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 showing 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;106:1083-1091.)

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.8 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.5,10 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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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. 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 nucleolar (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, 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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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tr>
<td>ANF</td>
<td>atrial natriuretic peptide</td>
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<tr>
<td>BNP</td>
<td>brain natriuretic peptide</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<td>E</td>
<td>embryonic day</td>
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<td>EB</td>
<td>embryoid body</td>
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<td>ES</td>
<td>embryonic stem</td>
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<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
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<td>LOT1</td>
<td>lost on transformation 1</td>
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<tr>
<td>MEF2C</td>
<td>myocyte enhancer factor 2C</td>
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<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
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<tr>
<td>MLC</td>
<td>myosin light chain</td>
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<tr>
<td>P</td>
<td>postnatal day</td>
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<tr>
<td>PLAG</td>
<td>pleomorphic adenoma gene</td>
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<tr>
<td>SRF</td>
<td>serum response factor</td>
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<td>ZRE</td>
<td>Zac1-response element</td>
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**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 po-
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

![Image](http://circres.ahajournals.org/)

**Figure 1.** Expression of Zac1 in the mouse 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.

![Image](http://circres.ahajournals.org/)

**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, NKX; 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.
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. 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

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, trypsin 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 [35S]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 [35S]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.
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 [35S]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 [35S]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 communoprecipitation 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 promoter/enhancer region in embryos at E8.5 (Figure 4C and 4D). These results 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–null embryos (Figure 4C and 4D). These results...
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 knockout neonatal mice showing a defect of 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.

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 down-regulated 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 reduced 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. To confirm that the targeted locus is a null allele, we reexamined Zac1 expression in knockout mice and could not detect Zac1 by Western blotting in the homozygous mutant mice. The 250-bp product represents the targeted allele-specific band. The 407-bp product is absent in the homozygous mutant mice. The 250-bp product represents the targeted allele-specific band. The 407-bp product is absent in the homozygous mutant mice.
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 (pleiomorphic 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-
eloid leukemia, and may in fact induce acute myeloid leukemia in cooperation with other fusion genes.\textsuperscript{16} PLAG1 and PLAGL2, therefore, have similar capabilities in tumorigenesis and have indistinguishable DNA-binding specificities, which are different from that of Zac1.\textsuperscript{17} Zac1/LOT1/PLAGL1 is lost in malignantly transformed rat ovarian surface epithelial cells, hence the name LOT1 (lost on transformation).\textsuperscript{14} However, Zac1 was also shown to regulate apoptosis and the cell cycle, accordingly named Zac1.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{18} In the murine genome, approximately 600 genes are potentially imprinted,\textsuperscript{19} and several theories have been proposed to explain why so many genes should be imprinted.\textsuperscript{20} The ovarian time bomb hypothesis states that imprinting occurs to prevent parthenogenesis from unfertilized oocytes, which can lead to malignant trophoblastic disease.\textsuperscript{21} Epigenetic abnormalities in imprinted regions have been implicated in a number of developmental disorders and carcinogenesis in mice and humans.\textsuperscript{22,23}

The maternally methylated CpG island of the murine and human Zac1 locus was identified in a screen for imprinted genes.\textsuperscript{24,25} Genetic and epigenetic defects in the Zac1 locus are also associated with Beckwith–Wiedemann syndrome.\textsuperscript{26} Although Beckwith–Wiedemann syndrome is generally characterized by exomphalos, macroglossia, and gigantism, cardiac manifestations are also known to occur, including congenital heart disease (ventricular septum defect, atrial septum defect, aortic stenosis) and cardiomyopathy.\textsuperscript{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\textsuperscript{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 \(\alpha\)-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.

**Sources of Funding**

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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 transcriptional activity and to interact physically with Nkx2-5.

**Determination of Zac1 binding sites within the ANF promoter was also performed.** Zac1 was found to exert strong synergistic transcriptional activity and to interact physically with Nkx2-5. Nkx2-5 also activated the transcriptional activity and to interact physically with Nkx2-5.
Zac1 Is an Essential Transcription Factor for Cardiac Morphogenesis
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Supplement Material

In Situ Hybridization

Whole-mount *in situ* hybridization was carried out as previously described\(^1\). Digoxigenin-labeled RNA probes were prepared by *in vitro* transcription. The full-length cDNA for murine *Zac1* (accession no. AK142210) was obtained by RT-PCR and subcloned into the pBluescript plasmid. The cDNAs for murine Nkx2-5, GATA4, ANP, MLC2-v, and MLC-2a were kindly provided by Dr. E.N. Olson and Dr. H. Yamagishi. The probes were transcribed with T3 or T7 RNA polymerase.

Animal study

Pregnant ICR wild-type mice were purchased from Japan CLEA. All experiments were approved by the Keio University Ethics Committee for Animal Experiments.

Immunostaining

Antibodies directed against Zac1 (G-18; Santa Cruz Biotechnology, Santa Cruz, CA), actinin (EA-53; Sigma, St. Louis, MO), Lamin A/C (#2032, Cell Signaling Technology), Rho-GDI (610255, BD Biosciences), phospho-histone H3 (9071; Cell Signaling) and phalloidin (Molecular Probes, Eugene, OR) were added to the sections, followed by overnight incubation at 4°C. Next, three 5-min washes in PBS were carried out, followed by the addition of secondary antibodies conjugated with Alexa 546 (Molecular Probes), and incubation for 1 h at room temperature. The sections were washed three times in PBS for 5 min each and then observed by confocal laser-scanning microscopy (LSM510; Carl Zeiss, Jena, Germany). The TUNEL assay was performed using the ApopTag Red In Situ Apoptosis Detection kit (Chemicon International) according to the manufacturer’s protocol.
Western blotting

COS7 cells were transfected with pcDNA3.1 Zac1 using Lipofectamine (Invitrogen, Carlsbad, CA). Cell extracts were isolated 24 h after transfection and separated into nuclear and cytosolic fractions. Fractionated protein lysates were resolved by SDS-PAGE and transferred to a PVDF membrane, followed by immunoblotting with rabbit anti-Zac1 antibody (Santa Cruz Biotechnology) at a dilution of 1:1,000 and horseradish peroxidase-conjugated anti-goat IgG, followed by development with the SuperSignal West Pico Chemiluminescent reagent (Pierce, Rockford, IL).

IP-western blot analysis

Total cell lysate was prepared from neonatal mouse hearts. IP-western blot analysis was performed essentially as described previously using anti-Zac1 and anti-Nkx2-5 for hearts lysate.

Plasmids

The Zac1-expressing plasmids were generated through conventional or PCR-based cloning. Deletion mutants were constructed by PCR-based mutagenesis and subcloning of the DNA fragments into the pcDNA3.1 expression vector. Site-directed mutagenesis was performed using the QuickChange kit (Stratagene, La Jolla, CA). The reporter plasmids (ANP-luciferase, BNP-luciferase, and α-MHC-luciferase) were kindly provided from Dr. E.N. Olson. The Zac1 promoter was cloned using PCR-based techniques from a BAC clone into the pGL3 basic vector (Promega, Madison, WI). For mammalian hybrid assay, pBIND vector and pG5luc vector were purchased from Promega.

Cell culture, transfection, and luciferase assay
COS-7 cells plated in DMEM with 10% FBS were transfected with Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Unless otherwise indicated, 100ng of reporter and 100ng of each activator plasmid were used. The DNA doses represented by the ramp symbol indicate 0, 30, 100 and 300ng of plasmid. The total amount of DNA per well was kept constant by adding the corresponding amount of expression vector without a cDNA insert. CMV-Renilla luciferase was used as an internal control, to normalize for variations in transfection efficiency. All the proteins were expressed at very similar levels, as confirmed by Western blotting.

**EMSA**

Nuclear extracts were collected from COS7 cells that overexpressed Zac1. Double-stranded oligonucleotides for the Zac1-binding sequence ‘(5'-GCATCTTCTGCTGGCCGC-3') were synthesized, and the two complementary oligonucleotides were annealed and labeled with [α-³²P]-dATP using the Klenow enzyme. Labeled probes were incubated with 5 ml of nuclear extracts and 2mg of poly(dI-dC) in 20 ml of binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol, 0.05% Nonidet P-40] for 30 min at room temperature. The protein/DNA mixture was resolved on a 5% polyacrylamide gel in 0.5 Tris borate/EDTA buffer at 4°C for 2 h at 150 V.

**ChIP assay**

For the *in vivo* ChIP experiments, extracts were prepared from five neonatal rat wild-type hearts for independent experiments. For the ChIP assays, we used the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, Lake Placid, NY) and followed the instructions of the supplier. Primer in PCR reactions is 5’-ACAAGCTTCGCTGGACTGAT-3’ and 5’-TCTCGGCTCCTCTCTGGTT-3’ (-148 +43), 5’-CCTGACTGCTAACAGGACA-3’ and 5’-
TGTCAGGGCCTCAAATAAG-3’ (-576 -398), 5’-GAGAGGAGCTGGGACCATGAG-3’ and 5’-TTGAAAGCGTGAGGACTTGA-3’ (-2907 -2728). The amplified region corresponded to the rat ANP promoter, which encompasses the Zac1-binding sites.

**Glutathione S-transferase (GST) pulldown assay**

Murine Zac1 cDNA and several DNA fragments encoding Zac1 were subcloned into the pGex-6P vector (Amersham Biosciences). GST fusion proteins were isolated by standard procedures. The plasmids that contained the deletion mutants of Nkx2-5 were gifted by Dr. I. Komuro. Proteins translated *in vitro* were labeled with [35S]-methionine in the coupled transcription-translation T7 reticulocyte lysate system (Promega), and assayed for binding to the GST-fusion proteins.

**RT-PCR and real-time quantitative PCR**

Total RNA was extracted using the Trizol reagent (Invitrogen), and RT-PCR was performed as described previously. At least five replicates were processed for each assay. GAPDH was used as an internal control. For quantitative analysis of Nkx2-5, GATA4, ANP, MLC2v, and MLC2a expression, the respective cDNA was used as the template in a TaqMan real-time PCR assay using the ABI Prism 7700 sequence detection system (Applied Biosystems) according to the manufacturer’s instructions. All samples were run in triplicate. The data were normalized to GAPDH expression. The primers and TaqMan probe for Nkx2-5, GATA4, ANP, MLC2v, MLC2a and Zac1 were Mm00657783_m1, Mm00484689_m1, Mm01255747_g1, Mm00440384_m1, Mm00491655_m1, and Mm00494251_m1, respectively.

**Generation of mutant mice**
The Zac1-mutated mice were generated by Lexicon Pharmaceuticals from ES cells that corresponded to OST181461 (OmniBank sequence tag) and that were targeted by gene trapping. The gene-trapping vector contained a retroviral 5'-end long terminal repeat (LTR), a splice acceptor sequence, neomycin gene (Neo), and partial first intron of the murine Bruton’s tyrosine kinase (Btk) gene as the 3'-trapping component, rather than a selectable marker, which was regulated by the 3'-phosphoglycerate kinase 1 (PGK-1) gene promoter, a splice donor sequence, and a 3'-LTR. Retroviral infection, selection, and screening of the ES cells were performed as previously described. The gene-trapping vector was inserted at the third intron of the Zac1 gene (corresponding to OST181461) in the ES cells, as detected by inverse-PCR. ES cells were selected for blastocyst injection into C57BL/6 mice to produce chimeric mice. Heterozygous and homozygous animals were analyzed along with littermate control animals.

**Statistical Analyses**

Values are presented as mean ± SEM. Statistical significance was evaluated with the unpaired Student $t$ test for comparisons between 2 mean values. A chi squared analysis for comparisons between 2 groups. Comparisons between >3 groups were performed with ANOVA. A value of $P<0.05$ was considered significant. *$p<0.05$, **$P<0.01$, NS; not significant.
References

