90α/β and Cdc37 in the p38α complex was confirmed by Western immunoblots (Figure 1B). As a control for specificity, another known Hsp90 co-chaperone, Hsc70 was not detected[30].

To determine whether p38α and Hsp90-Cdc37 complex interact directly, we performed co-IP using recombinant proteins. As shown in Figure 1C, Hsp90 interacts much stronger with FLAG-DNp38α in the presence of Cdc37, with Hsp90β showing a higher affinity than

Hsp90α. In contrast, Cdc37 was able to interact with either wildtype (WT) or dominant negative mutant (DN) of p38α in the absence of Hsp90 (Figure 1D). Therefore, p38α appears to bind directly to Cdc37 which can subsequently recruit p38α into Hsp90-Cdc37 complex, a scheme previously demonstrated for other Hsp90-Cdc37 client protein kinases [17, 31]. Consistently, p38 and Hsp90-Cdc37 in total protein extract prepared from heart tissue had broad distribution pattern on a sucrose gradient with significant overlap (Online Figure IA). However, when the p38 immunocomplex was isolated from heart and separated on the same sucrose gradient, Hsp90 was detected only in the same fractions when both Cdc37 and p38 were present, while a separate pool of p38 and Cdc37 co-migrated together without Hsp90 (Online Figure IB). All of these evidences suggest that p38 specifically interacts with Hsp90-Cdc37 complex via direct binding with Cdc37.

Molecular basis of interaction between p38α and Cdc37

In order to determine the Cdc37 binding domain on p38α, we created a number of GST-tagged truncation mutants of p38α and performed co-IP assays with Cdc37 (Figure 2A). The results indicated that the N-terminus of p38α contains a putative Cdc37 binding motif. Based on the sequence analysis, p38 MAP kinase isoforms share a common GXGXYG motif at their N-terminus, which is reported to function as an ATP binding site[32], while JNK and ERK have GXGXQG and GXGXPG respectively. Interestingly, GXGXF/YG is reported as a conserved Cdc37 binding site[23]. However, Y35A single mutation or Y35A and G31A double mutations on p38α did not abolish its binding to Cdc37, instead the GY double mutant appeared to bind Cdc37 stronger (Figure 2B). The VIWCI residues within the Hsp90-binding motif of Cdc37 are also reported to be critical for binding of another established client protein kinase Raf-1[33]. However, Cdc37 protein with mutation at V192A or both V192A and I193A had similar p38α binding capacity as the Cdc37WT (Figure 2C). Therefore, the direct interaction between N-terminal domain of p38α and Cdc37 appears to be mediated by so far uncharacterized motifs.

Hsp90-Cdc37 chaperone directly inhibits p38 autophosphorylation *in vitro*

To investigate the functional implication of Hsp90-Cdc37 interaction with p38, we first performed *in vitro* phosphorylation assay using recombinant p38α and the isolated p38α immunocomplex, but we did not detect *de novo* phosphorylation at the molecular weight of Hsp90 or Cdc37 (Online Figure II), suggesting that Hsp90 and Cdc37 are unlikely the direct substrates of p38α. To examine whether Hsp90-Cdc37 chaperone complex is able to directly regulate p38α kinase activity, *in vitro* phosphorylation assay was performed using purified recombinant proteins (Figure 3). As reported previously[34 35], we observed SB203580-sensitive autophosphorylation of recombinant p38α before co-incubation with Cdc37-Hsp90α/β (-incubation). However, overnight incubation with recombinant Cdc37 and Hsp90α/β prior to the kinase assay resulted in a marked decrease in SB203580 sensitive autophosphorylation of p38α (+ incubation). Such inhibition of p38α autophosphorylation by Hsp90-Cdc37 was blocked by the addition of Hsp90 inhibitor geldanamycin (GA) during the kinase assay. Therefore, Hsp90-Cdc37 chaperone complex appears to be responsible for inhibiting p38α autophosphorylation without other cellular proteins and this inhibitory function is chaperone activity dependent.

Hsp90-Cdc37 inhibits p38 autophosphorylation *in vivo*

Our data suggests that Hsp90-Cdc37 may function to suppress the autophosphorylation of endogenous p38 kinase in cells. However, it is well established that Hsp90 also regulates the stability and the activity of many of its client kinases via ATP dependent chaperone activity[36-39]. As shown in Figure 4A, treatment with GA resulted in a robust induction of p38 activity within 5 minutes, which lasted for the duration of the experiment without significant

change in the total protein level of p38 (Figure 4A). In contrast, at 5 minutes post GA treatment, activities of upstream MKK3/6 and other MAPK pathways including ERK and JNK were not affected. At later time points, JNK activity was also induced to a modest level, along with phospho-Hsp27, a downstream target of p38, while ERK activity remained unaffected. Activation of p38 phosphorylation was also observed using 17-allylamino-17-demethoxygeldanamycin (17-AAG) as an alternative Hsp90 inhibitor (Online Figure IIIA).

Addition of p38 inhibitor SB203580 along with GA effectively diminished early induction of p38 phosphorylation, a characteristic feature of p38 kinase autophosphorylation. After longer GA treatment, an increase in phospho-MKK4 was observed, and the level of p38 phosphorylation became less sensitive to SB203580, suggesting an increased contribution of canonical p38 activation at later time points due to perhaps secondary and non-specific effects of Hsp90 inhibition. Phospho-p38 level in the presence or absence of GA and SB203580 were also quantified to demonstrate the SB-sensitive activity of p38 in NRVM (Figure 4C). Furthermore, GA-induced autophosphorylation was observed in the MKK3/6 DKO MEFs (Figure 4D and 4E), again supporting MKK-independent activation of p38. In agreement with this scheme, T180 but not Y182 of p38 was selectively phosphorylated at 5 minutes post GA treatment, while only slight increase in phospho-Tyr-p38 was observed 15 minutes after GA treatment (Online Figure IVA). Furthermore, Hela cells expressing p38αT180A showed blunted response to GA, further supporting the notion that T180 is the site for GA induced p38 autophosphorylation (Online Figure IVB).

To investigate the impact of p38 activation status on its interaction with Hsp90/Cdc37, we studied endogenous p38 kinase interaction with Cdc37 in Hela cells which also demonstrated GA induced p38 autophosphorylation (Figure 5A). Upon Hsp90 inhibition, total p38 protein in the Cdc37 immunocomplex was reduced relative to the untreated cells while phospho-p38 was not detected in Cdc37 immunocomplex under either basal or stimulated conditions (Figure 5B). This observation suggests that Hsp90-Cdc37 preferentially interacts with non-phosphorylated p38 and Hsp90 inhibition promotes p38 autophosphorylation associated with its release from the Hsp90-Cdc37 complex.

Cdc37 expression is both sufficient and necessary for regulating basal p38 activity

From these biochemical studies, we can speculate that Hsp90-Cdc37 chaperone complex is a negative regulator for basal p38 activity. To demonstrate the functional significance of Hsp90-Cdc37 mediated regulation of basal p38 activity, we used two different Adv-shCdc37 constructs to achieve selective knockdown of Cdc37 in HeLa cells. As shown in Figure 6A, shCdc37 expression resulted in a dosage dependent decrease in Cdc37 protein level in parallel with an increase in phospho-p38. Knockdown of Cdc37 protein also reduced p38 interaction with Hsp90 based on co-IP assay (Online Figure V). On the other hand, overexpression of Cdc37 in HeLa cells significantly reduced the basal p38 activity and blocked p38 autophosphorylation induced by GA (Figure 6B). Therefore, it appears that Hsp90-Cdc37 chaperone complex is both necessary and sufficient to regulate the basal activity of endogenous p38 by negatively suppressing its autophosphorylation. However, the elevated basal p38 activity in Cdc37 knock-down cells was no longer very sensitive to p38 inhibition (Figure 4B). The underlying reason is unclear but may be partially due to toxicity inflicted by Lipofectamine treatment.

Hsp90-Cdc37 specifically regulates non-canonical p38 pathway without significant impact on canonical p38 activation

Using H_2O_2 as an established p38 inducer via canonical pathway, we observed that overexpression of Cdc37 did not have a significant effect on p38 activation and its downstream signaling induced by H_2O_2, suggesting that Hsp90-Cdc37 complex has no impact on canonical

p38 pathway (Online Figure VI). To study this further, HeLa cells were co-transfected with constitutively active MKK3 (MKK3bE) and Cdc37. As expected, MKK3bE led to a significant induction of phospho-p38 in the presence or absence of p38 inhibitor (Figure 6C). However, co-expression of Cdc37 did not affect p38 induction by MKK3bE, suggesting that canonical p38 activation pathway is not affected by Cdc37 (Figure 6C).

It has been shown that TAK1 binding protein-1 (TAB1) promotes autophosphorylation of p38α through direct interaction[13]. Consistently, expression of TAB1β which lacks the TAK1 binding domain [15], was sufficient to induce p38 activation in HeLa cells (Figure 6D). To determine whether Cdc37 can regulate TAB1β mediated autophosphorylation of p38, Cdc37 and TAB1β were co-expressed in HeLa cells (Figure 6D). The results showed that the p38 activation by TAB1β was reduced by the exogenous Cdc37, suggesting that Cdc37 overexpression can negatively regulate p38 autophosphorylation induced by TAB1β. Together with the previous results, our data indicate that Hsp90-Cdc37 chaperone complex selectively regulates the non-canonical p38 activation pathway, but not the MKK-dependent canonical activation pathway.

Role of p38 in Hsp90-Cdc37 inhibition induced cardiomyocyte apoptosis

Hsp90 is reported to have a potent cytoprotective function in the heart[40-42]. As expected, treating NRVM with Hsp90 inhibitor GA resulted in massive cell death within 6 hours (Figure 7A). Consistent with the increased cell death, the expression levels of both IL-6 and TNFα were increased in NRVM treated with GA (Online Figure VII). Similarly, COS7 cells treated with GA showed a significant increase in TUNEL positive apoptotic cells (Figure 7B). However, the relative low frequency of TUNEL+ cells (1.1%) suggest that apoptosis may not be the only cause to trigger massive cell death induced by GA. Consistent with its effect on p38 autophosphorylation, 17-AAG treatment also led to similar cell death though to a lesser extent (Online Figure IIIB). Expression of DN38α was sufficient to block p38 dependent downstream signaling as demonstrated by the reduced p-Hsp27 and p-MK2 (Figure 7C). DNp38α expression in NRVM significantly delayed GA induced cell death up to 12 hours (Figure 7D). The reduction in cell death by DNp38α correlated with a decrease in the level of TUNEL positive cells suggesting that p38 activation contributed to GA induced apoptosis (Figure 7E). The loss of efficacy of DNp38α in suppressing apoptosis at higher dose of GA treatment (9 μmol/L) also suggests that the contribution of p38 activation in HSP90 mediated cell death regulation is partial. This is consistent with the earlier data which shows delayed, but not complete inhibition of cell death in the presence of DNp38α.

Discussion

In this report, we have demonstrated that Hsp90-Cdc37 chaperone complex is a part of the cardiac p38 signaling complex via direct interaction between p38 and Cdc37. Recombinant Hsp90 and Cdc37 are sufficient to attenuate the intrinsic autophosphorylation activity of the recombinant p38 kinase in test tubes. In intact myocytes, Hsp90 inhibition or Cdc37 knockdown leads to significant induction of p38 autophosphorylation. Cdc37 is both sufficient and necessary to regulate the basal p38 activity, as well as Hsp90 inhibition- or TAB1β-mediated p38 autophosphorylation, while p38 activation induced by H_2O_2 or MKK3 is not hindered. Finally, p38α activity contributes to cell death induced by Hsp90 inhibition in cultured cardiomyocytes or COS cells. These data lead us to conclude that Hsp90-Cdc37 chaperone complex is a specific regulator of non-canonical pathway for p38α by suppressing basal autophosphorylation.

Among its vast variety of client proteins, Hsp90 is known to regulate both protein stability and activity of its target kinases [43, 44]. Early studies attempting to identify Hsp90 client kinases by assessing protein degradation in response to Hsp90 inhibition have excluded p38 MAPK as an

Hsp90 client kinase based on the observation that p38 kinase protein level was not affected. Yet, increases in p38 phosphorylation in response to Hsp90 inhibition has been reported[45], and Hsp90 has also been shown to promote autophosphorylation of some of the client protein kinases, such as GSK-3β[46]. p38 homologue in *S. pombe*, Spc1, is reported to directly bind to yeast Cdc37[47], and Cdc37 has been suggested to modulate kinase activity independent of Hsp90[48]. The data reported here from both *in vitro* and cellular experiments clearly demonstrate that mammalian Hsp90 also interacts with p38 through Cdc37. Hsp90 activity is not required for p38 protein stability as no change in the total p38 protein level was observed up to 6 hours of Hsp90 inhibition. Rather, Hsp90-Cdc37 complex functions to suppress p38 autophosphorylation. This finding not only adds p38 kinase to the list of Hsp90 client proteins, but also reveals a novel function of the Hsp90-Cdc37 chaperone complex as a negative regulator of kinase autophosphorylation to the established large repertoire of Hsp90-Cdc37 functions. This newly identified mechanism may have general implications in other unidentified Hsp90-Cdc37 client protein kinases that also possess intrinsic autophosphorylation properties.

Although we have demonstrated that Cdc37 directly binds to p38α both *in vitro* and *in vivo*, the underlying molecular basis is unclear. It appears that the N-terminal domain of p38α contains weak Cdc37 binding activity when it is expressed in its truncated form, but mutations within a putative Cdc37 binding motif (GXGXYG) failed to disrupt full-length p38α interaction with Cdc37. Indeed, GY double mutant appeared to bind stronger to Cdc37 (Figure 2B), suggesting that protein conformation in the N-terminal domain, which is known to go through conformational change upon autophosphorylation, may be involved in this interaction[8, 35]. Likewise, although Cdc37 is reported to require V192 and I193 residues[33] to interact with a client protein kinase Raf-1, mutations of these sites did not affect its p38α interaction. Clearly, more efforts are needed to identify the responsible motifs involved in p38α-Cdc37 interaction.

Autophosphorylation is an intrinsic property of p38 kinase although its physiological role remains uncertain[8, 9],. TAB1 and ZAP-70 are reported to positively promote p38 autophosphorylation thus represent a non-canonical pathway for p38 activation under different upstream stimuli with different functional outcome[13-16]. Previous studies have revealed much of the regulatory components for canonical p38 pathway, including upstream MKKs, downstream targets and negative modulating phosphatases [10-12]. However, the regulatory components specific for the non-canonical pathway remain elusive. Our report here has provided the first evidence that Cdc37 expression has no impact on p38 activation induced by H_2O_2 or MKK3, but significantly attenuates TAB1 mediated autophosphorylation of p38, suggesting that Hsp90-Cdc37 chaperone complex is a non-canonical pathway specific regulator for p38. The molecular basis for this specificity is unclear. We showed that phosphorylated p38 was not detectable in the Hsp90-Cdc37 immunocomplex and the total p38 protein present in the Hsp90-Cdc37 complex was reduced upon Hsp90 inhibition. This observation suggests that phosphorylated p38 loses the ability to bind to Cdc37. Thus, we can speculate that p38 autophosphorylation is inhibited by Hsp90-Cdc37 by conferring a change in p38 protein conformation. While this change does not affect its recognition and phosphorylation by upstream kinases, it hinders p38 autophosphorylation induced by non-canonical activator, such as TAB1.

Hsp90 inhibition induced cell death is an emerging therapeutic strategy to treat cancer and the underlying mechanisms mainly involve the loss of pro-survival proteins, such as AKT, Raf-1, Cdk4, and p53[49, 50]. Our data suggest that activation of p38 via autophosphorylation may also play a significant role in Hsp90 inhibition induced cell death accompanied by increased expression of inflammatory cytokines including IL-6 and TNFα, thus, providing yet another possible downstream mechanism in Hsp90 mediated cell death regulation. Although our data

suggests Hsp90 inhibition induced cell death involves apoptosis, other form of cell death, including necrosis and autophagy cannot be excluded. The full scope of both physiological and pathological role of Hsp90-Cdc37 complex in p38 mediated stress signaling remains to be determined. However, autophosphorylation of p38 in response to ischemia and its contribution to cardiac injury have been reported. Ischemia-reperfusion on mkk3-/- heart demonstrated TAB1-mediated autophosphorylation of p38, which appeared to play a role in necrosis of cardiomyoctes [14]. Autophosphorylated p38 was also reported to mediate glucose uptake through GLUT4 induced by ischemia[51] and TAB1-p38 interaction was implicated in myocyte apoptosis regulation by cGMP-depending kinase I [52]. On the other hand, Hsp90 inhibition exacerbated cardiac injury induced by doxorubicin, a known p38 activator in heart [53] and over-expression of Hsp90 protected ischemia/reperfusion injury in heart[54]. Therefore, it can be speculated that Hsp90-Cdc37 mediated p38 autophosphorylation may play a role in ischemia-reperfusion injury in heart. Our data in Online Figure VII showed a significant induction of inflammatory cytokines by Hsp90 inhibition in NRVM, suggesting that Hsp90-Cdc37 mediated non-canonical p38 activity may have different downstream effect vs. TAB1 mediated activation in heart which does not appear to induce inflammatory gene induction [15]. Our data also suggest the presence of numerous sarcomere-associated proteins in cardiac p38 complex (Online Table I), yet their functional relevance to Hsp90-Cdc37 mediated p38 activity remains unknown. Further studies with targeted manipulation of Hsp90-Cdc37-p38 interaction will be needed to fully establish the functional relevance of this new stress signaling pathway.

Other than chaperone function, Hsp90 can also serve as a scaffold protein to coordinate/integrate multiple signal transduction activities in different cellular processes[55]. We and others have demonstrated in previous studies that cardiac p38 kinase also functions to regulate diverse cellular processes including gene transcription[7], proliferation[56], apoptosis[6, 57], and cardiac contractility[58, 59]. Canonical and non-canonical pathways for p38 activation can have major differences in intracellular localization, downstream targets and ultimate functional outcome[15]. In short, identification of Hsp90-Cdc37 as an additional component in non-canonical p38 signaling pathway will help to elucidate the molecular network and intricate regulatory mechanisms for this important stress signal pathway.

Novelty and Significance

What is known?

- p38 MAPK is a stress-induced signaling pathway that is involved in regulation of various cellular activities including proliferation, apoptosis, cell growth, gene transcription, differentiation, and cell motility during development and pathological processes, including heart failure.
- p38 MAPK is activated by two mechanisms; a canonical pathway mediated by upstream kinases and a non-canonical pathway mediated by interacting proteins, such as TAB1.
- Canonical p38 activity is known to be highly regulated by the balance of upstream kinases and phosphatases; however, the functional significance and the regulatory mechanisms of non-canonical p38 activity remain poorly understood.

What new information does this article contribute?

- The Hsp90-Cdc37 chaperone complex interacts with p38 and negatively regulates p38 autophosphorylation, which is essential to maintain low p38 activity in the absence of stress stimuli.
- Hsp90-Cdc37 specifically regulates TAB1-mediated non-canonical activation of p38, but has no effect on MKK-dependent canonical activation of p38.

- Cell death induced by Hsp90-Cdc37 inhibition is contributed by p38 activation in cardiomyocytes.

Novelty and Significance

As a stress-response pathway, p38 MAPK is under tight regulation in basal states; upon activation, it can lead to many downstream effects via canonical vs. non-canonical pathways. Previous work has focused mostly on the regulatory mechanism of the canonical p38 pathway; the underlying mechanisms for the regulation of non-canonical p38 pathway are unknown. In this report, we identified that Hsp90-Cdc37 is part of the p38 complex, and functions as a negative regulator of p38 autophosphorylation. While MKK-dependent p38 activation through the canonical pathway is not affected, the non-canonical activation of p38 through autophosphorylation is greatly enhanced upon inhibition or loss of Hsp90-Cdc37. We also show that p38 activation induced by Hsp90 inhibition plays a role in apoptosis. Therefore, we have established that Hsp90/Cdc37-mediated suppression of p38 autophosphorylation is a critical mechanism for non-canonical p38 kinase regulation and that p38 is a new client protein and regulatory target for Hsp90/Cdc37 in stress signaling. This is the first report to demonstrate the molecular and functional link between these two important cell signaling molecules, thereby revealing a new connection between two major nodes in stress signaling network. The mechanism discovered herein may play a significant role in stress-induced cardiac injury and other pathological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Non-standard abbreviations and acronyms

MAP kinase	mitogen activated protein kinase
HSP	heat shock protein
GA	geldanamycin
LC-MS/MS	liquid chromatography-mass spectrometry
GST	glutathione S-transferase
TUNEL	terminal dexoynucleotidyl transferase dUTP nick end labeling
IP	immunoprecipitation
DMSO	dimethyl sulfoxide
LacZ	gene coding for beta-galactosidase
DAPI	4′,6-diamidino-2-phenylindole

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Protein	Queries Matched	Peptide Matched	Coverage (%)	MOWSE Score
Hsp90β/Hspcb	20	16	30.4	401
Hsp90α/Hspca	8	7	13.4	216
Cdc37	7	6	21.1	160

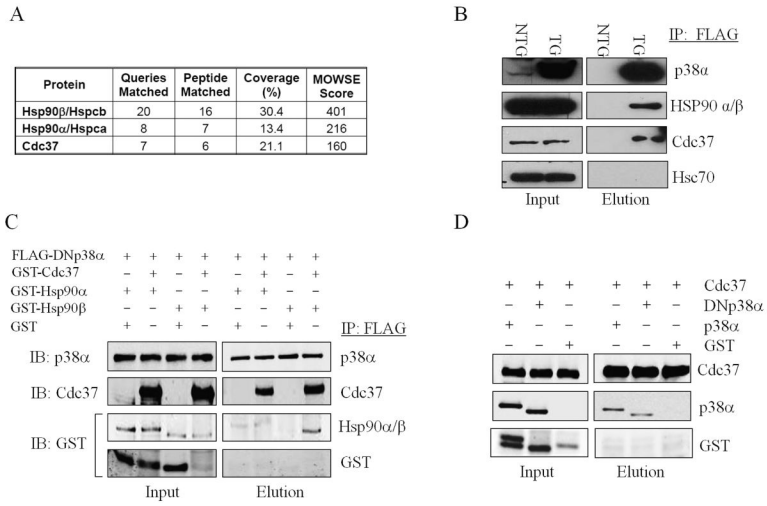

Figure 1. Interaction between Hsp90-Cdc37 and p38α protein

A) Hsp90α, Hsp90β, and Cdc37 are identified in DNp38α immunocomplex by LC-MS/MS. The total numbers of identified peptide sequences (Queries Matched), total number of identified peptides (Peptide Matched), the percentage of sequence covered by the identified peptides (Coverage %), and peptide mass spectra fingerprinting score based on MASCOT database (MOWSE, >40 indicates a significant match) are shown. **B)** Immunoblots on immunocomplex isolated with anti-FLAG antibody (IP: FLAG) from DNp38α transgenic (TG) and non-transgenic (NTG) control hearts using antibodies as labeled. **C)** Co-IP using purified recombinant proteins with anti-FLAG after incubating FLAG-DNp38α protein with GST-Cdc37, GST-Hsp90α and GST-HSP90β as indicated, followed by immunoblots with anti-GST, anti-p38α, anti-Cdc37 and anti-Hsp90α/β. Only small molecular weight section containing GST (28KD) was shown for the GST blots. Note the GST signal from lane 2 of the Input panel is from the breakdown product of GST-Hsp90α. **D)** IP with anti-Cdc37 after incubating recombinant GST-Cdc37 with GST-DNp38α, GST-p38α or GST alone as a control, followed by immunoblot using anti-Cdc37, anti-p38α and anti-GST as indicated. Only small molecular weight section containing GST (28KD) was shown for the GST blots. Note the GST signals from lanes 1 and 2 of the Input panel are from the breakdown product of GST-p38.

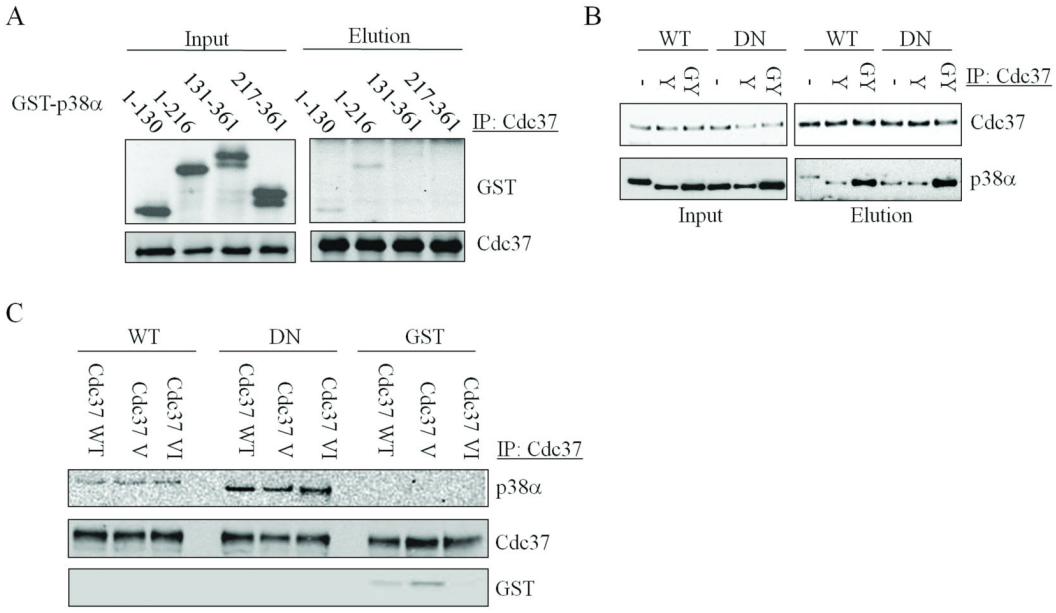

Figure 2. Specific interaction between Cdc37 and p38α

A) IP with anti-Cdc37 after incubating Cdc37 with GST-tagged truncated p38α as labeled, followed by immunoblots with anti-GST and anti-Cdc37 as indicated. All the experiments were repeated at least three times. **B)** 0.1 μg of purified recombinant p38α with Y35A (Y) or Y35A and G31A (GY) mutations were subjected to co-IP with Cdc37 using anti-Cdc37, and analyzed by immunoblots as indicated. **C)** Cdc37 with single mutation at V131A (Cdc37 V) or double mutations at V192A and I193A (Cdc37 VI) were mixed with recombinant WT- or DN-p38α and immunoprecipitated using anti-Cdc37 followed by immunoblots as indicated.

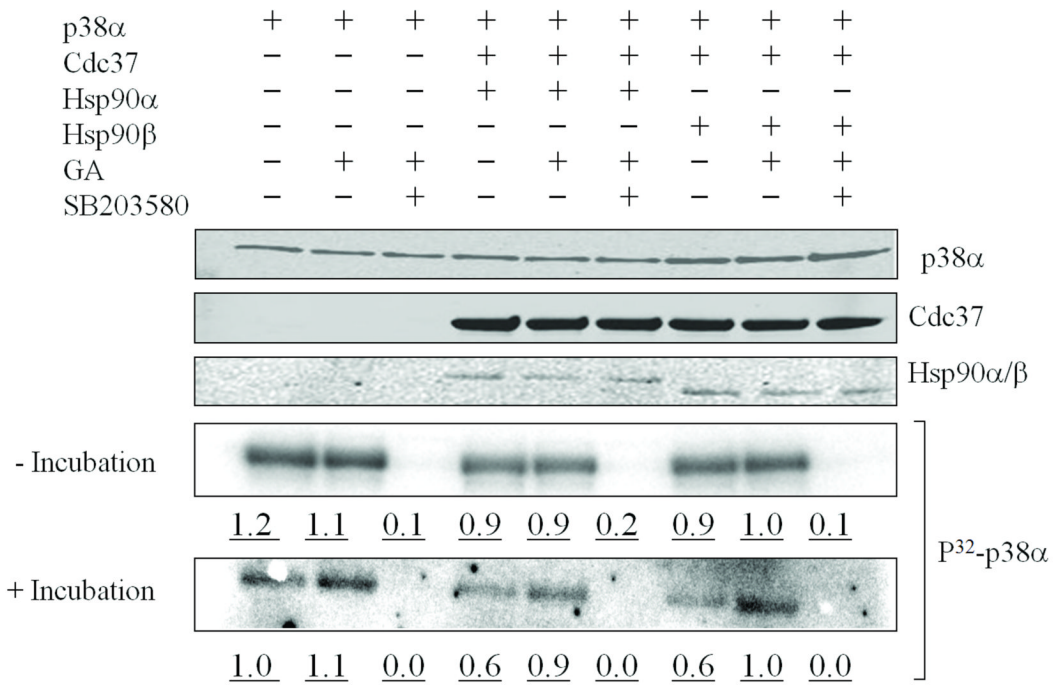

Figure 3. p38α activity assay *in vitro*

Recombinant Hsp90α or Hsp90β plus Cdc37 were mixed and incubated in kinase buffer with [γ-^{32}P]-ATP in the presence or absence of 5 μmol/L SB203580 or 10 μmol/L GA for 30 minutes at 30°C. The top three panels show immunoblots of the total reaction mixtures using antibodies as indicated. The bottom two panels show autoradiograms of ^{32}P-labeled p38. Quantification for the ^{32}P-labeled p38 is indicated at the bottom of each autoradiogram. This experiment was repeated twice with similar results. The "– Incubation" samples were identical to the "+ Incubation" samples except the overnight pre-incubation at 4 °C prior to incubation at 30°C.

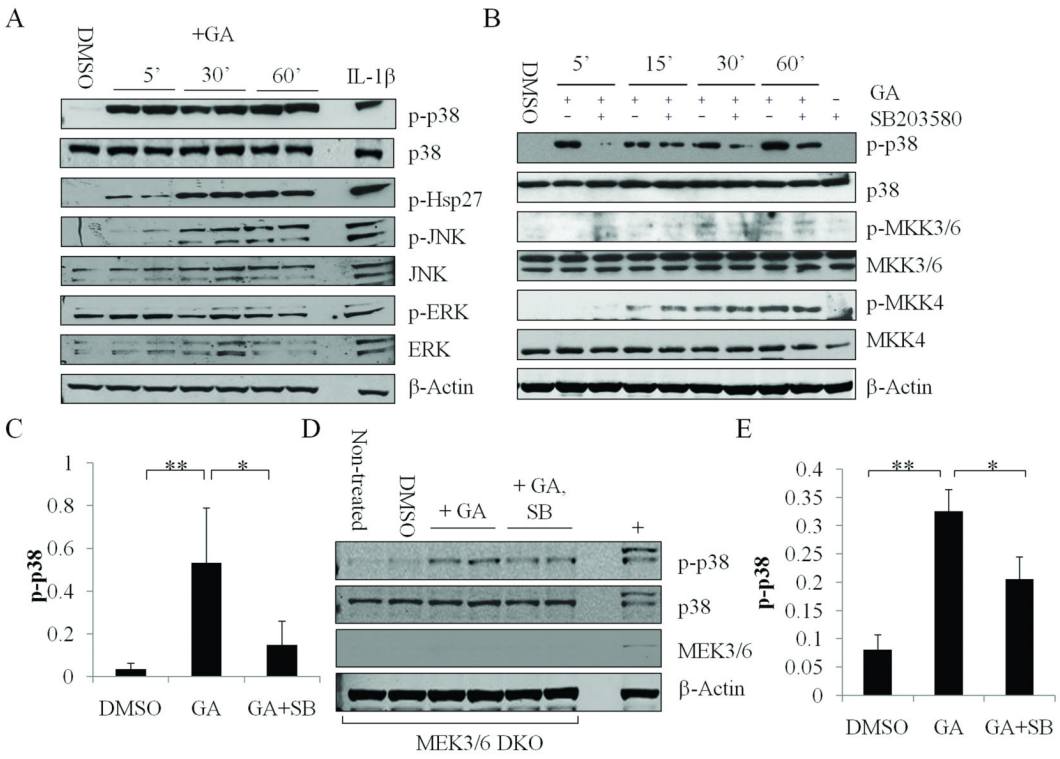

Figure 4. Impact of Hsp90 inhibition on p38 activity

A) NRVM were treated with 1.8 μmol/L geldanamycin (GA) for different time periods in minutes as indicated. Immunoblots for total protein lysates collected from duplicate samples at each time point are shown using specific antibodies as labeled. NRVM treated with equal volume of DMSO and interleukin-1β (IL-1β) were included as basal and positive controls for p38 activation respectively. **B)** NRVM were pre-incubated with 1μmol/L SB203580 or DMSO for 20 minutes prior to 1.8 μmol/L GA treatment for different time periods. Immunoblots were performed from the total protein lysates using antibodies as labeled. **C)** p-p38 level normalized to background level was quantified for NRVM treated with DMSO, GA, and GA+SB for 5 minutes (n=5, * $p<0.05$, ** $p< 0.005$). **D)** MKK3/6 DKO MEFs (DKO) were pre-treated with 1 μmol/L SB203580 for 30 minutes, followed by 1.8 μmol/L GA or 0.01% DMSO for another 30 minutes. Immunoblots on the total cell lysates was performed with antibodies as indicated. Non-treated WT MEF (+) was run on the side as a positive control for the immunoblots. **E)** p-p38 level normalized to background level was quantified for DKO MEF treated with DMSO, GA, and GA+SB for 30 minutes (n=4, * $p<0.05$, ** $p<0.001$).

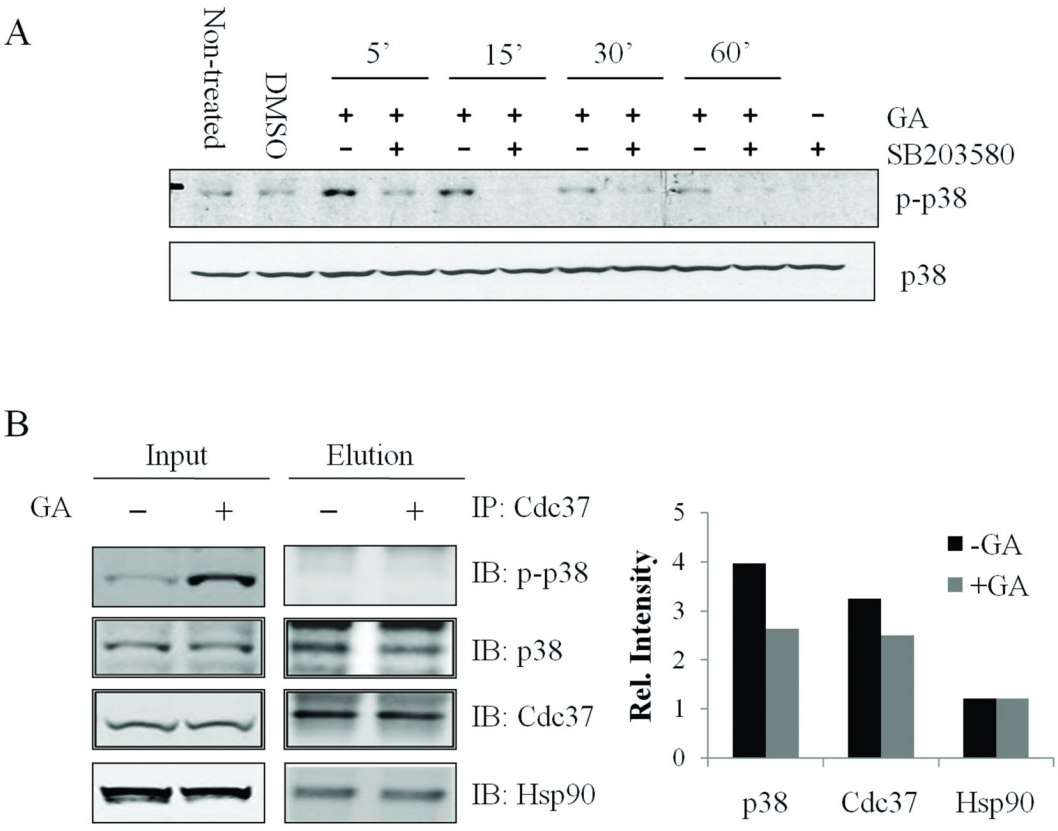

Figure 5. Impact of Hsp90 inhibition on p38 activity and interaction with Cdc37 in HeLa cells

A) Immunoblots for phospho- and total p38 on HeLa cells treated with 1.8μmol/L GA for time periods as indicated in the presence of absence of pre-treatment with 1μmol/L SB203580. **B)** Co-IP assay on HeLa cell extract with or without 1.8μmol/L GA treatment for 15 min. Immunoprecipitation with anti-Cdc37 was followed by immunoblots with antibodies as indicated. Relative intensity of the signals of total p38, Cdc37 and Hsp90 in the elution blots were quantified and shown in a bar graph.

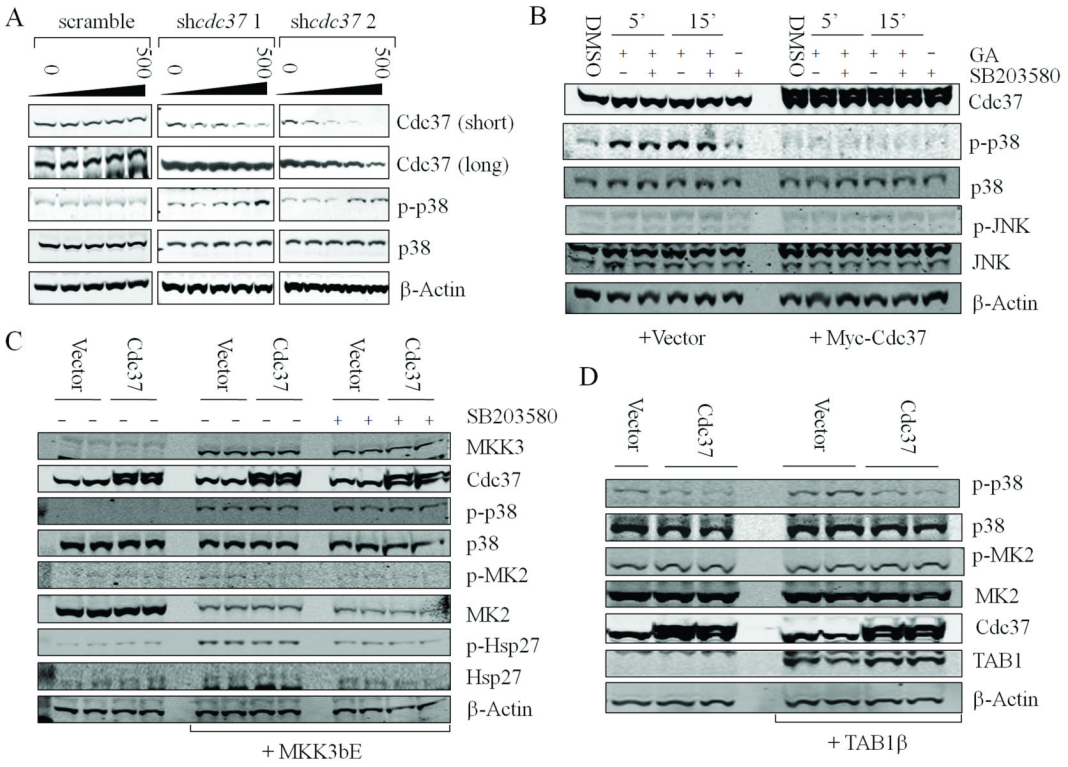

Figure 6. Functional impact of Cdc37 expression on canonical and non-canonical p38 activation

A) HeLa cells were infected with different MOI (0 to 500) of adenoviral vectors expressing scramble or two different shCdc37 constructs (shCdc37-1 and shCdc37-2) for 48 hours. Immunoblots for Cdc37, phospho-p38 and total-p38 were performed using specific antibodies as indicated. β-Actin was used as a loading control. **B)** HeLa cells were transfected with vector or Myc-Cdc37 for 24 hours, followed by treatment with 1.8μmol/L GA for 15 minutes with or without 1μmol/L SB203580 added 20 minutes before the GA treatment. Immunoblots for Cdc37, phospho- and total-p38, as well as phospho- and total-JNK were performed as indicated. **C)** HeLa cells were transfected with constitutively active MKK3 (MKK3bE) with or without Cdc37, and treated with 1μmol/L SB203580 for 30 minutes. Immunoblots for p38 and its downstream substrates are shown as indicated. **D)** Cdc37 was expressed in HeLa cells with TAB1β. Immunoblots are shown with β-Actin as a control.

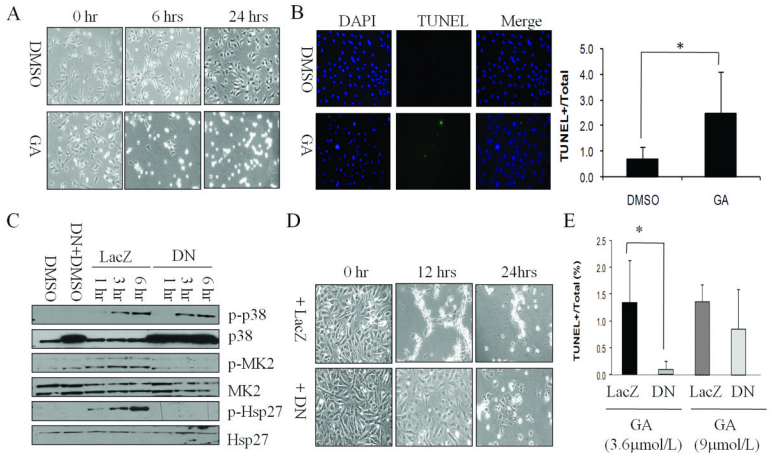

Figure 7. Hsp90 and p38 activity in myocyte survival

A) NRVM was treated with 9 μmol/L GA or DMSO as a control. Representative images of cell viability were recorded at 0, 6, and 24 hours post treatment as indicated. **B)** TUNEL staining on COS7 treated with 9 μmol/L GA for 6 hours. Quantification of TUNEL positive cells from three different experiments. * $p < 0.01$, student's *t* test. **C)** NRVM was infected with adv-LacZ or Adv-DNp38α (DN) for 48 hours followed by 9 μmol/L GA treatment for specific periods as indicated. Immunoblots for phospho- and total p38 were performed. Phospho-MK2 and phospho-Hsp27 were assessed as p38 downstream activities. **D)** COS7 cells were infected with Adv-LacZ or Adv-DNp38α as indicated for 48 hours prior to treatment with 9 μmol/L GA for 0, 12, and 24 hours. Representative images of cell viability are shown at 0, 12 and 24 hours post treatment as indicated. **E)**. Quantification of TUNEL positive apoptotic COS7 cells among different treatment groups (LacZ vs. DNp38α) at two doses of GA (3.6 μmol/L vs. 9 μmol/L) as indicated. * $p < 0.05$.