Complex genetic pathways intimately regulate heart development and function. 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. Those include Nkx2.5, a homeobox protein, MEF2C, a member of the myocyte enhancer factor-2 (MEF2) family of MADS-box transcription factors, and GATA4, a GATA family zinc finger protein. Some of the well-characterized cardiac genes regulated by these cardiac transcription factors include cardiac-specific atrial natriuretic factor and cardiac α-actin.

Two distinct sources of cardiac precursor cells from the “primary” and “secondary heart fields” participate in heart formation. 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. Loss-of-function studies have defined genes whose activity is required in secondary heart field progenitors for morphogenesis of outflow tract and right ventricle. Among the genes with key function in secondary heart field is Isl1, a LIM/homeodomain transcription factor.

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. 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 outcomes such as heart failure.

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 a novel cardiac Isl1-interacting protein (CIP). CIP was identified from a bioinformatic search for novel cardiomyocyte-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 cofactor 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 myocyte enhancer factor 2C. 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 as a novel regulator of cardiac hypertrophy. (Circ Res. 2012;110: 818-830.)

Key Words: cardiomyocyte hypertrophy • cardiac development • Isl1-interacting protein • transcription factor
consequences that may lead to heart failure and sudden death.

Cardiac hypertrophy occurs in response to a variety of mechanical, hemodynamic, hormonal, and pathological stimuli. 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. 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 and histone deacetylases to remodel chromatin, has been proven as another mean of hypertrophic regulation. Cardiac hypertrophy is also accompanied by reactivation of a set of cardiac fetal genes, including those that encode atrial natriuretic factor, B-type natriuretic peptide, and β myosin heavy chain. Reactivation of these fetal genes suggests that molecular pathways that control heart development are reemployed to regulate hypertrophic growth. The pathophysiology of cardiac hypertrophy has been extensively studied for decades and much is known about signaling pathways that activate cardiac hypertrophy.

We previously identified myocardin as a SAP (SAF-A/B, Acinus, PIAS) domain transcriptional regulator. Myocardin is expressed in both cardiomyocytes and a subset of vascular and visceral smooth muscle cell types. Myocardin does not to bind DNA directly, but rather forms a stable ternary complex with serum response factor during cardiomyocyte hypertrophy. Epigenetic regulation of gene expression, including histone modification by histone acetyltransferases and histone deacetylases to remodel chromatin, has been proven as another mean of hypertrophic regulation. However, relatively less is known about how cardiac hypertrophy is repressed.

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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. Immunochemistry 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 (Figure 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′ untranslated region of the mouse Cip transcript. Whole-mount in situ hybridization first demonstrated that CIP expression is restricted to the heart of embryonic day 9.5 mouse embryos, with highest expression level detected in the ventricle (Figure 2A). In situ hybridization on tissue sections of staged mouse embryos revealed that Cip expression was first detected in the heart of embryonic day 8.5 mouse embryos (Figure 2B). Cip continued to be restricted to the heart from embryonic day 9.5 to embryonic day 15.5 (Figure 2C, D, E, F, G). An apparently positive signal was also detected in the trunk of embryonic day 9.5 embryo, which might represent transient expression of CIP in presomitic mesoderm (Figure 2C). From embryonic day 11.5 to embryonic day 15.5, Cip expression appeared to be higher in the ventricles of embryonic hearts (Figure 2E, F, G). Myocardial Cip expression continued in the adult heart (Figure 2H).

Because the heart consists 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 (Figure 3A, B). The presence of Cre will lead to the deletion...
of the mT cassette and the activation of the downstream membrane-targeted enhanced green fluorescent protein (EGFP) (mG) cassette (Figure 3A). The membrane-targeted EGFP can be utilized as a marker for fluorescence-activated cell sorting (Figure 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 (Figure 3B). Immunohistochemistry confirmed the labeling of cardiomyocytes in Rosa-mT-mG/cTNT-Cre embryos (Figure 3C). Hearts were dissected out from embryonic day 10.5 Rosa-mT-mG/cTNT-Cre embryos and digested. EGFP-positive cardiomyocytes and EGFP-negative noncardiomyocytes were separated by fluorescence-activated cell sorting (Figure 3D). Quantitative reverse-transcriptase polymerase chain reaction detected high expression level of Myh6 (\(\text{Myh6}^H9251\)) and CIP in the EGFP-positive cell sample but not in EGFP-negative noncardiomyocytes were separated by fluorescence-activated cell sorting (Figure 3D). Quantitative reverse-transcriptase polymerase chain reaction detected high expression level of Myh6 (\(\alpha\)-myosin heavy chain) and CIP in the EGFP-positive cell sample but not in EGFP-negative cell sample, whereas endothelial markers Flk1, platelet/endothelial cell adhesion molecule 1, and fibrotic tissue marker Postn were highly expressed in EGFP-negative cell sample but not in the EGFP-positive cell sample (Figure 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 Methods for details) to further map the expression of Cip in the heart (Online Figure III). LacZ reporter gene expression was restricted to the developing heart at embryonic day 10.5 and embryonic day 13.5, recapitulating endogenous expression pattern of Cip and highlighting the cardiac specific expression of this gene (Figure 4A, K). Immunohistochemistry analyses clearly demonstrated that the expression of Cip, as marked by \(\beta\)-galactosidase, overlapped with that of Nkx2-5, cardiac troponin T (TNNT2), and cardiac actin (ACTN2) in cardiomyocytes (Figure 4B, C, D, G, J, yellow arrow heads). Cip-expressing cells did not coexpress WT1, a marker of epicardium, or platelet/endothelial cell adhesion molecule, a marker of endothelial cells (Figure 4E, F, H, I, white arrows and arrow heads; n>3). Together these studies clearly demonstrate that Cip is specifically expressed in cardiomyocytes during embryogenesis. Interestingly, we detected CIP expression in the skeletal muscle of neonatal mice (Online Figure IV, middle). However, the expression of CIP in skeletal muscle appears to be transient, because we did not detect its expression in embryonic skeletal muscle (Online Figure IV, left) or in adult skeletal muscle tissue using the lacZ reporter (Online Figure IV, right) or by Northern blot analyses (Figure 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 embryonic day 10.5 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.\(^{23,45}\)

To confirm the interaction of CIP and Isl1, we performed coimmunoprecipitation 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 (Figure 5A). To further confirm direct interaction between CIP and Isl1, we per-
formed in vitro GST–fusion protein pull-down assays. Isl1 was 35S-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 (Figure 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 coimmunoprecipitation assays. As shown in Figure 5C, the interaction between CIP and Isl1 is readily detected in full-length (1–309) and both C-terminal 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 and 200 are required for CIP to interact with Isl1 (Figure 5C). Similarly, we examined domains in the Isl1 protein that are needed for the interaction with CIP. We found that aa 1 to 133 fragment is sufficient to mediate the interaction, whereas the aa 79 to 349 region failed to interact (Figure 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.23 We investigated whether CIP and Isl1 are coexpressed in mouse embryos. The expression of Isl1 was examined in Cip-lacZ reporter embryos. As shown in Figures 5E and 5F, both LacZ and Isl1 were expressed in cardiomyocytes located in the outflow tract of embryonic day 10.5 mouse embryos (Figure 5E, F, white arrowheads). However, Isl1 is not expressed in the cardiomyocytes of atrium or in the left ventricle, where CIP is expressed (Figure 5F, yellow arrows). Given that Isl1 is expressed in cardiac progenitor cells of the second heart field and newly formed cardiomyocytes in the outflow tracts,23 these results confirmed that CIP and Isl1 are coexpressed in the cardiac progenitor cells of embryonic hearts.

CIP Is a Transcription Cofactor 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 cotransfected into HEK293T cells (Figure 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.23 Isl1 activates the expression of the Mef2c gene by directly binding to its anterior heart field...
enhancer.\textsuperscript{46} We tested whether CIP participates in regulation of the \textit{Mef2c} anterior heart field enhancer. As expected, \textit{Isl1} constantly activated the \textit{Mef2c} enhancer luciferase reporter, although its activity does not appear to be potent (Figure 5I). CIP alone did not significantly affect \textit{Mef2c} enhancer activity. However, coexpression of \textit{Isl1} (the full-length, 1–309) and CIP inhibited \textit{Isl1}-mediated \textit{Mef2c} enhancer luciferase activity (Figure 5I). Further analyses, using different CIP deletion mutants, showed that the aa 1 to 200 and the aa 100 to 309 constructs (but not the aa 1–100) were able to repress \textit{Isl1}-mediated activation of the \textit{Mef2c} enhancer. These observations indicate that direct interaction of CIP and \textit{Isl1} is necessary for CIP to repress \textit{Isl1}. Our results suggest that CIP is a transcriptional repressor and represses the transcriptional activity of \textit{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 atrial natriuretic factor promoter activity in conjunction with the transcription factor serum response factor.\textsuperscript{36} We found that CIP significantly repressed myocardin-mediated activation of the atrial natriuretic factor–luciferase reporter (Figure 5J). Similarly, CIP also repressed serum response factor-dependent activation of the atrial natriuretic factor–luciferase reporter (Figure 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 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, \textit{Mef2c} was previously shown to be a key regulator of cardiac hypertrophy and dilated cardiomyopathy.\textsuperscript{11,47–50} Because CIP repressed the \textit{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, atrial natriuretic peptide and B-type natriuretic peptide, well-known hypertrophy markers, were dramatically upregulated in transverse aortic constriction mice at day 3 and day 7 (Figure 6A). In contrast, \textit{Cip} transcripts were substantially reduced in transverse aortic constriction mice. Similarly, expression of CIP was reduced in a second well-characterized cardiac hypertrophy model.\textsuperscript{27}
Figure 5. CIP interacts with Isl1 and represses its transcription activity. A. Coimmunoprecipitation assays showing the interaction between Flag-tagged CIP and Myc-tagged Isl1. HEK-293T cells were cotransfected Flag-CIP, Myc-Isl1, or both. Anti-Flag antibody was used to precipitate Flag-CIP from the cell lysate, and the precipitated proteins were detected with anti-Myc antibody. B. Western blots showing the expression levels of Flag-CIP and Myc-Isl1. C. Summary table showing the interaction between CIP and Isl1 deletion mutants. D. Western blots showing the interaction between Flag-Isl1 and Myc-CIP. E. Confocal microscopy images showing the expression of Isl1 and β-galactosidase in the heart. F. Histograms showing the relative activity of UAS-Luc reporter in different conditions. G. Bar graph showing the relative activity of UAS-Luc reporter in different conditions. H. Bar graph showing the relative activity of MEF2C-Luc reporter in different conditions. I. Bar graph showing the relative activity of MEF2C-Luc reporter in different conditions. J. Bar graph showing the relative activity of ANF-Luc reporter in different conditions.
in which transgenic expression of activated calcineurin stimulates dramatic cardiac hypertrophy (Figure 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, endothelin-1, and phenylephrin (PE) (Figure 6C). Quantitative reverse-transcriptase polymerase chain reaction analyses of PE-treated cardiomyocytes corroborated this finding and indicated that CIP expression is regulated at the transcriptional level (Figure 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**

These observations established an inverse correlation between the expression of CIP and cardiomyocyte 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. PE treatment dramatically induced cardiomyocyte hypertrophy, as measured by increased cell surface area (Figure 6F). Overexpression of CIP did not result in significant change to cardiomyocytes. However, CIP significantly attenuated PE-induced hypertrophy (Figure 6E). Quantification of cardiomyocyte size confirmed the observation (Figure 6F). At the level of gene expression, PE induced expression of hypertrophic marker genes atrial natriuretic peptide, B-type natriuretic peptide, and β-myosin heavy chain, as expected. PE-induced expression of hypertrophic marker genes was inhibited by CIP (Figure 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 cofactor 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 interactions. Our data suggest that a direct interaction between these two proteins is needed for CIP to repress the transactivity of Isl1. Isl1 is expressed in the second heart field and is required for the morphogenesis of embryonic heart. Interestingly, Isl1-positive progenitor cells were shown to be multi-potent and give rise to cardiac, smooth muscle, and endothelial cell lineages. Given that CIP interacts with Isl1 and is expressed in cardiac progenitors, we speculate that CIP might participate in the function of Isl1 in cell fate determination during early cardiac development. Despite its pivotal role in development, less is known...
Figure 6. CIP represses cardiomyocyte hypertrophy. A, Gene expression of CIP and hypertrophic marker, atrial natriuretic peptide (ANP), and B-type natriuretic peptide (BNP), determined by quantitative polymerase chain reaction (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 (wild-type [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. No treated sample serves as a control. LIF, leukemia inhibitory factor; ET-1, endothelin-1; PE, phenylephrine. D, Quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) showing CIP expression in rat neonatal cardiomyocytes with or without 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-green fluorescent protein (GFP) (ad-GFP), to serve as controls, and treated with PE (or without treatment to serve as controls). Anti-α-actinin antibodies were used to indicate cardiomyocytes (red). DAPI marks nucleus. Bar=21 μm. F, Quantitative analysis of cardiomyocyte cell size. Approximately 300 cells positive for α-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 β-myosin heavy chain (β-MHC) when CIP was overexpressed in neonatal cardiomyocytes. n=4. *P<0.05.
about how Isl1 works at the molecular level. Isl1 is a LIM/homeodomain transcription factor and it synergistically activates insulin gene transcription with BETa2 in pancreatic beta cells. Furthermore, Isl1 forms a complex with Jak1 and Stat3 and triggers the tyrosine phosphorylation of Jak1 and its kinase activity. Isl1 works upstream of the sonic hedgehog signaling and the MEF2C transcription factor in the heart. Our data showed that the transcriptional activity of Isl1, although 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 to CIP as an important regulator of cardiac gene expression and cardiac hypertrophy. Intriguingly, our data showed the capability of CIP to repress Isl1-mediated activation of the MEF2C enhancer. It had been shown that MEF2C was upregulated during pathological hypertrophy. Transgenic mice expressing a dominant-negative MEF2C displayed attenuated postnatal growth of myocardium. 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 in things from fish to humans, 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). 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/cofactors, including retinoblastoma transcriptional regulator (RB), germ cell-less, steroid response element binding protein, C-Fos, and zinc finger protein 239. 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. LMNA-null mice display severe skeletal muscle atrophy and dilated cardiomyopathy and die by 8 weeks. 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.

Database Searching and Cloning of CIP
We screened for novel cardiac-specific genes in silico by performing a Basic Local Alignment Search Tool search with expressed sequence tags from mouse embryonic heart cDNA libraries in the database as described previously. One of the cDNA sequences identified in this screen (access number AA919489) corresponded to the 3’ untranslated region of the CIP transcript and was found in a cDNA library of embryonic day 13.5 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-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 chromosomes 9: 77 046 001 and 77 046 002) was generated and obtained from Texas A&M Institute for Genomic Medicine. The gene trap cassette containing the selectable marker β-geo, a functional fusion between the β-galactosidase and neomycin resistance gene, was inserted into the putative third intron of the CIP gene (Online Figure III).

In Situ Hybridization and Northern Analysis
Whole-mount and section in situ hybridization and Northern analyses were performed as described. The 273 nt cDNA fragment corresponding to the 3’ untranslated region of the CIP gene was used as a probe to perform both whole-mount and tissue section in 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% fetal bovine serum in a 5% CO2 atmosphere at 37°C. Luciferase reporter constructs fused with the MEF2C enhancer were as reported and are generous gifts of Dr. Brian Black (University of California, San Francisco). Transfections were performed with either FuGENE6 (Roche) or lipofectamine (Invitrogen) reagents according to manufacturer instructions. Unless otherwise indicated, 100 ng of reporter plasmid and 100 ng of each activator plasmid were applied; 48 hours after transfection, cell extracts were pre-
pared 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 contained full-length CIP cDNA and was constructed as previously described.51

Tissue Dissociation and Cell Sorting of Mouse Embryos

The embryonic day 10.5 embryonic hearts were isolated by microdissection and dissociated to single cells by collagenase digestion as previously described.64 Isolated cells were fluorescence-activated cell-sorted into green fluorescent protein-positive and green fluorescent protein-negative populations. Sorted cells were collected into Trizol (Invitrogen) and frozen at −20°C for RNA isolation.

Immunocytochemistry and β-Galactosidase Staining

Stage mouse embryos were dissected out, collected, and fixed in 4% paraformaldehyde at 4°C for 4 hours. After washing in phosphate-buffered saline, embryos were treated in 15% and 30% sucrose for 2 hours each and embedded in OCT. Approximately 5-µm to 8-µm cryostat sections were collected on positively charged slides. Sections were washed in phosphate-buffered saline, blocked in 5% serum/phosphate-buffered saline, and subjected to immunostaining.

Antibody sources were as follows: green fluorescent protein (Invitrogen); platelet/endothelial cell adhesion molecule (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); and 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); and β-galactosidase (MP Biomedical). Alexa-488 and Alexa-594 secondary antibodies (Invitrogen). Fluorescently stained slides were counterstained with DAPI and imaged with an FV1000 confocal microscope (Olympus). For β-galactosidase staining, samples were stained with a solution containing 5 mmol/L isopropyl-β-D-thiogalactopyranoside (50 µmol/L) and 2 mg/ml of X-gal substrate at 37°C for 12 hours after fixation.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction and Western Blot Analyses

Total RNAs were isolated using Trizol reagent (Invitrogen) from cells and tissue samples. For quantitative reverse-transcriptase polymerase chain reaction, 2.0-µg RNA samples were reverse-transcribed to cDNA by using random hexamers and moloney murine leukemia virus reverse-transcriptase (Invitrogen) in a 20-µL reaction system. In each analysis, 0.1 µL cDNA pool was used for quantitative polymerase chain reaction. For Western blot analyses, cell extractions were cleared by 10 000 g centrifugation for 10 minutes at 4°C. Samples were subsequently separated by SDS/PAGE and transferred to polyvinylidene fluoride membranes that were incubated with 5% milk and anti-FLAG (1:1000; Sigma), anti-Myc (1:1000; Santa Cruz), and β-tubulin (1:10 000; Sigma) overnight at 4°C and then washed three times with tris-buffered saline tween-20 buffer before adding secondary antibody. Polyclonal antibodies against the CIP protein were generated by immunizing rabbits with CIP proteins that were expressed and purified as GST-CIP fusion proteins in bacterial. Specific protein bands were visualized by using enhanced chemiluminescence (Invitrogen) reagents.

Commmunoprecipitation Assays

COS7 cells were transiently transfected with plasmids encoding FLAG-tagged CIP and Myc-tagged Isl1 proteins with FuGENE6 (Roche). Cells were harvested 48 hours after transfection in lysis buffer composed of 1X phosphate-buffered saline containing 0.5% Triton X-100, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 150 mmol/L 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 were 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 2X yeast-extract tryptone medium to an optical density of 1.0. Isopropyl-β-D-thiogalactopyranoside (50 µmol/L) was then added to the culture to induce protein expression. After being shaken at room temperature for 4 hours, the cells were harvested and the GST protein was purified with glutathione beads. Isl1 proteins translated in vitro were labeled with 35S methionine by using a TNT T7 reticulocyte lysate system (Promega). Glutathione beads conjugated with GST fusion protein were incubated with 10 µL of TNT product at 4°C for 2 hours in 500 µL of GST-binding buffer (20 mmol/L Tris, pH 7.3/150 mmol/L NaCl/0.5% Nonidet P-40/protease inhibitor/1 mmol/L phenylmethylsulfonyl fluoride). The beads were washed three times with GST-binding buffer; 50 µL of SDS loading buffer was then added to the beads. After boiling, 20 µL was loaded onto an SDS/PAGE gel and analyzed by autoradiography.

Cardiomyocyte Culture

Neonatal rat cardiomyocytes were prepared as previously described.51 Briefly, 18 hours after plating, cells were changed into serum-free medium and infected with adenovirus (Ad-GFP for control and Ad-CIP) at a multiplicity of infection of 25. Twenty-four hours later, cells were treated with hypertrophic agents PE. Cells were harvested 24 hours after PE treatment for RNA isolation or 48 hours after PE treatment for immunostaining.

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Disclosures

None.

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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 cardiac isl1-interacting protein (CIP) 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. Combinatorial 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.
CIP, a Cardiac Isl1-Interacting Protein, Represses Cardiomyocyte Hypertrophy
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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 1. 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 β-geo, a functional fusion between the β-galactosidase and neomycin resistance gene, was inserted into the putative third intron of the CIP gene (Online Figure III).

In Situ Hybridization and Northern Analysis
Whole-mount and section in situ hybridization and Northern analyses were performed as described 1,2. 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.

For whole-mount in situ hybridization, E9.5 mouse embryos were dissected in PBS buffer and fixed in M buffer (100mM MOPS pH 7.4, 2mM EGTA, 1mM MgSO4, and 3.7% formaldehyde) at 4°C overnight. After bleaching in M buffer with 6% hydrogen peroxide for 48 hr at room temperature, embryos were dehydrated through an ascending methanol series in PBS (25, 50, 75 and 100% methanol) and stored in 100% methanol at -20°C until needed. Embryos were rehydrated through a descending methanol series in PBT (PBS plus 1% Tween 20) and PBT, permeabilized in PBT containing proteinase K (10µg/ml in PBT) for 30 minutes at room temperature. After post-fixing in 4% paraformaldehyde in PBT, embryos were washed three times in PBT, rinsed once each in 0.1M triethanolamine (pH 8) and in 0.1M triethanolamine plus acetic anhydride (2.5µl/ml). After incubation with prehybridization solution (0.3M NaCl, 20mM Tris pH 8.0, 1mM EDTA, 0.1M DTT, 1X Denharts, 10% dextran sulfate, 50% formamide, 250µg/ml yeast tRNA, and 100µg/ml salmon sperm DNA) for 2 hr at 60°C, embryos were hybridized with DIG-labeled riboprobes in hybridization solution (prehybridization solution plus 0.5-1µg/ml riboprobe) at 60°C overnight. After hybridization, embryos were washed two times (30 min each) with prehybridization solution at the hybridization temperature, followed by washing two times (30 min each)
with 50% formamide, 2X SSC at the same temperature. Embryos were then treated with RNase A (1µg/ml) and RNase T1 (1 unit/ml) for 30 min at 37°C. After a final wash with 0.2X SSC, 1% Tween 20 at 60°C, embryos were incubated with PBT plus 10% heat inactivated sheep serum (Sigma, St. Louis, MO) for 2 hr at room temperature and then with alkaline phosphatase conjugated Fab anti-DIG solution (1 to 2000 dilution in PBT plus 10% sheep serum) overnight at 4°C. Embryos were washed at least 8 times with PBT (1 hour each) and left in PBT overnight at 4°C to completely wash out excess amounts of alkaline phosphatase conjugated Fab. Color reaction was performed by washing embryos three times for 10 min each with buffer A containing 100 mM Tris pH 9.5, 50mM MgCl2, 100mM NaCl, 1% Tween 20, and 2mM levamisol, followed by incubating with detection solution (buffer A plus 4.5µl/ml nitroblue tetrazolium and 3.5µl/ml 5-bromo-4-chloro-3-indolyl-phosphate toluidinium) for various times at room temperature. When the color reaction was complete, embryos were washed twice with buffer A, once with PBT, and then post-fixed in 4% paraformaldehyde in PBT.

For Northern blot analysis, adult mouse multiple tissue northern blot purchased from Clontech (Palo Alto, CA) was used. A DNA probe containing 3'-UTR of the CIP gene was labeled with a-32P-dATP using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). The hybridization was performed at 68°C for 1 hour in an ExpressHyb hybridization solution (Clontech, Palo Alto, CA). The filter was then washed twice in 2×SSC, 0.05% SDS at room temperature for 30 minutes each, followed by 30 minutes at 50°C in 0.1×SSC, 0.1% SDS. The filter was then exposed to X-ray film at -70°C with the presence of intensifying screens.

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

Hela, COS7 and HEK293T cells were cultured in DMEM supplemented with 10% FBS in a 5% CO2 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) 3. 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) was constructed as previously described 4. The CIP adenoviral expression construct (Ad-CIP) contained a cDNA encoding amino acids 1-309 of mouse CIP. Ad-CIP adenoviral constructs were N-terminal FLAG tagged.

**Tissue Dissociation and Cell Sorting of Mouse Embryos**

E10.5 embryonic hearts were isolated by microdissection and dissociated to single cells by collagenase digestion as previously described 5. 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.

**Immuonochemistry and β-Gal Staining**
Staged mouse embryos were dissected out, collected and fixed in 4% paraformaldehyde 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 μm 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); β-galatosidase (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 β-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 μg RNA samples were reverse-transcribed to cDNA by using random hexamers and MMLV reverse transcriptase (Invitrogen) in 20 μl reaction system. In each analysis, 0.1 μl cDNA pool was used for quantitative PCR. For Western blot analyses, cell extractions were cleared by 10,000×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); β-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 PBS containing 0.5% Triton X-100, 1 mM EDTA, 1 mM PMSF, and complete protease inhibitors (Roche). After a brief sonication and removal of cellular debris by 10,000×g centrifugation for 10 min at 4°C, 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
The cells were grown at 37°C in 2×YT medium to an optical density of 1.0. Isopropyl-β-D-thiogalactopyranoside (50 μM) 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 35S methionine by using a TNT T7 reticulocyte lysate system (Promega). Glutathione beads conjugated with GST fusion protein were incubated with 10 μl of TNT product at 4°C for 2 h in 500 μl 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 μl of SDS loading buffer was then added to the beads. After boiling, 20 μl was loaded onto an SDS/PAGE gel and analyzed by autoradiography.

**Cardiomyocyte Culture**

Neonatal rat cardiomyocytes were prepared as previously described. Briefly, Neonatal rat cardiomyocytes were isolated by enzymatic disassociation of 1- to 2-day old neonatal rat hearts with the Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Corp.). Cardiomyocytes were plated differentially for 2 hours to remove fibroblasts. Cells were plated on 1% gelatin-coated plates in medium containing 10% horse serum and 5% fetal calf serum (FCS). 18 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 agent, phenylephrine (PE), by changing to the PE-contained serum-free medium. Cells were harvested 24 hours after PE treatment for RNA isolation or 48 hours after PE treatment for immunochemistry.

**REFERENCES:**


Online Figure I

Supplemental Material

Online Figure I. Sequence alignment between the putative AT_hook DNA binding domain in CIP and other known AT_hook DNA binding domain.
Online Figure II

Amino acid sequence alignment of CIP orthologs in multiple species. The AT hook DNA binding domain (in box) is highly conserved during evolution.
Online Figure III. Targeting strategy of CIP reporter line. The expression of β-gal-Neo (geo) fusion gene is driven by endogenous CIP promoter. An internal ribosome entry site (IRES) allows for translation initiation of β-gal-Neo fusion gene.
Online Figure IV. β-gal staining of forelimb/hindlimb in multiple stages of CIP reporter line showing the transient expression of CIP in skeletal muscle.