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## CIP, a cardiac Isl1-interacting protein, represses cardiomyocyte hypertrophy

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### Abstract

**Rationale**—Mammalian heart has minimal regenerative capacity. In response to mechanical or pathological stress, the heart undergoes cardiac remodeling. Pressure and volume overload in the heart cause increased size (hypertrophic growth) of cardiomyocytes. Whereas the regulatory pathways that activate cardiac hypertrophy have been well established, the molecular events that inhibit or repress cardiac hypertrophy are less known.

**Objective**—To identify and investigate novel regulators that modulate cardiac hypertrophy.

**Methods and Results**—Here, we report the identification, characterization and functional examination of CIP, a novel cardiac Isl1-interacting protein. CIP was identified from a bioinformatic search for novel cardiac-expressed genes in mouse embryonic hearts. CIP encodes a nuclear protein without recognizable motifs. Northern blotting, in situ hybridization and reporter gene tracing demonstrated that CIP is highly expressed in cardiomyocytes of developing and adult hearts. Yeast-two-hybrid screening identified Isl1, a LIM/homeodomain transcription factor essential for the specification of cardiac progenitor cells in the second heart field, as a co-factor of CIP. CIP directly interacted with Isl1 and we mapped the domains of these two proteins which mediate their interaction. We show that CIP represses the transcriptional activity of Isl1 in the activation of the MEF2C enhancer. The expression of CIP was dramatically reduced in hypertrophic cardiomyocytes. Most importantly, overexpression of CIP repressed agonist-induced cardiomyocyte hypertrophy.

**Conclusions**—Our studies therefore identify CIP a novel regulator of cardiac hypertrophy.

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## Keywords

Isl1-interacting protein; transcription factor; cardiac development; cardiomyocyte hypertrophy

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## INTRODUCTION

Complex genetic pathways intimately regulate heart development and function<sup>1</sup>. Much of our current understanding of how cardiac gene expression is controlled is at the level of transcriptional regulation, in which multiple tissue-specific transcription factors have been implicated in the control of gene expression during cardiomyocyte differentiation<sup>2</sup>. Those include Nkx2.5, a homeobox protein<sup>3-5</sup>, MEF2C, a member of the myocyte enhance factor-2 (MEF2) family of MADS-box transcription factors<sup>6-11</sup>, and GATA4, a GATA family zinc finger protein<sup>12, 13</sup>. Some of the well-characterized cardiac genes regulated by these cardiac transcription factors include cardiac-specific atrial natriuretic factor (ANF) and cardiac  $\alpha$ -actin<sup>13, 14</sup>.

Two distinct sources of cardiac precursor cells from the “primary” and “secondary heart fields” participate in heart formation<sup>15-17</sup>. Whereas the primary heart field is essential for the formation of the initial heart tube, additional cardiac precursor cells recruited from the secondary heart field contribute to the future right ventricle and outflow tract<sup>18, 19</sup>. Loss-of-function studies have defined genes whose activity is required in secondary heart field progenitors for morphogenesis of outflow tract and right ventricle<sup>18, 20</sup>. Among the genes with key function in secondary heart field is *Isl1*, a LIM/homeodomain transcription factor<sup>21-24</sup>.

One of the major responses of the heart to biomechanical stress and pathological stimuli is to undergo cardiac hypertrophy, an increase in the thickness of the cardiac ventricular wall. At a cellular level, cardiac hypertrophy is defined as an increase of cardiomyocyte size<sup>25, 26</sup>. Initially, cardiac hypertrophy is an adaptive response that maintains cardiac output in the face of increased workload. However, chronic activation of hypertrophic pathways is associated with adverse consequences that may lead to heart failure and sudden death<sup>27</sup>.

Cardiac hypertrophy occurs in response to a variety of mechanical, hemodynamic, hormonal, and pathologic stimuli<sup>28-30</sup>. Numerous studies have demonstrated that many signaling pathways and transcriptional networks that normally regulate different aspects of cell proliferation, differentiation and survival are also involved in the induction of cardiac hypertrophy<sup>1, 29, 31</sup>. Hypertrophic growth involves control of cardiomyocyte gene expression at multiple molecular levels. Key regulators of gene expression in cardiac myocytes such as members of the MEF2 and GATA families are involved in the control of gene expression during cardiomyocyte hypertrophy. Epigenetic regulation of gene expression, including histone modification by histone acetyltransferases (HATs) and histone deacetylases (HDACs) to remodel chromatin, has been proved another mean of hypertrophic regulation<sup>32, 33</sup>. Cardiac hypertrophy is also accompanied by reactivation of a set of cardiac fetal genes, including those that encode atrial natriuretic factor (ANF), B-type natriuretic peptide (BNP), and  $\beta$ MHC<sup>26</sup>. Reactivation of these fetal genes suggests that molecular pathways that control heart development are redeployed to regulate hypertrophic growth<sup>34</sup>. The pathophysiology of cardiac hypertrophy has been extensively studied for decades and much is known about signaling pathways that activate cardiac hypertrophy<sup>26, 30</sup>. However, relatively less is known about how cardiac hypertrophy is repressed.

We previously identified myocardin as a SAP (SAF-A/B, Acinus, PIAS) domain transcriptional regulator<sup>35, 36</sup>. Myocardin is expressed in both cardiomyocytes and a subset

of vascular and visceral smooth muscle cell types<sup>35, 36</sup>. Myocardin does not bind DNA directly, but forms a stable ternary complex with SRF bound to DNA<sup>37, 38</sup>. As a transcriptional cofactor of SRF, myocardin potently transactivates CArG box-containing reporter genes<sup>36, 39, 40</sup>. Genetic studies revealed that myocardin and MRTFs play critical roles in a variety of biological processes, including vascular smooth muscle development, aortic vessel patterning, mammary myoepithelium formation and others<sup>41-43</sup>. In this study, we report the identification and characterization of a novel cardiac-specific nuclear protein, named CIP. CIP interacts with the LIM/homeodomain transcription factor Isl1. We showed that the expression of CIP was repressed in hypertrophic hearts, and most importantly, CIP represses cardiomyocyte hypertrophy.

## RESULTS

### Identification of CIP, a novel gene from mouse hearts

We searched expressed sequence tag (EST) databases for novel sequences present in cardiac cDNA libraries. Previously, this approach succeeded in identifying myocardin as a novel cardiac- and smooth muscle-specific transcription co-factor for serum response factor (SRF)<sup>36</sup>. An additional candidate EST sequence recovered in the search (GenBank Accession Number AA919489), contained a short 273 nucleotide tag that was derived from a mouse embryonic day 13 (E13.5) heart cDNA library. Using this sequence as a probe to screen a mouse embryonic heart cDNA library, we identified a larger cDNA sequence of 1336 nucleotides encoding a putative protein of 309 amino acids, which we name CIP (Cardiac Isl1-interesting Protein) (Fig. 1A). Genbank Database search indicated that this cDNA clone is similar to RIKEN cDNA clone 2310046A06Rik, and is a novel, alternative slicing-produced isoform of a recently reported protein called MLIP (Muscle-enriched A-type lamin interacting protein)<sup>44</sup>. In addition to the putative lamin-A interaction domain in its N-terminus, CIP contains an AT-hook DNA binding domain in its C-terminus (aa 212-224), suggesting that CIP could function as a DNA-binding transcriptional regulator (Figs. 1A, C; Online Figure I). Furthermore, the CIP protein contains a putative asparagine (Asn) glycosylation site (aa 223-226), a poly serine stretch (aa 225-229) and a tyrosine (Tyr) phosphorylation site (aa 254-260) (Fig. 1A). Northern blot analysis using an adult mouse multiple tissue blot demonstrated that the expression of CIP is restricted to the heart. Two major transcripts, ~1.4K and ~3.4K nucleotides, respectively, were detected in the heart, likely represent products of alternative splicing (Fig. 1B). The cDNA sequence we have cloned represents the ~1.4 kb transcript, whereas the sequences of the ~3.4 kb remain to be identified.

The *Cip* gene contains at least eight exons and spans over 245 kb on mouse chromosome 9. *Cip* is evolutionary conserved from fish to human (Online Figure II). Sequencing of *Cip* RT-PCR products identified complex alternative splicing (Fig. 1C). The functional significance of those isoforms is currently not clear.

Next, we examined the subcellular location of the CIP protein. We transiently overexpressed Flag-CIP fusion proteins in COS7 and Hela cells. Immunocytochemistry assays revealed that the Flag-CIP fusion proteins are primarily located in the nuclei of transfected cells. We also observed a weaker distribution of the Flag-CIP proteins in the cytoplasm (Fig. 1D).

### CIP expression is restricted to cardiomyocytes of developing hearts

To define the expression pattern of *Cip* during development, *in situ* hybridization was performed using an antisense probe to the 3' UTR of the mouse *Cip* transcript. Whole mount *in situ* hybridization first demonstrated that CIP expression is restricted to the heart of embryonic day (E9.5) mouse embryos, with highest expression level detected in the

ventricle (Fig. 2A). *In situ* hybridization on tissue sections of staged mouse embryos revealed that *Cip* expression was first detected in the heart of E8.5 mouse embryos (Fig. 2B). *Cip* continued to be restricted to the heart from E9.5 to E15.5 (Fig. 2C, D, E, F, G). An apparently positive signal was also detected in the trunk of E9.5 embryo, which might represent transient expression of CIP in presomitic mesoderm (Fig. 2C). From E11.5-E15.5, *Cip* expression appeared to be higher in the ventricles of embryonic hearts (Fig. 2E, F, G). Myocardial *Cip* expression continued in the adult heart (Fig. 2H).

Since the heart is constituted of cardiomyocyte, cardiac fibroblast, smooth muscle cell, endothelial cell and epicardial cell, we performed additional experiments to determine exactly in which cell type/lineage the CIP is expressed. First, we utilized a genetic approach in which the Rosa-mT-mG reporter line was used to trace the expression of CIP in different cell types. The Rosa-mT-mG mice possess loxP sites on both sides of a membrane-targeted tdTomato (mT) cassette and express strong red fluorescence in all tissues and cell types (Fig. 3A, B). The presence of Cre will lead to the deletion of the mT cassette and the activation of the downstream membrane-targeted EGFP (mG) cassette (Fig. 3A). The membrane-targeted EGFP can be utilized as a marker for FACS sorting (Fig. 3D). To label and sort out the cardiomyocytes, the Rosa-mT-mG mice were bred with the cTNT-Cre mice, in which the Cre recombinase is driven by the cardiomyocyte-specific cardiac troponin T promoter (Fig. 3B). Immunohistochemistry confirmed the labeling of cardiomyocyte in Rosa-mT-mG/cTNT-Cre embryos (Fig. 3C). Hearts were dissected out from E10.5 Rosa-mT-mG/cTNT-Cre embryos and digested. EGFP positive cardiomyocytes and EGFP negative non-cardiomyocytes were separated by FACS sorting (Fig. 3D). Quantitative RT-PCR detected high expression level of Myh6 ( $\alpha$ -myosin heavy chain) and CIP in EGFP-positive cell sample but not in EGFP-negative cell sample, while endothelial markers Flk1, Pecam1 and fibrotic tissue marker Postn were highly expressed in EGFP-negative cell sample but not EGFP-positive cell sample (Fig. 3E, F, G),  $n = 3$ . These data, together with the results of *in situ* hybridization, suggested that CIP is expressed in cardiomyocytes of developing hearts.

Next, we used a *Cip* gene trap mouse line in which a LacZ reporter gene was inserted into the *Cip* genomic locus (See Material and Methods for detail) to further map the expression of *Cip* in the heart (Online Figure III). LacZ reporter gene expression was restricted to the developing heart at E10.5 and E13.5, recapitulating endogenous expression pattern of *Cip* and highlighting the cardiac specific expression of this gene (Fig. 4A, E).

Immunohistochemistry analyses clearly demonstrated that the expression of *Cip*, as marked by  $\beta$ -gal, overlapped with that of Nkx2-5, cardiac troponin T (TNNT2), and cardiac actin (ACTN2) in cardiomyocytes (Fig. 4B, C, D, G, J, yellow arrow heads). *Cip* expressing cells did not co-express WT1, a marker of epicardium, nor PECAM, a marker of endothelial cells (Fig. 4E, F, H, I, white arrows and arrow heads),  $n > 3$ . Together these studies clearly demonstrate that *Cip* is a specifically expressed in cardiomyocytes during embryogenesis. Interestingly, we detected CIP expression in the skeletal muscle of neonatal mice (Online Figure IV, middle panel). However, the expression of CIP in skeletal muscle appears to be transient, since we did not detect its expression in embryonic skeletal muscle (Online Figure IV, left panel) or in adult skeletal muscle tissue using the lacZ reporter (Online Figure IV, right panel) or by Northern blot analyses (Fig. 1B).

### CIP interacts with cardiac transcriptional factor Islet1

To identify putative CIP-interacting proteins, we performed yeast two-hybrid screening. The bait consisted of full-length CIP protein fused with the yeast GAL4 DNA binding domain. The bait construct did not autonomously activate transcription in yeast. We screened a mouse E10.5 embryonic cDNA prey library and identified more than 30 positive clones, one of which encoded Islet1 (ISL1), a LIM/homeodomain transcription factor essential for differentiation of second heart field cardiac progenitors<sup>23, 45</sup>.

To confirm the interaction of CIP and ISL1, we performed co-immunoprecipitation (Co-IP) assays. HEK293T cells were transfected with expression plasmids for FLAG-CIP, Myc-ISL1, or both. FLAG immunoprecipitates of cells expressing both constructs contained ISL1 protein, as demonstrated by Western blotting with anti-Myc antibodies. As negative controls, ISL1 was not detected in FLAG immunoprecipitates of cells transfected with each construct alone (Fig. 5A). To further confirm direct interaction between CIP and ISL1, we performed in vitro GST-fusion protein pull down assays. ISL1 was <sup>35</sup>S labeled in a cell-free translation system, and the labeled ISL1 protein was then incubated with either GST-CIP fusion protein or GST protein alone (to serve as a negative control). As expected, GST-CIP fusion protein, but not GST alone, bound ISL1 (Fig. 5B).

Next, we attempted to map the domains of these two proteins that mediate their interaction. We made series of deletion mutants and tested their interaction using Co-IP assays. As shown in Fig. 5C, the interaction between CIP and Isl1 is readily detected in full-length (1-309) and both C- and N-terminal truncated mutants (1-200 and 100-309). However, further C-terminal deletion (1-100) abolished the interaction, suggesting that regions between aa 100 to 200 are required for CIP to interact with Isl1 (Fig. 5C). Similarly, we examined domains in the Isl1 protein which are needed for the interaction with CIP. We found that aa 1-133 fragment is sufficient to mediate the interaction, whereas the aa 79-349 region failed to interact (Fig. 5D). These data indicate that the N-terminal region of the Isl1 protein is essential for its interaction with the CIP protein.

ISL1 is expressed in the cardiac progenitors and marks the second heart field. It was previously reported that ISL1 is not expressed in the cardiomyocytes of adult hearts<sup>23</sup>. We investigated whether CIP and ISL1 are co-expressed in mouse embryos. The expression of ISL1 was examined in *Cip-lacZ* reporter embryos. As shown in Fig. 5E and 5F, both LacZ and ISL1 were expressed in cardiomyocytes located in the outflow tract (OFT) of E10.5 mouse embryos (Fig. 5E, and 5F, white arrowheads). However, ISL1 is not expressed in the cardiomyocytes of atrium nor left ventricle, where CIP is expressed (Fig. 5F, yellow arrows). Given that ISL1 is expressed in cardiac progenitor cells of second heart field and newly formed cardiomyocytes in the outflow tracts<sup>23</sup>, these results confirmed that CIP and ISL1 are co-expressed in the cardiac progenitor cells of embryonic hearts.

### CIP is as transcription co-factor of Isl1 and regulates its transcriptional activity

Because CIP was localized to the nucleus, we asked whether it possessed transcriptional activity. We fused the full-length CIP protein to the DNA binding domain of yeast GAL4 protein. Interestingly, this fusion protein repressed the GAL4-dependent reporter when co-transfected into HEK293T cells (Fig. 5G). Similar, we observed that GAL4-CIP repressed the reporter in HeLa and COS7 cells (data not shown).

Loss-of-function studies indicate that *Isl1* is essential for cardiac development<sup>23</sup>. ISL1 activates the expression of the *Mef2c* gene by directly binding to its anterior heart field enhancer<sup>46</sup>. We tested whether CIP participates in regulation of the *Mef2c* anterior heart field enhancer. As expected, ISL1 constantly activated the *Mef2c* enhancer luciferase reporter, though its activity does not appear to be potent (Fig. 5I). CIP alone did not significantly affect *Mef2c* enhancer activity. However, co-expression of ISL1 (the full-length, 1-309) and CIP inhibited ISL1-mediated *Mef2c* enhancer luciferase activity (Fig. 5I). Further analyses, using different CIP deletion mutants, showed that the aa 1-200 and the aa 100-309 constructs (but not the aa 1-100) were able to repress Isl1-mediated activation of the *Mef2c* enhancer. These observations indicate that direct interaction of CIP and Isl1 is necessary for CIP to repress Isl1. Our results suggest that CIP is a transcription co-factor and represses the transcriptional activity of ISL1.

Next, we tested whether CIP represses the activity of other transcriptional regulators. Myocardin is a transcription factor specifically expressed in cardiac- and smooth muscle that potently stimulates ANF promoter activity in conjunction with the transcription factor SRF<sup>36</sup>. We found that CIP significantly repressed myocardin-mediated activation of the ANF-luciferase reporter (Fig. 5J). Similarly, CIP also repressed SRF-dependent activation of the ANF luciferase reporter (Fig. 5J). Together, our results indicated that CIP is a putative transcriptional repressor expressed in the heart.

### The expression of CIP is dysregulated in hypertrophic hearts

The heart undergoes cardiac remodeling in response to cardiac injury and/or stress. Increased pressure or volume overload in the heart results in cardiac hypertrophy, a process in which the size of cardiomyocytes increases without an increase in the number of cardiomyocytes. Interestingly, MEF2C was previously shown to be a key regulator of cardiac hypertrophy and dilated cardiomyopathy<sup>11, 47-50</sup>. Since CIP repressed the *Mef2c* enhancer activity, we hypothesized that CIP regulates cardiac hypertrophy. We first examined whether hypertrophic signals regulate cardiac *CIP* expression. We used a transverse aortic constriction to induce pressure overload and subsequent pathological cardiac hypertrophy. As expected ANP and BNP, well-known hypertrophy markers, were dramatically upregulated in Transverse Aortic Constriction (TAC) mice at day 3 (D3) and day 7 (D7) (Fig. 6A). In contrast, *Cip* transcripts were substantially reduced in TAC mice. Similarly, expression of CIP was reduced in a second well-characterized cardiac hypertrophy model<sup>27</sup>, in which transgenic expression of activated calcineurin stimulates dramatic cardiac hypertrophy (Fig. 6B).

In vivo cardiac hypertrophy models are complex and encompass both direct effects of inducing stimuli and secondary effects of myocardial responses, including cardiac fibrosis and failure. To directly examine the effect of hypertrophic stimuli on cardiomyocyte *Cip* expression, we used the well-established neonatal rat cardiomyocyte hypertrophy model. We treated neonatal rat cardiomyocytes with a panel of hypertrophic agonists, and measured the effect on *Cip* expression. We found that the CIP protein level was significantly reduced in neonatal rat cardiomyocytes treated with the hypertrophic agonists leukemia inhibitory factor (LIF), endothelin (ET)-1 and phenylephrin (PE) (Fig. 6C). Quantitative RT-PCR analyses of PE-treated cardiomyocytes corroborated this finding and indicated that CIP expression is regulated at the transcriptional level (Fig. 6D). These results demonstrate that the expression of CIP is repressed in hypertrophic cardiomyocytes and hearts, in vitro and in vivo.

### CIP represses cardiomyocyte hypertrophy

The above observation established an inverse correlation between the expression of CIP and cardiac hypertrophy and suggests that CIP could directly regulate cardiomyocyte hypertrophy. To test this hypothesis, we overexpressed CIP in cardiomyocytes by adenoviral gene transfer. Neonatal rat cardiomyocytes were isolated, cultured in vitro and treated with PE to induce hypertrophy as previously described<sup>51</sup>. PE treatment dramatically induced cardiomyocyte hypertrophy, as measured by increased cell surface area (Fig. 6E). Overexpression of CIP did not result in significant change to cardiomyocytes. However, CIP significantly attenuated PE-induced hypertrophy (Fig. 6E). Quantification of cardiomyocyte size confirmed the observation (Fig. 6F). At the level of gene expression, PE induced expression of hypertrophic marker genes ANP, BNP and  $\beta$ -MHC, as expected. PE-induced expression of hypertrophic marker genes was inhibited by CIP (Fig. 6G). Together, our results suggest that CIP negatively regulates cardiomyocyte hypertrophy.

## DISCUSSION

In the present study, we identified CIP as a novel cardiac-specific expressed nuclear protein. We demonstrated that CIP expression is restricted to cardiomyocytes. We further characterized CIP as a transcription co-factor of LIM/homeodomain transcription factor ISL1. We found that CIP physically interacts with ISL1 and represses its transcriptional activity. Functionally, we established that CIP represses cardiomyocyte hypertrophy.

We identified ISL1 as a CIP interacting protein via unbiased yeast two-hybrid screening and subsequently confirmed their physical and functional interaction. Our data suggest that a direct interaction between these two proteins is needed for CIP to repress the transactivity of Is11. Is11 is expressed in the second heart field and is required for the morphogenesis of embryonic heart<sup>23</sup>. Interestingly, Is11+ progenitor cells were shown multipotent and give rise to cardiac, smooth muscle and endothelial cell lineages<sup>22, 52</sup>. Given that CIP interacts with Is11 and is expressed in cardiac progenitors, we would speculate that CIP might participate in the function of Is11 in cell fate determination during early cardiac development. Despite its pivotal role in development, less is known about how Is11 works at molecular level. Is11 is a LIM/homeodomain transcription factor and it synergistically activates insulin gene transcription with BETA2 in pancreatic beta cells<sup>53</sup>. Furthermore, Is11 forms a complex with Jak1 and Stat3 and triggers the tyrosine phosphorylation of Jak1 and its kinase activity<sup>54</sup>. Is11 works upstream of the sonic hedgehog signaling and the MEF2C transcription factor in the heart<sup>21, 46</sup>. Our data showed that the transcriptional activity of Is11, though not very strong when compared with many other transcription factors, was repressed by CIP. This suggests another layer of regulation of this transcription factor.

In addition to its unique cardiac expression pattern, several lines of evidences point CIP an important regulator of cardiac gene expression and cardiac hypertrophy. Intriguingly, our data showed the capability of CIP to repress Is11-mediated activation of the MEF2C enhancer. It had been shown that MEF2C was upregulated during pathological hypertrophy<sup>50</sup>. Transgenic mice expressing a dominant-negative MEF2C displayed attenuated postnatal growth of myocardium<sup>48, 49</sup>. Given the critical role of MEF2C in cardiac development and hypertrophy, it is reasonable to predict that CIP is also involved in this process. Consistent with this view, we found that the expression level of CIP was consistently downregulated in cardiac hypertrophy models, both *in vivo* and *in vitro*. Most importantly, we provide evidence to demonstrate that overexpression of CIP partially represses agonist-induced cardiomyocyte hypertrophy. It remains to be determined, using genetic approaches, whether CIP participates in the regulation of cardiac gene expression during heart development and whether dysregulation of CIP expression will lead to cardiac pathophysiological condition or human cardiovascular disease. It will also be important to understand how CIP participates in the regulatory pathways of cardiac hypertrophy.

CIP is evolutionarily conserved in vertebrate. Its orthologs were found from fish to human, suggesting it has conserved biological function and may play an important role in cardiac development and disease. CIP appears to stand as a single member of this class of protein, and no homologous protein is encoded in the mouse genome. Recently, another splice variant in the locus of 2310046A06Rik was identified as a lamin interacting protein (MLIP)<sup>44</sup>. MLIP was shown to interact directly with Lamin A/C (LMNA), a nuclear envelope protein. Interestingly, LMNA is known to interact with many transcription factors/co-factors, including retinoblastoma transcriptional regulator (RB)<sup>55</sup>, germ cell-less (GCL)<sup>56</sup>, sterol response element binding protein (SREBP1)<sup>57</sup>, FOS<sup>58</sup>, and MOK2<sup>59</sup>. However, the functional significance of such interaction remains to be fully understood. LMNA is essential for the function of heart and human patients with LMNA mutation often

exhibit adult-onset dilated cardiomyopathy accompanied with conduction-system disease<sup>60</sup>. LMNA null mice display severe skeletal muscle atrophy and dilated cardiomyopathy, and die by 8 weeks<sup>61, 62</sup>. These observations suggest that the LMNA interacting protein CIP/MLIP could be part of the network that regulates gene expression in LMNA-related dilated cardiomyopathy. It will be interesting to understand how CIP/MLIP modulates the function of LMNA, and most importantly, whether CIP/MLIP is directly related to human cardiac and muscle diseases.

Our data demonstrate that CIP is expressed in the developing and adult heart. More specifically, we show that CIP expression is restricted to cardiomyocytes and cardiac progenitors during development. We have also observed transient CIP expression in the skeletal muscle. However, the level of CIP expression in skeletal muscle appears to be low, when compared with that of cardiac muscle. Moreover, skeletal muscle expression of CIP is not detected at all stages of embryos or postnatal mice. It will be interesting to investigate whether CIP plays a role in skeletal muscle development and function.

## MATERIALS AND METHODS

### Database Searching and Cloning of CIP

We screened for novel cardiac-specific genes in silico by performing a BLAST search with ESTs from mouse embryonic heart cDNA libraries in the database as described previously<sup>36</sup>. One of the cDNA sequences identified in this screen (Access number AA919489) corresponded to the 3' untranslated region (3'-UTR) of the CIP transcript and was found in a cDNA library of E13.5 mouse embryonic hearts. This short cDNA fragment (273 nt) was used as a probe to screen a mouse embryonic heart cDNA library. Several cDNA clones were identified. Among them, the longest clone is 1336 nt in length. Polymerase chain reaction (PCR)-based cloning was further used to identify additional cDNA isoforms.

### CIP reporter mice

The CIP reporter line with a retroviral gene trap cassette inserted into mouse genome chromosome 9 (between chr9: 77,046,001 and 77,046,002) was generated and obtained from Texas A&M Institute for Genomic Medicine (TIGM). The gene trap cassette containing the selectable marker  $\beta$ -geo, a functional fusion between the  $\beta$ -galactosidase and neomycin resistance gene, was inserted into the putative third intron of the CIP gene (Fig. S3).

### In Situ Hybridization and Northern Analysis

Whole-mount and section in situ hybridization and Northern analyses were performed as described<sup>36, 63</sup>. The 273 nt cDNA fragment corresponding to the 3'-UTR of the CIP gene was used as a probe to perform both whole-mount and tissue section in situ hybridization on staged mouse embryos.

### Constructs, Cell Culture, and Luciferase Reporter Assays

Hela, COS7 and HEK293T cells were cultured in DMEM supplemented with 10% FBS in a 5% CO<sub>2</sub> atmosphere at 37°C. Luciferase reporter constructs fused with the MEF2C enhancer were as reported and is a generous gift of Dr. Brian Black (University of California, San Francisco)<sup>46</sup>. Transfections were performed with either FuGENE6 (Roche) or Lipofectamine (Invitrogen) reagents according to manufacturers instruction. Unless otherwise indicated, 100 ng of reporter plasmid and 100 ng of each activator plasmid were applied. 48 hours after transfection, cell extracts were prepared and luciferase activity was determined. For luciferase assay, normalized luciferase expression from triplicate samples in 12-well plates relative to LacZ expression was calculated, and the results are expressed as



fold activation over the value relative to the control (Luciferase reporter and empty pcDNA). The CIP adenoviral expression construct (Ad-CIP) contained full-length CIP cDNA and was constructed as previously described<sup>51</sup>.

### Tissue dissociation and cell sorting of mouse embryos

The E10.5 embryonic hearts were isolated by microdissection and dissociated to single cells by collagenase digestion as previously described<sup>64</sup>. Isolated cells were FACS sorted into GFP positive and GFP negative populations. Sorted cells were collected into Trizol (Invitrogen) and frozen at -20°C for RNA isolation.

### Immunocytochemistry and $\beta$ -gal staining

Staged mouse embryos were dissected out, collected and fixed in 4% PFA at 4°C for 4 hours. After washing in PBS, embryos were treated in 15% and 30% sucrose for 2 hours each and embedded in OCT. About 5–8  $\mu$ m cryostat sections were collected on positively charged slides. Sections were washed in PBS, blocked in 5% serum/PBS, and subjected to immunostaining. Antibody sources were as follows: GFP (Invitrogen); PECAM (BD Biosciences); Nkx2-5 (Santa Cruz); Wt1 (Santa Cruz); Cardiac Troponin T (Tnnt2) (generous gift of Dr. Jim Lin, University of Iowa); Cardiac Troponin I (Tnni3) (Santa Cruz); Actn2 (Sigma); Isl1 (DSHB, University of Iowa);  $\beta$ -galactosidase (MP Biomedical). Alexa-488 and 594 secondary antibodies (Invitrogen). Fluorescently stained slides were counterstained with DAPI and imaged with an FV1000 confocal microscope (Olympus). For  $\beta$ -gal staining, samples were stained with a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal substrate at 37°C for 12 hours after fixation.

### Quantitative RT-PCR and Western blot analyses

Total RNAs were isolated using Trizol Reagent (Invitrogen) from cells and tissue samples. For Quantitative RT-PCR, 2.0  $\mu$ g RNA samples were reverse-transcribed to cDNA by using random hexamers and MMLV reverse transcriptase (Invitrogen) in 20  $\mu$ l reaction system. In each analysis, 0.1  $\mu$ l cDNA pool was used for quantitative PCR. For Western blot analyses, cell extractions were cleared by 10,000 $\times$ g centrifugation for 10 min at 4°C. Samples were subsequently separated by SDS/PAGE and transferred to PVDF membranes that were incubated with 5% milk and Anti-FLAG (1:1,000, Sigma); Anti-Myc (1:1,000, Santa Cruz);  $\beta$ -tubulin (1:10,000, Sigma) overnight at 4°C and then washed three times with TBST buffer before adding secondary antibody. Polyclonal antibodies against the CIP protein were generated by immunizing rabbits with CIP proteins which were expressed and purified as GST-CIP fusion proteins in bacterial. Specific protein bands were visualized by using ECL (Invitrogen) reagents.

### Coimmunoprecipitation Assays

COS7 cells were transiently transfected with plasmids encoding FLAG-tagged CIP and Myc-tagged Isl1 proteins with FuGENE6 (Roche). Cells were harvested 48 h after transfection in lysis buffer composed of 1X PBS containing 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF, 150 mM of NaCl and complete protease inhibitors (Roche). After a brief sonication and removal of cellular debris by centrifugation, FLAG-tagged CIP proteins were precipitated with anti-FLAG antibodies and protein A/G beads and associated proteins analyzed by Western blotting with anti-Myc antibodies.

### *In vitro* GST Protein-Binding Assays

Plasmids encoding a GST fusion with CIP were transformed into BL21 plus cells (Stratagene). The cells were grown at 37°C in 2 $\times$ YT medium to an optical density of 1.0.

Isopropyl- $\beta$ -D-thiogalactopyranoside (50  $\mu$ M) was then added to the culture to induce protein expression. After being shaken at room temperature for 4 h, the cells were harvested and the GST protein was purified with glutathione beads. Isl1 proteins translated *in vitro* were labeled with  $^{35}$ S methionine by using a TNT T7 reticulocyte lysate system (Promega). Glutathione beads conjugated with GST fusion protein were incubated with 10  $\mu$ l of TNT product at 4°C for 2 h in 500  $\mu$ l of GST-binding buffer (20 mM Tris, pH 7.3/150 mM NaCl/0.5% Nonidet P-40/protease inhibitor/1 mM phenylmethylsulfonyl fluoride). The beads were washed three times with GST binding buffer. 50  $\mu$ l of SDS loading buffer was then added to the beads. After boiling, 20  $\mu$ l was loaded onto an SDS/PAGE gel and analyzed by autoradiography.

### Cardiomyocyte culture

Neonatal rat cardiomyocytes were prepared as previously described<sup>51</sup>. Briefly, eighteen hours after plating, cells were changed into serum-free medium and infected with adenovirus (Ad-LacZ for control and Ad-CIP) at a multiplicity of infection (m.o.i.) of 25. 24 hours later, cells were treated with hypertrophic agents phenylephrine (PE). Cells were harvested 24 hours after PE treatment for RNA isolation or 48 hours after PE treatment for immunochemistry.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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### Non-standard Abbreviations and Acronyms

<b>ACTN2</b>	cardiac actin
<b>ANF</b>	atrial natriuretic factor
<b>BNP</b>	brain natriuretic factor
<b>cDNA</b>	complementary DNA
<b>CIP</b>	cardiac Isl1-interacting protein
<b>Co-IP</b>	co-immunoprecipitation
<b>E</b>	embryonic
<b>EST</b>	expressed sequence tag
<b>ET</b>	endothelin
<b>FACS</b>	fluorescence-activated cell sorting
<b>HATs</b>	histone acetyltransferases
<b>HDACs</b>	histone deacetylases
<b>IP</b>	immunoprecipitation

<b>ISL1</b>	Islet1
<b>LIF</b>	leukemia inhibitory factor
<b>LMNA</b>	Lamin A/C
<b>Luc</b>	luciferase
<b>MEF2</b>	Myocyte Enhancer Factor 2
<b>MYC</b>	myosin heavy chain
<b>PE</b>	phenylephrine
<b>qPCR</b>	quantitative polymerase chain reaction
<b>TAC</b>	transverse aortic constriction
<b>TNNT2</b>	cardiac troponin T
<b>UTR</b>	untranslated region
<b>WB</b>	Western blot

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### Novelty and Significance

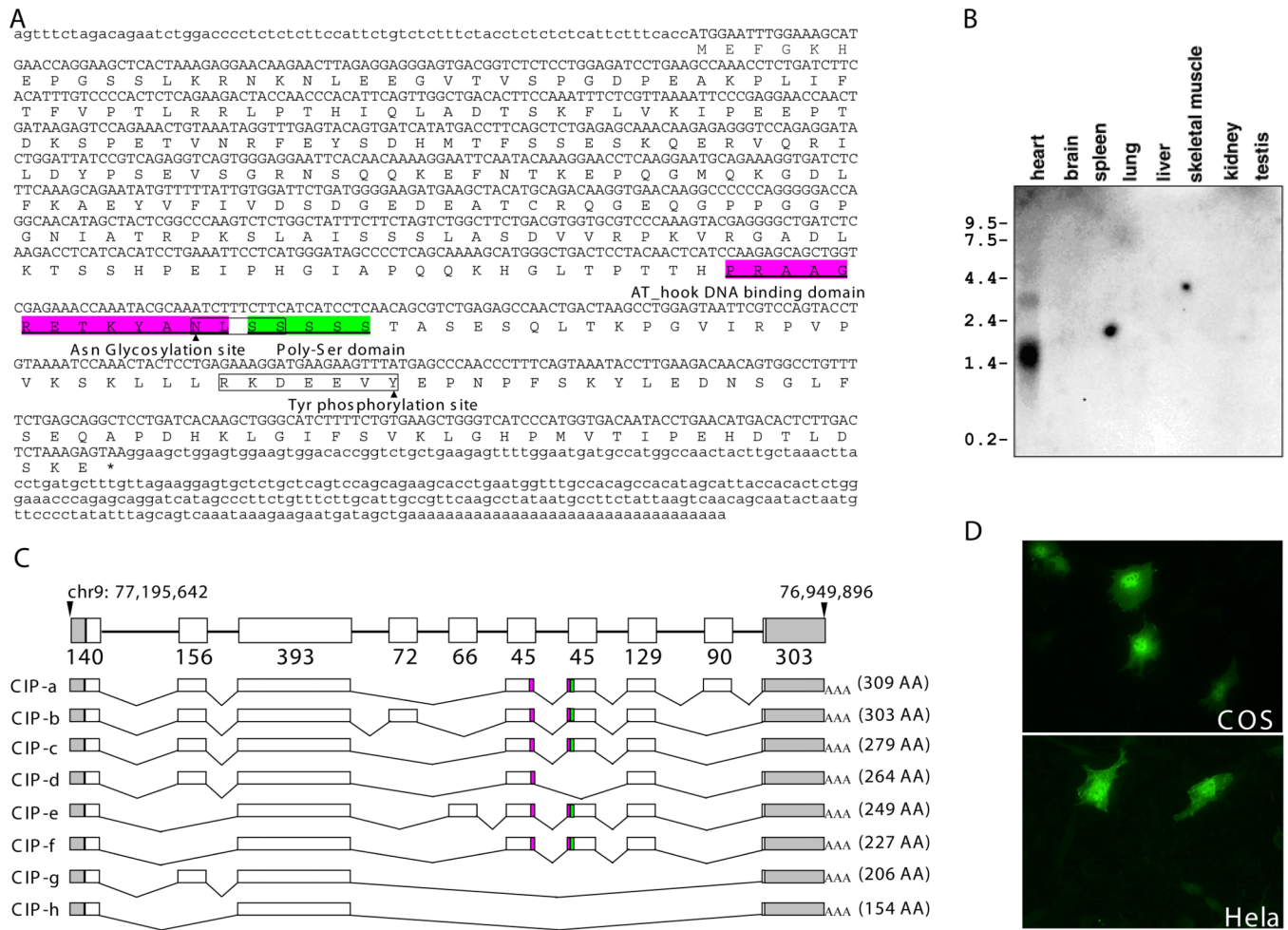
#### What Is Known?

- Cardiac hypertrophy is one of the most common responses of the heart in response to mechanical stress and pathological stimuli
- Chronic activation of pathological hypertrophy often leads to heart failure and sudden death
- Several signaling pathways and transcriptional networks activate cardiac hypertrophy
- Mechanisms underlying the repression of hypertrophy are not well understood.

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

- This study identified CIP (cardiac Isl1-interacting protein) as a novel cardiac-specific nuclear protein.
- CIP is dynamically regulated in response to cardiac hypertrophy.
- Overexpression of CIP represses cardiomyocyte hypertrophy.

Congestive heart failure is a common outcome of a variety of primary cardiovascular disease entities, including coronary artery disease, and hypertension. Nevertheless, mechanisms underlying the progression of compensated hypertrophy to heart failure remain unclear. Although the pathophysiology of cardiac hypertrophy has been extensively studied and much is known about signaling pathways that activate cardiac hypertrophy, processes leading to the repression of cardiac hypertrophy are less well understood. In this study, we identify CIP as a novel gene expressed in embryonic and adult hearts. Combinational approaches demonstrated that CIP expression is limited to cardiomyocytes, and that it is not expressed in other cell types (fibroblast, endothelial cells or vascular smooth muscle cells) of the heart. Our results show that CIP physically interacts with Isl1, a transcription factor essential for heart development and the fate of cardiac progenitors. Functionally, we found that CIP is a transcriptional repressor that represses agonist-induced cardiomyocyte hypertrophy and the expression of hypertrophic marker genes. These findings suggest that CIP may be a potential target in the development of future therapies for cardiac hypertrophy and heart failure.



**Figure 1. Identification of the CIP gene**

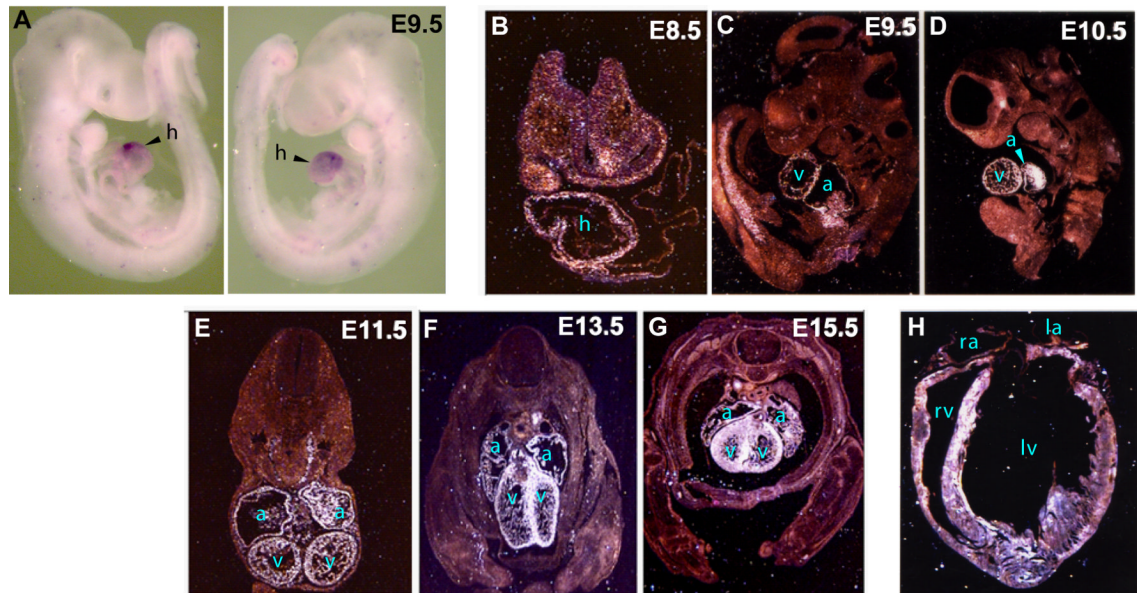
(A) Nucleotide sequences and deduced amino acid sequences. Nucleotides for open reading frame (ORF) are shown in uppercase; Nucleotides for both 5' and 3' untranslated regions (UTRs) are shown in lowercase. The corresponding amino acids (in single letter code) are shown below. Amino acids underlined indicate the putative AT\_hook domain and poly-Ser domain. The predicted amino acid modifications are also indicated and their recognition motifs are shown in boxes.

(B) Northern blotting analysis of CIP expression using RNAs of different tissues from adult mice.

(C) Gene structure and alternative splicing of the mouse CIP gene. Empty boxes mark ORF, gray boxes indicate UTR. The color boxes in alternative splicing variants indicate putative domains shown in Figure 1A.

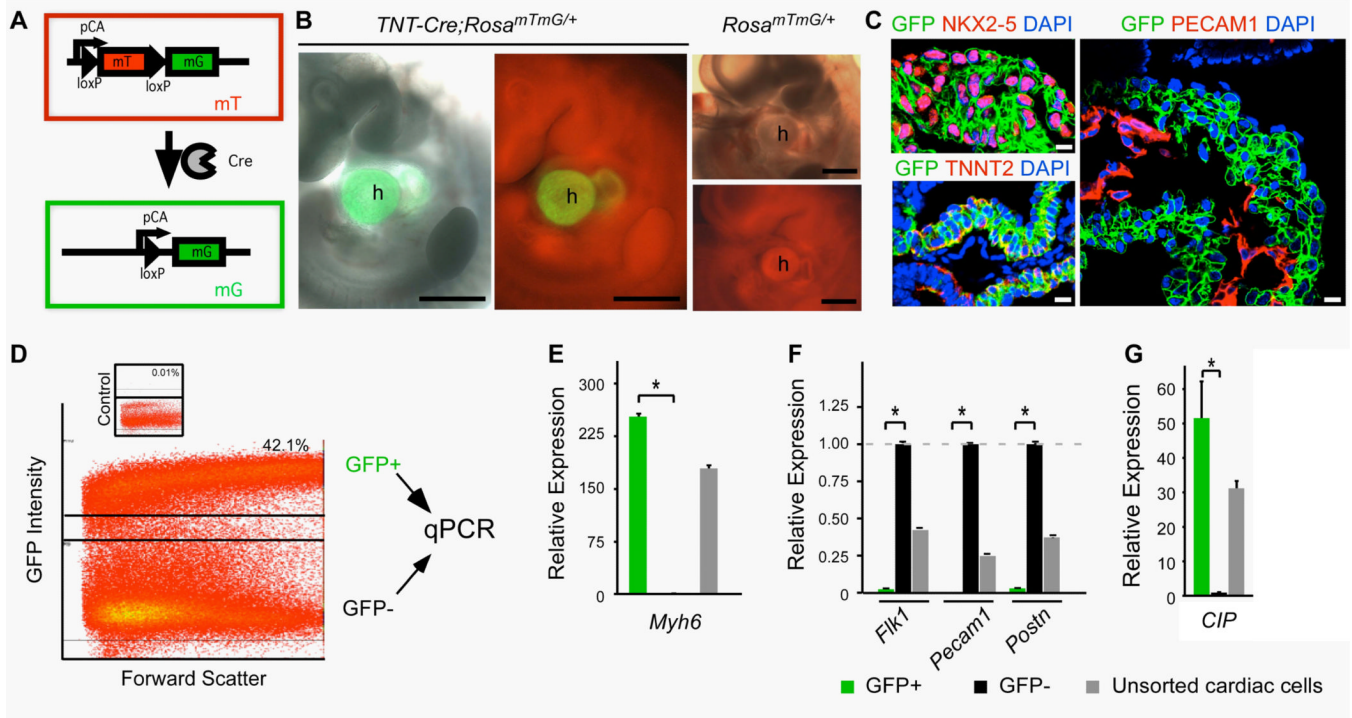
(D) CIP is a nuclear protein. Immunofluorescence detecting the nuclear localization of the FLAG-CIP fusion proteins in transfected COS and HeLa cells.





**Figure 2. Expression of CIP gene in embryonic and adult mouse tissues**

(A) Whole mount in situ hybridization detecting CIP transcript expression in E9.5 mouse embryos. *In situ* hybridization detecting CIP transcript expression on sections of staged mouse embryos (B-G) and in an adult mouse heart (H). (B, E, F, G) transverse sections; (C, D, H) sagittal sections. h: heart; a: atrium; la: left atrium; lv: left ventricle; ra: right atrium; rv: right ventricle; v: ventricle.



**Figure 3. Quantitative RT-PCR analyses of CIP expression in cardiomyocytes of mouse embryos** (A) Schematic of the *Rosa<sup>mTmG/+</sup>* reporter system. In the presence of Cre recombinase, which deletes the mT cassette, the expression of membranous tomato red (mT) turns into membranous green fluorescence protein (mG).

(B) When the *Rosa<sup>mTmG/+</sup>* reporter line was crossed with a cardiomyocyte specific Cre line (TNT-Cre), membranous GFP is detected in E10.5 heart (h) whereas there is no green signal in the heart of control littermate. Bar = 500  $\mu$ m.

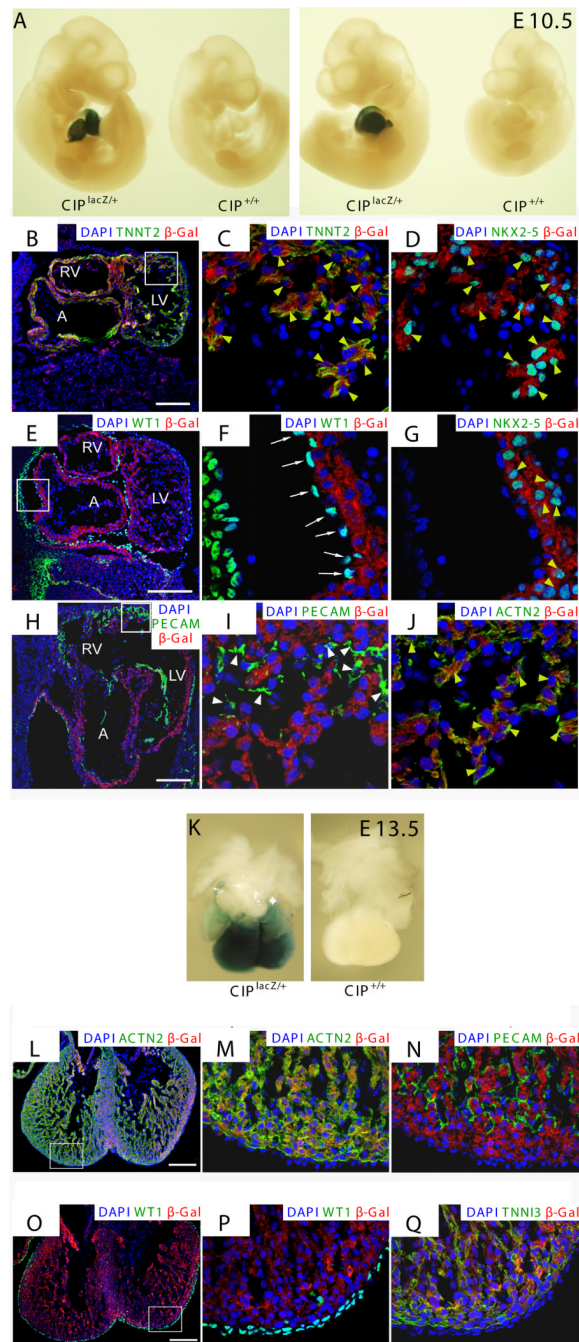
(C) Membranous GFP is detected in NKX2.5 positive and TNNT2 positive myocardium, but not the PECAM1 positive endocardium. Bar = 10  $\mu$ m.

(D) FACS isolation of GFP positive and negative cell populations from TNT-Cre; *Rosa<sup>mTmG/+</sup>* hearts. Insert shows that less than 0.01% GFP positive cells were found in the hearts of control littermates.

(E) GFP-positive population is highly enriched for cardiomyocyte specific marker *Myh6*, documented by quantitative RT-PCR assays.

(F) GFP-positive population expresses very low level of endothelial marker *Flk1* or *Pecam1*, and fibrotic tissue marker *Postn*, indicating the specificity of cell sorting.

(G) *CIP* is highly expressed in the GFP-positive cardiomyocytes, but not in GFP-negative non-cardiomyocytes. Expression in GFP negative population is set as 1. \*  $P < 0.01$ , three embryos were used ( $n=3$ ) for analysis in each group.



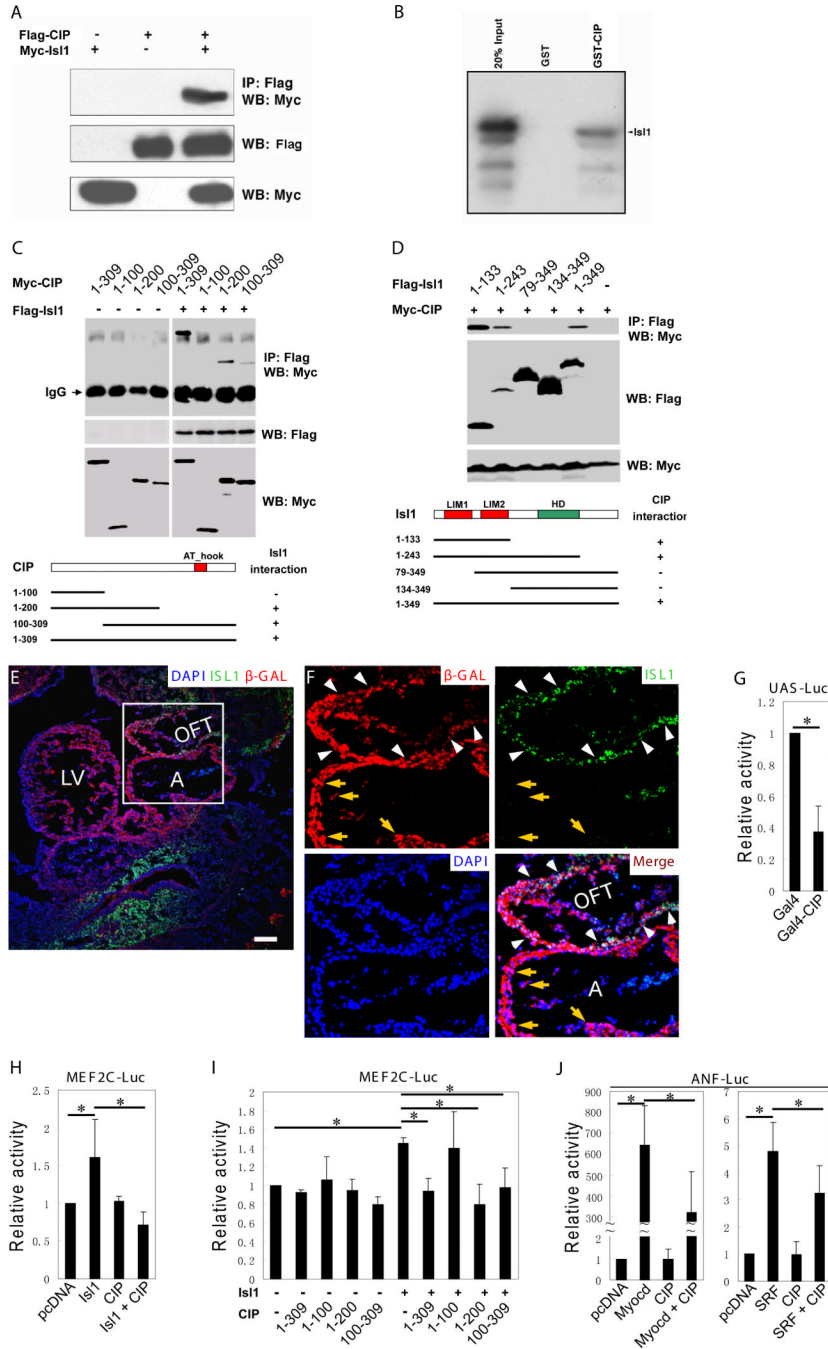
**Figure 4. Characterization of CIP expression using a LacZ reporter gene in mouse embryos**

(A) Whole mount  $\beta$ -gal staining of mouse E10.5 embryos harboring a LacZ cassette inserted in the CIP locus ( $CIP^{lacZ/+}$ ), or the control ( $CIP^{+/+}$ ). Positive  $\beta$ -gal staining is only detected in the heart.

(B-J) Immunohistochemistry using indicated antibodies on tissue sections of E10.5  $CIP^{lacZ/+}$  embryo. Note that CIP positive cells (marked by antibodies against the  $\beta$ -gal and is in red) overlay with Nkx2-5 (D, G), cardiac  $\alpha$ -actin (ACTN2) (J) and cardiac troponin T (TNNT2) (B, C) positive cardiomyocytes (yellow arrowheads) but not that of WT1-positive epicardium (white arrows) (E, F) nor PECAM-positive fibroblasts (white arrowheads) (H, I). DAPI (blue) staining marks the nucleus.

(K) Whole mount  $\beta$ -gal staining of mouse E13.5 embryonic hearts harboring a LacZ cassette inserted in the CIP locus ( $CIP^{lacZ/+}$ ), or the control ( $CIP^{+/+}$ ).

(L-Q) Immunohistochemistry using indicated antibodies on tissue sections of E13.5  $CIP^{lacZ/+}$  embryo. Note that CIP positive cells (marked by antibodies against the  $\beta$ -gal) overlay with cardiac  $\alpha$ -actin (ACTN2) and cardiac troponin T (TNNT2) positive cardiomyocytes not that of WT1 positive epicardium, nor PECAM positive fibroblasts. DAPI staining marks the nucleus. (B, E, H, bar=100  $\mu$ m; C, D, F, G, I, J, bar=10  $\mu$ m; L, O, bar=200  $\mu$ m, M, N, P, Q, bar=40  $\mu$ m. Data are presented as representative images derived from >3 embryos. A, atrium; LV, left ventricle; RV, right ventricle.



**Figure 5. CIP interacts with Isl1 and represses its transcription activity**

(A) Co-immunoprecipitation assays showing the interaction between Flag-tagged CIP and Myc-tagged Isl1. HEK-293T cells were co-transfected Flag-CIP, Myc-Isl1, or both. Anti-Flag antibody was used to immunoprecipitate (IP) cell extracts and anti-Myc antibody was used in Western blot (WB) to detect Myc-Isl1 protein in the complex. 10% cell lysate was used as input to demonstrate the expression of tagged proteins (button two panels).

(B) In vitro GST-fusion protein pull down assays showing the direct interaction between CIP and Isl1. Isl1 protein was synthesized *in vitro* and labeled with S-35. GST-CIP protein, but not GST protein, pulled down S-35 labeled Isl1. 20% of labeled Isl1 protein was load as control (Input).

(C) Map the interaction domains of the CIP protein that mediate the interaction between Isl1 and CIP using co-immunoprecipitation assays. HEK-293T cells were co-transfected Flag-Isl1, Myc-CIP full-length (1-309) or indicated truncated mutants, or both. Anti-Flag antibody was used to immunoprecipitate (IP) cell extracts and anti-Myc antibody was used in Western blot (WB) to detect Myc-CIP protein in the complex. 10% cell lysate was used as input to demonstrate the expression of tagged proteins (lower two panels). The interaction domains are summarized in the bottom diagram.

(D) Map the interaction domains of Isl1 proteins which mediate the interaction between Isl1 and CIP using co-immunoprecipitation assays. HEK-293T cells were co-transfected Myc-CIP, Flag-Isl1 full-length (1-349) or indicated truncated mutants, or both. Anti-Flag antibody was used to immunoprecipitate (IP) cell extracts and anti-Myc antibody was used in Western blot (WB) to detect Myc-CIP protein in the complex. 10% cell lysate was used as input to demonstrate the expression of tagged proteins (lower two panels). The interaction domains are summarized in the bottom diagram.

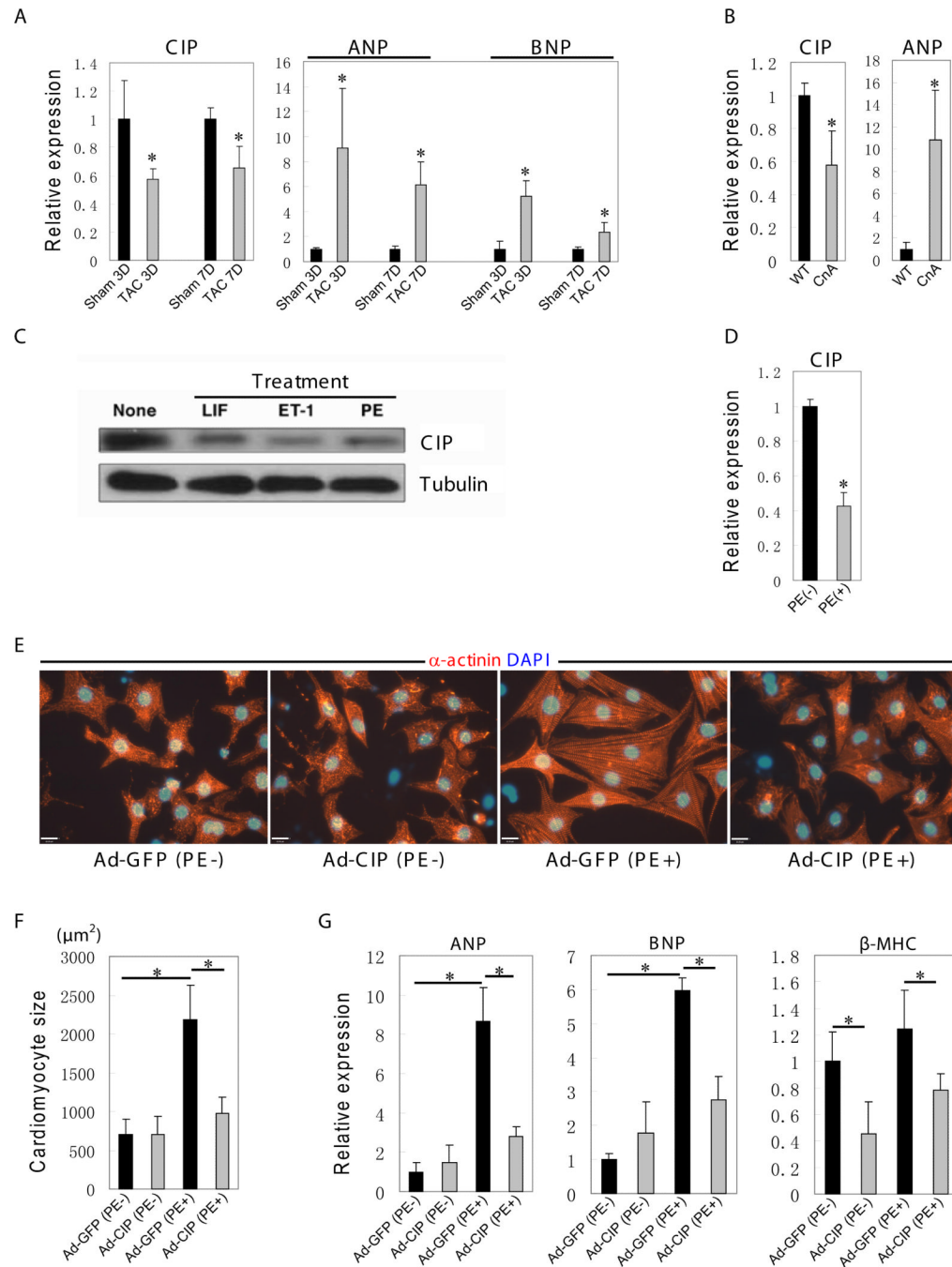
(E) Isl1 and CIP are co-expressed in cardiomyocytes of outflow tract of mouse embryos. Immunohistochemistry to demonstrate that Isl1 (green) and  $\beta$ -gal positive CIP (red) expressing cells are co-located in the cardiomyocytes of outflow tract (OFT) of E10.5 mouse embryos (arrowheads). DAPI staining marks the nucleus. Bar = 50  $\mu$ m. A, atrium; LV, left ventricle; OFT, outflow tract.

(F) Enlargement of boxed area in (E) to show that Isl1 and CIP expression is overlapped in the OFT cardiomyocytes (white arrowheads). The expression of Isl1 (green), CIP (as marked by  $\beta$ -gal positive cells, red) DAPI (blue) and merged images were shown. Isl1 and CIP positive cells were indicated by white arrowheads. Note that Isl1 is not expressed in the cardiomyocytes of atrium where CIP is expressed (yellow arrows). (G) CIP is a transcriptional repressor. HEK293T cells were transiently transfected with expression vectors encoding the CIP fused to GAL4 (1-147) and the pL8G5-luciferase reporter, which contains binding sites for the GAL4 DNA binding domain. Luciferase activity is expressed as relative activity in which the control was assigned a value of 1. Data represent the mean  $\pm$  s.d. from at least three independent experiments in triplicate. \*P<0.05.

(H) CIP represses the transcription activity of Isl1. MEF2C enhancer-luciferase reporter was co-transfected with indicated expression plasmids in HEK293T cells and luciferase activity was measured. Results were presented as relative luciferase activity in which the control was assigned a value of 1. Data represent the mean  $\pm$  s.d. from at least three independent experiments in triplicate. \*P<0.05.

(I) Define the domains of the CIP protein that mediate the repression of Isl1 transcription activity. MEF2C enhancer-luciferase reporter was co-transfected with indicated expression plasmids (full-length isl1, full-length or truncated CIP mutants) in HEK293T cells and luciferase activity was measured. Results were presented as relative luciferase activity in which the control was assigned a value of 1. Data represent the mean  $\pm$  s.d. from at least three independent experiments in triplicate. \*P<0.05.

(J) CIP represses myocardin- and SRF-mediated transactivation of the ANF-luciferase reporter. The ANF-luciferase reporter was co-transfected with indicated expression plasmids in HEK293T cells and luciferase activity was measured. Results were presented as relative luciferase activity in which the control was assigned a value of 1. Data represent the mean  $\pm$  s.d. from at least three independent experiments in triplicate. \*P<0.05.



**Figure 6. CIP represses cardiomyocyte hypertrophy**

(A) Gene expression of CIP and hypertrophic marker, ANP and BNP, determined by qPCR in heart samples from Sham group and Transverse Aortic Constriction (TAC) group at two different time point (3 days and 7 days). n=4.

(B) Gene expression of CIP and hypertrophic marker ANP determined by qPCR in heart samples from 1 month old Myh6-calcineurin transgenic mice (CnA) and control littermates (WT). n=3.

(C) Western blotting analysis showing decreased expression of CIP proteins in neonatal rat cardiomyocytes, which were induced to develop hypertrophy by different hypertrophic

agonists *in vitro*. None treated sample serves as a control. LIF, leukemia inhibitory factor; ET-1, Endothelin-1; PE, phenylephrine.

(D) Quantitative RT-PCR showing CIP expression in rat neonatal cardiomyocytes with or without phenylephrine (PE) treatment. \*P<0.05.

(E) CIP represses PE-induced hypertrophy in neonatal cardiomyocytes. Representative images of cardiomyocytes infected with adenoviral-CIP (ad-CIP) or adenoviral-GFP (ad-GFP) (to serve as controls) and treated with phenylephrine (PE) (or without treatment to serve as controls). Anti  $\alpha$ -actinin antibodies were used to mark cardiomyocytes (red). DAPI marks nucleus. Bar = 21  $\mu$ m.

(F) Quantitative analysis of cardiomyocyte cell size. ~300 cells positive for  $\alpha$ -actinin from each treatment were randomly chosen for surface area measurement. \*P<0.05.

(G) Quantitative RT-PCR showing the downregulation of PE-induced expression of hypertrophic marker ANP, BNP and  $\beta$ -MHC when CIP was overexpressed in neonatal cardiomyocytes. n=4. \*P<0.05. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide;  $\beta$ -MHC,  $\beta$ -myosin heavy chain.