

Dissociation of Cardiogenic and Postnatal Myocardial Activities of GATA4

Joseph M. Gallagher,^a Hiba Komati,^b Emmanuel Roy,^c Mona Nemer,^{b,c} and Branko V. Latinkić^a

School of Biosciences, Cardiff University, Museum Avenue, Cardiff, United Kingdom^a; Laboratory of Cardiac Development and Differentiation, Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, Ontario, Canada^b; and Graduate Program in Biomedical Sciences, University of Montréal, Montréal, Québec, Canada^c

Transcription factor GATA4 is a critical regulator of the embryonic and postnatal heart, but the mechanisms and cofactors required for its diverse functions are not fully understood. Here, we show that whereas the N-terminal domain of GATA4 is required for inducing cardiogenesis and for promoting postnatal cardiomyocyte survival, distinct residues and domains therein are necessary to mediate these effects. Cardiogenic activity of GATA4 requires a 24-amino-acid (aa) region (aa 129 to 152) which is needed for transcriptional synergy and physical interaction with BAF60c. The same region is not essential for induction of endoderm or blood cell markers by GATA4, suggesting that it acts as a cell-type-specific transcriptional activation domain. On the other hand, a serine residue at position 105, which is a known target for mitogen-activated protein kinase (MAPK) phosphorylation, is necessary for GATA4-dependent cardiac myocyte survival and hypertrophy but is entirely dispensable for GATA4-induced cardiogenesis. We find that S105 is differentially required for transcriptional synergy between GATA4 and serum response factor (SRF) but not other cardiac cofactors such as TBX5 and NKX2.5. The findings provide new insight into GATA4 mechanisms of action and suggest that distinct regulatory pathways regulate activities of GATA4 in embryonic development and postnatal hearts.

Vertebrate heart development is a complex, multistage process that begins with specification during gastrulation and continues well after birth. Throughout embryonic development, cells fated to give rise to the heart undergo well-orchestrated molecular events that control their proliferation, migration, and differentiation. Virtually all aspects of heart development are regulated by a set of conserved cardiac transcription factors, which have been well characterized by a combination of genetic and biochemical approaches. They include the zinc finger proteins GATA4/GATA5/GATA6 (GATA4/5/6), the MADS-domain factors MEF2 and serum response factor (SRF), the NKX2.5 homeodomain protein, the basic helix-loop-helix (bHLH) proteins HAND1 and -2, and the T-box factors TBX2/TBX5/TBX20 (33, 36). Spatial and temporal specificity may be achieved through the formation of multiprotein complexes that contain a subset of these factors along with other inducible or ubiquitous transcriptional regulators (10, 11, 26, 31). Generation of animal models lacking specific factors has started to shed light on the *in vivo* interactions of these proteins at various stages of cardiogenesis (20, 22, 31). Moreover, biochemical analyses of disease-causing human mutations are providing some insight into structural determinants of multiprotein complex formation (13).

GATA4, a member of the evolutionarily conserved GATA proteins, has emerged as a critical regulator of cardiogenesis either directly in cardiac precursors or through its role in the adjacent endoderm, where it can modulate cardiogenic factors like bone morphogenetic proteins (BMPs) (32). Mouse embryos lacking GATA4 die at embryonic day 8.5 (E8.5) to E9 due to abnormal heart morphogenesis (27) while mice with hypomorphic *Gata4* alleles show reduced viability due to a spectrum of congenital heart diseases (38). Loss of GATA4 specifically in endothelial or myocardial cells also leads to congenital heart defects (CHD), underscoring the important role for GATA4 in both cell lineages (48, 50). Human genetics have confirmed the essential role of GATA4

in valve and septal formation as evidenced by the finding of GATA4 mutations in association with several forms of CHD (39). Loss- and gain-of-function studies have also shown that GATA4 is a potent inducer of cardiogenesis. For example, GATA4 down-regulation blocks embryonic stem cell cardiogenesis at an early stage while ectopic GATA4 enhances cardiogenesis therein and formation of beating cardiomyocytes (15). In *Xenopus* embryos, gain of function of GATA4 is sufficient to induce cardiogenesis in embryonic ectoderm (21). More recently, GATA4 in combination with cofactors such as BAF60c and TBX5 was shown to be sufficient to induce cardiogenesis in heterologous murine cells (17, 44). BAF60c is a broadly expressed subunit of the SWI/SNF-like BAF chromatin remodeling complex, with high levels found in the heart (7, 24, 47). *In vitro* BAF60c mediates interaction of cardiac transcription factors GATA4, NKX2.5, and TBX5 with the SWI/SNF complex ATPase Brg1, resulting in strong transcriptional synergy (24). The interaction of BAF60c with cardiac transcription factors may underlie its essential role in heart development (24). Despite this recent progress, how GATA4 interacts with BAF60c and more generally how it induces cardiogenesis are incompletely understood.

In addition to its role during development, GATA4 is an essential regulator of the postnatal and adult heart, where it modulates

Received 17 February 2012 Accepted 24 March 2012

Published ahead of print 2 April 2012

Address correspondence to Mona Nemer, Mona.nemer@uottawa.ca, or Branko V. Latinkić, LatinkicB@Cardiff.ac.uk.

J.M.G. and H.K. contributed equally to the article and are co-first authors. M.N. and B.V.L. contributed equally to the article.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/MCB.00218-12

growth and survival of the terminally differentiated myocytes (1, 5, 35), and is essential for the heart adaptive response to stress and ischemic injury (35, 37, 40). In particular, GATA4 appears to be essential, and its upregulation is sufficient to promote myocyte survival, in part through transcriptional regulation of the antiapoptotic Bcl-X_L and Bcl-2 genes (1, 19). Similarly, GATA4 is an essential nuclear effector of numerous signaling pathways that are activated by hormones and growth factors that cause myocyte enlargement and cardiac hypertrophy (5, 37, 46). Whether such gain-of-function effects necessitate the presence of specific amino acid residues or protein domains and whether those are identical to the domains required for the cardiogenic activity of GATA4 remain to be determined. For example, the activity of GATA4 is upregulated in response to signaling pathways critical for embryogenesis and for cardiac hypertrophy, such as the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, which phosphorylates GATA4 at S105 (5, 23). However, the importance of this residue in the various GATA4 functions has not yet been determined.

In this report we have directly tested the potential link between the mechanisms by which GATA4 exerts its survival, hypertrophic, and cardiogenic functions through structure-function analysis. We mapped a 24-amino-acid (aa) region essential for cardiogenesis and for transcriptional synergy and physical interaction with BAF60c. In addition we determined that phosphorylation of S105 is required for hypertrophy and survival of cardiac myocytes but not for cardiogenesis. The results provide insight into the distinct mechanisms through which multiple roles of GATA4 are mediated.

MATERIALS AND METHODS

Xenopus embryos and explants. All animal work was approved by Cardiff University's Ethical Review Committee and was undertaken under a license from the Home Office of the United Kingdom. *Xenopus* embryos were obtained and cultured as previously described (43). Explants (stage 8.5) were dissected and cultured in 75% normal amphibian medium (NAM), supplemented with gentamicin sulfate (50 µg/ml; Sigma). *Xenopus* GATA4 (xGATA4) and GATA1 templates for *in vitro* transcription have been described previously (21). All rat GATA4 (rGATA4) constructs were linearized with Asp718 (Roche). Capped mRNA was synthesized using a mMessageMachine kit (Ambion/ABI), and RNA was subsequently purified on Sephadex G50 columns (GE Healthcare). RNA samples were injected into embryos using an IM-300 Microinjector (Narishige Scientific). RNA (400 pg) was coinjected with a mix of rhodamine-dextran (20 mg/ml) and dextran-biotin (25 mg/ml) lineage tracers (Invitrogen) (21, 43) in 10 nl/embryo at the 1- to 2-cell-stage. For ectopic cardiac tissue formation assay, 2 nl (80 pg) of RNA was injected in one anterior dorsal blastomere at the 8- to 16-cell stage. These precursors of the central nervous system are away from the developing heart and were chosen to assay genuine ectopic cardiac tissue induction. Translation-blocking morpholino oligonucleotides against GATA4 were used as described previously (16), and their effectiveness was confirmed by phenotypic analysis of sibling embryos.

Reverse transcription-PCR (RT-PCR). Total RNA was isolated from samples using the acid guanidinium thiocyanate-phenol-chloroform method (6). Approximately 20 animal caps were used per sample, and cDNA was synthesized using Moloney murine leukemia virus (MMLV) reverse transcriptase (RT) and random hexamers (both Invitrogen) according to the manufacturer's instructions. Primer sequences and cycling conditions were as described previously (41). PCR was performed with GoTaq enzyme (Promega). Whole-mount *in situ* hybridization was carried out as described previously (43), using the digoxigenin (Roche)-la-

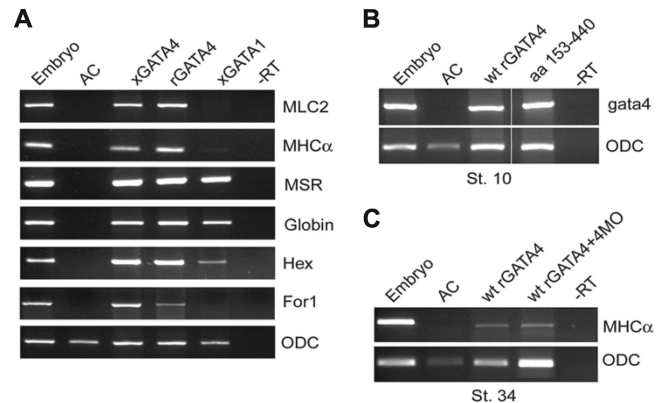


FIG 1 Rat GATA4 induces cardiac tissue in *Xenopus* animal caps independently of endogenous xGATA4. (A) Animal cap explants injected with 500 pg of indicated mRNAs have been analyzed for expression of cardiac (MLC2 and alpha-myosin heavy chain [MHC α]), endothelial (MSR), blood (globin), and liver (For1) markers at stage 34. Hex marks both liver and endothelia (34). rGATA4 and xGATA4 induce all markers tested, whereas xGATA1 induces only blood and endothelia. (B) rGATA4 induces endogenous GATA4 expression at stage 10, approximately 3 h after activation of zygotic gene expression. (C) Morpholino oligonucleotides against xGATA4 (4MO) do not prevent induction of cardiac markers by rGATA4. ODC, ornithine decarboxylase, loading control; AC, control animal caps; embryo, cDNA from sibling control embryos; -RT, no reverse transcriptase control for the embryo sample.

beled antisense riboprobe for MLC2 (3). Color was developed using BM purple (Roche).

Immunohistochemistry on blastula stage *Xenopus* embryos was performed as described previously (43), using rat monoclonal antihemagglutinin (anti-HA)-horseradish peroxidase (HRP) (1:2,500; Roche) and diaminobenzidine (DAB) substrate (Sigma). Images were taken on a Leica MZ16 stereomicroscope with a DFC300FX Leica camera and were processed with Adobe Photoshop software.

Western blotting. Western blotting of *Xenopus* protein extracts was performed as described previously (9). Total cell extracts were prepared from 1 to 2 µl of lysis buffer-animal cap or from cultured cells. Ten-microliter samples were resolved by polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore) and blotted by standard techniques. Antibodies used were rat monoclonal anti-HA-HRP (1:2,500; Roche) and, as a loading control, anti-Erk (1:2,500; Santa Cruz). Secondary antibody was goat anti-rabbit antibody-HRP (1:5,000; Chemicon). Detection was achieved using a chemiluminescent detection kit (Pierce). Western blotting on nuclear extracts from cardiac myocytes or other cell lines overexpressing various GATA4 proteins was performed as previously described (5). HA antibody at a 1/500 dilution and Bcl-X_{S/L} (S-18) antibody at 1/1,000 were both from Santa Cruz. Visualization was performed using an anti-rabbit horseradish peroxidase-conjugated antibody (Sigma).

Recombinant DNA. For gain-of-function studies in *Xenopus*, rat GATA4 sequences together with an N-terminal HA tag were generated by high-fidelity PCR and were cloned in pCS2 vector via BamHI/EcoRI sites. Primer sequences are available upon request. Adenoviral constructs expressing HA-tagged GATA4 (GATA4 wild type [WT], GATA4 with the S105A mutation [GATA4-S105A], and GATA4 consisting of residues 201 to 440 [GATA4(201–440)]) in recombinant replication-deficient type 5 adenovirus (Ad5) were generated using an AdEasy XL adenoviral vector system (Stratagene). cDNAs of the corresponding rat GATA4 constructs were subcloned into Ad5 shuttle vector pshuttle using HindIII and EcoRV, and the adenoviruses were generated through recombination with the pAdEasy-1 as described previously (8). Transformant bacteria were selected for kanamycin resistance, and recombination was identified via restriction enzyme digestion. Recombinants were then amplified using

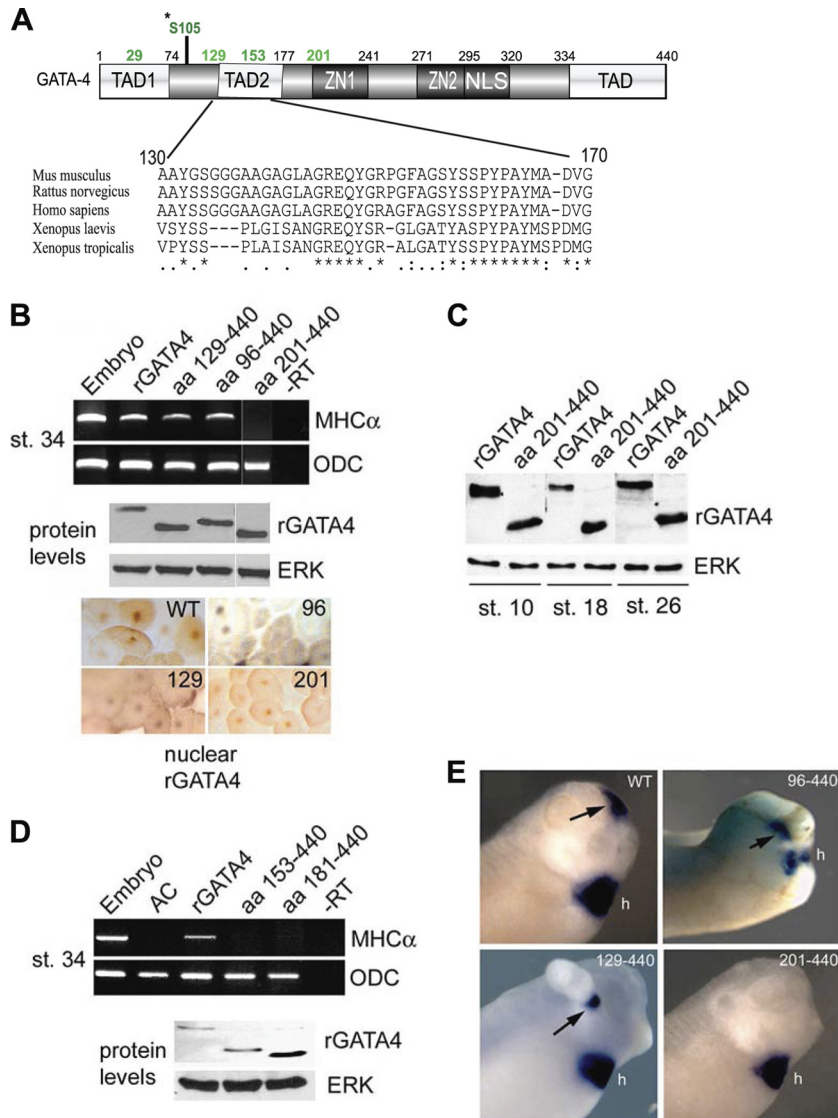


FIG 2 The rGATA4 domain of aa 129 to 153 is required for cardiogenesis. (A) Schematic representation of GATA4 protein showing the position of mutations/deletions in green. NLS, nuclear localization signal. (B and D) Explants injected with indicated rGATA4 variants were tested for expression of alpha-myosin heavy chain (MHC α) at stage 34 (top) and by Western blotting for the levels of rGATA4 protein at stage 10 (below). Panel B (bottom) shows nuclear localization as determined by whole-mount immunohistochemistry with anti-HA-HRP at stages 6 to 8. (C) Noncardiogenic rGATA4(201–440) protein is stable throughout the cultivation period. Samples collected at indicated stages were analyzed for rGATA4 and ERK by Western blotting. rGATA4 protein was detected by anti-HA (B and C) or anti-human GATA4 antibodies (D). (E) Stage 34 embryos injected with indicated mRNAs in anterior ectodermal precursor blastomeres were analyzed for MLC2 expression, highlighting the heart (h) and ectopic MLC2 expression (arrow). More than 50 embryos were analyzed for each construct, with 15 to 20% showing ectopic expression in the case of cardiogenic constructs.

the recombination-deficient *Escherichia coli* strain Epicurian Coli XL10-Gold. Purified recombinant adenoviral plasmid DNA was digested with PacI to expose the terminal repeats and was later transfected to AD-293 cells in which deleted viral assembly genes are complemented *in vivo*. The viruses were produced in bulk, and titers were determined as reported previously (8).

Electrophoretic mobility shift assays. DNA binding of GATA4 variants was assessed as described previously (46). Nuclear extracts were obtained from cultured 293T cells overexpressing GATA4 constructs, and the -120 atrial natriuretic factor (ANF) GATA site was used as a probe.

Luciferase assays. *Xenopus* embryos were injected with indicated RNAs together with 30 pg each of 2XGATALuc, a luciferase reporter driven by two GATA elements in front of BNP $_{-50\text{bp}}$ (14) and TK-RL

(Promega). Dual luciferase assays (Promega) were carried out on extracts from approximately 20 animal caps per sample that were incubated for 3 h after excision at stage 9.

NIH 3T3 cells were cultured and transfected, and luciferase assays using ANF reporter plasmids and GATA4, NKX2.5, Tbx5, MEF2C, and SRF expression vectors were carried out as previously described (8, 10). All experiments were repeated at least three times in duplicate.

Cardiomyocyte cultures and infections. *Ex vivo* experiments were done on primary cultures of rat neonatal cardiomyocytes as previously described (5). Cardiomyocytes were plated and kept overnight in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. The next day, cells were extensively washed, and the medium was replaced with serum-free, hormone-free medium. Cardiomyocytes were infected at a multiplicity of infection (MOI) of 5 with either adeno-LacZ, adeno-

GATA4-WT, adeno-GATA4-S105A, or adeno-GATA4(201–440) as described previously (1, 4). Treatment with doxorubicin (Dox) was as described previously (1). Immunofluorescence on cardiomyocytes was performed as previously described (8), using a rabbit polyclonal HA antibody (dilution, 1/500) from Santa Cruz and phalloidin-Alexa Fluor 488 (dilution, 1/400). Apoptosis was detected by a terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay as recommended in the Apoptag kit (Chemicon). An average of 10 random fields with 100 nuclei per field were analyzed.

Coimmunoprecipitation. Coimmunoprecipitation of HA-GATA4 constructs and endogenous BAF60c was carried out using nuclear extracts of 293T cells overexpressing the relevant proteins. Nuclear extracts were prepared as previously described (28). Coimmunoprecipitation reactions were carried out on 60 μ g of nuclear extracts using 2 μ g of rabbit BAF60c antibody. Proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes, and subjected to immunoblotting using either anti-HA antibody to reveal tagged GATA4 proteins or anti-BAF60c antibody to reveal endogenous BAF60c protein.

Statistical analysis. Data are means \pm the standard error of the mean (SEM), with a *P* value of <0.05 by a Student's *t* test being considered statistically significant.

RESULTS

Mammalian GATA4 induces cardiac differentiation in pluripotent *Xenopus* embryo explants. In addition to promoting cardiomyocyte growth and survival, GATA4 can induce cardiogenesis, an activity that reflects its role in early heart development. To test the requirement of the N-terminal region for GATA4-induced cardiogenesis, we used a *Xenopus* animal pole explant assay (animal caps). This assay was established for the *Xenopus* GATA4, which, when ectopically expressed in animal caps, respecifies them from epidermal to cardiomyocyte fate (21). As expected from a high degree of sequence conservation (61% overall amino acid identity and 71% similarity), the rat GATA4 behaves indistinguishably from the *Xenopus* GATA4 in animal cap assays as it induces cardiomyocyte (as well as blood, endothelium, and liver) markers (Fig. 1A). In contrast, the hematopoietic factor GATA1 induces only blood and endothelium markers, attesting to the specificity of the assay (Fig. 1A). An early target of the exogenous GATA4 in animal caps is the endogenous *gata4* gene (Fig. 1B). As it is possible that rGATA4 induces cardiac differentiation markers through early activation of the endogenous GATA4, the assay may not accurately reflect the true cardiogenic activity of the exogenous rGATA4 protein. We addressed the question of requirement of xGATA4 for cardiogenesis triggered by rat GATA4 by using previously described antisense morpholino oligonucleotides against xGATA4 (16). As shown in Fig. 1C, our results demonstrate that the endogenous GATA4 protein is not required for cardiogenesis induced by the rat GATA4, arguing that the animal cap assay reflects intrinsic cardiogenic activity of the rGATA4 protein.

The N-terminal 129 amino acids of GATA4 are dispensable for cardiogenic activity. We next tested the cardiogenic activity of GATA4 mutants lacking part of or the entire N-terminal domain, schematically represented in Fig. 2A. As shown in Fig. 2B, the GATA4(201–440) mutant, which lacks the entire N-terminal activation domain, was defective in cardiogenic activity. However, and as previously reported (4), it localized to the nucleus (Fig. 2B) and was stable at several time points during cultivation of animal cap explants (Fig. 2C), indicating that lack of cardiogenic activity is not caused by mislocalization, reduced protein stability, or a loss of cells expressing it. To further delineate the cardiogenic do-

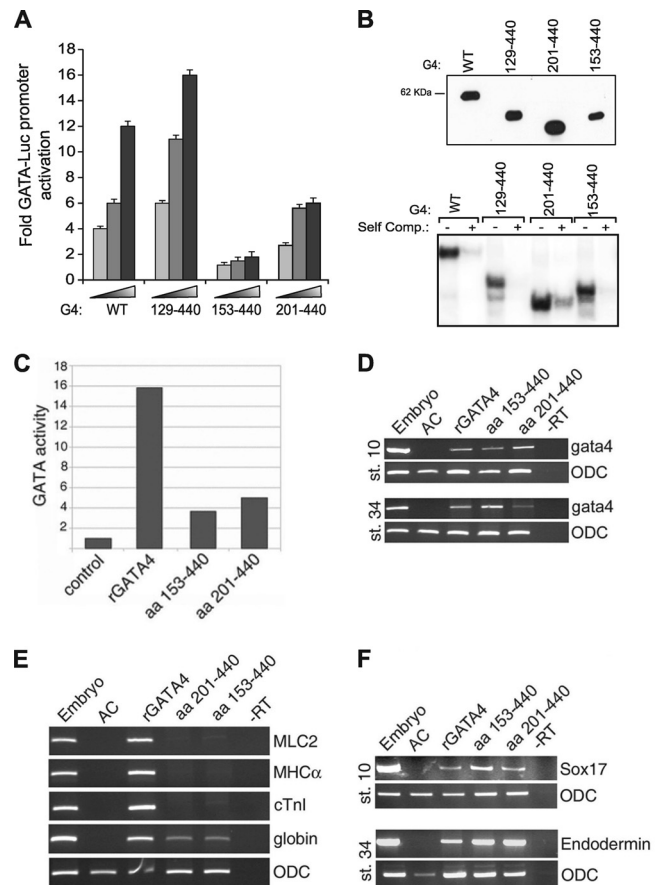


FIG 3 Noncardiogenic mutants GATA4(153–440) and GATA4(201–440) retain transcriptional activity. (A) Transcriptional activity of various GATA4 (G4) N-terminal deletion mutants. Cotransfections in NIH 3T3 cells were carried out on 1.5 μ g of the (GATA)₃-Luc reporter, and 25, 50, and 100 ng of each GATA4 construct. (B) Western blot showing protein levels of expression of GATA4 constructs (top) and gel shift assay showing the ability of GATA4 mutants to bind DNA (bottom). The probe used is the GATA element at position –120 on the Nppa (ANF) gene promoter. (C) Removal of the first 153 or 201 amino acids from rGATA4 attenuates, but does not abolish, the ability to activate a GATA site-driven reporter. Embryos were injected with a firefly luciferase reporter under the control of two GATA sites, *Renilla* luciferase plasmid driven by the thymidine kinase (TK) promoter and indicated mRNAs. Control, DNA alone (no RNA). Animal caps were collected 3 h after excision. GATA activity is firefly luciferase activity normalized for *Renilla* luciferase activity, expressed as fold activation over the control sample. A representative of three repeated experiments is shown. (D) Noncardiogenic mutants consisting of GATA4 aa 153 to 440 and 201 to 440 can induce the endogenous *gata4* gene both early (stage 10) and late (stage 34), as revealed by RT-PCR analysis. (E and F) rGATA4 mutants consisting of GATA4 aa 153 to 440 and 201 to 440 cannot induce cardiomyocyte-specific markers MLC2, MHC α , and cardiac troponin I (cTnI) but retain the capacity to induce globin, a marker of blood (albeit at a lower level) as well as endodermal markers Sox17 at stage 10 and endodermis at stage 34. RT-PCR analysis was performed on stage 34 animal caps. AC, control animal caps.

main(s), smaller N-terminal deletions were tested. Removal of the first 96 or 129 amino acids had no effect on the cardiogenic activity of GATA4 but further deletion at aa 152 or 181 abrogated cardiogenic activity (Fig. 2D).

The same rGATA4 mutants were also tested in an independent *in vivo* assay for their capacity to induce ectopic cardiac tissue in *Xenopus* embryos when overexpressed in anterior ectodermal pre-

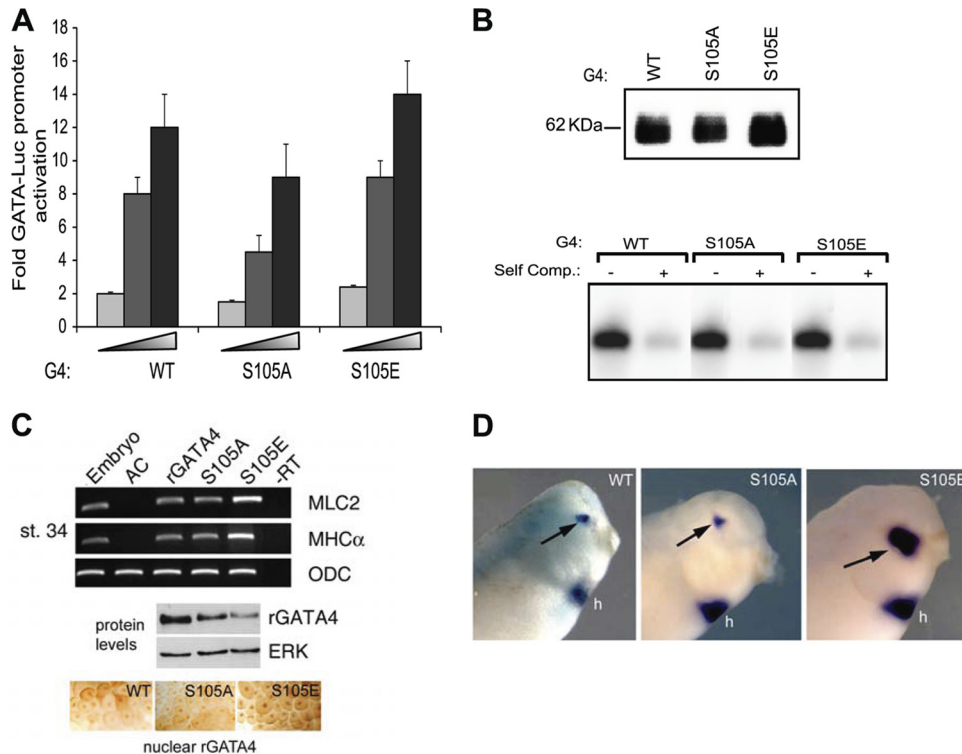


FIG 4 S105 phosphorylation is not required for cardiogenesis by rGATA4. (A) Transcriptional activity of GATA4 S105A and S105E mutants. Cotransfections in NIH 3T3 cells were carried out on 1.5 μ g of the (GATA)₃-Luc reporter, and 25, 50, and 100 ng of each of the GATA4 constructs. (B) Western blot showing nuclear GATA4 protein levels in transfected cells (top) and gel shift assay showing the ability of GATA4 mutants to bind DNA (bottom). The probe used is the GATA element at position -120 on the Nppa promoter. (C) S105A and S105E rGATA4 variants induce at stage 34 markers of cardiomyocyte differentiation MLC2 and MHC α . S105E induces a greater amount of cardiac markers than the WT or S105A rGATA (top). A Western blot shows levels of rGATA4 variants at stage 10, detected with anti-HA antibody (middle). Nuclear localization as determined by whole-mount immunohistochemistry with anti-HA-HRP is shown at stages 6 to 8 (bottom). (D) Indicated rGATA4 variants induce ectopic MLC2 expression in stage 34 embryos. More than 50 embryos were examined for each construct, and 15 to 20% showed ectopic expression. AC, control animal caps.

cursors. We found that the cardiogenic activity of rGATA4 mutants in animal cap explants perfectly correlated with their ability to induce ectopic cardiac tissue *in vivo* (Fig. 2E), suggesting that context-dependent modulation of cardiogenic activity of GATA4 is unlikely.

Noncardiogenic GATA4 mutants might be inactive simply because their transactivation function was severely compromised by deletion of one or more N-terminal activation domains (30). When assayed by using a GATA site-dependent luciferase reporter, transcriptional activity of noncardiogenic mutants GATA4(153–440) and GATA4(201–440) was shown to be attenuated (Fig. 3A and C) although their DNA binding was not affected (Fig. 3B). Remarkably, the noncardiogenic mutants were able to induce the endogenous *gata4* gene (Fig. 3D) as well as the markers of blood and endoderm (Fig. 3E and F). This result suggests that the determinants for specification of cardiomyocyte, blood, and endodermal cell fates by GATA4 are separable. In addition, the reporter assay suggests that, in contrast to cardiomyocyte induction, the induction of blood and endoderm does not require the intact transcriptional activity of GATA4.

The lack of cardiogenic activity of the GATA4(153–440) and GATA4(201–440) mutants was confirmed by examining the expression of additional cardiomyocyte-specific markers such as MLC2 and troponin I (Fig. 3E). Together, the results suggest that

amino acids 129 to 153 of GATA4 are specifically required for its cardiogenic activity. Since neither altered expression, nuclear accumulation, nor DNA binding could account for loss of cardiogenic activity when aa 129 to 153 were deleted, we reasoned that disruption of cofactor(s) interaction may underlie loss of cardiogenic activity.

S105 phosphorylation is not required for cardiogenesis induced by GATA4. As stated earlier, S105 is a major MAPK phosphorylation site. Although it resides outside the cardiogenic domain defined above, we tested its requirement for cardiogenesis, given the important role of MAPK in heart development. Our results demonstrate that a nonphosphorylatable S105A mutation is as efficient at inducing cardiogenesis as the native GATA4 protein as evidenced by similar levels of cardiac marker induction (Fig. 4). Interestingly, the phosphomimetic mutation S105E consistently increased the level of GATA4-induced cardiac markers (Fig. 4C and D). Thus, S105 phosphorylation may not be required for cardiogenesis but may instead have a positive modulatory effect.

N-terminal cardiogenic region of GATA4 (aa 129 to 152) is required for interaction with BAF60c. When tested for its ability to cooperate with NKX2.5, TBX5, and MEF2A in transcriptional activation, a GATA4 mutant lacking the first 152 aa was unable to support significant synergy with any of them (Fig. 5A and B). This result could not be attributable to impaired physical interactions

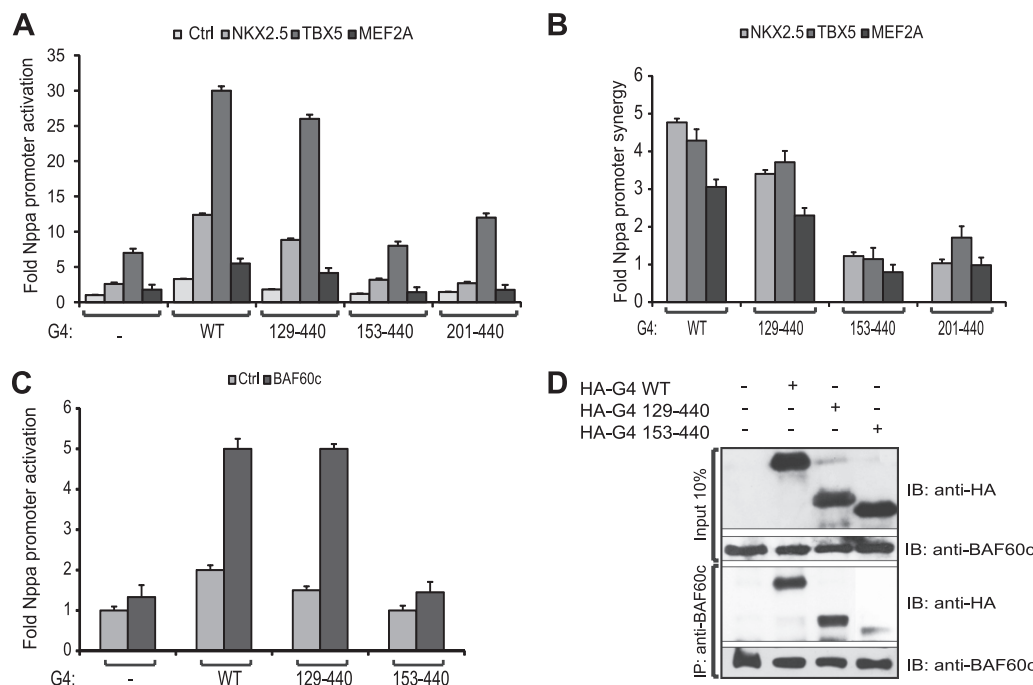


FIG 5 GATA4 regions required for functional cooperativity with cardiac cofactors. (A) Cotransfections in NIH 3T3 cells using 1.5 μ g of the Nppa promoter, 15 ng of each of the GATA4 constructs, and 50 ng each of NKX2.5, TBX5, and MEF2A. The data are the mean \pm SEM of two to three experiments carried out in duplicate. (B) Histograms showing the fold synergy for each GATA4 mutant. Synergy was calculated from the data in panel A. (C) Cotransfections in NIH 3T3 using 1.5 μ g of the Nppa promoter, 15 ng of each of the GATA4 constructs, and 250 ng of BAF60c. The data are the mean \pm SEM of three experiments carried out in duplicate. (D) Immunoprecipitation (IP) of endogenous BAF60c with rabbit anti-BAF60c (kind gift from Puri) followed by detection of transfected HA-tagged GATA4 mutants. IB, immunoblotting.

with the mutant GATA4 proteins lacking aa 129 to 153 as earlier studies established that in all three cases the GATA4-interacting domain resides in the second zinc finger (2, 10, 28). We then assessed the ability of all GATA4 proteins to interact with BAF60c, the cardiac-enriched subunit of BAF chromatin remodeling complexes, that was shown to be essential for the ectopic cardiogenic activity of GATA4 (44). The GATA4(153–440) mutant was unable to synergize with BAF60c, providing the potential underlying cause for its lack of cardiogenic activity (Fig. 5C). Because BAF60c was shown to potentiate cardiogenic function by physically interacting with GATA4 and enhancing its transcriptional activation of cardiac genes, we checked the ability of GATA4 N-terminal deletions to interact with BAF60c. Our results show that both GATA4-WT and the GATA4(129–440) mutant strongly interact with BAF60c protein. In contrast, the GATA4(153–440) mutant shows greatly reduced protein-protein interaction, suggesting a mechanism of action of the region of aa 129 to 152 in GATA4 cardiogenic function (Fig. 5D).

S105 is required for functional synergy with SRF and for hypertrophic and cell survival-promoting activities. Previously, we showed that ectopic expression of GATA4 in postnatal cardiomyocytes induced cytoskeletal reorganization and hypertrophy. This effect mimicked the response of cardiomyocytes to hypertrophic stimuli which require GATA4 presence and lead to MAPK-mediated S105 phosphorylation (5). To determine if the N-terminal activation domain of GATA4 and phosphorylation of S105 within it are required for this effect, we generated adenovirus vectors expressing HA-tagged wild-type GATA4, as well as HA-tagged mutants that delete the entire N terminus (aa 201 to 440) or har-

bor a point mutation at S105 (S105A). Infecting the neonatal cardiomyocytes with these constructs led to comparable exogenous nuclear protein levels, as assessed by Western blotting (Fig. 6B). As expected, overexpression of GATA4-WT induced myofibrillar reorganization and myocyte hypertrophy (Fig. 6A and C). Myocytes expressing GATA4-S105A and GATA4(201–440) showed reduced myofibrillar and growth response compared to GATA4 WT (Fig. 6A and C).

Because GATA4 can rescue cardiomyocytes from doxorubicin (Dox)-induced apoptosis (1), we tested the effect of the different GATA4 mutants on cell survival using a TUNEL assay to measure myocyte apoptosis. Overexpression of GATA4 significantly attenuated Dox-induced myocyte apoptosis, as evidenced by the reduction in the percentage of apoptotic nuclei (Fig. 6D). In contrast, cells infected with the GATA4-S105A and GATA4(201–440) vectors showed significantly decreased survival in the absence of Dox ($P < 0.05$) compared to cells infected with the WT GATA4 adenovirus or with the control LacZ adenovirus. Furthermore, neither of the mutant GATA4 vectors could rescue Dox-induced apoptosis, as evidenced by the significant increase in the percentage of TUNEL-positive nuclei (Fig. 6D). To elucidate the mechanism(s) underlying loss of prosurvival activity, we tested the transcriptional activity of the different constructs on the antiapoptotic gene Bcl-X_L. As shown in Fig. 6E (right panel) GATA4 WT, but not the mutants, activates Bcl-X_L promoter. Moreover, Western blot analysis showed upregulated Bcl-X_L protein levels in myocytes expressing GATA4 WT but not in cells expressing GATA4-S105A and GATA4(201–440) mutants (Fig. 6E, left panel), leading to a higher ratio of the short pro-

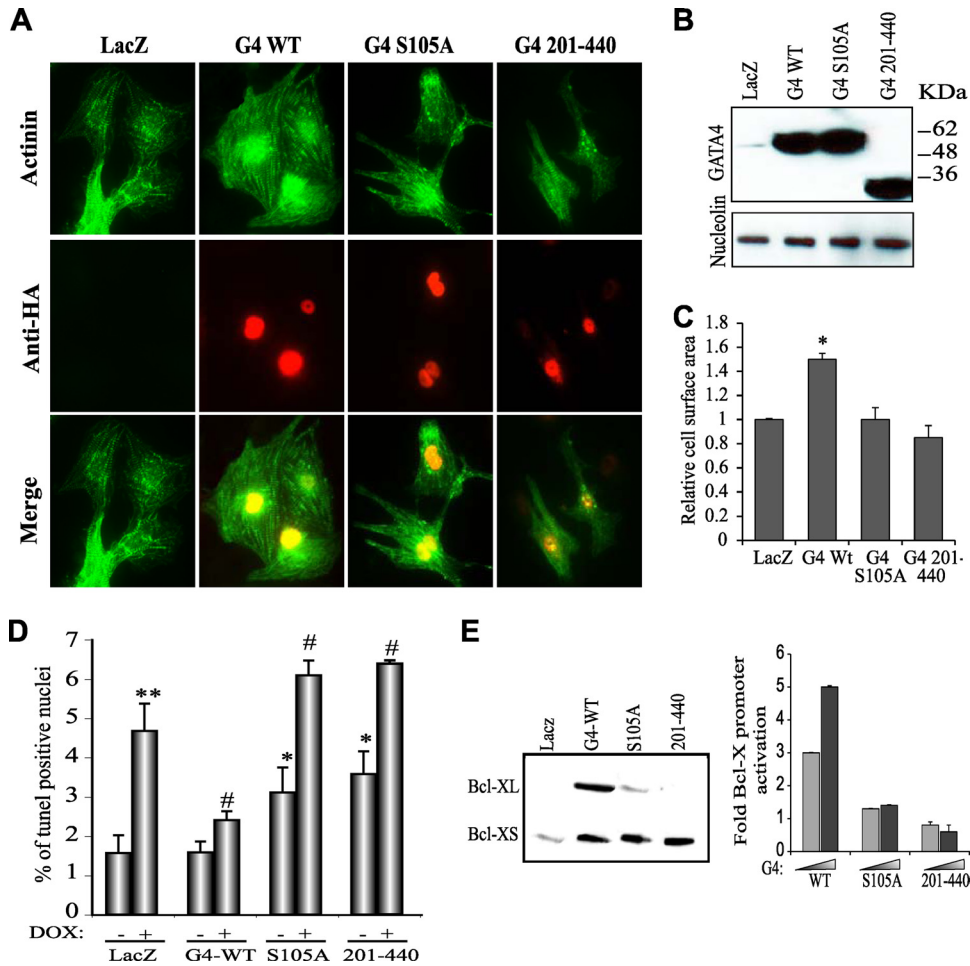


FIG 6 GATA4 N-terminal domain is essential for myofibrillar reorganization and myocyte survival. (A) Immunofluorescence in cardiomyocyte infected with the indicated adenoviral constructs. The sarcomeric alpha-actinin was labeled green, and the exogenous GATA4 proteins detected with the anti-HA antibody were labeled in red. Notice the increase in size of myocytes expressing the WT GATA4 (G4) but not the GATA4(201–440) mutant with an N-terminal deletion or the S105A GATA4 mutant. (B) Western blot confirming similar nuclear overexpression of indicated HA-tagged GATA4 proteins in cardiomyocytes infected with the corresponding adenoviral vectors. (C) Relative cell surface area was measured across 10 fields (magnification, $\times 40$) in three separate experiments. *, $P < 0.05$ versus LacZ, GATA4-S105A, and GATA4(201–440). (D) Quantification of TUNEL assays in cardiomyocytes infected with the indicated adenoviruses in the presence or absence of 300 nM Dox. Notice the apoptotic effect of both GATA4-S105A and GATA4(201–440) mutants. *, $P < 0.05$ versus LacZ; **, $P < 0.01$ versus LacZ; #, $P < 0.05$ versus LacZ+Dox. (E) The left panel shows a Western blot of Bcl-X proteins in nuclear extracts of cardiomyocytes expressing the indicated constructs. At right, 1.5 μ g of Bcl-X-luciferase reporter was cotransfected with 25 and 50 ng of each GATA4 construct. The data are the mean \pm SEM of two to three experiments carried out in duplicate.

apoptotic Bcl-X_S form over the antiapoptotic Bcl-X_L form. Thus, GATA4 growth and survival effects require the N-terminal transcriptional activation domain (TAD) and the presence of S105 phospho-residue therein.

To better understand the differential effect of S105 on cardiogenesis versus growth and survival, we tested the effect of mutating it to a nonphosphorylatable alanine residue or replacing it by the phosphomimetic mutation S105E on functional interaction with GATA4 cofactors linked to cardiogenesis (TBX5, MEF2, and NKX2.5) or to hypertrophy (SRF). Interestingly, the S105A mutant specifically affected the synergy of GATA4 with SRF but not with NKX2.5, TBX5, or MEF2 (Fig. 7A and B). Even though S105 phosphorylation was not required for synergy of GATA4 with TBX5 and NKX2.5, the phosphomimetic mutation (S105E) showed enhanced synergy, a result consistent with its enhanced ability to induce cardiogenesis (Fig. 4).

DISCUSSION

In this report we have provided evidence that GATA4 induction of cardiogenesis, on one hand, and postnatal cardiomyocyte hypertrophy and cell survival, on the other, may involve distinct mechanisms of action.

Amino acids 129 to 152 of GATA4 are required for cardiogenesis and for interaction with BAF60c. Functional domains of the GATA family proteins have been studied extensively *in vitro*, but relatively little is known about their roles *in vivo*. Of note, differences between *in vitro* and *in vivo* results were reported for GATA1: while the N terminus contains a transactivation domain that is essential in heterologous cells, the deletion of this region of the protein had no major effect on its ability to rescue GATA1-deficient mice and to promote hematopoiesis in embryonic stem (ES) cells (42, 49). Similarly, we found no evidence of an essential role of the *in vitro* defined transactivation domain I (aa 1 to 74) of

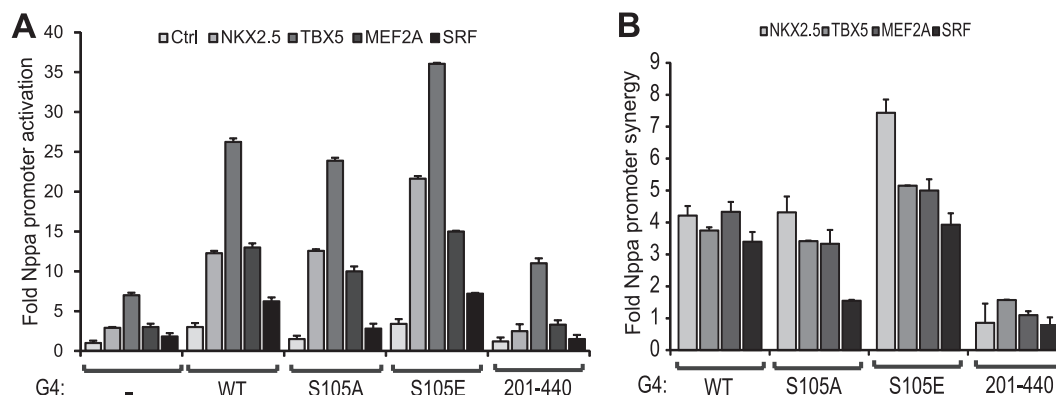


FIG 7 GATA4-S105 is required for functional cooperativity with SRF. (A) Cotransfections in NIH 3T3 using 1.5 μ g of the Nppa promoter, 15 ng of each of the GATA4 constructs, and 50 ng each of NKX2.5, TBX5, MEF2A, and SRF. The data are the mean \pm SEM of three experiments carried out in duplicate. (B) Histograms showing the fold synergy for each GATA4 mutant. Synergy was calculated from the data in panel A.

GATA4 (30) in cardiogenesis. In contrast, we have identified a 24-aa region located between aa 129 to 152 required for GATA4-dependent induction of cardiogenesis, which lies within activation domain II previously mapped to aa 130 to 177 (25). This finding suggests a physiological role for activation domain II. The activation domain II was reportedly required for transcriptional synergy between GATA4 and other cardiogenic regulators although it is not involved in physical interactions with any of them (2, 10, 28). Our study uncovers a novel function of the region of aa 129 to 152 in cardiogenesis and suggests that functional and physical interaction with BAF60c may be the molecular basis for action. BAF60c, a component of a multiprotein chromatin remodeler, SWI/SNF, was shown to interact with GATA4 and to stimulate its transcriptional activity (24). *In vivo*, BAF60c synergizes with GATA4 and GATA5 in inducing ectopic cardiac tissue (25, 44). GATA4 interacts with the ATPase Brg1, the motor of the core SWI/SNF complex, but this interaction is enhanced by BAF60c, providing a possible mechanism for activation of GATA4 target genes. Brg1 was shown to interact with the Zn fingers of GATA factors (18), and in this study we show that BAF60c interaction with GATA4 requires the N-terminal region of aa 129 to 152. Together, these findings suggest that GATA4 might interact, perhaps simultaneously, with two components of the SWI/SNF complex via different domains. In a recent study on the mechanisms of interaction between BAF60c with the myogenic determinant MyoD, Forcales et al. have suggested a two-step model: first, BAF60c mediates interaction of MyoD with its target promoters in chromatin, and then, upon signal-induced phosphorylation by p38, BAF60c recruits the ATPase-containing SWI/SNF complex, resulting in target gene activation and muscle differentiation (12). Whether an analogous dynamic and similarly regulated model for interaction of GATA4 with BAF60c and SWI/SNF complex takes place during cardiac specification will be interesting to investigate.

Cardiac specificity of the GATA4 region of aa 129 to 152 is also suggested by our results showing that noncardiogenic mutants GATA4(153–440) and GATA4(201–440) retain the capacity to induce endoderm and blood markers. Our data also indicate that noncardiogenic forms of GATA4 perform these activities, despite their greatly reduced capability to interact with BAF60c. Therefore, these results further support the essential role of GATA4-

BAF60c interaction in cardiogenesis and suggest that lineage-specific activities of GATA4 may be promoted by distinct parts of the protein, utilizing different mechanisms and cofactors.

S105 phosphorylation mediates synergy with SRF and myocyte survival. Our study has shown that S105A mutation abolishes GATA4 antiapoptotic activity and significantly reduces its hypertrophic effect, but it has no detectable impact on GATA4 cardiogenic activity either in explants or *in vivo*. These results suggest that cardiac hypertrophy is not simply a result of reactivation of the embryonic program in adult myocytes and instead imply that the mechanisms underlying these two GATA4-regulated programs are distinct. These findings are consistent with a recent report showing that mice with a *Gata4* knock-in S105A mutation have a compromised stress response but show no detectable embryonic defects (45). Furthermore, our results based on gain-of-function experiments in isolated cardiomyocytes indicate that the impaired cardiac response is likely due to defective cardiomyocyte homeostasis. Although they observed increased fibrosis and cardiac dilation—two processes associated with cardiomyocyte loss—van Berlo et al. (45) did not note changes in apoptosis in GATA4-S105 relative to wild-type mice after 2 weeks of pressure overload; given our findings of the importance of S105 for GATA4-dependent myocyte survival, it is possible that the time point chosen for the analysis did not allow detection of increased apoptosis which may have happened at an earlier stage in these mice before overt cardiac remodeling. Interestingly, intact S105 is specifically required for transcriptional synergy between GATA4 and SRF. Both GATA4 and SRF (35) have been shown to be regulators of cardiac hypertrophy and myocyte survival. Moreover, a ternary complex involving GATA4-SRF binding to target promoters was shown to mediate transcriptional upregulation in response to hypertrophic stimulation (29). Our findings suggest that S105 phosphorylation is essential for GATA4-SRF cooperativity and point to a potential role for SRF-GATA4 synergy in promoting cardiomyocyte cell survival.

Lastly, although our results indicate that S105 phosphorylation is not essential for induction of cardiac differentiation by GATA4, they suggest that this modification may enhance cardiogenic activity of GATA4. This role of S105 modification in cardiogenesis may be the result of increased GATA4 transcriptional activity, which may be mediated by functional interaction with NKX2.5

TABLE 1 Summary of biochemical and functional properties of GATA4 mutants

GATA4 protein	Biochemical property							Functional activity		
	DNA binding	Transcriptional activation	Cooperativity				BAF60c	Cardiogenic	Hypertrophic	Survival
			NKX2.5	TBX5	MEF2A	SRF				
WT	++	++	++	++	++	++	++	++	++	++
GATA4-S105A	++	+	++	++	++	+/-	ND ^a	++	+/-	-
GATA4-S105E	++	++	+++	++	++	++	ND	+++	ND	ND
GATA4(129-440)	++	++	++	++	++	ND	++	++	ND	ND
GATA4(153-440)	++	+/-	-	-	-	ND	-	-	ND	ND
GATA4(201-440)	++	+/-	-	+/-	-	-	ND	-	-	-

^a ND, not determined. +/-, very low; +, low; ++, strong; +++, very strong.

and/or TBX5 as the S105E mutation enhances synergy between these factors, whereas the S105A mutation has no effect. Taken together with the results from hypertrophy, cardiomyocyte survival, and biochemical assays (summarized in Table 1), we propose that regulation of GATA4 activities via phosphorylation of S105 does not operate as a simple on/off switch between activities. Instead, there appears to be a hierarchy of effects, where S105 phosphorylation is essential for promoting cell survival and is important for mediating hypertrophy but plays only a nonessential modulatory role in cardiogenesis.

In addition to their versatility in regulating multiple aspects of heart development and homeostasis, cardiac transcription factors are also important in noncardiac cell types, a property that has raised a question about the mechanisms through which they achieve cellular specificity. Our study, focusing on GATA4, suggests that specific domains and posttranslational modifications may be differentially deployed to achieve distinct cellular outputs. This would be consistent with human genetics since individuals harboring GATA4 point mutations linked to congenital heart disease do not generally show other organ defects. How different contexts determine differential utilization of GATA4 domains is not entirely clear and will undoubtedly be the focus of future research.

ACKNOWLEDGMENTS

We are grateful to Chantal Lefebvre and Janie Beaugard for technical assistance. We thank Pier Lorenzo Puri for sharing the BAF60c antibody and Ian Scott for the expression vector.

This work was supported by a grant from the Canadian Institute of Health Research to M.N. and by a project grant from the British Heart Foundation (PG07/013/22365) to B.V.L. H.K. was a CIHR fellow, and M.N. held a Canada Research Chair in Molecular Biology.

REFERENCES

- Aries A, Paradis P, Lefebvre C, Schwartz RJ, Nemer M. 2004. Essential role of GATA-4 in cell survival and drug-induced cardiotoxicity. *Proc. Natl. Acad. Sci. U. S. A.* 101:6975–6980.
- Bruneau BG, et al. 2001. A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* 106:709–721.
- Chambers AE, et al. 1994. The RSRF/MEF2 protein SL1 regulates cardiac muscle-specific transcription of a myosin light-chain gene in *Xenopus* embryos. *Genes Dev.* 8:1324–1334.
- Charron F, Paradis P, Bronchain O, Nemer G, Nemer M. 1999. Cooperative interaction between GATA-4 and GATA-6 regulates myocardial gene expression. *Mol. Cell. Biol.* 19:4355–4365.
- Charron F, et al. 2001. Tissue-specific GATA factors are transcriptional effectors of the small GTPase RhoA. *Genes Dev.* 15:2702–2719.
- Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform-extraction. *Anal. Biochem.* 162:156–159.
- Debril MB, et al. 2004. Transcription factors and nuclear receptors interact with the SWI/SNF complex through the BAF60c subunit. *J. Biol. Chem.* 279:16677–16686.
- Debrus S, et al. 2005. The zinc finger-only protein Zfp260 is a novel cardiac regulator and a nuclear effector of α 1-adrenergic signaling. *Mol. Cell. Biol.* 25:8669–8682.
- Dorey K, Hill CS. 2006. A novel Cripto-related protein reveals an essential role for EGF-CFCs in nodal signalling in *Xenopus* embryos. *Dev. Biol.* 292:303–316.
- Durocher D, Charron F, Warren R, Schwartz RJ, Nemer M. 1997. The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J.* 16:5687–5696.
- Durocher D, Nemer M. 1998. Combinatorial interactions regulating cardiac transcription. *Dev. Genet.* 22:250–262.
- Forcales SV, et al. 2012. Signal-dependent incorporation of MyoD-BAF60c into Brg1-based SWI/SNF chromatin-remodelling complex. *EMBO J.* 31:301–316.
- Garg V, et al. 2003. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature* 424:443–447.
- Grepin C, et al. 1994. A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Mol. Cell. Biol.* 14:3115–3129.
- Grepin C, Nemer G, Nemer M. 1997. Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. *Development* 124:2387–2395.
- Haworth KE, Kotecha S, Mohun TJ, Latinkic BV. 2008. GATA4 and GATA5 are essential for heart and liver development in *Xenopus* embryos. *BMC Dev. Biol.* 8:74. doi:10.1186/1471-213X-8-74.
- Ieda M, Fu JD, gado-Olguin P, Vedantham V, Hayashi Y, et al. 2010. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell* 142:375–386.
- Kadam S, Emerson BM. 2003. Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes. *Mol. Cell* 11:377–389.
- Kobayashi S, et al. 2006. Transcription factor gata4 regulates cardiac BCL2 gene expression in vitro and in vivo. *FASEB J.* 20:800–802.
- Laforest B, Nemer M. 2011. GATA5 interacts with GATA4 and GATA6 in outflow tract development. *Dev. Biol.* 358:368–378.
- Latinkic BV, Kotecha S, Mohun TJ. 2003. Induction of cardiomyocytes by GATA4 in *Xenopus* ectodermal explants. *Development* 130:3865–3876.
- Lavallee G, et al. 2006. The Kruppel-like transcription factor KLF13 is a novel regulator of heart development. *EMBO J.* 25:5201–5213.
- Liang Q, et al. 2001. The transcription factor GATA4 is activated by extracellular signal-regulated kinase 1- and 2-mediated phosphorylation of serine 105 in cardiomyocytes. *Mol. Cell. Biol.* 21:7460–7469.
- Lickert H, et al. 2004. Baf60c is essential for function of BAF chromatin remodelling complexes in heart development. *Nature* 432:107–112.
- Lou X, Deshwar AR, Crump JG, Scott IC. 2011. Smarcd3b and Gata5 promote a cardiac progenitor fate in the zebrafish embryo. *Development* 138:3113–3123.
- McBride K, Charron F, Lefebvre C, Nemer M. 2003. Interaction with GATA transcription factors provides a mechanism for cell-specific effects of c-Fos. *Oncogene* 22:8403–8412.
- Molkentin JD, Lin Q, Duncan SA, Olson EN. 1997. Requirement of the

- transcription factor GATA4 for heart tube formation and ventral morphogenesis. *Genes Dev.* 11:1061–1072.
28. Morin S, Charron F, Robitaille L, Nemer M. 2000. GATA-dependent recruitment of MEF2 proteins to target promoters. *EMBO J.* 19:2046–2055.
 29. Morin S, Paradis P, Aries A, Nemer M. 2001. Serum response factor-GATA ternary complex required for nuclear signaling by a G-protein-coupled receptor. *Mol. Cell. Biol.* 21:1036–1044.
 30. Morrisey EE, Ip HS, Tang Z, Parmacek MS. 1997. GATA-4 activates transcription via two novel domains that are conserved within the GATA-4/5/6 subfamily. *J. Biol. Chem.* 272:8515–8524.
 31. Nadeau M, et al. 2010. An endocardial pathway involving Tbx5, Gata4, and Nos3 required for atrial septum formation. *Proc. Natl. Acad. Sci. U. S. A.* 107:19356–19361.
 32. Nemer G, Nemer M. 2003. Transcriptional activation of BMP-4 and regulation of mammalian organogenesis by GATA-4 and -6. *Dev. Biol.* 254:131–148.
 33. Nemer M. 2008. Genetic insights into normal and abnormal heart development. *Cardiovasc. Pathol.* 17:48–54.
 34. Newman CS, Chia F, Krieg PA. 1997. The XHex homeobox gene is expressed during development of the vascular endothelium: overexpression leads to an increase in vascular endothelial cell number. *Mech. Dev.* 66:83–93.
 35. Oka T, et al. 2006. Cardiac-specific deletion of Gata4 reveals its requirement for hypertrophy, compensation, and myocyte viability. *Circ. Res.* 98:837–845.
 36. Olson EN. 2006. Gene regulatory networks in the evolution and development of the heart. *Science* 313:1922–1927.
 37. Pikkarainen S, et al. 2003. GATA-4 is a nuclear mediator of mechanical stretch-activated hypertrophic program. *J. Biol. Chem.* 278:23807–23816.
 38. Pu WT, Ishiwata T, Juraszek AL, Ma Q, Izumo S. 2004. GATA4 is a dosage-sensitive regulator of cardiac morphogenesis. *Dev. Biol.* 275:235–244.
 39. Rajagopal SK, et al. 2007. Spectrum of heart disease associated with murine and human GATA4 mutation. *J. Mol. Cell. Cardiol.* 43:677–685.
 40. Rysa J, et al. 2010. GATA-4 is an angiogenic survival factor of the infarcted heart. *Circ. Heart Fail.* 3:440–450.
 41. Samuel LJ, Latinkic BV. 2009. Early activation of FGF and nodal pathways mediates cardiac specification independently of Wnt/beta-catenin signaling. *PLoS One* 4:e7650. doi:10.1371/journal.pone.0007650.
 42. Shimizu R, Takahashi S, Ohneda K, Engel JD, Yamamoto M. 2001. In vivo requirements for GATA-1 functional domains during primitive and definitive erythropoiesis. *EMBO J.* 20:5250–5260.
 43. Sive HL, Grainger RM, Harland RM (ed). 2000. Early development of *Xenopus laevis*: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 44. Takeuchi JK, Bruneau BG. 2009. Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. *Nature* 459:708–711.
 45. van Berlo JH, Elrod JW, Aronow BJ, Pu WT, Molkentin JD. 2011. Serine 105 phosphorylation of transcription factor GATA4 is necessary for stress-induced cardiac hypertrophy in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 108:12331–12336.
 46. Wang J, et al. 2005. Convergence of protein kinase C and JAK-STAT signaling on transcription factor GATA-4. *Mol. Cell. Biol.* 25:9829–9844.
 47. Wang W, et al. 1996. Diversity and specialization of mammalian SWI/SNF complexes. *Genes Dev.* 10:2117–2130.
 48. Watt AJ, Battle MA, Li J, Duncan SA. 2004. GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 101:12573–12578.
 49. Weiss MJ, Yu CN, Orkin SH. 1997. Erythroid-cell-specific properties of transcription factor GATA-1 revealed by phenotypic rescue of a gene-targeted cell line. *Mol. Cell. Biol.* 17:1642–1651.
 50. Zeisberg EM, et al. 2005. Morphogenesis of the right ventricle requires myocardial expression of Gata4. *J. Clin. Invest.* 115:1522–1531.