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# Circulation Research

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## Promotion of CHIP-Mediated p53 Degradation Protects the Heart From Ischemic Injury

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# Promotion of CHIP-Mediated p53 Degradation Protects the Heart From Ischemic Injury

Atsuhiko T. Naito, Sho Okada, Tohru Minamino, Koji Iwanaga, Mei-Lan Liu, Tomokazu Sumida, Seitaro Nomura, Naruhiko Sahara, Tatsuya Mizoroki, Akihiko Takashima, Hiroshi Akazawa, Toshio Nagai, Ichiro Shiojima, Issei Komuro

**Rationale:** The number of patients with coronary heart disease, including myocardial infarction, is increasing and novel therapeutic strategy is awaited. Tumor suppressor protein p53 accumulates in the myocardium after myocardial infarction, causes apoptosis of cardiomyocytes, and plays an important role in the progression into heart failure.

**Objectives:** We investigated the molecular mechanisms of p53 accumulation in the heart after myocardial infarction and tested whether anti-p53 approach would be effective against myocardial infarction.

**Methods and Results:** Through expression screening, we found that CHIP (carboxyl terminus of Hsp70-interacting protein) is an endogenous p53 antagonist in the heart. CHIP suppressed p53 level by ubiquitinating and inducing proteasomal degradation. CHIP transcription was downregulated after hypoxic stress and restoration of CHIP protein level prevented p53 accumulation after hypoxic stress. CHIP overexpression in vivo prevented p53 accumulation and cardiomyocyte apoptosis after myocardial infarction. Promotion of CHIP function by heat shock protein (Hsp)90 inhibitor, 17-allylamino-17-demethoxy geldanamycin (17-AAG), also prevented p53 accumulation and cardiomyocyte apoptosis both in vitro and in vivo. CHIP-mediated p53 degradation was at least one of the cardioprotective effects of 17-AAG.

**Conclusions:** We found that downregulation of CHIP level by hypoxia was responsible for p53 accumulation in the heart after myocardial infarction. Decreasing the amount of p53 prevented myocardial apoptosis and ameliorated ventricular remodeling after myocardial infarction. We conclude that anti-p53 approach would be effective to treat myocardial infarction. (*Circ Res.* 2010;106:00-00.)

**Key Words:** myocardial infarction ■ CHIP ■ p53 ■ hypoxia

The number of patients with coronary heart disease has been increasing and cardiovascular diseases are the leading cause of deaths in the Western world. Despite the development of pharmacological and nonpharmacological interventions, 33% of the men and 43% of the women die within 5 years after myocardial infarction (MI).<sup>1</sup> Therefore, a novel therapeutic approach against coronary heart disease is awaited.

Apoptosis of cardiomyocytes is accompanied with acute coronary occlusion.<sup>2</sup> Because apoptotic loss of cardiomyocytes causes heart failure,<sup>3</sup> inhibition of apoptosis has been suggested as an additional therapeutic approach to coronary heart disease.<sup>4</sup> In mice, overexpression of antiapoptotic Bcl-2 protein or genetic deletion of proapoptotic Bax protein have been reported to prevent apoptosis and reduce

infarct size,<sup>5-8</sup> implicating that antiapoptotic approach is effective for prevention of ventricular remodeling after myocardial infarction.

The tumor suppressor p53 is an important transcription factor that regulates cell cycle progression, cellular senescence, and apoptosis. Under physiological condition, p53 protein level is maintained low, but is elevated when cells are stressed or damaged.<sup>9</sup> The mechanism for keeping p53 protein level low involves several E3 ubiquitin ligases such as MDM2,<sup>10,11</sup> COP1,<sup>12</sup> and Pirh2.<sup>13</sup> Importantly, the expression of these proteins were positively regulated by p53, suggesting the role for negative-feedback loop against p53 elevation.

Protein level of p53 is also kept low in the heart but it is elevated when cardiac cells are exposed to hypoxia.<sup>14-16</sup>

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**Non-standard Abbreviations and Acronyms**

<b>17-AAG</b>	17-allylamino-17-demethoxy geldanamycin
<b>CHIP</b>	carboxyl terminus of Hsp70-interacting protein
<b>HIF</b>	hypoxia-inducible factor
<b>HRE</b>	hypoxia-responsive element
<b>Hsp</b>	heat shock protein
<b>HW/BW</b>	heart weight/body weight
<b>MI</b>	myocardial infarction
<b>PARP</b>	poly(ADP-ribose)polymerase
<b>siRNA</b>	small interfering RNA

We have recently reported that elevation of p53 causes the development of pressure overload-induced heart failure.<sup>16</sup> We have also observed the elevation of p53 protein levels after myocardial infarction and shown that p53 gene deletion improved cardiac function after myocardial infarction,<sup>16</sup> suggesting that the inhibition of p53 might become a novel therapeutic strategy for ischemic heart diseases.

As an initial approach for the investigation of anti-p53 therapy, we searched for an endogenous p53 antagonist in the heart. Through expression screening, we found that CHIP (carboxyl terminus of Hsp70-interacting protein) is an endogenous p53 antagonist that keeps p53 level low in the heart. We also found that CHIP downregulation is involved in the mechanism of p53 accumulation in the heart after myocardial infarction. Facilitating CHIP-mediated p53 degradation prevented apoptosis of cardiomyocytes and ameliorated ventricular remodeling in the postinfarct heart. The present study revealed the mechanism of p53 accumulation in the heart after myocardial ischemia and suggested that anti-p53 approach would be effective to treat myocardial infarction.

**Methods****Expression Cloning**

Expression cloning was performed as described previously<sup>17</sup> using PG13-Luc (kind gift from B. Vogelstein) as a reporter plasmid. Initially, cDNA expression library from human heart (Invitrogen) was separated into small pools that contain ≈100 clones each. cDNA clones that downregulate PG13 activity were isolated by sib-selection.

**Cell Culture**

COS7 and HEK293 cells are from ATCC and cultured in DMEM containing 10% FBS (Invitrogen). Neonatal rat cardiomyocytes were isolated from 1-day-old Wistar rats and cultured as described previously.<sup>18</sup> Cardiomyocytes were exposed to hypoxic stress by culturing under CoCl<sub>2</sub> or by culturing in hypoxic chamber (<1% O<sub>2</sub>; PO<sub>2</sub>, 18 to ≈20 mm Hg).

**Animals**

All protocols were approved by Chiba University review board. CHIP knockout mice and cardiac-specific inducible hypoxia-inducible factor (HIF)-1 knockout mice were described.<sup>16,19,20</sup> Heterozygous CHIP knockout mice were used in this study because homozygous knockout mice were perinatally lethal.<sup>20</sup> Cardiac-specific CHIP transgenic mice were generated by pronuclear injection

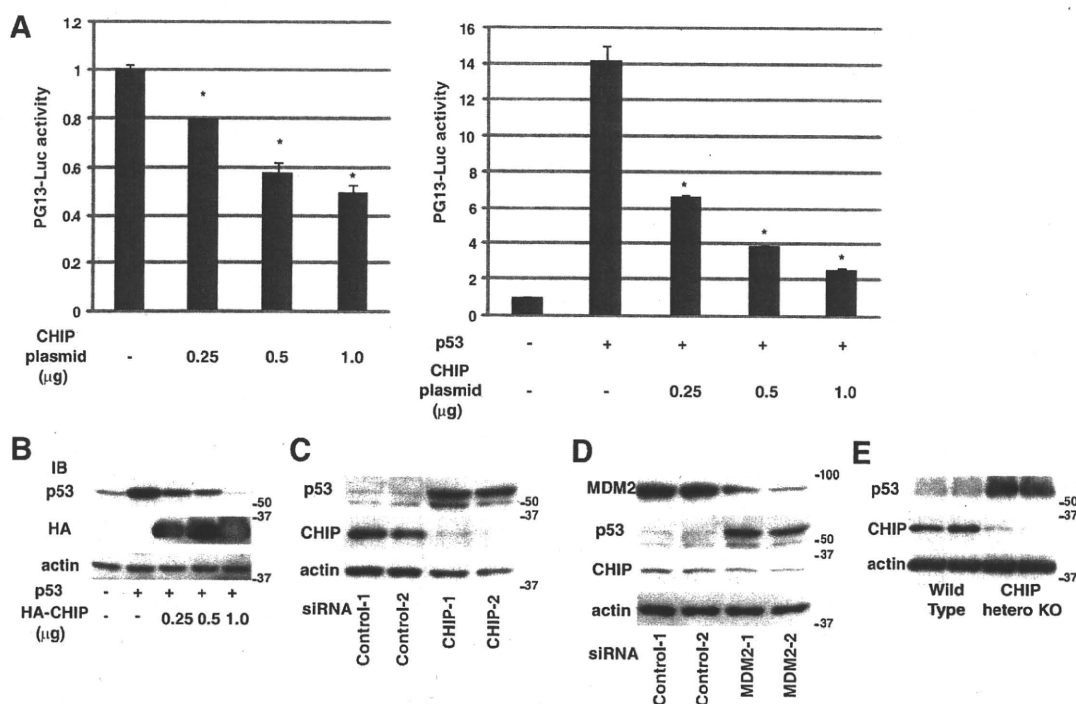
of αMHC-HA-CHIP transgene construct. Coronary artery ligation was performed on 10-week old male mice as described previously.<sup>21</sup>

**Statistical Analysis**

Data are expressed as means ± SE. The significance of differences among means was evaluated using analysis of variance (ANOVA), followed by Fisher's protected least significant difference test and Dunnett's test for multiple comparisons. Significant differences were defined as  $P < 0.05$ .

**Results****Identification of CHIP As a Novel p53 Antagonist From Heart cDNA Library**

To elucidate novel p53 antagonists in the heart, we performed expression screening by expressing cDNA pools in COS7 cells together with a reporter plasmid, PG13-luciferase, which contains 13 copies of p53 binding site upstream of luciferase gene and responsive to wild-type p53 dependent transcription. From the screening of 500 cDNA pools, each containing around 100 individual cDNA clones obtained from human heart cDNA library, we found 5 pools that suppress the PG13 activity. Individual cDNA clone that downregulates the PG13 activity was identified by sib-selection. One of the molecules that was highly expressed in the heart (Figure 1, A, in the Online Data Supplement, available at <http://circres.ahajournals.org>) was CHIP (also called STUB1 [Stip1 homology and U-box containing protein]), a chaperone-interacting protein with E3 ubiquitin ligase activity.<sup>22</sup> Transfection of CHIP suppressed endogenous and exogenous (by overexpression of p53) PG13 activity (Figure 1A) and decreased the protein levels of p53 (Figure 1B) in a plasmid dose-dependent manner in COS7 cells. Direct interaction between CHIP and p53 was confirmed both at the exogenous level in COS7 cells (Online Figure 1, B) and at the endogenous level in cardiomyocytes (Online Figure 1, C). Western blotting using anti-ubiquitin antibody after immunoprecipitation with p53 revealed that overexpression of CHIP increased poly-ubiquitinated p53 (which appears as a smear) (Online Figure 1, D). The proteasomal inhibitor MG132 restored p53 protein level that was suppressed by CHIP (Online Figure 1, E), indicating that CHIP directs p53 for proteasome-mediated degradation. When CHIP was knocked down in cardiomyocytes using small interfering (si)RNA, p53 expression was upregulated (Figure 1C), and p53 protein levels following CHIP knockdown were comparable to those induced by the knockdown of MDM2, a well known E3 ubiquitin ligase for p53 (Figure 1D). CHIP protein level was not changed by knockdown of MDM2 (Figure 1D). p53 protein levels were also markedly elevated in the heart of CHIP heterozygous mice (Figure 1E). These results suggest that CHIP induces degradation of wild-type p53 protein in cardiomyocytes, which is consistent with previous reports in other cells (H1299 cells and U2OS cells).<sup>23,24</sup> In addition, we revealed that CHIP is a crucial negative regulator that keeps p53 protein levels low in the heart under physiological conditions.



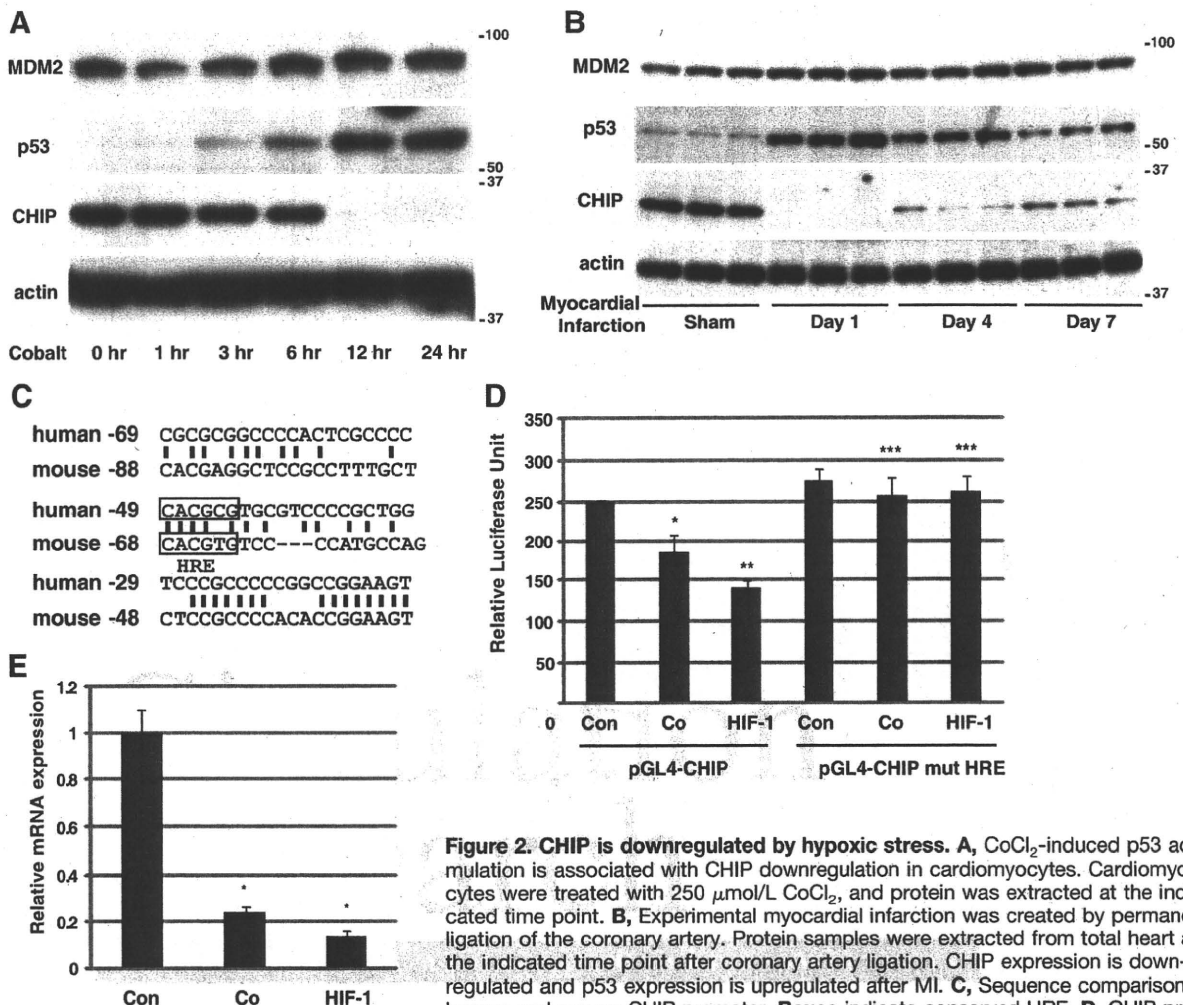
**Figure 1. CHIP is a crucial negative regulator of p53 expression in the heart.** **A**, Transfection of CHIP expressing plasmid suppressed endogenous (left) and exogenous (right) p53 transcriptional activity. \* $P < 0.01$  vs control;  $n = 5$ . **B**, CHIP decreases p53 protein levels in COS7 cells. IB indicates immunoblot. **C**, p53 expression is upregulated by CHIP knockdown in cardiomyocytes. siRNAs specific to CHIP (CHIP-1 and CHIP-2), or control siRNA were transfected into cultured cardiomyocytes and protein levels of CHIP and p53 were examined by Western blotting. CHIP-1 and CHIP-2 represent 2 different siRNAs against CHIP. Control-1 is a commercially available control RNA, and control-2 is a scrambled control RNA. **D**, p53 upregulation is also observed by MDM2 knockdown. siRNAs specific to MDM2 (MDM2-1 and MDM2-2) or control siRNA were transfected into cultured cardiomyocytes, and protein levels of CHIP, p53, and MDM2 were examined by Western blotting. The extent of p53 upregulation by MDM2 knockdown was comparable to that induced by CHIP knockdown. **E**, Total protein of wild-type and CHIP heterozygous mice were analyzed by Western blotting. p53 expression is upregulated in the heart of CHIP heterozygous mice.

### Molecular Mechanisms of Hypoxia-Induced p53 Accumulation

As CHIP regulates p53 status in the heart, we speculated that CHIP might be involved in the molecular mechanism of hypoxia-induced p53 accumulation in the heart. Cobalt chloride ( $\text{CoCl}_2$ ) increases HIF-1 activity through preventing HIF-1 $\alpha$  protein degradation and is widely used as a hypoxia mimicking reagent.<sup>25,26</sup> Treatment of cardiomyocytes with  $\text{CoCl}_2$  (250  $\mu\text{mol/L}$ ) increased p53 protein level with a marked downregulation of CHIP protein level (Figure 2A). Notably, the expression of MDM2 was rather increased in this experimental condition. Because transcriptional regulation of MDM2 is known to be upregulated by p53 as a part of negative-feedback loop, increased MDM2 expression after  $\text{CoCl}_2$  treatment may possibly be attributable to this feedback system against p53 elevation. Accumulation of p53 and downregulation of CHIP were also observed when cardiomyocytes were cultured in hypoxic chamber for 24 hours (Online Figure I, F). We confirmed that both treatments increased nuclear HIF-1 $\alpha$  protein that binds to HIF-1 $\alpha$  binding oligonucleotide by commercially available ELISA system (Online Figure I, G). We also analyzed the expression of p53 and CHIP in the heart after MI. p53 protein levels were increased on day 1 after MI and remained upregulated thereafter, whereas expression levels of CHIP were markedly downregulated on day 1, and remained at lower levels than

those of controls (Figure 2B and analyzed in Online Figure II, A and B). In contrast, MDM2 protein levels were slightly increased after MI (Figure 2B). The inverse correlation between CHIP and p53 protein level implies the possible involvement of CHIP downregulation in the initiation of p53 accumulation after acute hypoxic stress. Other E3 ubiquitin ligases whose transcription is regulated by p53, such as MDM2, might work to reverse p53 level after initial accumulation of p53 as a feedback system to prevent further detrimental effects that might be elicited by chronic p53 elevation.

To investigate why CHIP is downregulated after hypoxic insult, we tested whether HIF-1 mediates hypoxia-induced downregulation of CHIP, because HIF-1 is known to downregulate some of its target genes through hypoxia-responsive element (HRE).<sup>27-30</sup> Human CHIP promoter (from -329 bases upstream of transcription start site to +39 bases downstream of transcription start site) that contains a conserved HRE at -49 (Figure 2C) was cloned upstream of luciferase reporter gene (pGL4-CHIP). pGL4-CHIP activity was significantly suppressed by both  $\text{CoCl}_2$  treatment (24 hours) and HIF-1 $\alpha$  overexpression in COS7 cells (Figure 2D). When a mutation was introduced into HRE at -49 (pGL4-CHIP-mutHRE), the luciferase activity was no longer responsive to hypoxic stress or HIF-1 $\alpha$  overexpression (Figure 2D), suggesting that CHIP gene expression is downregu-

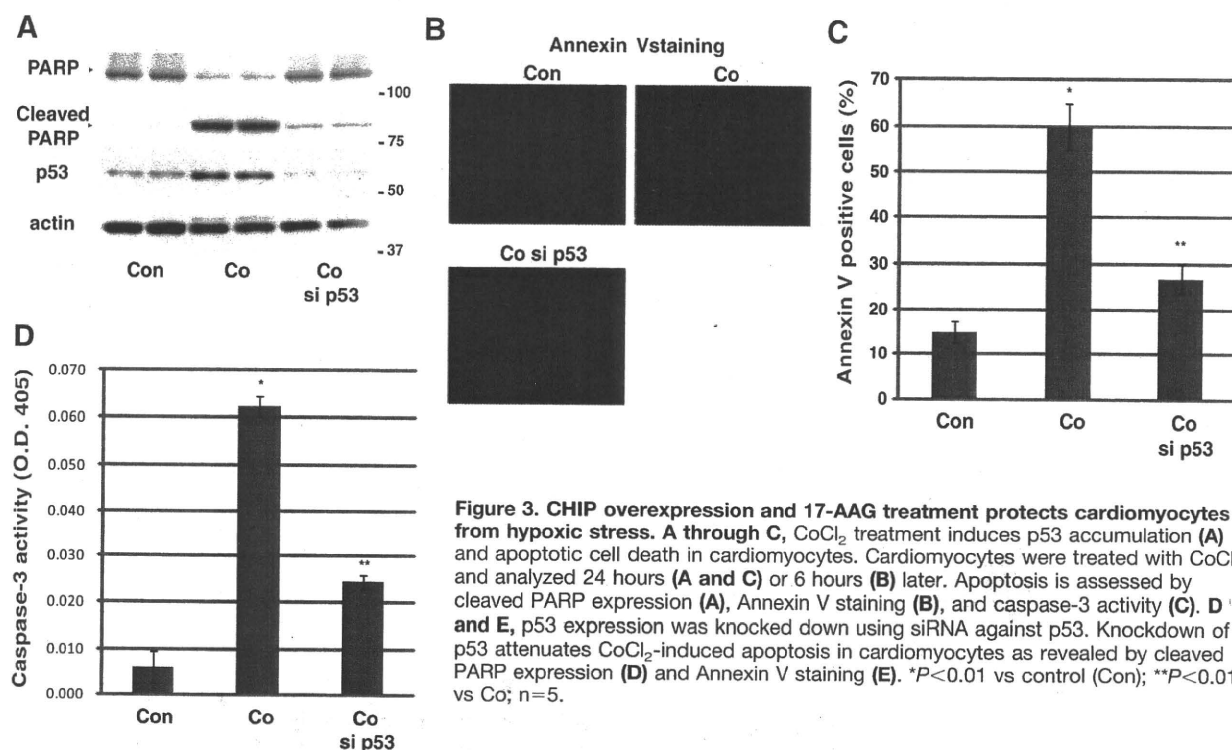


**Figure 2. CHIP is downregulated by hypoxic stress.** **A**,  $\text{CoCl}_2$ -induced p53 accumulation is associated with CHIP downregulation in cardiomyocytes. Cardiomyocytes were treated with  $250 \mu\text{mol/L}$   $\text{CoCl}_2$ , and protein was extracted at the indicated time point. **B**, Experimental myocardial infarction was created by permanent ligation of the coronary artery. Protein samples were extracted from total heart at the indicated time point after coronary artery ligation. CHIP expression is downregulated and p53 expression is upregulated after MI. **C**, Sequence comparison of human and mouse CHIP promoter. Boxes indicate conserved HRE. **D**, CHIP promoter activity is downregulated by  $\text{CoCl}_2$  treatment and HIF-1 $\alpha$  overexpression, and mutations (CACGTG to CTGGCG) introduced into HRE at -49 abrogated this response. CHIP promoter sequence from human genomic DNA (-329 to +39 from transcription start site) was cloned upstream of luciferase gene. Mutation was introduced using a kit from Stratagene. Luciferase assay was performed 24 hours after  $\text{CoCl}_2$  treatment or HIF-1 $\alpha$  overexpression. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P = \text{NS}$  vs control;  $n = 5$ . **E**, Real-time PCR analysis revealed mRNA level of CHIP was also downregulated by hypoxic stress (Co) and HIF-1 $\alpha$  overexpression. RNA was extracted 24 hours after  $\text{CoCl}_2$  treatment or HIF-1 $\alpha$  overexpression. \* $P < 0.01$ .

lated by HIF-1 at the transcriptional level through HRE. Real-time PCR analysis revealed that exposure of cardiomyocytes to  $\text{CoCl}_2$  (24 hours) and adenoviral overexpression of constitutively active HIF-1 $\alpha$  led to marked downregulation of CHIP mRNA levels (Figure 2E), further supporting our data that hypoxic stress downregulates CHIP levels. HIF-1 $\alpha$  gene is both required and sufficient for hypoxic stress-induced CHIP downregulation and p53 accumulation because knockdown of HIF-1 $\alpha$  attenuated the effects of  $\text{CoCl}_2$  treatment on expressions of p53 and CHIP (Online Figure III, A), and overexpression of constitutively active HIF-1 $\alpha$  suppressed CHIP expression and increased p53 expression in cardiomyocytes (Online Figure III, B). Furthermore, downregulation of CHIP protein levels after MI was attenuated in cardiac-specific inducible HIF-1 $\alpha$  conditional knockout mice<sup>16</sup> (Online Figure III, C). Collectively, these findings suggest that CHIP transcription is directly downregulated by hypoxia through HIF-1.

**CHIP Protects Cardiomyocytes From Hypoxia-Induced p53-Mediated Apoptosis of Cardiomyocytes**

Because hypoxia or p53 overexpression induces apoptotic cell death in cultured cardiomyocytes,<sup>14</sup> we next examined whether hypoxia-induced cardiomyocyte apoptosis is mediated by the HIF-1-CHIP-p53 pathway.  $\text{CoCl}_2$  treatment (24 hours) induced p53 accumulation and promoted apoptosis of cardiomyocytes as assessed by cleaved poly (ADP-ribose) polymerase (PARP) expression (Figure 3A), Annexin V staining (Figure 3B and 3C), and caspase-3 activity (Figure 3D).  $\text{CoCl}_2$ -induced apoptosis was p53-dependent, because knockdown of p53 in  $\text{CoCl}_2$ -treated cardiomyocytes attenuated hypoxia-induced cell death (Figure 3A through 3D). We next assessed whether overexpression of CHIP could rescue  $\text{CoCl}_2$ -induced apoptosis. Adenovirus-mediated overexpression of CHIP in cardiomyocytes markedly downregulated p53 expression and attenuated apoptosis in  $\text{CoCl}_2$ -treated



**Figure 3. CHIP overexpression and 17-AAG treatment protects cardiomyocytes from hypoxic stress.** A through C,  $\text{CoCl}_2$  treatment induces p53 accumulation (A) and apoptotic cell death in cardiomyocytes. Cardiomyocytes were treated with  $\text{CoCl}_2$  and analyzed 24 hours (A and C) or 6 hours (B) later. Apoptosis is assessed by cleaved PARP expression (A), Annexin V staining (B), and caspase-3 activity (C). D and E, p53 expression was knocked down using siRNA against p53. Knockdown of p53 attenuates  $\text{CoCl}_2$ -induced apoptosis in cardiomyocytes as revealed by cleaved PARP expression (D) and Annexin V staining (E). \* $P < 0.01$  vs control (Con); \*\* $P < 0.01$  vs Co;  $n = 5$ .

cardiomyocytes (Figure 4A through 4C). These results underscore our hypothesis that downregulation of CHIP is responsible for p53 accumulation after hypoxic stress. Moreover, forced expression of CHIP prevented hypoxia-induced cardiomyocyte apoptosis by inducing degradation of p53, suggesting that CHIP-mediated p53 degradation is a potential therapeutic target.

### 17-AAG Protects Cardiomyocytes From Hypoxia-Induced Apoptosis

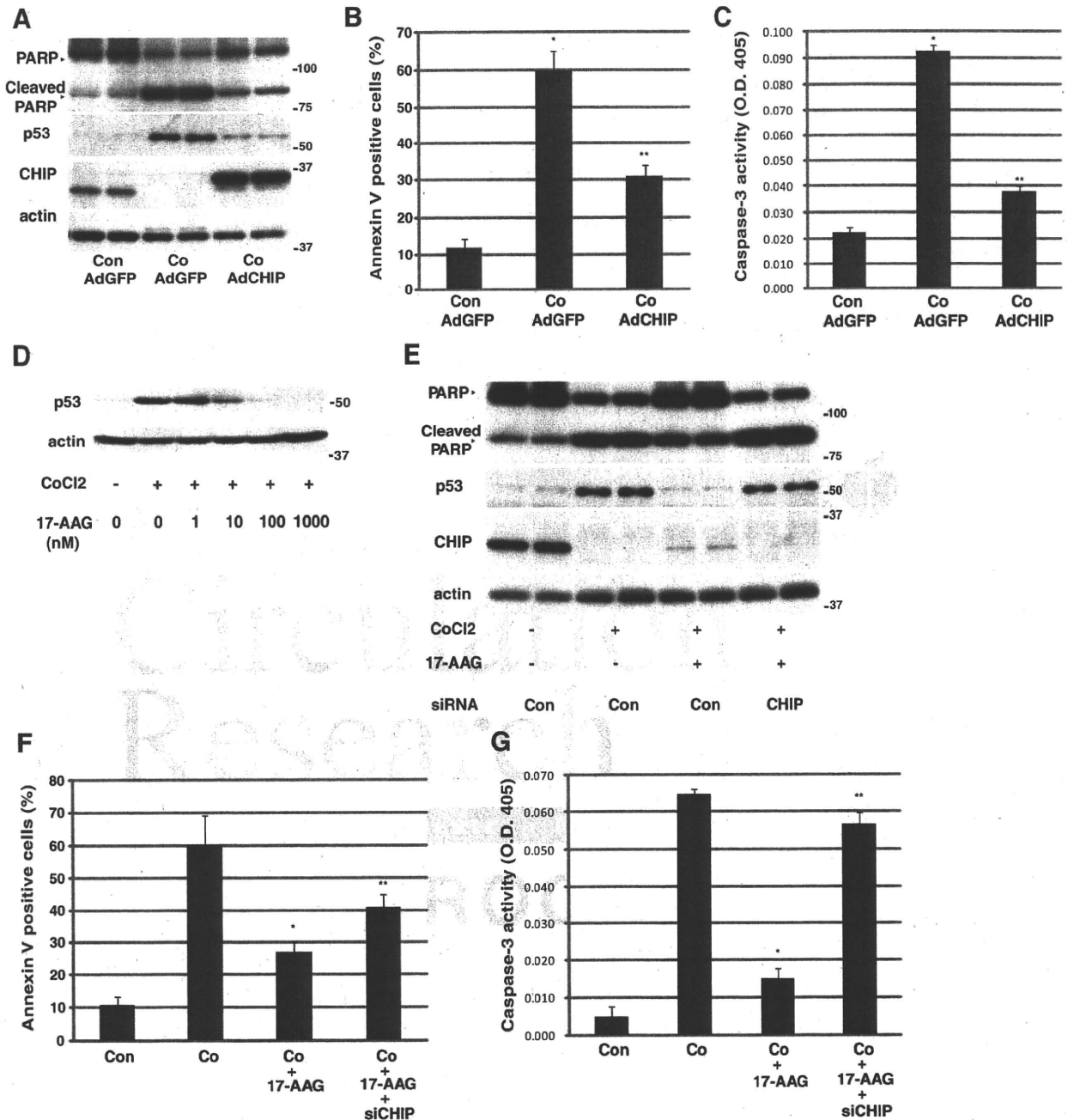
Inhibitors for heat shock protein (Hsp)90 have been shown to promote proteasomal degradation of CHIP client proteins and to be effective for the diseases caused by the accumulation of CHIP substrates.<sup>31,32</sup> We therefore examined whether an Hsp90 inhibitor 17-allylamino-17-demethoxy geldanamycin (17-AAG) induces degradation of p53 protein and protects cardiomyocytes from hypoxic stress. In cardiomyocytes treated with  $\text{CoCl}_2$ , 17-AAG downregulated p53 expression (Figure 4D). 17-AAG treatment also suppressed hypoxia-induced cardiomyocyte apoptosis in a CHIP-dependent manner, because CHIP knockdown attenuated the protective effects of 17-AAG (Figure 4E through 4G). These results suggest that 17-AAG protects cardiomyocytes from hypoxic stress by promoting CHIP-mediated p53 degradation.

Interestingly, protein level of CHIP was increased by 17-AAG treatment (Figure 4E). As mRNA level of CHIP was not changed by 17-AAG treatment (Online Figure IV, A), we speculated that protein stability was affected by 17-AAG treatment. When protein translation was inhibited by cycloheximide, 17-AAG treatment dramatically extended the protein half-life of CHIP (Online Figure IV, B and C). 17-AAG also upregulated the protein stability of other proteins, Hsp70

and HSF-1 (Online Figure IV, B and C). Because 17-AAG exerted some antiapoptotic effects even in the cells of negligible CHIP protein level (Figure 4E and 4F), upregulation of these cardioprotective proteins<sup>33,34</sup> might mediate part of the effects of 17-AAG. It remains to be determined how 17-AAG prolongs protein half-life of certain kinds of proteins.

### CHIP and 17-AAG Prevent Apoptosis and Ventricular Remodeling After Myocardial Infarction

We next examined whether promotion of CHIP-mediated p53 degradation could attenuate ischemic cardiac injury also in vivo. For this purpose, transgenic mice which overexpress CHIP specifically in the heart (CHIP-Tg) (Figure 5A) were subjected to permanent coronary artery ligation. In CHIP-Tg mice, elevation of p53 protein levels (Figure 5B) and apoptotic cardiomyocyte death in the border zone of the infarct area (Figure 5B and 5C) were attenuated compared to wild-type littermates at 24 hours after the MI operation. Apoptotic death of the cardiomyocytes in the remote zone of the infarct was not changed between littermates (data not shown). We next examined whether this decrease in apoptotic cell death leads to attenuation of cardiac ventricular remodeling. At day 14, CHIP-Tg mice exhibited smaller heart weight/body weight (HW/BW) ratio, better contractility and less ventricular remodeling (Figure 5D and 5E) compared to wild-type littermates. These results provide an evidence for our hypothesis that CHIP downregulation is responsible for p53 accumulation after myocardial infarction, and suggests that CHIP overexpression is protective for the heart by preventing p53 accumulation and cardiomyocyte apoptosis after myocardial infarction.

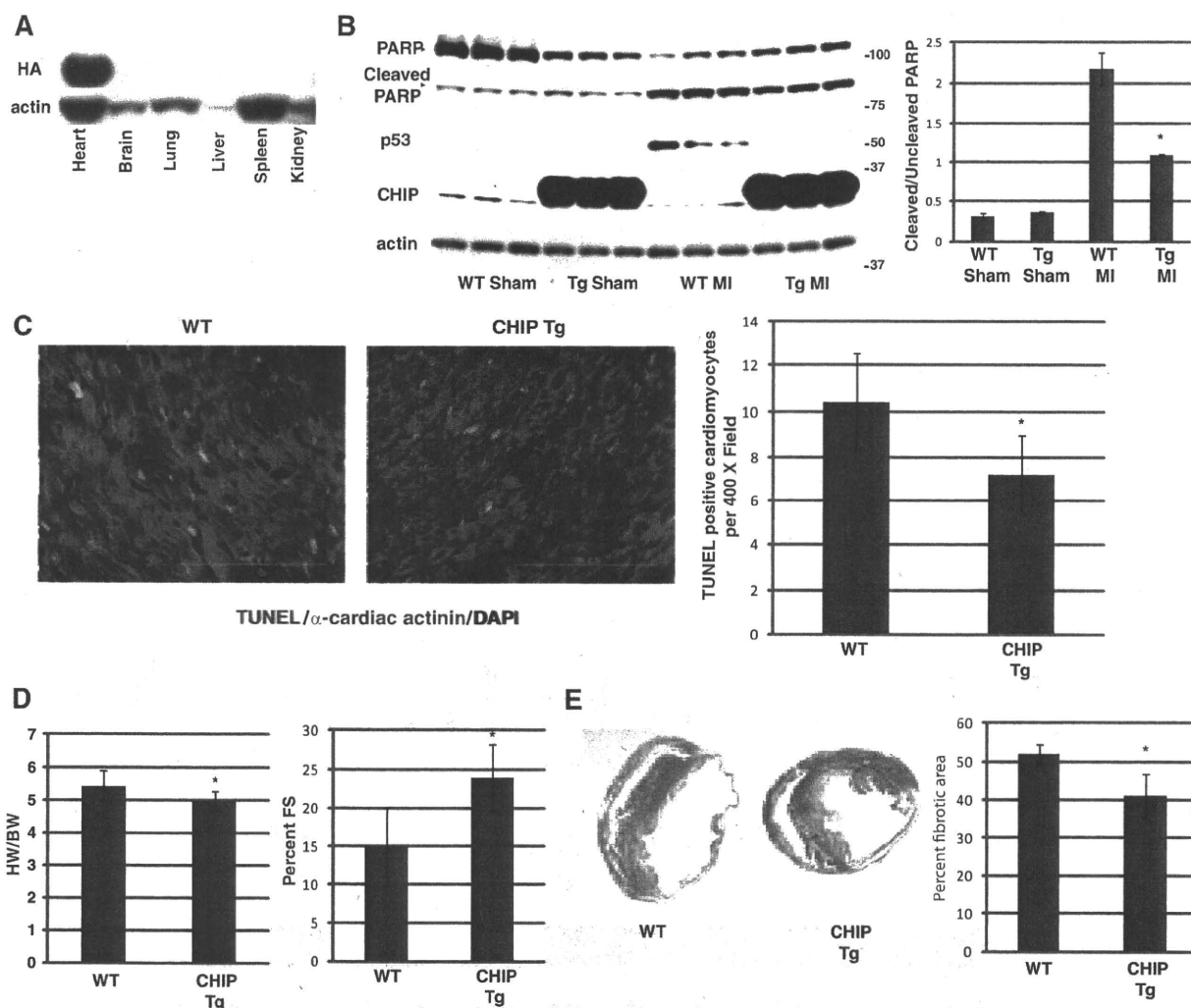


**Figure 4. Promoting CHIP-mediated p53 degradation is protective against hypoxic stress.** **A through C,** Overexpression of CHIP attenuates CoCl<sub>2</sub>-induced p53 accumulation (**A**) and apoptosis in cardiomyocytes. Cardiomyocytes were infected with adenovirus harboring green fluorescent protein (GFP) or CHIP. Twenty-four hours later, culture medium was changed and the cells were treated with CoCl<sub>2</sub>. Apoptosis was assessed by cleaved PARP expression (**A**), Annexin V staining (**B**), and caspase-3 activity (**C**). \**P*<0.01 vs control (Con)+AdGFP; \*\**P*<0.01 vs Co+AdGFP; n=5. **D,** 17-AAG downregulates p53 expression in cardiomyocytes. Neonatal rat cardiomyocytes were treated with CoCl<sub>2</sub> with or without 17-AAG at the indicated concentration. **E through G,** 17-AAG inhibits CoCl<sub>2</sub>-induced p53 accumulation (**E**) and apoptosis in cardiomyocytes, which is abrogated by CHIP knockdown. Neonatal rat cardiomyocytes were transfected with control siRNA or siRNA against CHIP. Twenty-four hours later, medium was changed and the cells were treated with CoCl<sub>2</sub> and/or 17-AAG. Apoptosis is assessed by cleaved PARP expression (**E**), Annexin V staining (**F**), and caspase-3 activity (**G**). \**P*<0.01 vs Co; \*\**P*<0.05 vs Co +17-AAG; n=3.

We also examined whether treatment with 17-AAG exerts similar cardioprotective effects. 17-AAG (10 mg/kg) or vehicle was intraperitoneally injected immediately after permanent coronary artery ligation. This single injection of 17-AAG effectively suppressed the elevation of p53 protein levels and apoptotic cell death in the border zone of the infarct area at 24 hours

after the operation (Figure 6A and 6B). As p53 protein level was kept elevated even 4 and 7 days after MI (Figure 2B), 17-AAG was injected every other days and we assessed whether 17-AAG treatment also leads to attenuation of ventricular remodeling, as observed in CHIP-Tg mice. At day 14, mice treated with 17-AAG exhibited smaller HW/BW ratio, better contractility,





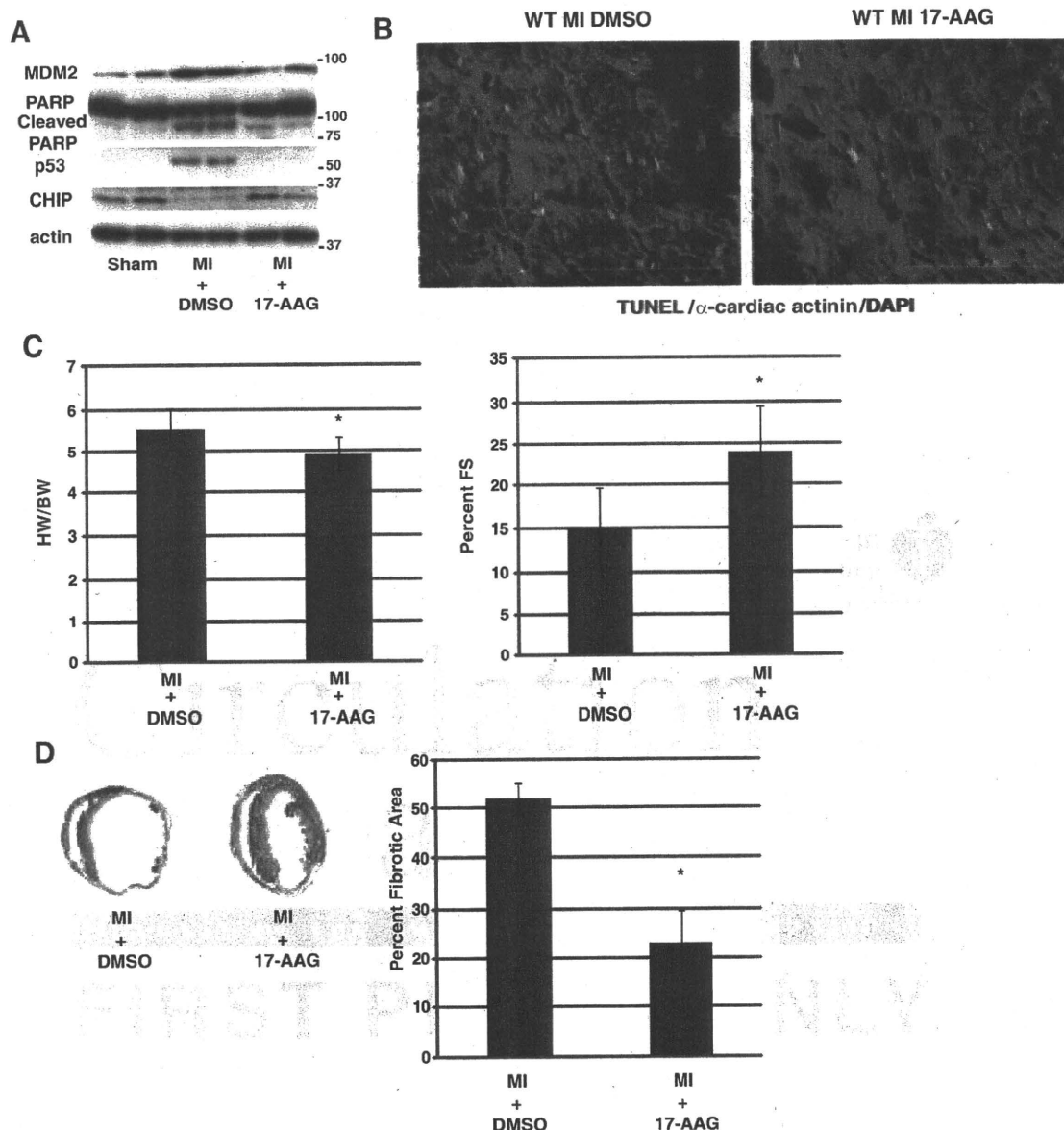
**Figure 5. Overexpression of CHIP attenuates ischemic cardiac injury in vivo.** **A**, Cardiac-specific expression of HA-tagged human CHIP in CHIP-Tg mice. **B** and **C**, p53 accumulation (**B**) and apoptosis 1 day after MI are reduced in CHIP-Tg mice. Apoptosis was assessed by cleaved PARP expression (**B**) and TUNEL staining (**C**). Cleaved PARP level was assessed by densitometric analysis on band intensity of cleaved PARP over un-cleaved PARP.  $P < 0.05$  vs WT;  $n = 3$ . WT indicates wild-type mice. **D** and **E**, Postinfarct cardiac remodeling is attenuated in CHIP-Tg mice ( $n = 15$ ). HW/BW ratio (**D**, left), contractile function (**D**, right), and percentage fibrotic area (**E**).  $*P < 0.01$  vs WT ( $n = 30$ ).

and less ventricular remodeling (Figure 6C and 6D). Interestingly, the effects of 17-AAG were greater than CHIP overexpression (compare Figures 5 and 6), suggesting that 17-AAG possesses cardioprotective activities that do not involve CHIP-mediated p53 degradation. As protein stability of cardioprotective proteins such as Hsp70 and HSF-1 was increased in vitro (Online Figure IV, B and C), we have examined the expression of these proteins in 17-AAG-treated mice. As expected, expression of these two proteins were increased by 17-AAG treatment (Online Figure IV, D), indicating that 17-AAG exerts its antiapoptotic effects by at least two mechanisms, one by inducing CHIP-mediated p53 degradation and the other by increasing cardioprotective heat shock proteins.

Finally, we examined the contribution of CHIP-mediated p53 degradation on the cardioprotective effects of 17-AAG. For that purpose we used CHIP heterozygous mice. There were no differences in cleaved PARP level (Figure 7A; compare WT Sham and Het Sham) or cardiac function between CHIP heterozygous mice and wild-type littermates at the basal level (Table). Following coronary artery ligation, however, apoptotic

cell death was observed more prominently in CHIP heterozygous mice as assessed by increased cleaved PARP level (Figure 7A; compare WT MI and Het MI) and increased TUNEL positive cells (Figure 7B). The level of p53 accumulation was comparable following myocardial infarction between wild-type and CHIP heterozygous mice, suggesting the presence of p53 independent mechanisms for enhanced apoptosis caused by CHIP haploinsufficiency. Chronically, CHIP heterozygous mice showed worse cardiac function and worse ventricular remodeling compared with wild-type mice (Figure 7C and 7D). 17-AAG treatment was less effective to reduce p53 protein level, cleaved PARP level (Figure 7A; compare Het MI and Het MI 17-AAG), and TUNEL positive cardiomyocytes in CHIP heterozygous mice, possibly as a result of CHIP haploinsufficiency. 17-AAG treatment had minimal effects on improvements of cardiac function and ventricular remodeling on CHIP heterozygous mice also in the chronic phase (Figure 7C and 7D).

However, we must emphasize that the effects of 17-AAG were not fully attributable to CHIP-mediated p53 degradation



**Figure 6. 17-AAG treatment attenuates ischemic cardiac injury in vivo.** **A**, Accumulation of p53 and cleaved PARP in the heart after MI are reduced by 17-AAG treatment. 17-AAG (10 mg/kg) or vehicle was injected immediately after coronary ligation. **B**, Apoptotic cardiomyocytes at 1 day after MI was reduced in 17-AAG-treated mice. Apoptosis was assessed by TUNEL staining. **C and D**, Postinfarct cardiac remodeling is attenuated by 17-AAG treatment ( $n=20$ ). HW/BW ratio (**C**, left), contractile function (**C**, right), and fibrotic area (**D**). \* $P<0.001$ ; vs MI+DMSO ( $n=30$ ).

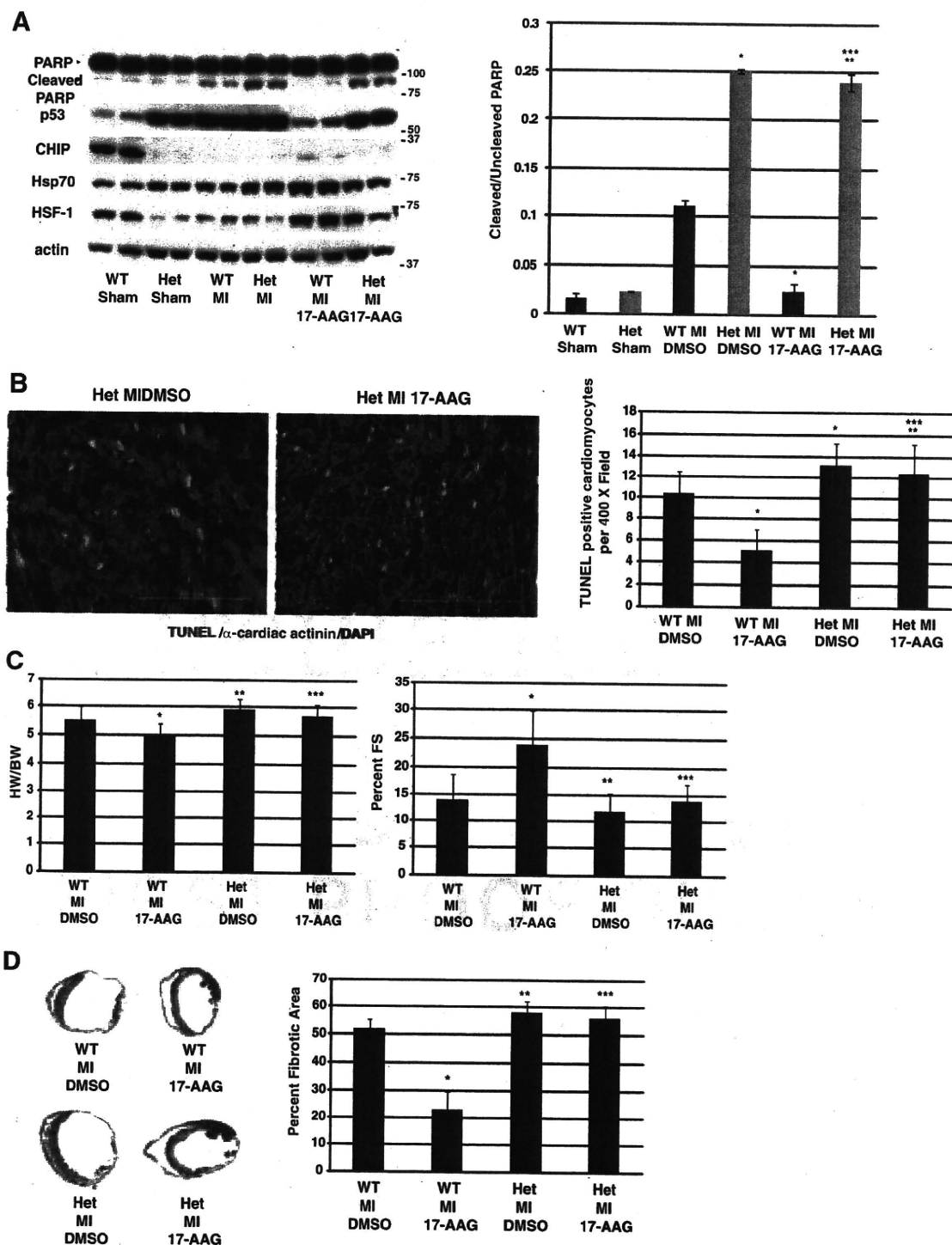
because upregulation of heat shock proteins by 17-AAG was also impaired in CHIP heterozygous mice (Figure 7A; compare WT MI 17-AAG and Het MI 17-AAG). Therefore, it would be fair to conclude that 17-AAG exerts multiple cardioprotective effects after myocardial infarction and at least one of its effects were mediated by promotion of CHIP-mediated p53 degradation.

### Discussion

In the present study, we found that accumulation of p53 protein after myocardial ischemia is initiated by HIF-1 dependent downregulation of CHIP level. We have found that CHIP overexpression decreased the amount of p53 and prevented myocardial apoptosis and ameliorated ventricular remodeling

after myocardial infarction. We have also found that Hsp90 inhibitor, 17-AAG, exerted similar antiapoptotic and cardioprotective effects after myocardial infarction and showed that these effects of 17-AAG was at least in part mediated by promotion of CHIP-mediated p53 degradation.

Although hypoxic stimuli have been reported to raise p53 protein levels in a variety of cell types, molecular mechanisms of p53 accumulation have been largely unknown. In the present study, we unveiled that downregulation of CHIP protein is critically involved in this process. We found that CHIP expression was downregulated after hypoxic stress through HIF-1-mediated suppression of *CHIP* promoter (Figure 2). We also found that overexpression of CHIP attenuated the p53 accumulation after hypoxic stress (Figures 4A and 5B). These results



**Figure 7.** The effect of 17-AAG was dependent on CHIP-mediated p53 degradation and upregulation of heat shock proteins. **A**, 17-AAG-induced reduction of p53 accumulation and PARP expression was not observed in the heart of CHIP heterozygous mice (Het). Notably, upregulation of heat shock proteins by 17-AAG was ameliorated in CHIP heterozygous mice. **B**, Apoptotic cardiomyocytes on 1 day after MI was reduced in 17-AAG-treated mice, but this antiapoptotic effect of 17-AAG after MI was ameliorated in CHIP heterozygous mice. Apoptosis was assessed by cleaved PARP expression (**A**) and TUNEL staining (**B**). \* $P < 0.01$  vs WT+MI+DMSO; \*\* $P < 0.01$  vs WT+MI+17-AAG; \*\*\* $P = NS$  vs Het+MI+DMSO  $n = 5$ . WT indicates wild-type mice; Het, CHIP heterozygous mice. **C**, 17-AAG-induced attenuation of postinfarct cardiac remodeling is less in CHIP hetero knockout mice than in wild-type mice. HW/BW ratio (**C**, left), contractile function (**C**, right), and fibrotic area (**D**). \* $P < 0.001$ ; \*\* $P < 0.05$  vs WT+MI+DMSO; \*\*\* $P = NS$  vs Het+MI+DMSO. WT+MI+DMSO:  $n = 30$ ; WT+MI+17-AAG:  $n = 20$ ; Het+MI+DMSO:  $n = 15$ ; Het+MI+17-AAG:  $n = 15$ . WT indicates wild-type mice.

**Table. Basal Characterization of the Mice Used in this Study**

	Body Weight (g)	LVEDD (mm)	LVESD (mm)	IVS (mm)	LVPW (mm)	%FS
Wild type	26.3±1.2	3.12±0.12	1.04±0.02	0.72±0.02	0.74±0.02	66.7±1.3
CHIP hetero KO	25.6±0.8	3.10±0.08	1.05±0.03	0.74±0.03	0.78±0.02	66.1±0.8
CHIP Tg	26.9±1.5	3.14±0.16	1.05±0.04	0.75±0.02	0.75±0.05	66.6±1.8

suggest that hypoxic stress downregulates CHIP, leading to decreased CHIP-mediated proteolysis of p53 protein and accumulation of p53 protein. This mechanism seems to be a 'fine-tuning' of HIF-1 activity because p53 protein has been reported to bind to and inhibit HIF-1 activity.<sup>16</sup> After hypoxia, first HIF-1 accumulates and induces angiogenic genes, to promote angiogenesis. Thereafter, as a negative feedback loop, HIF-1 induces downregulation of *CHIP* expression and p53 accumulates, then accumulated p53 inhibits HIF-1 activity.<sup>35</sup> In general, this feedback system might have an antitumor effect, because in many tumor cells HIF-1 induces feeding vessels in hypoxic tumors and promotes tumor growth. HIF-1-induced, CHIP-mediated p53 accumulation acts to suppress tumor growth by (1) suppressing HIF-1 activity and blocking neovascularization and (2) inducing p53-mediated apoptosis of tumor cells. However, in the heart, this negative feedback system worsens hypoxic situation by blocking neovascularization<sup>16</sup> and by inducing apoptosis (this study).

The important role of apoptosis in the progression of ventricular remodeling and the possibility of antiapoptotic approach against heart failure has already been elegantly shown by Wencker et al.<sup>3</sup> Antiapoptotic approach after myocardial infarction has been reported to be cardioprotective not only in ischemia-reperfusion model but also in permanent coronary ligation model.<sup>21,36,37</sup> Therefore, inhibition of apoptotic death does not only reduce initial infarct size but also prevents ventricular remodeling through inhibiting apoptosis in the border zone of the infarct.

Accumulation of p53 has been reported to initiate many proapoptotic triggers.<sup>38</sup> In the heart, p53 accumulates (Figure 3C) and p53-dependent apoptosis occurs<sup>39</sup> after permanent coronary occlusion. We have also observed that p53 gene deletion lead to less ventricular remodeling after myocardial infarction.<sup>16</sup> In the present study, we have shown that CHIP overexpression or 17-AAG treatment could prevent cardiomyocyte apoptosis and ameliorate ventricular remodeling after myocardial infarction. We have also shown some evidences that inhibition of p53 accumulation is at least one of the mechanisms for the effect of CHIP overexpression and 17-AAG. However, it should be noted that Hsp90 chaperones various proteins including prosurvival factors such as Akt/protein kinase B<sup>40</sup> in tumor cells and that Hsp90 inhibitors induced degradation of aberrantly overexpressed prosurvival factors in those tumor cells. Although the nature of the effects of 17-AAG seems to induce degradation of aberrantly expressed proteins, it is also possible and taken into account that 17-AAG could also induce degradation of prosurvival factors and play detrimental effects in cardiomyocytes.

In conclusion, our observations indicate that investigation of novel anti-p53 approach would open a way toward new treatment of myocardial infarction.

## Acknowledgments

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## Disclosures

None.

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### Novelty and Significance

#### What Is Known?

- Inhibition of myocardial apoptosis after myocardial infarction is cardioprotective.
- p53 expression is increased after myocardial infarction and induces cardiomyocyte apoptosis.

#### What New Information Does This Article Contribute?

- We identified CHIP as the endogenous p53 antagonist expressed in the heart.
- We found that CHIP downregulation is critically involved in the molecular mechanisms for p53 elevation after myocardial infarction.
- We showed several possibilities of the anti-p53 treatment after myocardial infarction.

Accumulation of tumor suppressor protein p53 in the myocardium causes the transition from adaptive cardiac hypertrophy to heart

failure. However, the mechanisms of p53 accumulation in the heart and its therapeutic implications have been elusive. Here we show that downregulation of the chaperone-associated E3 ubiquitin ligase CHIP (carboxyl terminus of Hsp70-interacting protein) mediates hypoxia-induced p53 accumulation in the heart and that promotion of CHIP-induced p53 degradation protects the heart from ischemic injury. Under physiological conditions, CHIP limited the p53 protein amount at low levels by inducing proteasomal degradation of p53. Under hypoxic conditions, hypoxia inducible factor-1 (HIF-1) down-regulated CHIP, resulting in the accumulation of p53. Overexpression of CHIP or administration of an Hsp90 inhibitor promoted CHIP-mediated p53 degradation and attenuated ischemic cardiac injury. These results indicate that CHIP is a crucial negative regulator of p53 in the heart and suggest that promotion of CHIP-mediated p53 degradation could be a novel therapeutic strategy for heart diseases.

## **Detailed Methods**

### **Expression Cloning.**

Expression cloning was performed as described previously <sup>1</sup> using PG13-Luc (A kind gift from B. Vogelstein) as a reporter plasmid. Initially, cDNA expression library from human heart (Invitrogen) was separated into small pools that contain ~100 clones each. cDNA clones that downregulate PG13 activity were isolated by sib-selection.

### **Plasmids and Transfection assay.**

Transfection into COS7 cells were performed using Fugene HD (Roche). HA-tag was introduced to the N-terminus of human CHIP using PCR and subcloned into pCAGGS vector <sup>2</sup>. Flag-tagged p53 was from B. Gellersen <sup>3</sup>. Adenoviral vector of HA-tagged CHIP was created using AdEasy Vector System (Qbiogen) and infected as described previously<sup>4</sup>. Human CHIP promoter (-329 to +39 from TSS) was cloned into pGL4-basic (Promega). Mutations of the HRE site (CACGTG to CTGGCG) were introduced using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Stealth siRNA against rat CHIP, MDM2, HIF-1 $\alpha$ , and p53 were designed and purchased from Invitrogen. Sequences for each siRNAs are; rat CHIP-1: ugguguaguacacugccacaagugg; rat CHIP-2: cucaucauaacucuccaucucuagc; rat MDM2-1: agcuaaggaaauucaggaucuccc; rat MDM2-2: auagucgucacucuccugugacagg; rat HIF1 $\alpha$ : uagugcuuccaucagaaggacuugc; rat Tp53: uuaagggugaaauuucuccaucga. Negative controls for siRNA was purchased from Invitrogen (Medium GC). Transfection of siRNA was performed using Lipofectamine RNAiMax (Invitrogen).

### **Cell culture.**

COS7 and HEK293 cells are from ATCC and cultured in DMEM containing 10% FBS (Invitrogen). Neonatal rat cardiomyocytes were isolated from 1-day old Wistar rats and

cultured as described previously <sup>5</sup>. Cardiomyocytes were exposed to hypoxic stress by culturing under  $\text{CoCl}_2$  (250  $\mu\text{M}$ ) or by culturing in hypoxic chamber (<1%  $\text{O}_2$ ,  $\text{PO}_2$  18~20mmHg).

### **Evaluation of apoptosis**

Caspase-3 activity was examined by using CaspACE™ Assay System, Colorimetric (Promega). Annexin V staining was performed as previously described <sup>4</sup> using Annexin V-Cy3 apoptosis detection kit (Bio Vision). TUNEL staining on fresh frozen section of the heart was performed using In situ apoptosis detection kit (TaKaRa) and fluorescence was enhanced using anti-fluorescein rabbit IgG Alexa488 conjugated (Molecular Probe). The section was counterstained with  $\alpha$ -sarcomeric actinin (SantaCruz) and DAPI. We have performed TUNEL staining 24 hours after coronary artery ligation because myocardial apoptosis was most prominent 24 hours after the operation (unpublished observations).

### **Luciferase assays**

The reporter plasmid, PG13-Luc, pGL4-CHIP, or pGL4-CHIP-mutHRE was transfected into COS7 cells using Fugene HD (Roche) or to neonatal rat cardiomyocytes using Lipofectamine 2000 (Invitrogen). pRL-tk encoding Renilla luciferase was co-transfected as an internal control. Luciferase assay was carried out 24 hours after transfection of the effector plasmids or addition of drugs using dual-luciferase assay system (Promega).

### **RNA analysis**

Total RNA extraction and DNase treatment was performed using SV total RNA isolation Kit (Promega). RNA was DNase treated and reverse transcribed using QuantiTect Reverse Transcription Kit (QIAGEN). Real time quantitative PCR was performed using

Universal Probe Library (UPL) (Roche) and Light Cycler TaqMan Master kit (Roche). Relative levels of gene expression were normalized to the rat GAPDH (for rat cardiomyocytes) or human  $\beta$ -actin (for HEK293 cells) expression using the comparative Ct method according to the manufacturer's instructions. Primer sequences and UPL number were; rat GAPDH: Fwd aatgtatccgtgtggatctga, Rev gcttcaccaccttcttgatgt and UPL No. 80; Human beta actin: Fwd ggaaatcgctgcgtgacatta, Rev ccgtcaggcagctcgtag and UPL No. 80; rat CHIP: Fwd ctcaaggagcagggaaacc, Rev aagtggttccgggtgat and UPL No. 70; human p21: Fwd cgaagtcagttcctgtggag, Rev catgggtctgacggacat and UPL No. 82; human PUMA: Fwd gacctcaacgcacagtaacga, Rev gagattgtacaggacctcca and UPL No. 68; human MDM2: Fwd tctgatagtattcccttctcttg, Rev tgttcacttacaccagcatcaa and UPL No. 21.

#### **Protein analysis.**

Whole cell lysates (50-100  $\mu$ g) were separated by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane and incubated with primary antibodies followed by incubation with a HRP-conjugated secondary antibody (Jackson). Membrane was developed using ECL-Plus (Amersham). Densitometric analysis for the band intensity was performed using ImageJ. Immunoprecipitation was performed using specific antibodies and Protein A/G agarose (SantaCruz). Antibodies used were; anti-CHIP (CHEMICON), anti-p53 (1C12) (Cell Signaling), anti-HA (Roche), anti-Flag (M2) (Sigma), anti-MDM2 (R&D), anti-ubiquitin (CHEMICON), anti-HIF-1 $\alpha$  (Novus), anti-PARP (Cell signaling), and anti-actin (Sigma).

HIF-1 $\alpha$  activity was determined using TransAM™ HIF-1 (ActiveMotif).

#### **Animals**

All protocols were approved by Chiba University review board. CHIP heterozygous mice



and cardiac-specific inducible HIF-1 knockout mice were described <sup>6-8</sup>. Unlike the CHIP-deficient mice described by Dai et al. <sup>9</sup>, our CHIP heterozygous mice were maintained on a pure C57BL/6 background. Very few homozygous knockout were obtained (2.9% of the alive embryo) but all of them were small in size and died before 1 week after birth. The reason for perinatal lethality in our mouse strain remains to be elucidated. Cardiac-specific CHIP transgenic mice were generated by pronuclear injection of  $\alpha$ MHC-HA-CHIP transgene construct. Two independent lines that express HA-tagged CHIP to the same level were obtained and both lines showed similar results. Cardiac specific expression of HA-tagged CHIP was confirmed by Western blotting. The basal cardiac parameters of the mice used in this study (CHIP heterozygous knockout mice and cardiac specific CHIP overexpressing transgenic mice) were shown in Table 1. Permanent coronary artery ligation was performed on 10-week old male mice as described previously <sup>4</sup>. We anesthetized mice by intraperitoneally injecting a mixture of 100 mg/kg ketamine and 5 mg/kg xylazine. 17-AAG (Alexis) was dissolved in DMSO and injected intraperitoneally right after coronary artery ligation.

#### **Physiological analysis and histological analysis.**

Echocardiography was performed as described previously <sup>8</sup>. Paraffin-embedded heart samples were sectioned and stained with Masson's Trichrome staining as previously described <sup>4</sup>.

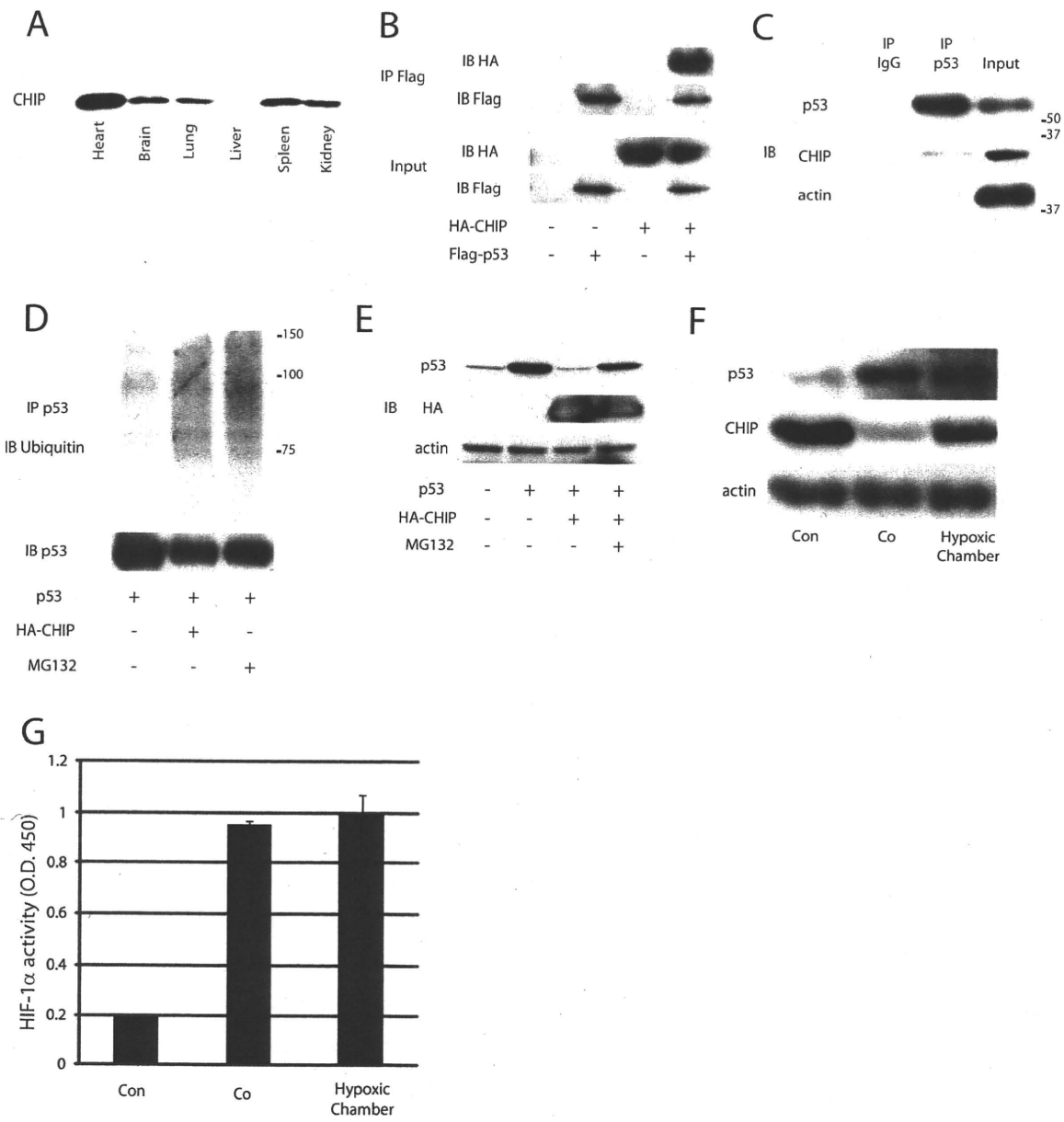
#### **Statistical analysis.**

Data are expressed as mean $\pm$ SE. The significance of differences among means was evaluated using analysis of variance (ANOVA), followed by Fisher's PLSD test and Dunnett's test for multiple comparisons. Significant differences were defined as  $P < 0.05$ .

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# Online Figure I



# Online Figure II

