predicts all-cause mortality, it is most predictive of death from progressive HF, which suggests that it is a manifestation of or exacerbating factor for LV dysfunction.[34]

Study Limitations

One of the limitations is the small sample size of the present study. First, there were only 3 deaths from progressive HF and 2 sudden deaths during the follow-up periods. Therefore, our observations need to be examined in a larger population and cutoff points for major clinical outcomes should be evaluated. Second, the use of β-blockers was less frequent in serum TN-C ≥78.4 ng/mL group than <78.4 ng/mL group. More patients in serum TN-C ≥78.4 ng/mL were unable to tolerate β-blockers because of hypotension, bradycardia, or other limiting symptoms. We cannot rule out the effects of chronic medications on the worse outcomes in serum TN-C levels ≥78.4 ng/mL group. Third, the results reported in the present study cannot be extrapolated to chronic HF and to ischemic cardiomyopathy. Further studies are needed in these specific settings.

Conclusions

The combined index of serum levels for TN-C and BNP at discharge predicts cardiac events of rehospitalization and cardiac death due to decompensated HF. Additionally, elevated serum TN-C levels reflect left ventricular and pulmonary vascular remodeling in patients with DCM.

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Cardiac Ankyrin Repeat Protein Gene (*ANKRD1*) Mutations in Hypertrophic Cardiomyopathy

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Objectives The purpose of this study was to explore a novel disease gene for hypertrophic cardiomyopathy (HCM) and to evaluate functional alterations caused by mutations.

Background Mutations in genes encoding myofilaments or Z-disc proteins of the cardiac sarcomere cause HCM, but the disease-causing mutations can be found in one-half of the patients, indicating that novel HCM-susceptibility genes await discovery. We studied a candidate gene, ankyrin repeat domain 1 (*ANKRD1*), encoding for the cardiac ankyrin repeat protein (CARP) that is a Z-disc component interacting with N2A domain of titin/connectin and N-terminal domain of myopalladin.

Methods We analyzed 384 HCM patients for mutations in *ANKRD1* and in the N2A domain of titin/connectin gene (*TTN*). Interaction of CARP with titin/connectin or myopalladin was investigated using coimmunoprecipitation assay to demonstrate the functional alteration caused by *ANKRD1* or *TTN* mutations. Functional abnormalities caused by the *ANKRD1* mutations were also examined at the cellular level in neonatal rat cardiomyocytes.

Results Three *ANKRD1* missense mutations, Pro52Ala, Thr123Met, and Ile280Val, were found in 3 patients. All mutations increased binding of CARP to both titin/connectin and myopalladin. In addition, *TTN* mutations, Arg8500His, and Arg8604Gln in the N2A domain were found in 2 patients, and these mutations increased binding of titin/connectin to CARP. Myc-tagged CARP showed that the mutations resulted in abnormal localization of CARP in cardiomyocytes.

Conclusions CARP abnormalities may be involved in the pathogenesis of HCM. (J Am Coll Cardiol 2009;54:334–42)
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Cardiomyopathy is a primary heart muscle disorder caused by functional abnormalities of cardiomyocytes. There are several clinical subtypes of cardiomyopathy, and the most prevalent subtype is hypertrophic cardiomyopathy (HCM) (1,2). HCM is characterized by hypertrophy and diastolic dysfunction of cardiac ventricles accompanied by cardiomyocyte hypertrophy, fibrosis, and myofibrillar disarray (1). Although the etiologies of HCM have not been fully elucidated, 50% to 70% of the patients with HCM have apparent family histories consistent with autosomal dominant genetic trait (3), and recent genetic analyses have revealed that a significant percentage of HCM is caused by mutations in the genes encoding for myofilaments and Z-disc proteins of the cardiac sarcomere, with the majority of mutations identified in *MYH7*-encoded beta

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myosin heavy chain and *MYBPC3*-encoded myosin-binding protein C (3).

Ankyrin repeat domain 1 (*ANKRD1*)-encoded cardiac adriamycin responsive protein (4), or cardiac ankyrin repeat protein (CARP) (5), is a transcription cofactor and an early differentiation marker of cardiac myogenesis, expressed in the heart during embryonic and fetal development. CARP expression is up-regulated in the adult heart at end-stage heart failure (6). In addition, increased CARP expression was found in hypertrophied hearts from experimental murine models (7,8). These observations suggest a pivotal role for CARP in cardiac muscle function in both physiological and pathological conditions. Although CARP is known to be involved in the regulation of gene expression in the heart, Bang et al. (9) demonstrated that CARP located to both the sarcoplasm and nucleus, suggesting a shuttling of CARP in cellular components. Within the I-band region of sarcomere, CARP bound to both the N2A domain of titin/connectin encoded by titin/connectin gene (*TTN*) and the N-terminal domain of myopalladin encoded by *MYPN*. Hence, titin/connectin and myopalladin function in part as anchoring proteins of "sarcomeric CARP" (9,10).

Titin/connectin is the most giant protein expressed in the striated muscles, which is involved in sarcomere assembly, force transmission at the Z-disc, and maintenance of resting tension in the I-band region (11,12). In cardiac muscle, there are 2 titin isoforms, N2B and N2BA. The N2B isoform contains a cardiac specific N2B domain, and the N2BA isoform contains both N2B and N2A domains. Both N2A and N2B domains, within the extensible I-band region, function as a molecular spring that develops passive tension; the expression of N2B isoform results in a higher passive stiffness than that of N2AB isoform. We previously reported an HCM-associated mutation localizing to the N2B domain (13), and Gerull et al. (14) reported other *TTN* mutations in the Z/I transition domain. These observations suggest that the I-band region of titin/connectin contains elastic components extending with stretch to generate passive force, which plays an important role in the maintenance of cardiac function.

Another protein that anchors CARP at the Z/I band is myopalladin, a cytoskeletal protein containing 3 proline-rich motifs and 5 Ig domains. The proline-rich motifs in the central part is required for binding to nebulin/nebulette, and the Ig domains at the N-terminus and C-terminus are involved in the binding to CARP and sarcomeric a-actinin, respectively (9). It has been suggested that myopalladin played key roles in sarcomere/Z-disc assembly, myofibrillogenesis, recruitment of the other Z/I-band elements, and signaling in the Z/I-band (9).

In this study, we analyzed unrelated patients with heretofore genotype-negative HCM for mutations in *ANKRD1* and found 3 mutations that showed abnormal binding to myopalladin and titin/connectin. In addition, we searched for mutations in the reciprocal CARP-binding N2A domain of titin/connectin and identified 2 HCM-associated mutations in *TTN* causing abnormal binding to CARP. We report here that abnormal CARP assembly in the cardiac muscles may be involved in the pathogenesis of HCM.

Abbreviations and Acronyms

Ab = antibody

ANKRD1 = ankyrin repeat domain 1

CARP = cardiac ankyrin repeat protein

cDNA = complementary deoxyribonucleic acid

Co-IP = coimmunoprecipitation

DAPI = 4′6-diamidino-2-phenylindole

DCM = dilated cardiomyopathy

HCM = hypertrophic cardiomyopathy

PCR = polymerase chain reaction

WT = wild type

Methods

Subjects. A total of 384 unrelated patients with HCM were included in this study. The patients were diagnosed based on medical history, physical examination, 12-lead electrocardiogram, echocardiography, and other special tests if necessary. The diagnostic criteria for HCM included left ventricular wall thickness >13 mm on echocardiography, in the absence of coronary artery disease, myocarditis, and hypertension. The patients had been analyzed previously for mutations in previously published myofilament- and Z-disc associated genes, and no mutation was found in any of the known HCM-susceptibility genes (15–18). Ethnically-matched healthy persons (400 from Japan, and 300 from the U.S.) were used as controls. Blood samples were obtained from the subjects after given informed consent. The protocol for research was approved by the Ethics Reviewing Committee of Medical Research Institute, Tokyo Medical and Dental University (Japan) and by the Mayo Foundation Institutional Review Board (U.S.).

Mutational analysis. Using intronic primers, each translated *ANKRD1* exon was amplified by polymerase chain reaction (PCR) from genomic DNA samples. *TTN* exons 99 to 104 corresponding to the N2A domain including binding domains to CARP and p94/calpain were amplified by PCR in exon-by-exon manner. Sequence of primers and PCR conditions used in this study are available upon request. PCR products were analyzed by direct sequencing or by denaturing high-performance liquid chromatography followed by sequencing analysis. Sequencing was performed using Big Dye Terminator chemistry (version 3.1, Applied Biosystems, Foster City, California) and ABI3100 DNA Analyzer (Applied Biosystems).

Coimmunoprecipitation (co-IP) assay. We obtained complementary deoxyribonucleic acid (cDNA) fragments of human *ANKRD1* and *TTN* by reverse-transcriptase PCR from adult heart messenger ribonucleic acid. A wild-type (WT) full-length CARP cDNA fragment spanned from bp249 to bp1208 of GenBank Accession No. NM_014391 (corresponding to aa1-aa319). Three equivalent mutant cDNA fragments containing C to G (Pro52Ala mutation), C to T (Thr123Met mutation), or A to G (Ile280Val mutation) substitutions were obtained by the primer-

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directed mutagenesis method. A WT *TTN* cDNA fragment encoding N2A domains (from bp25535 to bp26465 of NM_133378 corresponding to aa8437-aa8747) was obtained, and 3 *TTN* mutants carrying T to C (non–disease-associated Ile8474Thr polymorphism), G to A (HCM-associated Arg8500His mutation), or G to A (HCM-associated Arg8604Gln mutation) substitutions were created by the primer-mediated mutagenesis method. The cDNA fragments of *ANKRD1* were cloned into myc-tagged pCMV-Tag3 (Stratagene, La Jolla, California), and *TTN* and *MYPN* cDNA fragments were cloned into pEGFP-C1 (Clontech, Mountain View, California). These constructs were sequenced to ensure that no errors were introduced.

Cellular transfection and protein extractions were performed as described previously (19), and co-IP assays were performed using the Catch and Release version 2.0 Reversible Immunoprecipitation System according to the manufacturer's instructions (Millipore, Billerica, Massachusetts). Immunoprecipitates were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to a nitrocellulose membrane. After a pre-incubation with 3% skim milk in phosphate-buffered saline, the membrane was incubated with primary rabbit anti-myc polyclonal antibody (Ab) or mouse anti-GFP monoclonal antibody Ab (1:100, Santa Cruz Biotechnology, Santa Cruz, California), and with secondary goat anti-rabbit (for polyclonal Ab) or rabbit anti-mouse (for monoclonal Ab) IgG HRP-conjugated Ab (1:2,000, Dako A/S, Grostrup, Denmark). Signals were visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore) and Luminescent Image Analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan), and their densities were quantified by using Multi Gauge version 3.0 (Fujifilm, Tokyo, Japan). Numerical data were expressed as mean ± SEM. Statistical differences were analyzed using 1-way analysis of variance and the Student *t* test for paired values. Means were compared by independent sample *t* tests without correction for multiple comparisons. A p value <0.05 was considered to be statistically significant.

Indirect immunofluorescence microscopy. All care and treatment of animals were in accordance with "Guidelines for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publication 85-23, revised 1985) and subjected to prior approval by the local animal protection authority. Neonatal rat cardiomyocytes were prepared as described previously (19). Eighteen hours and 48 h after the transfection, cardiomyocytes were washed with phosphate-buffered saline, fixed for 15 min in 100% ethanol at −20°C. Transfected cells were incubated in blocking solution, and stained by primary rabbit anti-myc polyclonal Ab (1:100, Santa Cruz Biotechnology) and mouse anti–α-actinin monoclonal Ab (1:800, Sigma-Aldrich, St. Louis, Missouri), followed by secondary sheep anti-rabbit IgG FITC-conjugated Ab (1:500, Chemicon, Boronia, Victoria, Australia) and Alexa fluor 568 goat anti-mouse IgG (1:500, Molecular Probes, Fugeue, Oregon). All cells were mounted on cover-glass using Mowiol 4-88 Reagent (Calbiochem, Darmstadt, Germany) with 4′6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich), and images from at least 200 transfected cells were analyzed with an LSM510 laser-scanning microscope (Carl Zeiss Microscopy, Jena, Germany).

Results

Identification of *ANKRD1* (CARP) and *TTN* mutations in HCM. Eleven distinct sequence variations in *ANKRD1* were identified among the 384 patients with HCM (Fig. 1A). Four intronic variants, 2 nonsynonymous substitutions, and 1 synonymous variation were polymorphisms, because they were also found in the controls. A nonsense mutation (c.423C>T in exon 2 yielding Gln59ter) was found in 2 patients with familial HCM and was absent in the controls, but was not cosegregated with the disease in both families, suggesting that they were not associated with HCM. In contrast, 3 missense mutations, Pro52Ala (c.402C>G in exon 2), Thr123Met (c.616C>T in exon 4), and Ile280Val (c.1086A>G in exon 8), identified in 3 unrelated HCM patients, were not found in the controls.

Sequence variations in *TTN* at the N2A domain containing binding region to CARP and p94/calpain were searched for in the patients, and 8 variations were identified (Fig. 1B). An intronic variation and 3 synonymous variations were polymorphisms observed in the controls. Two nonsynonymous variations, Ile8474Thr (c.25645T>C in exon 99) and Asp8672Val (c.26239A>T in exon 102), were not associated with HCM, because Ile8474Thr was found in the controls and Asp8672Val did not cosegregate with the disease in a multiplex family. On the other hand, 2 missense mutations, Arg8500His (c.25723G>A in exon 99) and Arg8604Gln (c.26035G>A in exon 100), identified in familial HCM patients, were not found in the controls.

Clinical phenotypes. Clinical findings of the patients carrying the *ANKRD1* or *TTN* mutations are summarized in Table 1. All patients manifested with HCM except CM1288 II-2, who had mild cardiac hypertrophy. Her father had died suddenly of unknown etiology at the age of 30 years. Two unaffected brothers of the patient did not harbor the mutation (Fig. 1C). The proband patient with the *TTN* Arg8606Gln mutation (CM1480) (Table 1) showed asymmetric septum hypertrophy. A family study revealed that his father had unexplained sudden cardiac death. His son (CM1481) (Table 1) was affected and carried the same mutation (Fig. 1D).

Altered interaction between titin/connectin and CARP caused by the TTN or CARP mutations. To investigate the functional alterations caused by the CARP mutations in the binding to titin/connectin N2A domain, WT-, Pro52Ala-, Thr123Met-, or Ile280Val-CARP construct was cotransfected with the WT TTN-N2A construct into COS-7 cells. Western blot analyses of immunoprecipitates from the transfected cells demonstrated that HCM-associated CARP mutations signif-

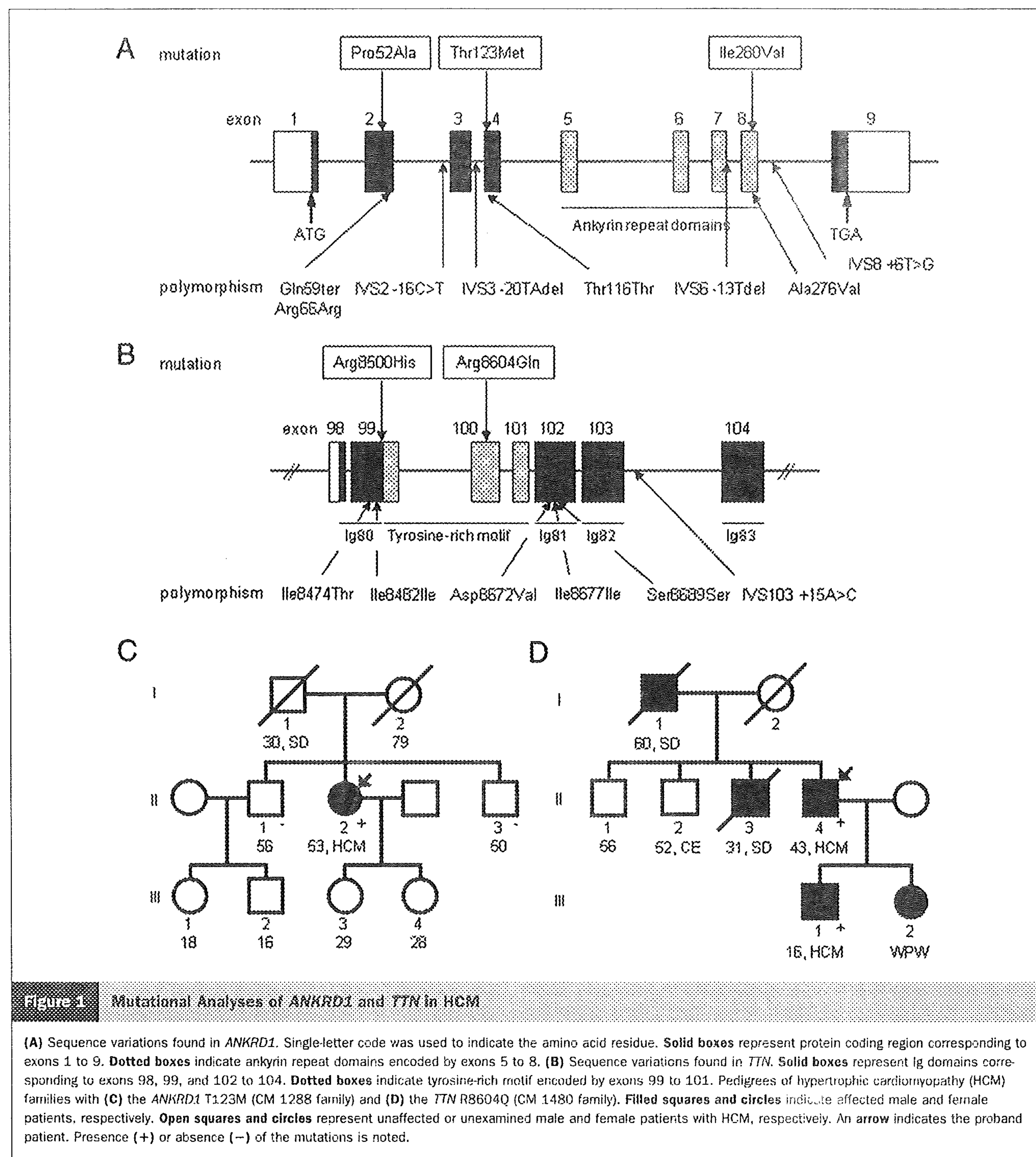

Figure 1 Mutational Analyses of *ANKRD1* and *TTN* in HCM

(A) Sequence variations found in *ANKRD1*. Single-letter code was used to indicate the amino acid residue. **Solid boxes** represent protein coding region corresponding to exons 1 to 9. **Dotted boxes** indicate ankyrin repeat domains encoded by exons 5 to 8. **(B)** Sequence variations found in *TTN*. **Solid boxes** represent Ig domains corresponding to exons 98, 99, and 102 to 104. **Dotted boxes** indicate tyrosine-rich motif encoded by exons 99 to 101. Pedigrees of hypertrophic cardiomyopathy (HCM) families with **(C)** the *ANKRD1* T123M (CM 1288 family) and **(D)** the *TTN* R8604Q (CM 1480 family). **Filled squares and circles** indicate affected male and female patients, respectively. **Open squares and circles** represent unaffected or unexamined male and female patients with HCM, respectively. An **arrow** indicates the proband patient. Presence (+) or absence (−) of the mutations is noted.

icantly increased binding to TTN-N2A (2.22 ± 0.76 arbitrary units [AU], $p < 0.05$; 1.98 ± 0.52 AU, $p < 0.01$; 2.16 ± 0.64 AU, $p < 0.05$, respectively) (Figs. 2A and 2B). Reciprocally, the effect of titin/connectin mutations in binding to CARP was assessed. The TTN-N2A constructs, WT-, HCM-associated mutants (Arg8500His- and Arg8604Gln-TTN), or non–disease-related variant (Ile8474Thr) TTN-N2A were cotransfected with WT CARP. Western blot analyses showed that Arg8500His and Arg8604Gln significantly increased the binding to CARP (2.78 ± 0.40 AU or 3.16 ± 0.40 AU, respectively, $p < 0.001$ in each case) (Figs. 2A and 2B), whereas the non–disease-related variant (Ile8474Thr) did not alter the binding (1.18 ± 0.11 AU), despite equal expression of proteins.

Altered interaction between myopalladin and CARP caused by the CARP mutations. Because CARP bound also to myopalladin, we investigated the effects of CARP mutations in binding to myopalladin. The WT or mutant

Table 1 Clinical Characteristics of Persons Carrying *ANKRD1* or *TTN* Mutations

ID	Mutation	Age (yrs), Sex	Age at Onset (yrs)	Clinical Diagnosis	Age at CE (yrs)	Family History of CM	NYHA Functional Class	LVEDD (mm)	LVESD (mm)	IVS (mm)	PW (mm)	%FS	%EF	Other Remarks
Mayo I	*ANKRD1* P52A	44, male	30	HCM	32	No	II	—	—	22	—	—	70	LVH on ECG; provocable gradient 100 mm Hg, but asymptomatic
Mayo II	*ANKRD1* P52A	65, male	41	HCM	54	No	III	38	16	14	14	—	84	Midventricular-apical hypertrophy with midventricular wall thickness up to 35 mm
CM1288 II-2	*ANKRD1* T123M	62, female	40	HCM	40	No	I	41	22	13	13	46	78	Lateral LVH (15 mm), LAD 37 mm. ECG: abnormal Q-wave in II, III, aVf, V_4–V_6
Mayo III	*ANKRD1* I280V	82, female	61	HCM	73	No	III	52	30	20	14	—	70	Septal ablation (relieved obstruction 73 mm Hg >22 mm Hg)
CM89	*TTN* R8500H	59, male	53	HCM	59	No	I	42	25	28	8	40	79	LVH (ASH)
CM1480 II-4	*TTN* R8604Q	52, male	43	HCM	43	Yes	I	41	24	18	10	41	80	LVH (ASH), atrial fibrillation ECG: inverted T-wave in V_4–V_6
CM1481 III-1	*TTN* R8604Q	25, male	16	HCM	16	Yes	I	45	27	22	9	40	66	LVH (ASH), ECG: inverted T-wave in V_1–V_3

CE = clinical examination; ECG = electrocardiogram; EF = ejection fraction; FS = fractional shortening; HCM = hypertrophic cardiomyopathy; ID = identification; IVS = intraventricular septal thickness; LAD = left anterior descending artery; LVEDD = left ventricular end-diastolic diameter; LVESD = left ventricular end-systolic diameter; LVH = left ventricular hypertrophy; NYHA = New York Heart Association; PW = posterior wall thickness.

CARP construct was cotransfected with a MYPN construct. Western blot analysis revealed that binding of mutant CARPs, Pro52Ala, Thr123Met, or Ile280Val to myopalladin was significantly increased (3.60 ± 0.67 AU, $p < 0.001$; 1.87 ± 0.47 AU, $p < 0.01$; or 2.48 ± 0.45 AU, $p < 0.001$, respectively) (Figs. 2C and 2D).

Altered localization of CARP caused by the mutations. To further investigate the functional consequence of the CARP mutations, we examined cellular distribution of the mutant CARP proteins expressed in neonatal rat primary cardiomyocytes. Cells were transfected with myc-tagged WT or mutant CARP constructs, coimmunostained for myc (a marker for CARP) and α-actinin (a marker for Z-disc). The WT and mutant myc-CARP proteins were expressed at a similar level in the transfected cells as assessed by Western-blot analyses, suggesting that the mutations did not affect the expression level and stability of CARP proteins (data not shown). Control cells expressing myc-tag alone showed negative staining for myc-tag with striated staining pattern of sarcomeric α-actinin at the Z-disc (data not shown). In premature cardiomyocytes containing Z-bodies (Z-disc precursors), myc-tagged WT CARP was mainly targeted to nucleus and colocalization of CARP with α-actinin, which formed patchy dense bodies in the cytoplasm, was observed (Figs. 3A to 3C). No apparent changes in localization of mutant CARP proteins were observed in the nascent and immature cardiomyocytes (Figs. 3D to 3L).

In the mature cardiomyocytes where Z-discs were well organized, myc-tagged WT CARP was assembled in the striated pattern at the Z-I bands and colocalized with α-actinin (Figs. 4A to 4C). It was found that most (≈90%) of mature cardiomyocytes did not contain nuclear CARP (Figs. 4A to 4C). On the other hand, higher intensity of CARP-related fluorescence at the Z-I bands and diffused localization in cytoplasm was observed in most (≈80%) of the mature cardiomyocytes expressing myc-tagged mutant CARPs, albeit that the Z-disc assembly was not impaired (Figs. 4D to 4L). Quite interestingly, myc-tagged mutant CARP proteins displayed localization within the nuclear and/or at nuclear membrane in ≈60% of mature cardiomyocytes (Figs. 4D to 4L).

Discussion

CARP encoded by *ANKRD1* is a nuclear transcription cofactor expressing in the embryonic hearts. Its expression progressively decreases in adult hearts (4,5) and reappears in the hypertrophied or failing adult heart (6,22), suggesting that CARP may be involved in the regulation of muscle gene expression. CARP also localizes in cardiac sarcomere although the roles of "sarcomeric CARP" are not fully elucidated. Several reports have demonstrated that CARP binds titin/connectin (10), myopalladin (9), and desmin (21) at the Z/I-region of sarcomere. In this study, we found that the HCM-associated *ANKRD1* mutations increased the binding of CARP to titin/connectin and myopalladin, and HCM-associated *TTN* mutations in its reciprocal

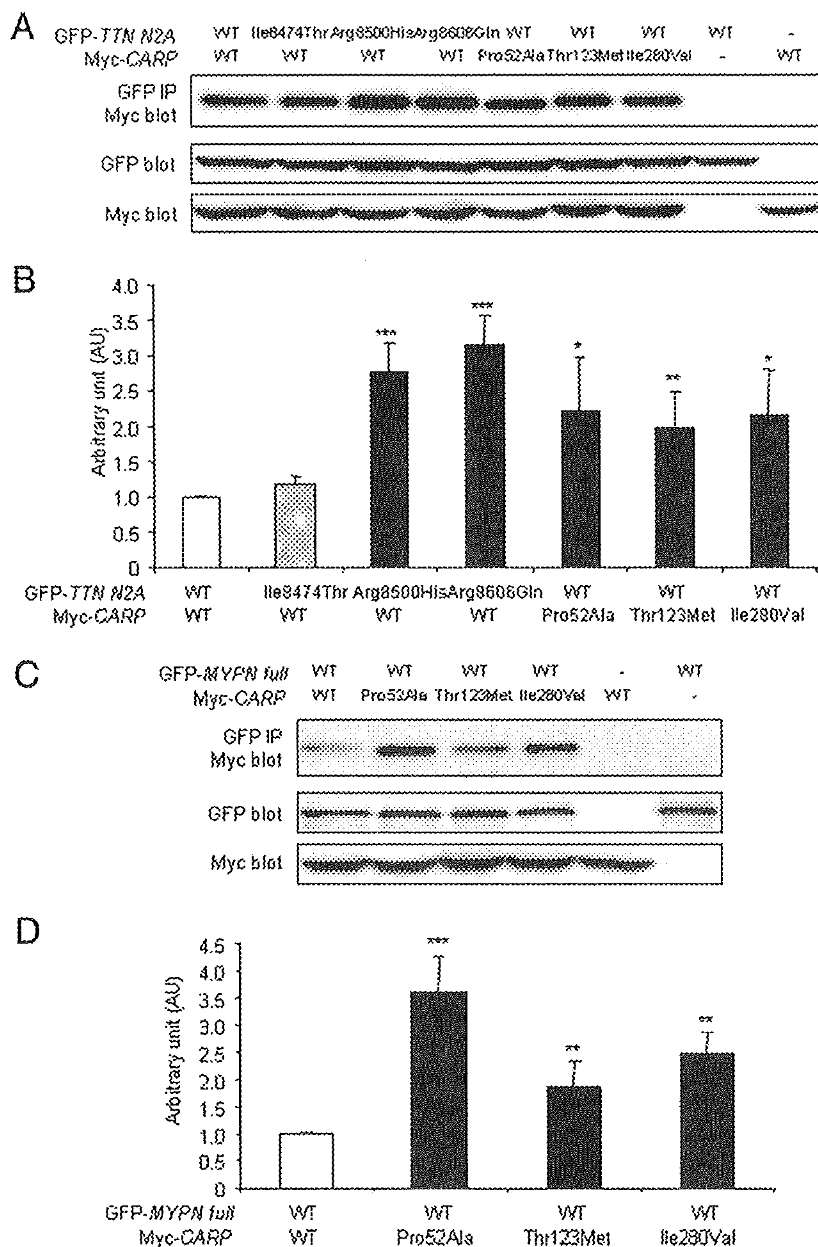

Figure 2 Binding of CARP to TTN and MYPN

Binding of cardiac ankyrin repeat protein (CARP) to titin/connectin (TTN) or myopalladin (MYPN) was analyzed by coimmunoprecipitation (co-IP) assays. **(A)** Myc-tagged CARPs coprecipitated with GFP-tagged TTN-N2A domain were shown **(top panel)**. Expressions of GFP-tagged TTN- N2A **(middle panel)** and myc-tagged CARP **(lower panel)** were confirmed by immunoblotting of whole cell supernatants. Binding pairs were wild-type (WT) CARP in combination with WT, I8474T, R8500H, or R8604Q mutant TTN-N2A , or WT TTN-N2A with WT, P52A, T123M, or I280V mutant CARP. **Dashes** indicate no GFP- or myc-tagged proteins (transfected only with pEGFP-C1 or pCMV-Tag3 vectors, respectively). **(B)** Densitometric data obtained in the co-IP assay. Data for WT CARP with WT TTN-N2A were arbitrarily defined as 1.00 arbitrary unit (AU). Data are represented as means ± SEM (n = 6 for each case). *p < 0.05 versus WT; **p < 0.01 versus WT; ***p < 0.001 versus WT. **(C)** Myc-tagged CARP coprecipitated with GFP-tagged full-length MYPN was detected by immunoblotting using anti-myc antibody **(top panel)**. Expressed amounts of GFP-tagged MYPN **(middle panel)** and myc-tagged CARP **(lower panel)** were confirmed as in **(A)**. Binding pairs were full-length WT-MYPN with WT, P52A, T123M, or I280V mutant CARP. **(D)** Densitometric analysis of myc-blotting data in **(C)**. Data were arbitrarily represented as intensities, and that for WT CARP with full length or N-terminal half WT MYPN were defined as 1.00 AU. Data are expressed as means ± SEM (n = 9 for each case). **p < 0.01 versus WT; ***p < 0.001 versus WT.

CARP N2A-binding domain increased the binding of titin/connectin to CARP. These observations in association with HCM suggested that the assembly or binding of sarcomeric CARP with titin/connectin and/or myopalladin would be required for the maintenance of cardiac function.

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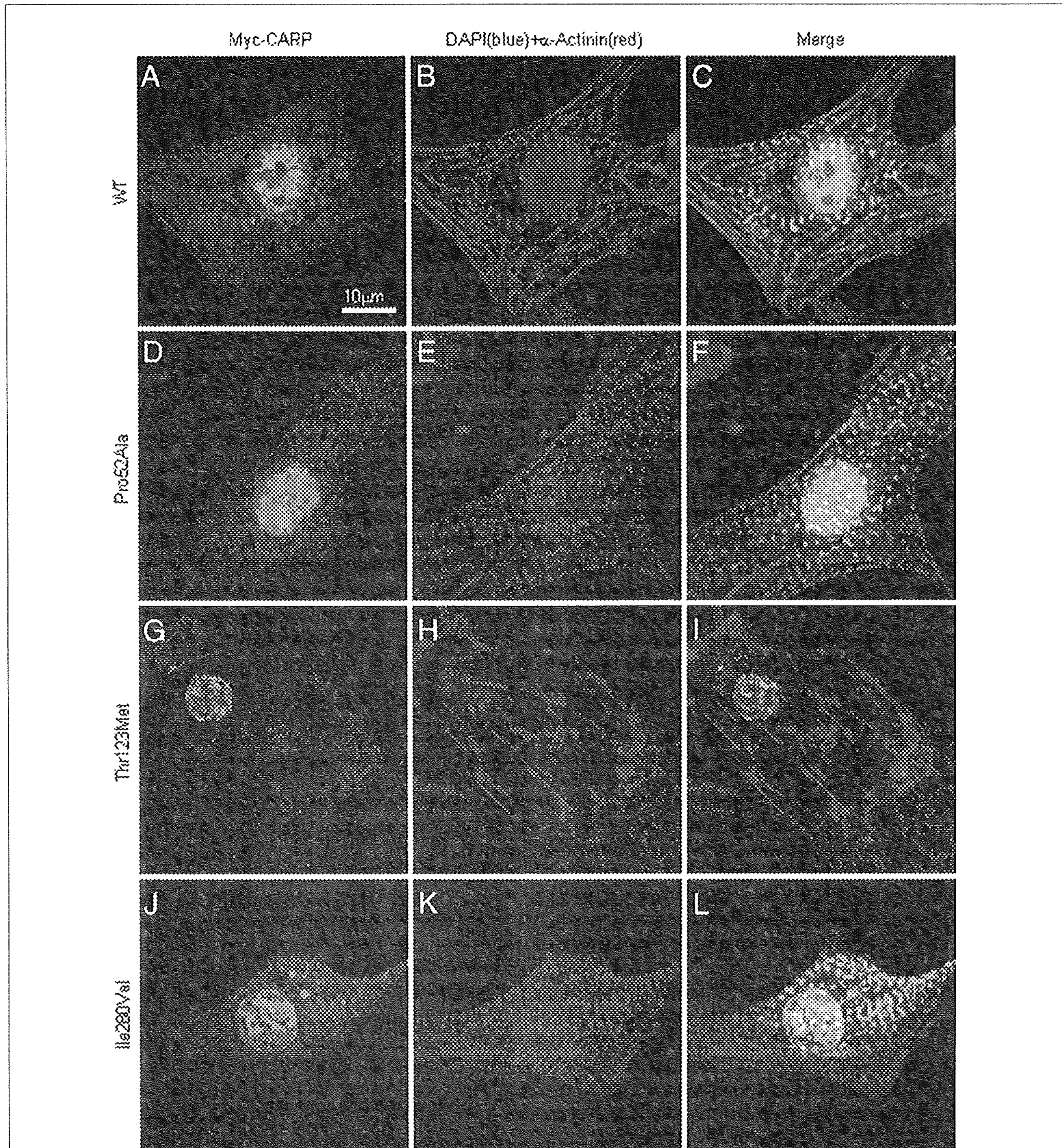

Figure 3 Distribution Myc-Tagged CARP in Immature Rat Cardiomyocytes

Neonatal rat cardiomyocytes transfected with myc-tagged wild-type (WT) **(A to C)** or mutant P52A **(D to F)**, T123M **(G to I)**, or I280V **(J to L)** cardiac ankyrin repeat protein (CARP) constructs were fixed 18 h after the transfection, and stained with 4′6-diamidino-2-phenylindole (DAPI) and anti–α-actinin antibody followed by secondary antibody **(B, E, H, K)**. Merged images **(C, F, I, L)** are shown. In the immature cardiomyocytes showing nascent myofibrils with Z bodies (Z-disc precursors), myc-tagged CARPs were preferentially localized to the nucleus, and mutant CARP showed relatively low expression in the cytoplasm. Scale bar = 10 μm.

In the nascent myofibrils, myc-tagged CARP proteins were detected within the nucleus irrespective of mutations. Because CARP is an early differentiation marker during heart development, recruitment of CARP into nuclei may be important in the embryonic gene expression. Interestingly, abnormal intranuclear accumulation of myc-tagged

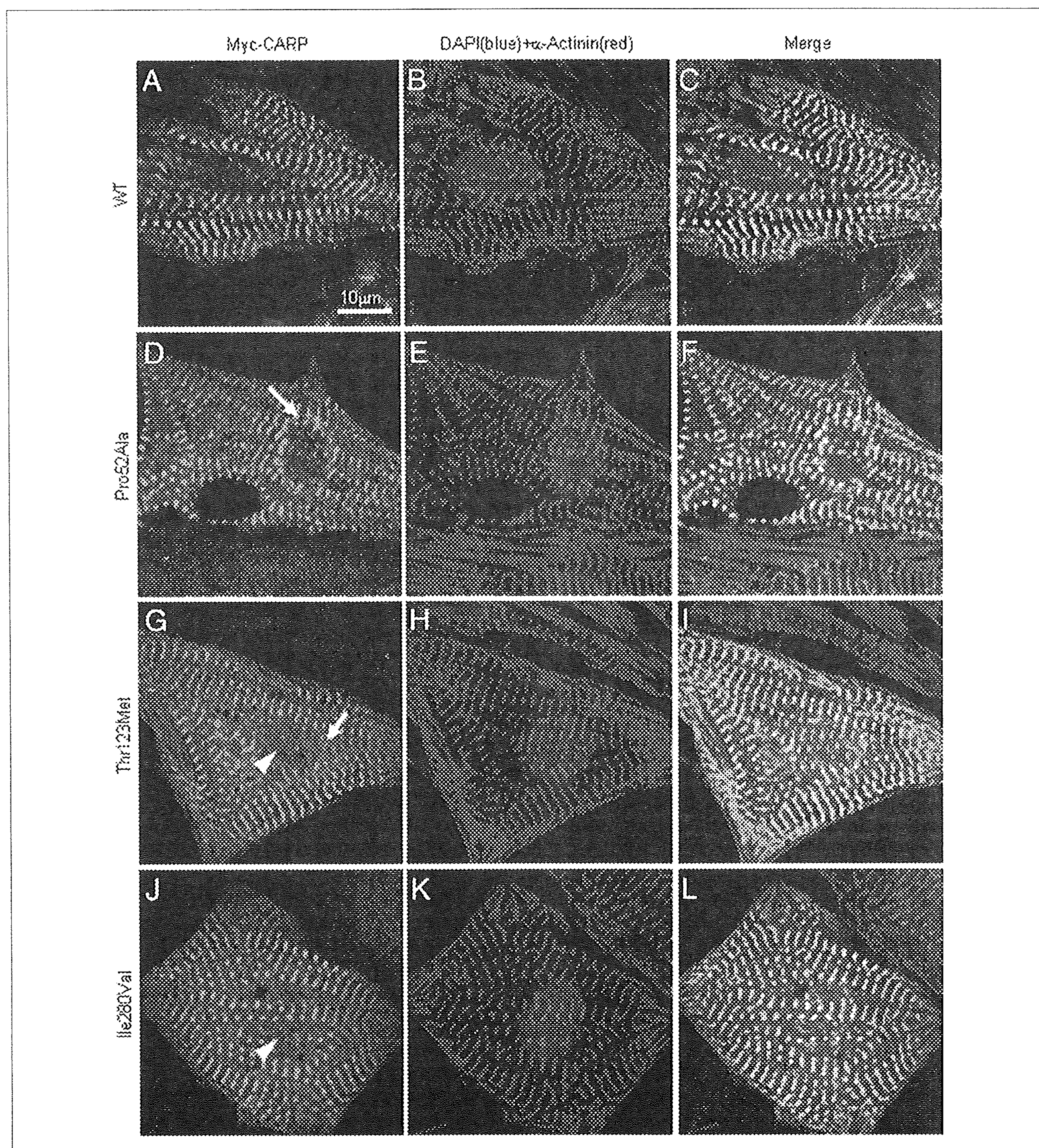

Figure 4 **Distribution of Myc-Tagged CARP in Mature Rat Cardiomyocytes**

Neonatal rat cardiomyocytes transfected with myc-tagged wild-type (WT) **(A to C)** or mutant P52A **(D to F)**, T123M **(G to I)**, or I280V **(J to L)** cardiac ankyrin repeat protein (CARP) constructs were fixed 48 h after the transfection, and stained with 4'6-diamidino-2-phenylindole (DAPI) and anti–α-actinin antibody followed by secondary antibody **(B, E, H, K)**. Merged images **(C, F, I, L)** are shown. In the mature cardiomyocytes showing myofibrils with Z-discs, normal localization of myc-tagged WT CARP at the Z-discs was observed **(A to C)**. In contrast, myc-tagged mutant CARP proteins showed intense localization at the I-discs (colocalization with α-actinin) and diffused localization in the cytoplasm **(D to F, G to I, J to L)**. In addition, myc-tagged mutant CARPs expressed at high levels around the nuclear membrane **(white arrows)** and/or in the nucleus **(white arrowheads)**. Scale bar = 10 μm.

mutant CARP proteins was observed in mature myofibrils. It is well known that the embryonic and fetal gene program of cardiac cytoskeletal proteins is initiated during the cardiac remodeling (22,23). Hence, one could hypothesize that nuclear CARP may cause embryonic/fetal gene expression in mature myofibrils, and this abnormal gene expression is a possible mechanism leading to the pathogenesis of HCM. It was reported that CARP negatively regulated expression of cardiac genes including *MYL2, TNNC1*, and *ANP* (4,5). Conversely, another report suggested that different expression level of CARP did not correlate with the altered expression of cardiac genes such as *MYL2, MYH7, ACTC, CACTN, TPM1, ACTN2*, and *DES* (24). Thus, the role of CARP as a regulator of cardiac gene expression remains to be resolved. During the preparation of this paper, Cinquetti et al. (25) reported other CARP mutations, rearrangements, or Thr116Met, in association with the cyanotic congenital heart anomaly known as total anomalous pulmonary venous return. These mutations were demonstrated to be associated with increased expression or stability of CARP. It is not clear whether the mutations associated with HCM altered expression or stability of CARP, although our data suggested that HCM-associated CARP mutations did not alter the stability. The molecular mechanisms underlying the CARP-related pathogenesis should be different between total anomalous pulmonary venous return and HCM.

Conclusions

We identified 3 missense CARP mutations in <1% of unrelated patients with HCM, which not only increased the binding of sarcomeric CARP to I-band components but also resulted in the mislocalization of CARP to the nucleus. Although the molecular mechanisms of HCM due to the CARP mutations remain to be elucidated, our findings imply that HCM may be associated with the abnormal recruitment of CARP in cardiomyocytes, leading to pathological hypertrophy.

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Key Words: hypertrophic cardiomyopathy ▪ mutation ▪ Z-disc ▪ cardiac ankyrin repeat protein ▪ titin/connectin.

Zac1 Is an Essential Transcription Factor for Cardiac Morphogenesis

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Rationale: The transcriptional networks guiding heart development remain poorly understood, despite the identification of several essential cardiac transcription factors.

Objective: To isolate novel cardiac transcription factors, we performed gene chip analysis and found that *Zac1*, a zinc finger–type transcription factor, was strongly expressed in the developing heart. This study was designed to investigate the molecular and functional role of Zac1 as a cardiac transcription factor.

Methods and Results: Zac1 was strongly expressed in the heart from cardiac crescent stages and in the looping heart showed a chamber-restricted pattern. Zac1 stimulated luciferase reporter constructs driven by *ANF*, *BNP*, or *αMHC* promoters. Strong functional synergy was seen between Zac1 and Nkx2-5 on the *ANF* promoter, which carries adjacent Zac1 and Nkx2-5 DNA-binding sites. Zac1 directly associated with the *ANF* promoter in vitro and in vivo, and Zac1 and Nkx2-5 physically associated through zinc fingers 5 and 6 in Zac1, and the homeodomain in Nkx2-5. *Zac1* is a maternally imprinted gene and is the first such gene found to be involved in heart development. Homozygous and paternally derived heterozygous mice carrying an interruption in the *Zac1* locus showed decreased levels of chamber and myofilament genes, increased apoptotic cells, partially penetrant lethality and morphological defects including atrial and ventricular septal defects, and thin ventricular walls.

Conclusions: Zac1 plays an essential role in the cardiac gene regulatory network. Our data provide a potential mechanistic link between Zac1 in cardiogenesis and congenital heart disease manifestations associated with genetic or epigenetic defects in an imprinted gene network. (*Circ Res.* 2010;106:00-00.)

Key Words: heart development ■ transcription factor ■ Zac1/Plagl1

The importance of transcription factors in development and cell differentiation has recently been underscored by the discovery that the introduction of 4 transcription factors into fibroblasts produces pluripotent stem cells.[1] Heart development is known to be regulated by a number of highly conserved transcription factors, although the mechanisms and logic of that regulation remain unclear. GATA4, myocyte enhancer factor (MEF)2C, serum response factor (SRF), Tbx5, and Nkx2-5 are expressed in the heart and play essential roles in its formation.[2–5] Furthermore, many of these transcription factors interact and act cooperatively and synergistically to direct cardiac developmental programs.[6] Despite their importance in cardiac development, however, none of the factors shows heart-specific expression, and it seems unlikely that a single factor determines cardiac cell fate.

We reported previously that transient treatment of differentiating embryonic stem cells with bone morphogenetic protein antagonists, efficiently induces cardiomyocyte differentiation.[7] Exploiting this system, we subsequently screened embryonic stem cell–derived cardiomyocytes for novel cardiac transcriptional factors using a gene chip analysis and found abundant cardiac expression of the zinc finger protein gene, *Zac1*. Zac1 was initially identified as an antiproliferative protein,[8] with subsequent studies implicating Zac1 in tumor suppression and organ development.[9,10] Furthermore, *Zac1* expression is regulated epigenetically during normal development. Imprinted genes are expressed from one allele according to their parent of origin, and this phenomenon is essential for mammalian embryogenesis. *Zac1* is a paternally expressed, imprinted gene.[10] Although imprinted genes are

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Non-standard Abbreviations and Acronyms	
ANF	atrial natriuretic factor
BNP	brain natriuretic peptide
ChIP	chromatin immunoprecipitation
E	embryonic day
EB	embryoid body
ES	embryonic stem
GST	glutathione *S*-transferase
LOT1	lost on transformation 1
MEF2C	myocyte enhancer factor 2C
MHC	myosin heavy chain
MLC	myosin light chain
P	postnatal day
PLAG	pleomorphic adenoma gene
SRF	serum response factor
ZRE	Zac1-response element

important for mammalian development, their roles in heart organogenesis are unknown.

In the present study, we investigated how Zac1 is involved in heart development. We show that Zac1 is an essential cardiac transcriptional factor, being highly expressed in mouse hearts from embryonic day (E)8.5 to adulthood in a chamber-restricted pattern. Zac1 was found to bind directly to the atrial natriuretic factor gene (*ANF/Nppa*) promoter in vitro and in vivo, and to possess potent transcriptional activity. Nkx2-5 and Zac1 bound to adjacent sites within the *ANF* promoter, physically interacted, and synergistically activated cardiac gene expression. The *Zac1* promoter was activated by Nkx2-5 in vitro, whereas Nkx2-5–null mice showed decreased *Zac1* expression. Genetic inactivation of *Zac1* in mice (paternal-mutated heterozygote-descendent mice) induced defective embryonic heart development and reduced expression of chamber and myofilament genes. Our results indicate that Zac1 is an essential transcription factor for cardiac morphogenesis. Moreover, this is the first report that an imprinting gene mutation causes abnormal development of the heart.

Methods

Experimental procedures for in situ hybridization, animal study, immunostaining, Western blotting, plasmids, cell culture, electrophoretic mobility-shift assay, chromatin immunoprecipitation (ChIP) assay, glutathione *S*-transferase (GST) pull-down assay, RT-PCR analysis, and statistical analyses are provided in the expanded Methods section in the Online Data Supplement, available at http://circres.ahajournals.org.

Results

Zac1 Expression in the Embryonic Heart

We used gene chip analysis to search for novel cardiac transcription factors. Initially, we screened for genes upregulated in Noggin-treated differentiating embryonic stem (ES) cells that contained conserved transcription factor motifs and then confirmed the expression in the heart by whole-mount in situ hybridization. We also analyzed the transcriptional potency of each identified factor in vitro using the *ANF* promoter as target gene. The *ANF* promoter is a marker of the developing chamber myocardium, and is responsive to various signals, including those controlling cardiac growth, remodeling and pathological overload.[11] We screened for upregulated genes by comparing cardiomyocyte-rich differentiating ES cell–derived embryoid bodies (EBs) and nontreated EBs at day 6 of culture. Three hundred fifty-three genes were upregulated (>4-fold) in Noggin-treated EBs. Among them, 13 genes encoded a recognizable conserved transcription factor motif and had not yet been analyzed in the context of heart development. These were analyzed for cardiac expression, and 6 genes were analyzed for *ANF* promoter transactivation.

In situ hybridization of staged mouse embryos showed weak expression of *Zac1* in the cardiac crescent and other embryonic sites at E7.75 and stronger heart expression at E8.5, E9.0 and E9.5 (Figure 1A). Expression at E8.5 was enriched in chamber myocardium. Immunostaining revealed Zac1 protein expression in the heart at E8.5, E9.5, and E10.5, with a heart expression pattern similar to that of α-Actinin, but included more extensive expression in mesenchyme dorsal to the heart tube, corresponding to the second heart field (SHF) (Figure 1B). Zac1 protein expression was also enriched in chamber myocardium at E9.5 and E10.5, being lower in nonchamber myocardium of the atrioventricular canal (Figure 1B). In COS7 cells, overexpressed Zac1 was localized to the nucleus, as assessed by immunohistochemistry with an anti-Zac1 antibody (Figure 1C). Fractionation of COS7 cells transfected with increasing amounts of expression vector followed by SDS-PAGE and immunoblotting confirmed the specific accumulation of Zac1 in the nuclear (Figure 1D).

Zac1 Is a Potent Activator of *Nppa* Gene Expression

We used the gene promoters from *ANF*, brain natriuretic peptide (*BNP/Nppb*), and α-myosin heavy chain (*α-MHC/Myh6*) to evaluate the transactivational potency of Zac1 in COS7 cells in comparison to that of cardiac transcription factors MEF2C, GATA4, and SRF. Zac1 activated these promoters in a manner similar to the other factors (Figure 2A), in the case of *ANF* >250-fold. We also performed the luciferase assay using neonatal rat ventricular cardiomyocytes (Online Figure I). In these cells, Zac1 increased *ANF* and *BNP* promoter activities, as did the other transcription factors; however, relative transactivation was not as strong as in COS7 cells. The α-MHC promoter did not significantly respond to any of the factors, likely because cardiac transcription factors including Zac1 are strongly expressed in these cardiomyocytes and the effect of additional expression is weak or insignificant, depending on the promoter. Although Zac1 has been identified as a transcription factor and its binding sequence reported,[12] homologous sequences were not identified in the *ANF* promoter. To show that the Zac1-dependent *ANF* promoter activation was regulated in a DNA-binding–dependent manner, we constructed a series of *ANF* promoter mutants and mapped the *cis*-regulatory sequence that mediates the response to Zac1 to the region from

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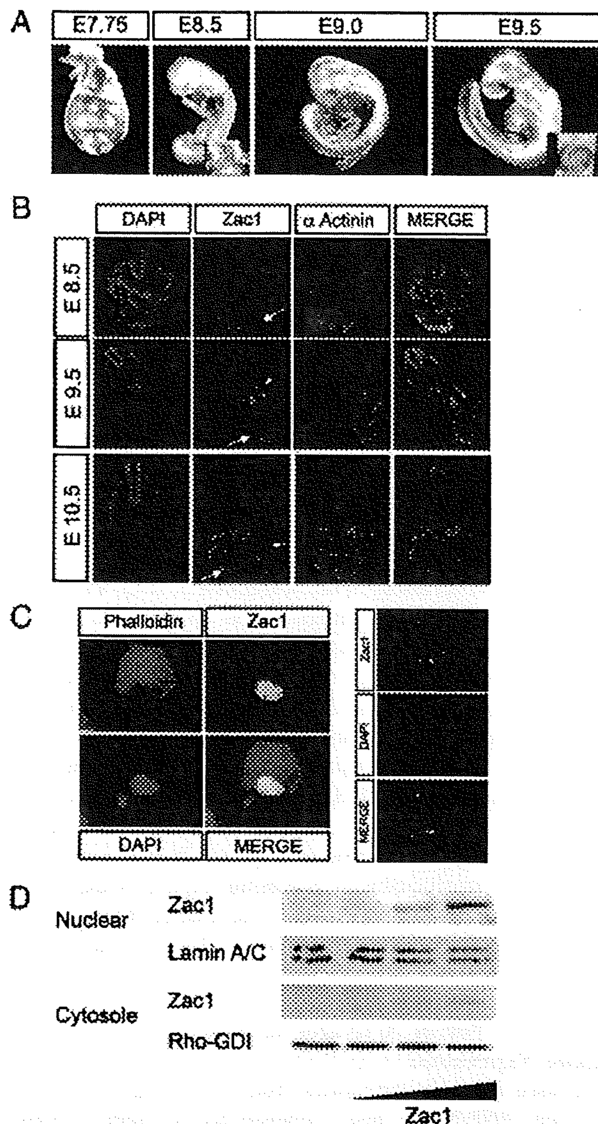

Figure 1. Expression of Zac1 in the murine embryonic heart. A, *Zac1* transcripts were detected in mouse embryos by whole-mount in situ hybridization. *Zac1* expression is weakly expressed in the cardiac crescent at E7.75 but detected throughout the heart at E8.5, E9.0, and E9.5. Frontal view of heart is shown in the **inset. B,** Immunostaining for the Zac1 protein in E8.5, E9.5, and E10.5 mouse embryos (transverse section). Zac1 protein is expressed in the heart enriched in chamber myocardium, whereas α-actinin is expressed throughout the heart and in the somites. Expression at E8.5 was enriched in chamber myocardium **(arrow)**. Zac1 expression included more extensive expression in mesenchyme dorsal to the heart tube, corresponding to the SHF **(arrowhead)**. Zac1 protein was also enriched in chamber myocardium **(arrow)** at E9.5 and E10.5, being lower in nonchamber myocardium of the atrioventricular canal **(short arrow)**. **C,** Immunostaining of Zac1 protein in transfected COS7 cells, showing expression in the nucleus. **D,** Subcellular location of Zac1 protein in transfected COS cells, as detected by Western blotting. The nuclear accumulation of Zac1 is proportional to the DNA dosage used for transfection. Lamin A/C is a nuclear protein control, and Rho-GDI is cytosolic protein control.

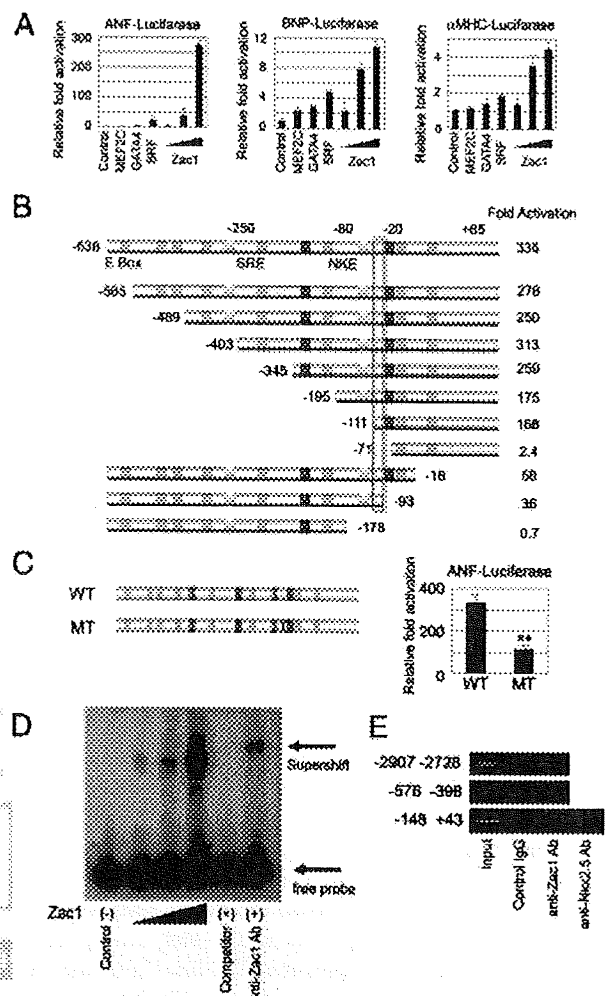

Figure 2. Zac1-transactivated ANF, BNP, and α-MHC genes. A, COS7 cells were cotransfected with a Zac1 expression plasmid and *ANF*, *BNP*, or *α-MHC*-luciferase reporter constructs. Values are expressed as the fold increase in luciferase activity compared to the empty expression plasmid (Control). **B,** COS7 cells were transfected with the Zac1 expression plasmid and the indicated *ANF* luciferase reporter constructs. Values are expressed as the fold increase in luciferase activity compared to the empty expression plasmid (Control). **Colored rectangles** indicate conserved transcription factor–binding site; **green box,** E box site; **blue box,** NKE; **yellow box,** SRF-binding element. **C,** COS7 cells were transfected with the Zac1 expression plasmid and the indicated *ANF* luciferase reporter constructs. The Zac1 response element is shown in blue (wild-type [WT]), and this element is mutated in the mutant (MT) promoter. **D,** Electrophoretic mobility-shift assay reveals the binding of Zac1 to radioactively labeled ZRE. Cold competitor interferes with the binding of Zac1 to the labeled ZRE. An antibody specific for Zac1 (anti-Zac1 Ab) supershifts the Zac1/ZRE complex. **E,** ChIP analysis reveals the binding of Zac1 and Nkx2-5 to the *ANF* promoter including the region −148 to +43 in vivo. PCR-amplified bands are apparent for the input DNA and anti-Zac1 antibody–precipitated DNA.

−111 to −93 (Figure 2B). The specific DNA sequence responsible for transactivation by Zac1 was further delineated by point mutagenesis. A Zac1-response element (ZRE) candidate sequence (GCCGCCG) within the *ANF* promoter was at least in part responsible for Zac1-dependent transactivation because mutation of this sequence to GTATATG attenuated responsiveness to Zac1 (Figure 2C). An electrophoretic mobility-shift assay was performed to determine whether Zac1 bound directly to this GCCGCCG sequence. The total amount of Zac1/DNA complex increased in proportion to the

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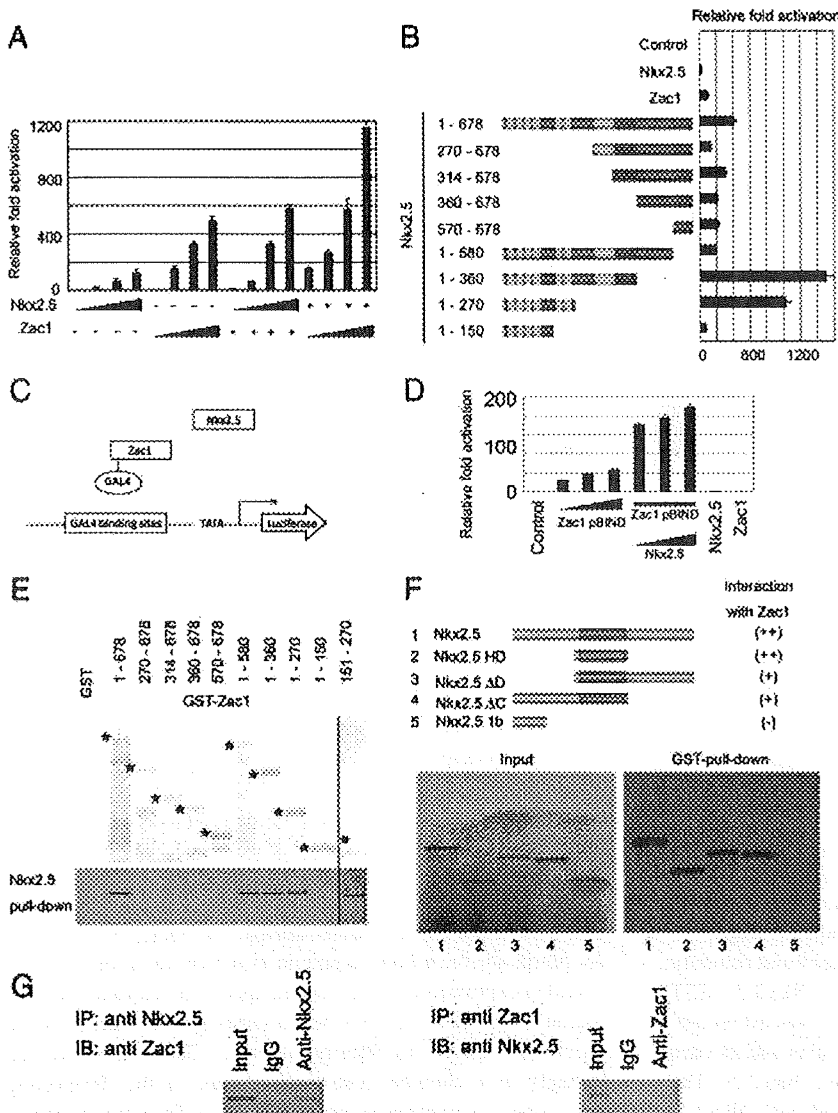

Figure 3. Zac1 and Nkx2-5 physically interact and synergistically activate *ANF* transcription. A, COS7 cells were transfected with ANF luciferase and expression vectors, encoding Nkx2-5 and Zac1. Both constructs synergistically activate *ANF* transcription (n=3). Nkx2-5 (10 to 300 ng); Zac1 (10 to 300 ng). **B,** Deletion mutants of *Zac1* were tested for their abilities to synergize with Nkx2-5 to activate *ANF* luciferase in COS7 cells. Values are expressed as fold increase in luciferase expression compared to the control. **Colored rectangles** indicate conserved protein motifs; **green box,** zinc finger motif; **blue and red boxes,** amelogenin motif; **brown box,** trypan PARP-like motif. **C,** Zac1 was fused with GAL4. A luciferase gene controlled by multiple GAL4-binding sites was used. Nkx2-5 cannot directly bind to GAL4 sites. **D,** Zac1-GAL4 increased the transactivation by DNA binding and Nkx2-5 increased this transactivation without direct DNA binding in presence of Zac1-GAL4. Wild-type Nkx2-5 and wild-type Zac1 alone did not show transactivation. **E,** GST-Zac1 deletion mutants were incubated with [^{35}S]methionine-labeled Nkx2-5 translated in vitro. The input Zac1 deletion mutant proteins are shown at **top.** Nkx2-5 proteins that bind to GST-Zac1 deletion mutants are shown at **bottom. F,** GST-Zac1 was incubated with [^{35}S]methionine-labeled Nkx2-5 deletion mutants translated in vitro. The input Nkx2-5 deletion mutant proteins are shown in the left panel. Nkx2-5 proteins that bind to GST-Zac1 deletion mutants are shown in the right panel. **G,** Coimmunoprecipitated proteins for Nkx2-5 or Zac1 were analyzed by immunoblotting using Zac1 or Nkx2-5 antibody. Nkx2-5 associated with Zac1 in neonatal heart extracts.

nuclear-localized Zac1 protein in COS7 cells at increasing DNA dosage. Furthermore, this complex was extinguished by the addition of cold competitor and was supershifted by the anti-Zac1 antibody (Figure 2D). To confirm that Zac1 binds to the *ANF* promoter in vivo, we used a ChIP assay. Cross-linked chromatin obtained from neonatal rat hearts was immunoprecipitated with the anti-Zac1 antibody. The precipitated chromatin DNA was then purified, and PCR analysis for enrichment of the target sequences revealed that Zac1 bound directly to the *ANF* promoter in vivo (Figure 2E). ChIP assay also showed that Nkx2-5 bound to same promoter region which includes an Nkx2-5–binding region (NKE). Zac1 did not bind to distant promoter regions which do not include a ZRE.

Zac1 Activates *ANF* Gene Expression Synergistically With Nkx2-5

The Zac1 DNA-binding site within the *ANF* promoter is adjacent to the reported binding site for Nkx2-5.[13] Therefore, we used the *ANF* promoter to ascertain whether Zac1 acts synergistically with Nkx2-5 to activate transcription. Vectors for these transcription factors were cotransfected at different DNA dosages into COS7 cells (Figure 3A). Zac1 activated the *ANF* promoter >1100-fold in a dose-dependent manner and this required the presence of Nkx2-5. Moreover, maximum activation by Nkx2-5 (>600 fold) required Zac1. To identify the protein domain of Zac1 that is involved in this synergistic activity with Nkx2-5, we cotransfected several mutated forms of Zac1 and Nkx2-5 into COS7 cells and measured the transcriptional activity of the *ANF* promoter (Figure 3B). Deletion of the 6 zinc finger domains in Zac1 (green domains in Figure 3B) reduced its ability to stimulate transcription. Notably, carboxyl-terminal deletion mutants 1 to 360 and 1 to 270, which potentially lack C-terminal repression domains, showed strong synergistic activities with Nkx2-5 (1000- to 1400-fold), which in turn was reduced by deletion of the zinc finger 5 and 6 domains (Figure 3B). Therefore, our data implicate zinc finger domains 5 and 6 of Zac1 in the functional interaction with Nkx2-5. To clarify the requirement of DNA binding for the interaction between

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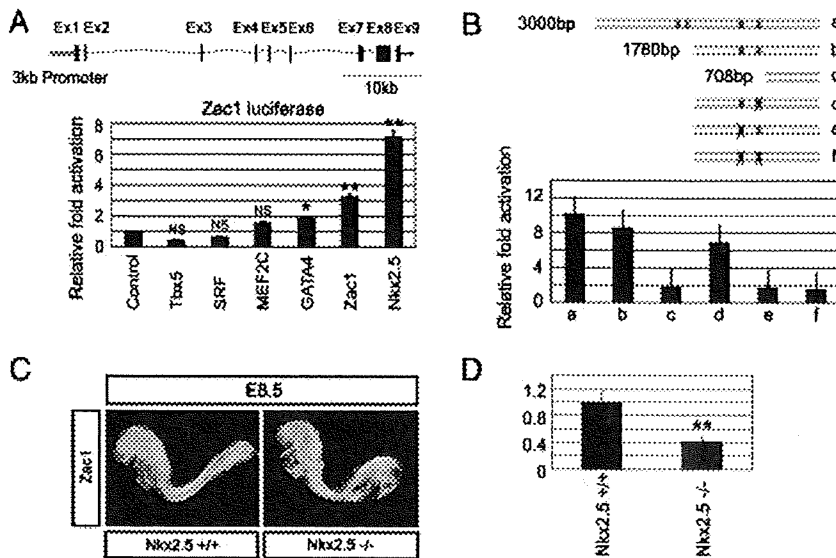

Figure 4. Nkx2-5 regulates *Zac1* gene expression. A, Structure of the mouse *Zac1* gene. The **red line** indicates the 3000-bp promoter used in this assay. COS7 cells were transfected with Zac1-luciferase and expression vectors, encoding Tbx5, SRF, MEF2C, GATA4, Zac1, and Nkx2-5. **B,** Four Nkx2-5–binding sites **(blue bar)** within the Zac1 3kb promoter/enhancer region are shown. Mutation of the third Nkx2-5–binding site **(e and f)** diminished the Nkx2-5–dependent Zac1 promoter transactivation. **C,** Detection of Zac1 transcripts by whole-mount in situ hybridization in wild-type and Nkx2-5 knockout embryos at E8.5. **D,** Quantitative RT-PCR analyses for Zac1 transcripts in wild-type and Nkx2-5$^{-/-}$ mice are shown.

Zac1 and Nkx2-5, we performed a mammalian 1-hybrid assay (Figure 3C). In this assay, Zac1, expressed as a fusion protein with the DNA-binding domain of the yeast transcription factor GAL4, was transfected with a luciferase vector under the control of multiple GAL4-binding sites (pBIND) and Nkx2-5 expression vector. Under these conditions, neither Nkx2-5 nor Zac1 could directly activate luciferase gene expression (Figure 3D). Zac1-GAL4 alone increased basal activity up to 50 fold, and Nkx2-5 increased this level of transactivation to a maximum of >200 fold (Figure 3D). These data suggest that a functional interaction between Zac1 and Nkx2-5 can occur in the absence of DNA binding.

To map the Nkx2-5–binding domain for Zac1 and to verify the physical interaction between Zac1 and Nkx2-5, GST pull-down experiments were performed using several recombinant GST-Zac1 deletion mutant fusion proteins and in vitro translated wild-type [^{35}S]methionine-labeled Nkx2-5. The wild-type Zac1-GST fusion protein interacted with Nkx2-5, as did the GST-Zac1 1 to 580, 1 to 360, 1 to 270, and 151 to 270 mutants, which encompass the zinc finger 5 and 6 domains (Figure 3E). The results indicate that these 2 zinc finger domains located within the N-terminal half of Zac1 are necessary and sufficient for association with Nkx2-5. To determine the domain of Nkx2-5 that interacts with wild-type Zac1, pull-down assays were performed with GST-conjugated full-length Zac1 and [^{35}S]methionine-labeled deletion mutants of Nkx2-5 translated in vitro. Wild-type and homeodomain-containing deletion mutants of Nkx2-5, including a homeodomain-only fragment, clearly interacted with Zac1, whereas an N-terminal fragment lacking the homeodomain did not (Figure 3F). The homeodomain of Nkx2-5 is therefore necessary and sufficient to mediate association with Zac1. These results demonstrate the importance of a protein–protein interaction between Zac1 and Nkx2-5 for gene activation in the heart. Although the Zac1 constructs amino acids 270 to 678, 314 to 278, 360 to 678, and 570 to 678 do not interact with Nkx2-5, they still show significant synergy with Nkx2-5. Because those mutants contain amino acids 570 to 678, we speculated that the 570 to 678 region of Zac1 was responsible for Zac1 dominant-active activity. Its mechanistic role is independent of a protein–protein interaction with Nkx2-5, and will be further investigated.

To demonstrate this more physiologically, we performed a coimmunoprecipitation assay to assess the existence of complexes between Nkx2-5 and Zac1 in nuclear extracts from neonatal rat hearts (Figure 3G). Coprecipitation of Zac1 with immunoprecipitated Nkx2-5, and of Nkx2-5 with immunoprecipitated Zac1, was observed.

Zac1 Is Expressed Downstream of Nkx2-5

As noted above, whole-mount in situ hybridization analysis revealed expression of *Zac1* transcripts in the cardiac crescent region in embryos at E7.5, when cardiogenic precursors are specified (Figure 1A). Shortly thereafter, Zac1 was expressed strongly in a chamber-restricted manner in the developing heart tube. To investigate the regulation of *Zac1* expression in the heart, we evaluated a 3000bp *Zac1* 5′ proximal, *cis*-regulatory fragment, which contained numerous putative cardiac transcription factor binding sites as predicted by the TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEARCH.html). Although Tbx5, SRF, and MEF2C had no significant effect on transcriptional activity, both Zac1 and Nkx2-5 specifically augmented *Zac1* expression (Figure 4A). The activity of Zac1 suggests autoregulation, perhaps in collaboration with Nkx2-5. To clarify the role on Nkx2-5 on *Zac1* promoter activation, we deleted or mutated several Nkx2-5–binding sites found within the 3kb promoter fragment (Figure 4B). Of the 4 consensus Nkx2-5–binding sites detected, mutation of the third site alone or in combination with other sites diminished Nkx2-5–dependent *Zac1* transactivation (Figure 4B). We also examined the expression of *Zac1* in *Nkx2-5*–null embryos to confirm that Nkx2-5 regulates *Zac1* expression in vivo. *Zac1* mRNA levels were downregulated, as assessed by whole mount in situ hybridization, and quantitative RT-PCR analysis indicated a reduction to approximately one-third of wild-type levels at E8.5 in *Nkx2-5*$^{-/-}$ embryos (Figure 4C and 4D). These results

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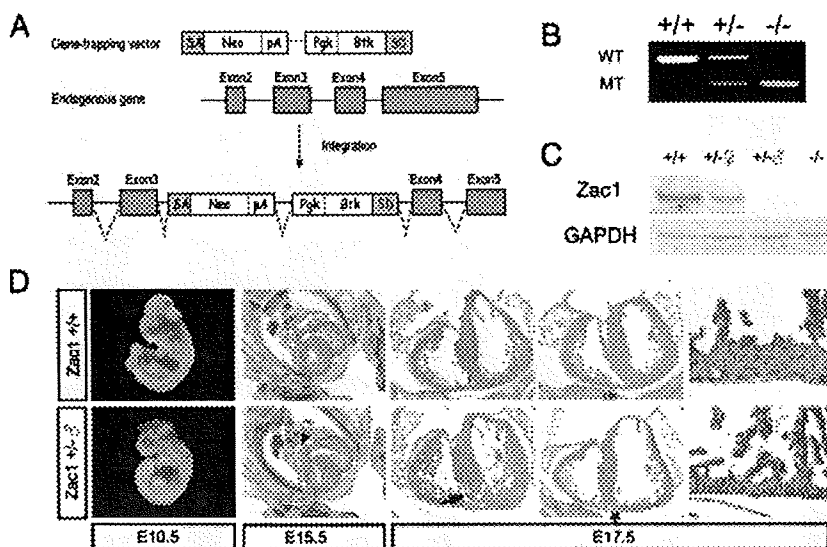

Figure 5. *Zac1* gene targeting–induced cardiac malformations. A, Schematic representation of the gene-trapping vector **(top)**, as well as wild-type **(middle)**, and interrupted Zac1 gene **(bottom)**. **B,** Genotyping by PCR. The wild-type allele yields a 407-bp product, which is absent in the homozygous mutant mice. The 250-bp product represents the targeted allele-specific band. **C,** Confirmation of Zac1 expression by Western blotting. **D,** Whole embryos at E10.5 and an embryonic heart at E15.5 and E17.5. Gross analysis of mutant embryos showed growth retardation and defective neural tube closure. Atrial septum defect **(arrowhead)** at E15.5, ventricular septum defect **(arrow)** at E17.5, and thin ventricular wall **(asterisk)** at E17.5 in Zac1-mutated embryos are shown compared to the wild-type controls.

indicate that Nkx2-5 induces and/or maintains *Zac1* expression in vivo, likely in a collaborative manner with Zac1 itself.

Partial Embryonic Lethality and Cardiac Malformation in *Zac1*-Null Embryos

To study the effect of *Zac1* mutation on mouse development, we assessed a mouse line carrying an interruption in *Zac1* generated by ES cell gene–trap methodology from Lexicon Pharmaceuticals. This mouse line contains an insertion in intron 3, which is predicted to induce a null mutation of the *Zac1* gene (Figure 5A). We confirmed the expected genomic mutation by PCR analysis (Figure 5B). Because *Zac1* shows only paternal expression, being a maternally imprinting gene, *Zac1* heterozygous animals descendent from male *Zac1* heterozygotes were indistinguishable from homozygous littermates. As we expected, Zac1 protein expression was totally abrogated in male *Zac1* mutant–descendent heterozygotes and homozygote mice (Figure 5C). Therefore, we deemed these 2 groups of mutant mice to be equivalent for the purpose of phenotypic analysis. At first we examined the gross phenotype of *Zac1* knockout mice which we generated and compared to the phenotypes previously described.[12] We confirmed that our *Zac1* knockout neonatal mice showed a similar phenotype with respect to overall weight loss, curly tail, and wrinkled skin. We analyzed 66 embryos at E10.5, 52 embryos at E15.5, and 89 embryos at E17.5. Gross examination of embryos at E10.5 revealed a defect of neural tube closure in 9% of *Zac1* mutants (Figure 5D). Histological analysis of the hearts of mutant embryos by serial sectioning along the anterior-posterior axis revealed an atrial septal defect in 42% of the mutant hearts at E15.5, as well as a ventricular septal defect involving fenestration of the muscular septum in 23% of the mutants and a thin ventricular wall in 26% at E17.5 (Figure 5D). At E17.5, we could not longer observe any of the neural tube defects evident in 9% of mutants at E10.5, suggesting a partially penetrant embryonic lethality before E17.5. Indeed, at E10.5, the expected Mendelian number of heterozygous embryos was observed (n=66). At E17.5, however, the number of heterozygous fetuses was reduced to 91% (n=89). These findings suggest neural developmental disorder as a cause of embryonic lethality in a low percentage of mutants. We also genotyped neonates at postnatal day (P)0 and P5 and adults at P90. At P0, the expected number of heterozygote mice was reduced to 91% (n=101). Although there seems to be approximately 10% reduction of heterozygous embryo, we could not obtain statistical significant differences compared to expected Mendelian ratios until P0 probably because of the limited number of embryos. At P5, this was further reduced to 44% (n=86) and at P90 was 40% (n=62), indicating an additional postnatal lethality. After P5, there are significant differences in this sample size. We did not observe any cardiac phenotypes at adult stages, suggesting that they were involved in the postnatal lethality. Varrault et al reported that approximately 30% to 50% of mutants survived to adulthood, with the percentage affected by genetic background, which is consistent with our own.[12] To confirm that the targeted locus is a null allele, we reexamined Zac1 expression in knockout mice and could not detect Zac1 by Western blot analysis using 2 different antibodies and quantitative RT-PCR analysis using independent primer sets and probes (data not shown).

The *Zac1* Mutant Mouse Shows Abnormal Cardiac Gene Expression and Patterning and a Significantly Increased Number of Apoptotic Cells in the Heart

Because *Zac1* mutant mice showed cardiac morphogenetic abnormalities, we examined the expression patterns of several cardiac genes in these mice. The expression patterns of cardiac-expressed transcription factors Nkx2-5 and GATA4 were unaffected (Figure 6A and 6B). By contrast, the expression levels of the cardiac-specific genes *ANF*, *MLC2v* (myosin light chain 2v), and *MLC2a* were significantly downregulated by both in situ hybridization and quantitative PCR (Figure 6C through 6E).

To clarify the mechanisms of cardiac malformation, we analyzed proliferation and apoptosis in the embryonic hearts. We found that *Zac1* mutant mice displayed a significantly

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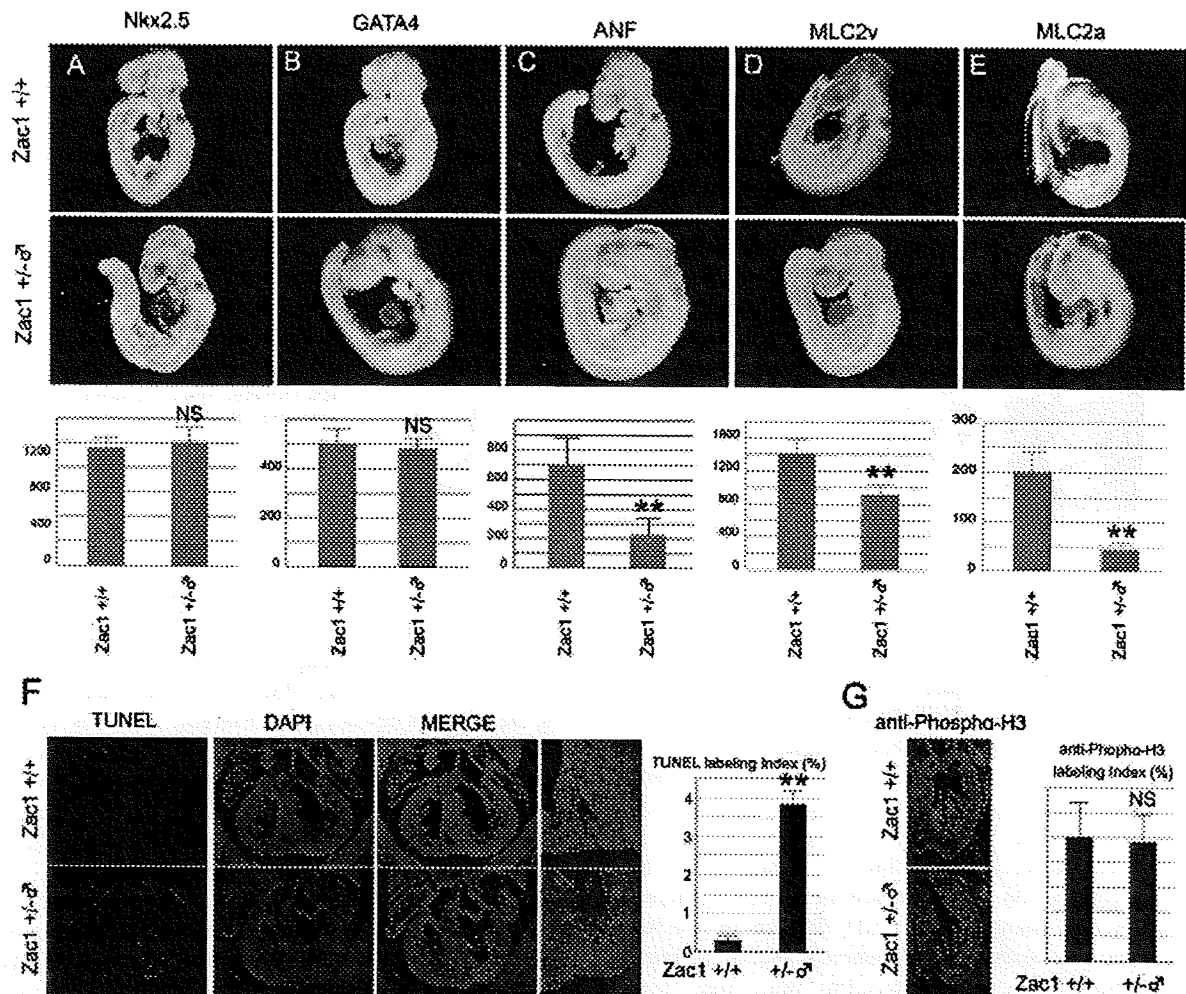

Figure 6. Altered gene expression in Zac1-mutated hearts. A and B, Expression levels of Nkx2-5 and GATA4 are normal in the *Zac1*-mutated heart, as assessed by whole-mount in situ hybridization and QT-PCR analysis. **C through E,** Expression levels of *ANF*, *MLC-2v*, and *MLC-2a* are decreased in the *Zac1*-mutated heart, as assessed by whole-mount in situ hybridization and QT-PCR analysis. **F,** Representative histological sections from the wild-type and *Zac1*-mutated hearts at E13.5 stained in the TUNEL assay. The numbers of positive cells in 5 different hearts of each genotype are shown. **G,** Representative histological sections from the wild-type and *Zac1*-mutated hearts at E13.5 stained with anti-phospho-histone H3 antibody. The numbers of positive cells in each 5 different hearts are shown.

increased number of apoptotic cells in the heart (Figure 6F). No such differences were observed in the number of proliferating cardiac cells (Figure 6G). *Zac1* is a known tumor suppressor gene, is frequently lost in multiple carcinomas, and promotes cell cycle and apoptosis.[9,14] However, many of those studies are performed in cancer cell, and there is no study in the heart. Therefore, we considered that Zac1 may have different, unique, and possibly opposite roles in cardiac development.

Discussion

In the present study, we identified the transcription factor Zac1 as an important to heart development. Initially, we used gene chip analysis of ES cell–derived cardiomyocytes to discover new cardiac-specific transcription factors.[7] Upregulated genes were tested for cardiac-specific expression and transcriptional potency using the *ANF* promoter, well studied as a cardiac target gene reflective of development and pathological hypertrophy. We confirmed Zac1 to be a strong transcriptional activator of cardiac gene in synergy with Nkx2-5 and that Zac1 itself is regulated by Nkx2-5. Analysis of a *Zac1* mutant mice verified that Zac1 is required for proper cardiac morphological development and gene expression.

The Zac1 Family of Transcription Factors

Zac1/LOT1/PLAGL1 is a member of the subfamily of PLAG (pleomorphic adenoma gene) transcriptional factors. The PLAG family genes were defined by the capacity of PLAG1 overexpression to induce pleomorphic adenomas.[15] The PLAG family comprises *PLAG1*, *Zac1/LOT1/PLAGL1*, and *PLAGL2*. These factors share high levels of homology, especially in their zinc finger amino-terminal regions, although they are functionally distinct. *PLAG1* is a protooncogene and a target of chromosomal rearrangements that results in tumorigenesis. *PLAGL2* is induced in human acute my-

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eloid leukemia, and may in fact induce acute myeloid leukemia in cooperation with other fusion genes.[16] PLAG1 and PLAGL2, therefore, have similar capabilities in tumorigenesis and have indistinguishable DNA-binding specificities, which are different from that of Zac1.[17] *Zac1/LOT1/PLAGL1* is lost in malignantly transformed rat ovarian surface epithelial cells, hence the name *LOT1* (lost on transformation).[14] However, Zac1 was also shown to regulate apoptosis and the cell cycle, accordingly named *Zac1*.[8] Subsequently, the gene symbol for this family member was designated as *PLAGL1*. Although having a similar protein structure, Zac1 appears to have an opposite function to PLAG1 and PLAGL2 in tumor formation and binds different DNA sequences.[17] Therefore, we speculated that there is no functional overlap between *Zac1* and the other *PLAG* family genes.

Imprinting Genes in the Heart

From a metaanalysis of microarray data, *Zac1* was found to be a member of an imprinted gene network.[12] Classically, both alleles of a gene were thought to be actively transcribed and functionally equivalent. Since the identification of the first autosomally imprinted genes in 1990s, researchers have tried to elucidate imprinting functions.[18] In the murine genome, approximately 600 genes are potentially imprinted,[19] and several theories have been proposed to explain why so many genes should be imprinted.[20] The ovarian time bomb hypothesis states that imprinting occurs to prevent parthenogenesis from unfertilized oocytes, which can lead to malignant trophoblastic disease.[21] Epigenetic abnormalities in imprinted regions have been implicated in a number of developmental disorders and carcinogenesis in mice and humans.[22,23]

The maternally methylated CpG island of the murine and human *Zac1* locus was identified in a screen for imprinted genes.[24,25] Genetic and epigenetic defects in the *Zac1* locus are also associated with Beckwith–Wiedemann syndrome.[26] Although Beckwith–Wiedemann syndrome is generally characterized by exomphalos, macroglossia, and giantism, cardiac manifestations are also known to occur, including congenital heart disease (ventricular septum defect, atrial septum defect, aortic stenosis) and cardiomyopathy.[27–31] Beckwith–Wiedemann syndrome is associated with a region of chromosome 11 in which many candidate disease genes are present including IGF-1 and $p57^{kip2}$. Although the molecular mechanisms underlying cardiac abnormalities seen in Beckwith–Wiedemann syndrome remain unknown, we have shown here a possible mechanistic link between *Zac1* and heart disease seen in the syndrome.

Regulation of Cardiac Gene Expression by Zac1

Our data show that Zac1 acts as a transcriptional activator for cardiac genes based on the following observations: (1) in development, Zac1 was highly expressed in the heart and enriched in chamber myocardium; (2) Zac1 bound directly to the *ANF* promoter and strongly activated the *ANF*, *BNP*, and *α-MHC* promoters; (3) Zac1 physically interacted with Nkx2-5 to synergistically activate cardiac gene expression; (4) Zac1 functioned as a downstream target of Nkx2-5 both in vitro and in vivo; (5) *Zac1* mutant mice showed cardiac gene expression abnormalities; and (6) *Zac1* mutant mice exhibited cardiac malformations.

A number of cardiac transcriptional factors collaborate in a complex manner to guide development and homeostasis in the heart. Nkx2-5, GATA4, Tbx5, MEF2C, and SRF are essential and potent cardiac transcriptional factors, regulating the expression of one another and serving to stabilize and reinforce the cardiac gene regulatory network. Zac1 expression was first observed at early stages of heart development, coincident with just after cardiac specification and expression of early transcription factors such as Nkx2-5. Our data also indicate that Nkx2-5 directly activates *Zac1* expression in the heart. We speculate that Zac1 and Nkx2-5 orchestrate and support the expression of other transcription factors and cofactors. In particular, cardiac transcription factors and Zac1 function together to stabilize the transcriptional machinery, in part by binding to adjacent sites within the promoter/enhancer regions of cardiac genes and also through direct protein–protein interaction. This robust transcriptional activation network promotes development and maturation of the heart. Our work establishes Zac1 as a new player in this network. Zac1 may provide a valuable entry point for genetic analysis heart growth and control of apoptosis and how these processes are controlled by the core, conserved transcription factor network.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Cardiac development is stringently regulated by various cardiac transcription factors, although many aspects of the underlying mechanisms remain to be elucidated.
- Mammals have evolved the intriguing process of gene imprinting, but it is not clear what roles gene imprinting plays in heart development and homeostasis.
-

What New Information Does This Article Contribute?

- We identify the maternally imprinted zinc finger–type transcription factor *Zac1* as a potent cardiac transcriptional activator.
- Our examination of homozygous and paternally derived heterozygous mice reveals several congenital cardiac malformations, indicating that *Zac1* is an essential transcription factor for cardiac morphogenesis.

Transcription factors play central roles in gene expression, organ morphogenesis, and pathogenesis. Although several essential cardiac transcription factors have been identified, the complex transcriptional networks in the heart are still poorly understood. To identify novel and potent cardiac transcription factors, we performed gene chip analysis using cardiomyocytes that were differentiated from ES cells. We found that the *Zac1* gene, which encodes a zinc finger–type transcription factor and is a maternally imprinted gene, was strongly expressed in the mouse embryonic heart. Zac1 is a potent transcriptional activator of several cardiac genes and binds directly to the ANF promoter. Binding sites for Zac1 within the ANF promoter were also determined. Zac1 was found to exert strong synergistic transcriptional activity and to interact physically with Nkx2-5. Nkx2-5 also activated the *Zac1* promoter, and Nkx2-5–null hearts showed decreased Zac1 expression. *Zac1*-mutated mice showed decreased levels of several cardiac-specific genes and increased numbers of apoptotic cells in the embryonic heart. The *Zac1*-mutated mice also exhibited severe cardiac deformities: an atrial septum defect, a ventricular septum defect, and thinning of the ventricular wall. Our results suggest a potential mechanistic link between genetic or epigenetic defects and congenital heart disease manifestations.

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