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The Kruppel-like transcription factor KLF13 is a novel regulator of heart development

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In humans, congenital heart defects occur in 1–2% of live birth, but the molecular mechanisms and causative genes remain unidentified in the majority of cases. We have uncovered a novel transcription pathway important for heart morphogenesis. We report that KLF13, a member of the Krüppel-like family of zinc-finger proteins, is expressed predominantly in the heart, binds evolutionarily conserved regulatory elements on cardiac promoters and activates cardiac transcription. KLF13 is conserved across species and knockdown of KLF13 in *Xenopus* embryos leads to atrial septal defects and hypotrabeculation similar to those observed in humans or mice with hypomorphic GATA-4 alleles. Physical and functional interaction with GATA-4, a dosage-sensitive cardiac regulator, provides a mechanistic explanation for KLF13 action in the heart. The data demonstrate that KLF13 is an important component of the transcription network required for heart development and suggest that KLF13 is a GATA-4 modifier; by analogy to other GATA-4 collaborators, mutations in KLF13 may be causative for congenital human heart disease.

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Introduction

Congenital heart malformations represent the single largest class of birth defects in humans and are the leading cause of mortality in infants under 1 year of age (Centers for Disease Control and Prevention, 2001). Epidemiologic studies as well as linkage analyses and candidate gene approaches point to a major role of genetic determinants in congenital heart disease (CHD). Despite remarkable progress in the past decade in elucidating the genetic blueprint of the heart, CHD-causing gene mutations in humans have only been identified in a minority of cases. As several of these genes encode transcription factors that are key regulators of cardiac development and gene expression (reviewed by Clark *et al*, 2006), furthering our understanding of cardiac transcription will likely translate into helpful clinical tools for the prevention, diagnosis and treatment of CHD.

Identification of the genetic pathways and critical regulators of heart formation has been achieved through genetic and biochemical approaches. For example, Tbx5 was first identified as the gene mutated in Holt–Oram syndrome (Basson *et al*, 1997); Nkx2.5 was isolated as the mammalian homologue of *Drosophila tinman*, the first transcription factor genetically shown to be essential for heart formation (Bodmer, 1993; Komuro and Izumo, 1993; Lints *et al*, 1993). Nkx2.5 mutations were subsequently linked to a variety of congenital heart malformations which overlap with those observed in Holt–Oram syndrome. The finding that Tbx5 and Nkx2.5 act cooperatively to regulate common targets has provided a molecular framework for understanding how mutations in different genes can cause similar phenotypes (Bruneau *et al*, 2001). Remarkably, both Nkx2.5 and Tbx5 interact with GATA-4 (Durocher *et al*, 1997; Garg *et al*, 2003) and mutations in GATA-4 have also been linked to CHD (Garg *et al*, 2003; Nemer *et al*, 2006).

Initially identified as one of the key transcription factors required for expression of the cardiac natriuretic peptide genes NPPA (ANP, natriuretic peptide precursor A) and NPPB (BNP, natriuretic peptide precursor B) (Grépin *et al*, 1994; Charron *et al*, 1999), GATA-4 has turned out to be a critical regulator of various aspects of embryonic and postnatal heart development (Grépin *et al*, 1997; Molkentin *et al*, 1997; Charron *et al*, 2001; Crispino *et al*, 2001). *In vitro*, cardiac progenitors lacking GATA-4 fail to upregulate Nkx2.5 and other regulators of cardiogenesis; they do not differentiate into cardiomyocytes and undergo apoptosis at an early stage (Grépin *et al*, 1997). *In vivo,* mice homozygous for a targeted GATA-4 allele do not survive past embryonic day 9 and display *cardiac bifida* (Molkentin *et al*, 1997). Cell- and stage-specific gene deletion has revealed essential functions of GATA-4 in endocardial as well as myocardial differentiation (Watt *et al*, 2004; Zeisberg *et al*, 2005). Studies in zebrafish and *Drosophila* suggest that GATA-4 is a competence factor required for establishing cardiac cell fate (Serbedzija *et al*, 1998; Klinedinst and Bodmer, 2003). This essential early role of GATA-4 could be explained by the finding that it is an upstream activator of Nkx2.5 and several other transcription factors required for cardiogenesis and heart morphogenesis (Lien *et al*, 1999; McFadden *et al*, 2000). Additionally, GATA-4, along with Nkx2.5, is required for cell response to cardiac inducers like BMPs (Monzen *et al*, 1999) and is itself a transcriptional activator of BMP-4 (Nemer and Nemer, 2003). Thus, GATA-4 appears to play a central role in positive feedback loops at the earliest stages of cardiac cell fate determination. Consistent with this, upregulation of GATA-4

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enhances cardiogenesis *in vitro* (Grépin *et al*, 1997) and ectopic GATA-4 expression in *Xenopus* embryonic ectoderm is sufficient to induce cardiac differentiation (Latinkic *et al*, 2003).

GATA-4 is also an essential dosage-sensitive regulator of postnatal cardiomyocyte survival and homeostasis (Charron *et al*, 1999, 2001; Aries *et al*, 2004). Mice with 70% reduction in GATA-4 display cardiac malformations and reduced cardiomyocyte proliferation (Pu *et al*, 2004), whereas mice with 50% reduction survive into adulthood but have impaired cardiac stress response and compromised myocyte survival (Aries *et al*, 2004). Consistent with this dosage sensitivity, genetic studies revealed that, in addition to Nkx2.5 and Tbx5, other GATA-4 collaborators such as FOG2 are essential regulators of heart morphogenesis (Tevosian *et al*, 2000). A knock-in mutation in GATA-4 that disrupts its interaction with FOG2 results in severe cardiac defects reminiscent of those observed in mice with reduced GATA-4 levels (Crispino *et al*, 2001). The importance of protein:protein interaction for GATA-4 function is further evidenced by the evolutionary conservation of several of them in *Drosophila* where the activity of the GATA-4 orthologue Pannier is modulated through interaction with the *Drosophila* FOG protein U-shaped (Fossett *et al*, 2001) and the NK protein *Tinman* (Gajewski *et al*, 1999). As a consequence, modulators of GATA-4 levels or activity, including interacting partners, can be predicted to play important roles in cardiogenesis.

In this study, we used the BNP promoter to identify a novel GATA-4 interacting pathway critical for cardiac gene transcription and heart development. The results identify a new GATA-4 interacting pathway and reveal a role for members of the Krüppel-like transcription factor (KLF) family of zinc-finger proteins in heart morphogenesis.

Results

A CACCC box-containing element is required for cardiac transcription

Previously, we showed that the proximal promoter of the gene coding for the cardiac BNP is sufficient for maximal cardiac transcription (Grépin *et al*, 1994). Other than the GATA elements, sequence alignment revealed the presence of an evolutionarily conserved CACCC box centered at −75 bp (Figure 1A). This motif lies within a highly conserved DNA fragment and is flanked by a GATA element on one side and a YY1 binding site (Bhalla *et al*, 2001) on the other side (Figure 1A). Deletion or mutation of the CACCC site significantly decreased BNP promoter activity in postnatal cardiomyocytes consistently, resulting in 20% residual activity in atrial cells and 50% activity in ventricular cells (Figure 1B). These results identified the CACCC box as an important cardiac regulatory element. Moreover, the more pronounced contribution of this element in atrial versus ventricular cardiomyocytes suggested that its cognate binding protein is asymmetrically expressed in heart chambers or that it functionally interacts with other regulators in a chamber-specific manner.

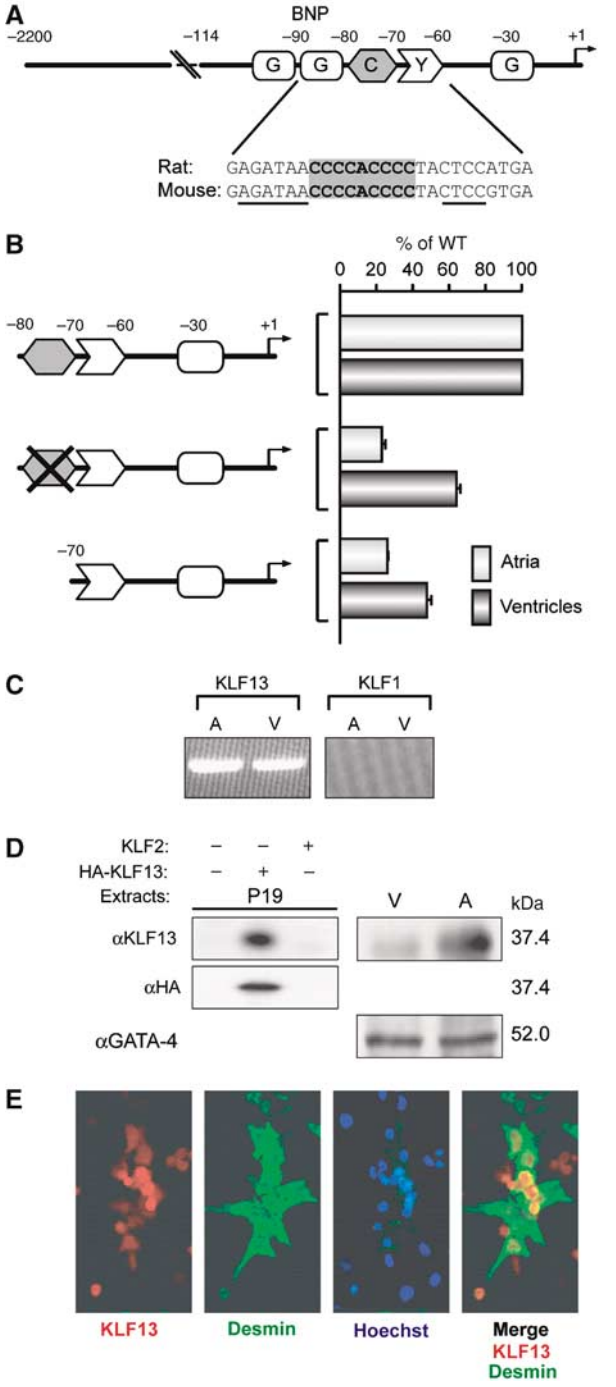

Figure 1 Identification of a novel transcription pathway in cardiomyocytes. (**A**) Schematic representation of the BNP promoter showing the conservation of the GATA-CACC-YY1 elements. G = GATA; C = CACC; Y = YY1. (**B**) Transient transfections of BNP-luc reporters in primary neonate atrial or ventricular cardiomyocyte cultures. The results are the mean ± s.d. of at least four independent duplicate experiments. (**C**) RT–PCR amplification of KLF1 and KLF13 transcripts from RNA obtained from atrial (A) or ventricular (V) cardiomyocyte cultures. (**D**) Western blot analysis of recombinant HA-KLF13 in P19 cells (left panel) or in primary cardiomyocyte extracts (right panel). (**E**) Immunohistochemical colocalization of KLF13 and desmin in primary cardiomyocyte cultures.

A tissue-specific member of the KLF family is a transcriptional activator of cardiac genes

CACCC boxes were first recognized as tissue-specific regulatory elements on erythroid genes, where they recur in combination with GATA and NF-E2 motifs (Walters and Martin, 1992; Cantor and Orkin, 2002). This was followed by the isolation and characterization of erythroid Krüppel-like factor (EKLF/KLF1). KLF1, the CACCC box-interacting protein in

erythroid cells (Nuez *et al*, 1995; Perkins *et al*, 1995), was the founding member of a large family of zinc-finger-containing transcription factors, whose important roles in cell differentiation and proliferation are being elucidated (Suske *et al*, 2005). *In silico* searches of EST databases identified KLF13 as potentially the most relevant member in the heart. Published reports of KLF13 expression are not conclusive and its function remains undefined. Northern blot analysis showed restricted expression in cardiac and skeletal muscle (Asano *et al*, 1999), but RT–PCR analysis detected KLF13 transcripts in several mouse tissues (Scohy *et al*, 2000). Whether this broader pattern reflects low-level expression in blood vessels is unclear (Asano *et al*, 2000; Song *et al*, 2002; Martin *et al*, 2003). During mouse embryonic development, KLF13 was found to be highly expressed in the heart and the cephalic mesenchyme (Martin *et al*, 2001).

Analysis of KLF13 transcripts confirmed expression in atrial and ventricular cardiomyocytes (Figure 1C). To further establish the ontogeny and spatial distribution of KLF13 in the developing mouse embryo, a rabbit anti-KLF13 antibody was generated against the N-terminal 135 aa, which is the most divergent domain across the various KLF family members (less than 30% homology). Western blot analysis confirmed that this antibody specifically recognized recombinant KLF13 but not other family members (Figure 1D and data not shown). The antibody also detected endogenous KLF13 in nuclear cardiomyocyte extracts and showed higher abundance in atrial versus ventricular cells (Figure 1D, right panel); the prevalence of KLF13 in atrial cells may thus explain the more important contribution of the CACCC box to BNP promoter activity in atrial cardiomyocytes (Figure 1B). The presence of KLF13 within cardiomyocytes was further confirmed by the localization of nuclear KLF13 within desmin-positive cells and in desmin-negative cardioblasts (Figure 1E).

Spatiotemporal expression of KLF13 was analyzed using histological sections of staged murine embryos (Figure 2). Immunoreactivity was detected at E9.5, mostly in the heart and the epidermis (data not shown). At E10.5, KLF13 staining was found predominantly in the atrial myocardium and endocardial layer (Figure 2A). By E12.5, staining was evident in atria and ventricles (Figure 2B), but the signal remained stronger in the atria (Figure 2B, middle panel). Within the ventricle, KLF13-positive cells were present predominantly in the trabeculae (Figure 2B, right panel) and this pattern persisted at later stages (Figure 2C). In addition, KLF13 immunoreactivity was evident in the cardiac cushions of the atrioventricular region (Figure 2A, right panel, and Figure 2D) and the truncus arteriosus (data not shown). Postnatally, KLF13 was downregulated, but positive cells were still detected within the atrial and ventricular myocardium but the highest expression was in the valves and the interventricular septum (Figure 2E).

As development progressed, KLF13 was also found outside the heart. Apart from epithelial cells (Figure 2H), KLF13 was present in brain mesenchyme (Figure 2F), dorsal ganglions (Figure 2I) and skeletal muscles (Figure 2J). At E14.5, the endothelial cells of the vascular vessels in the liver started showing KLF13 immunoreactivity (Figure 2G). This expression pattern is consistent with that previously reported for KLF13 transcripts during murine embryogenesis (Martin *et al*, 2001).

Next, we examined the transcriptional properties of KLF13. P19 cell extracts showed endogenous binding to the BNP CACCC probe, which was greatly enhanced in extracts overexpressing KLF13 and was efficiently eliminated by the addition of cold self but not mutated probe (Figure 3A). No specific DNA–protein complex could be detected on the probe harboring mutation in the CACCC box (Figure 3A, right panel). The CACCC probe also bound proteins present in cardiomyocyte extracts, which co-migrated with the CACCC/KLF13 complex and were abrogated by addition of the anti-KLF13 antibody (Figure 3B, right panel).

Next, the ability of KLF13 to act as a transcriptional activator of BNP was investigated using transient cotransfection assays. KLF13 dose-dependently activated the BNP promoter up to 15-fold in several non-cardiac cell types including the myoblasts C2C12 cells and NIH3T3 fibroblasts (Figure 3C and data not shown). This activation was almost abolished upon mutation of the CACCC box, whereas mutation of the neighboring YY1 motif had no adverse effect on the response to KLF13 (Figure 3C). Interestingly, cotransfection of KLF13 and BNP reporter constructs into rat atrial and ventricular cardiomyocytes revealed that KLF13 activation may be context-dependent with efficient activation achieved in atrial but not in ventricular cardiomyocytes (Figure 3D).

This result may point to differential interaction of KLF13 with coactivators or corepressors in the different heart chambers. We conclude that KLF13 is a bona fide transcription factor involved in atrial expression of the BNP gene.

KLF13 interacts functionally and physically with GATA-4

Given the sequence and spatial conservation of the GATA and CACCC elements on the BNP promoter, we tested for functional cooperativity between the GATA and KLF13 pathways. Cotransfection of a rat BNP promoter construct into P19 cells with KLF13, GATA-4 or both resulted in synergistic transcriptional activation (Figure 4A). Deletion analysis revealed that both N- and C-terminal activation domains were required for synergy with KLF13. GATA-4 binding to DNA was also required, as mutation of the C-terminal zinc-finger, which impairs DNA binding, abrogates KLF13/GATA-4 cooperativity (Figure 4A). This cooperativity reflected physical interaction between both proteins, as evidenced by co-immunoprecipitation (Figure 4B) and pull-down (Figure 4C) assays. Structure–function analysis revealed that KLF13 associates with GATA-4 mostly via its N-terminal zinc-finger, a domain previously shown to be the site of interaction with the FOG proteins (Crispino *et al*, 1999, 2001). This was evidenced by the ability of maltose binding protein (MBP)-KLF13 to retain a GATA-4 protein containing the N- but not the C-terminal zinc-finger (1–266) and a GATA-4 protein harboring a mutation in the C-terminal zinc-finger (mutZN2). On the other hand, a GATA-4 mutant containing the C-terminal zinc-finger and activation domains (244–440) failed to be retained on the MBP-KLF13 column; addition of the N-finger (200–440) restored KLF13 interaction (Figure 4C). To determine whether KLF13 and FOG2 recognize similar residues within the GATA-4 N-terminal zinc-finger, we tested the ability of KLF13 to functionally interact with GATA-4 mutants defective in FOG interaction. Residues E215 and E217 on GATA-4 are equivalent to E203 and V209 of GATA-1 within the N-terminal of the first zinc-finger. These residues were shown to be required for FOG interaction with GATA-1 (Crispino *et al*, 1999). The V217G

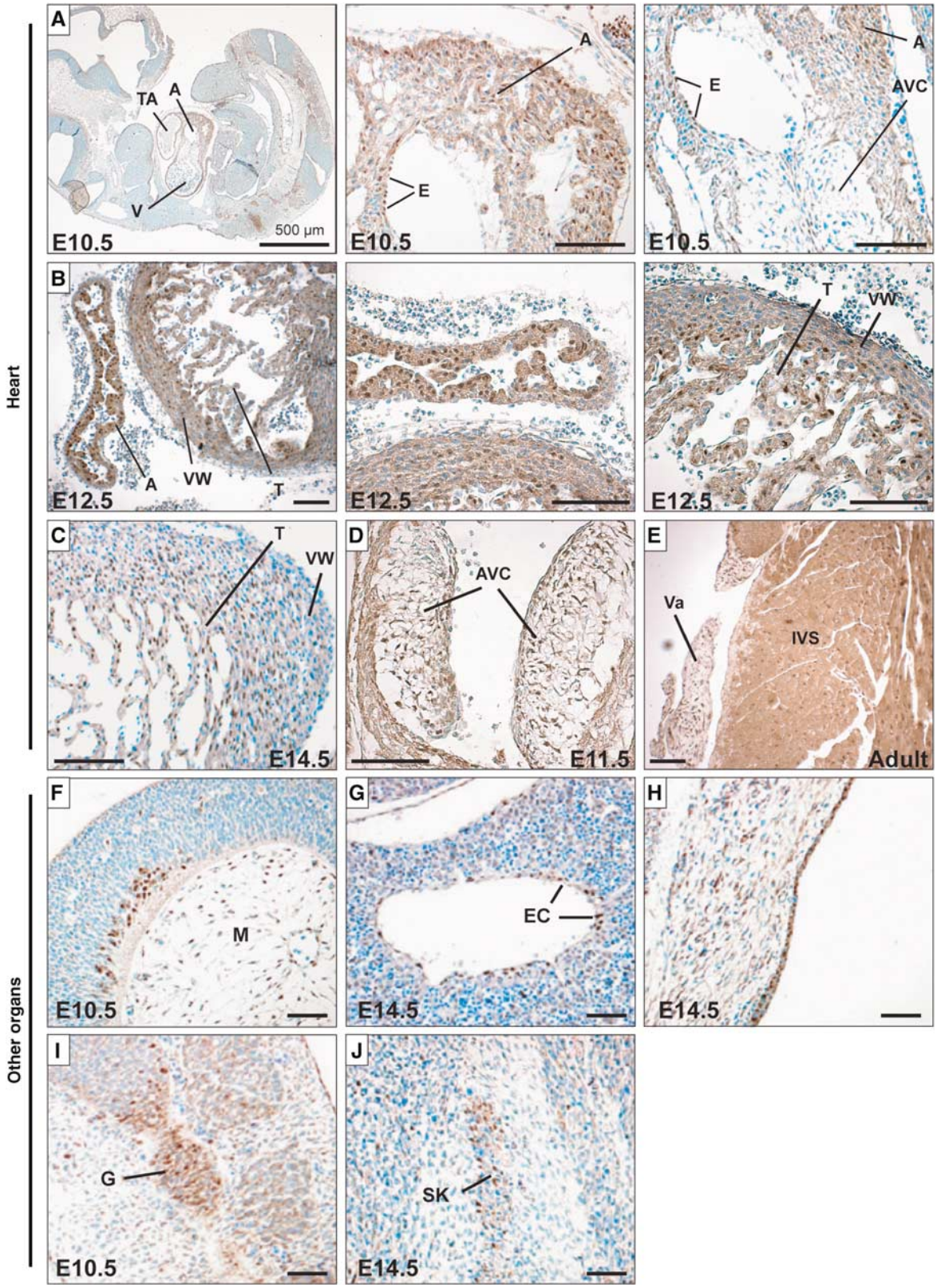

Figure 2 Spatiotemporal expression of KLF13 in mouse embryos. Immunohistochemical localization of KLF13 in staged murine embryos using immunoperoxidase staining. (**A–E**) Heart sections; (**F**) brain, (**G**) liver, (**H**) the epithelial layer of the dermis, (**I**) a dorsal ganglion and (**J**) skeletal muscle. A, atria; V, ventricles; TA, truncus arteriosus; E, endocardium; AVC, atrioventricular cushion; VW, ventricular wall; T, trabeculae; IVS, interventricular septum; Va, valve; M, mesenchyme; EC, endothelial cells; G, ganglion; SK, skeletal muscle. If not specified, scale bar represents 100 μm.

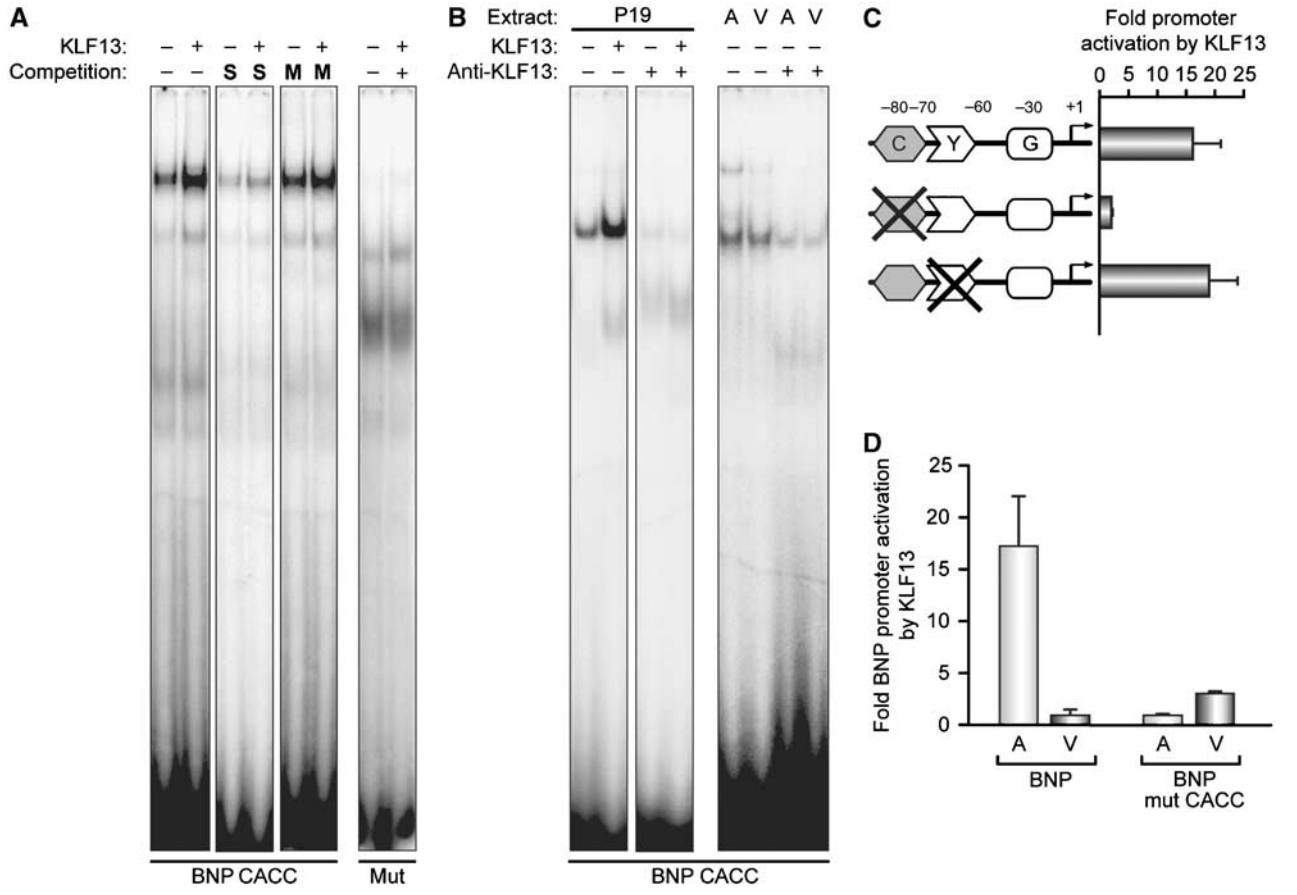

Figure 3 KLF13 is a bona fide transcriptional activator. (**A**) Binding of recombinant and endogenous KLF13 to the BNP CACCC box using P19 nuclear extracts transfected with empty or KLF13 expression vectors, and (**B**) cardiomyocyte nuclear extracts from atrial (A) or ventricular (V) cultures. KLF13 is present at low level in untransfected P19 cells and the binding is increased in cells transfected with KLF13. Binding is abrogated by the addition of 100-fold excess of unlabeled self probe (S) but not a mutated probe (M) that no longer interacts with KLF13 (right panel). (B) Cardiomyocyte nuclear extracts contain a DNA binding complex that co-migrates with that of recombinant KLF13. Note how addition of the KLF13 antibody disrupts both recombinant (middle panel) and endogenous (right panel) complex formation. (**C**) Cotransfection assays in C2C12 cells show that KLF13 can transactivate BNP-luc constructs containing an intact CACCC element. The results shown are the mean of two experiments carried out in duplicate. Similar results were obtained in NIH3T3 cells. (**D**) KLF13 is a context-dependent activator of BNP. Cotransfections were carried out in atrial (A) or ventricular (V) cardiomyocyte cultures using the wild type or CACCC mutated BNP promoter constructs shown in (C). The results are the mean$\pm$s.d. of two independent duplicate experiments carried out simultaneously on cultures prepared from the same neonate rat hearts.

mutant was also shown to abrogate FOG2–GATA-4 interaction (Crispino *et al*, 2001). As shown in Figure 4A, this mutant as well as GATA-4 E215D was as effective as the intact GATA-4 protein at synergizing with KLF13, suggesting that KLF13 and FOG2 have different recognition motifs on GATA-4. This was further confirmed by the ability of FOG2 to dose-dependently inhibit GATA-4/KLF13 synergy (Figure 4D), whereas KLF13 synergy with the V217G mutant was insensitive to FOG2 addition (Figure 4E). We conclude that KLF13 and FOG2 contact distinct residues within the N-terminal zinc-finger of GATA-4.

Finally, we tested the potential role of KLF13 as a more global regulator of cardiac transcription. Several other cardiac promoters that are known GATA-4 targets were found to contain CACCC motifs within their regulatory sequences. This includes the ANP promoter, which contains three evolutionarily conserved CCACC boxes centered around -535, -515 and -360 bp. Interestingly, the -360 motif (CCCACACCCA) maps to a cardiac-specific *cis*-element (McBride *et al*, 1993). The proximal α-cardiac actin promoter and also the β-myosin heavy chain proximal and distal enhancers, which contribute to basal cardiac activity as well as to α1-adrenergic response, contain CCACA motifs (Kariya *et al*, 1993). In transient cotransfection assays, KLF13 alone activated transcription from the ANP, α-cardiac actin and β-myosin heavy chain promoters by 8- to 12-fold (Figure 4F and data not shown). The simultaneous addition of KLF13 and GATA-4 leads to superactivation of all three promoters (Figure 4F and data not shown). Interestingly, KLF13 was also able to physically (Figure 4C) and functionally (Figure 4F) interact with the other cardiac GATA factor, GATA-6, to synergistically activate these promoters. Together, the data indicate that KLF13 is a novel transcriptional activator of cardiac genes and a collaborator of cardiac GATA factors. Given the essential role of GATA-4 in the heart, this raised the possibility that KLF13 may be involved in heart development.

Isolation of Xenopus KLF13 cDNA

To obtain insight into the functional role of KLF13 in early heart development, we turned to the amphibian *Xenopus laevis*. However, as *X. laevis* KLF13 had not been cloned, we used an *in silico* approach to first identify the *Xenopus tropicalis* KLF13. The mouse KLF13 sequence was used to

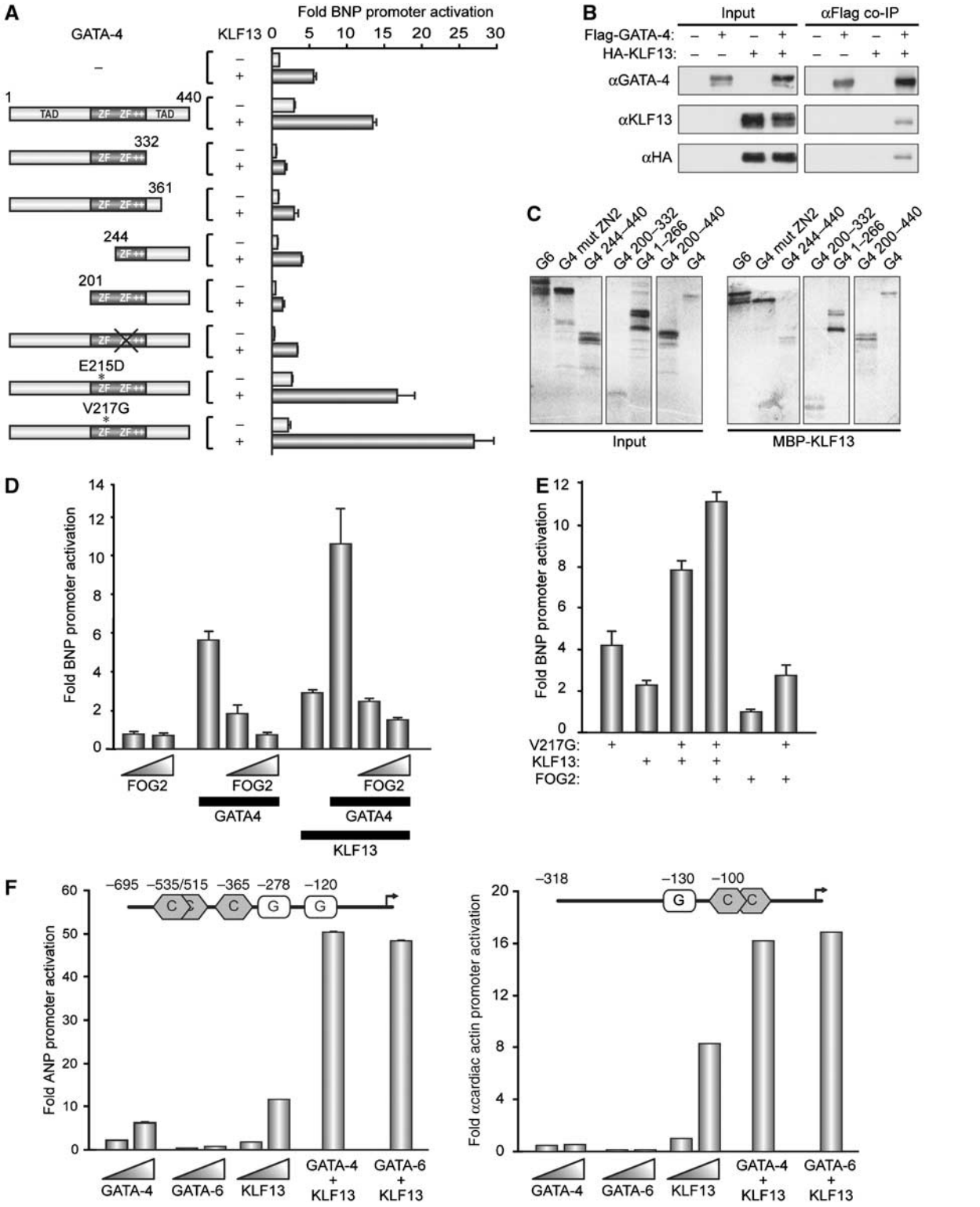

Figure 4 KLF13 is a GATA-4 interacting partner. (**A**) Cotransfections were carried out in NIH3T3 cells using 1.5 μg of BNP-luc reporter, 10 ng of GATA-4 and 50 ng of KLF13 expression vectors. The data shown are the mean of $n=4\pm$s.d. All GATA-4 mutants express equivalent levels of nuclear HA-tagged proteins that bind DNA (McBride *et al*, 2003). (**B**) Co-immunoprecipitations were carried out using 293T cells expressing Flag-GATA-4 and HA-KLF13 proteins alone or in combination. The left panel shows Western blots of the nuclear extracts used, with the indicated antibodies. The right panel is a Western blot of the anti-Flag immunoprecipitates. (**C**) Pull-down assays using *in vitro*-translated wild-type and mutant GATA-4 (G4) and GATA-6 (G6) proteins. The two bands observed with GATA-6 correspond to alternate ATG usage (Brewer *et al*, 2002). Note how GATA-4_{1-266}, which contains the N- but not the C-terminal zinc-finger, is retained on the MBP-KLF13 column. The results shown were reproduced on three different occasions. (**D, E**) KLF13 and FOG2 recognize distinct domains within the N-terminal zinc-finger of GATA-4. Cotransfections were carried out as in (A). (**F**) KLF13 activation of the ANP (left) and cardiac actin (right) promoters in NIH3T3 cells. The luciferase reporters used are shown schematically. G is a GATA site and C is a CACCC box.

search the *X. tropicalis* genome assembly for a KLF13 homologue and the information was used to isolate a *Xenopus leavis* KLF13 cDNA. During the course of this study, a second *X. laevis* KLF13 allele was identified in the Japanese EST database XDB. The deduced protein sequence of both alleles of XKLF13 shares 61% overall identity with murine KLF13 (Figure 5A). Chromosomal synteny and intraspecies phylogeny were consistent with the identification of this KLF cDNA as the *Xenopus* homologue of KLF13. Alignment of mouse and *X. tropicalis* genomic regions using VISTA browser revealed almost perfect synteny, as the genes surrounding KLF13 occupy the same relative position as on human chromosome 15 (KIAA1018, MTMR10 and TRPM1) and mouse chromosome 7 (data not shown). Reciprocal BLAST analyses of *Xenopus* and mouse KLF13 proteins identified the respective homologue as the best match. This is in line with the comparison of the deduced XKLF13 protein sequence to sequences corresponding to the other *Xenopus* KLF family

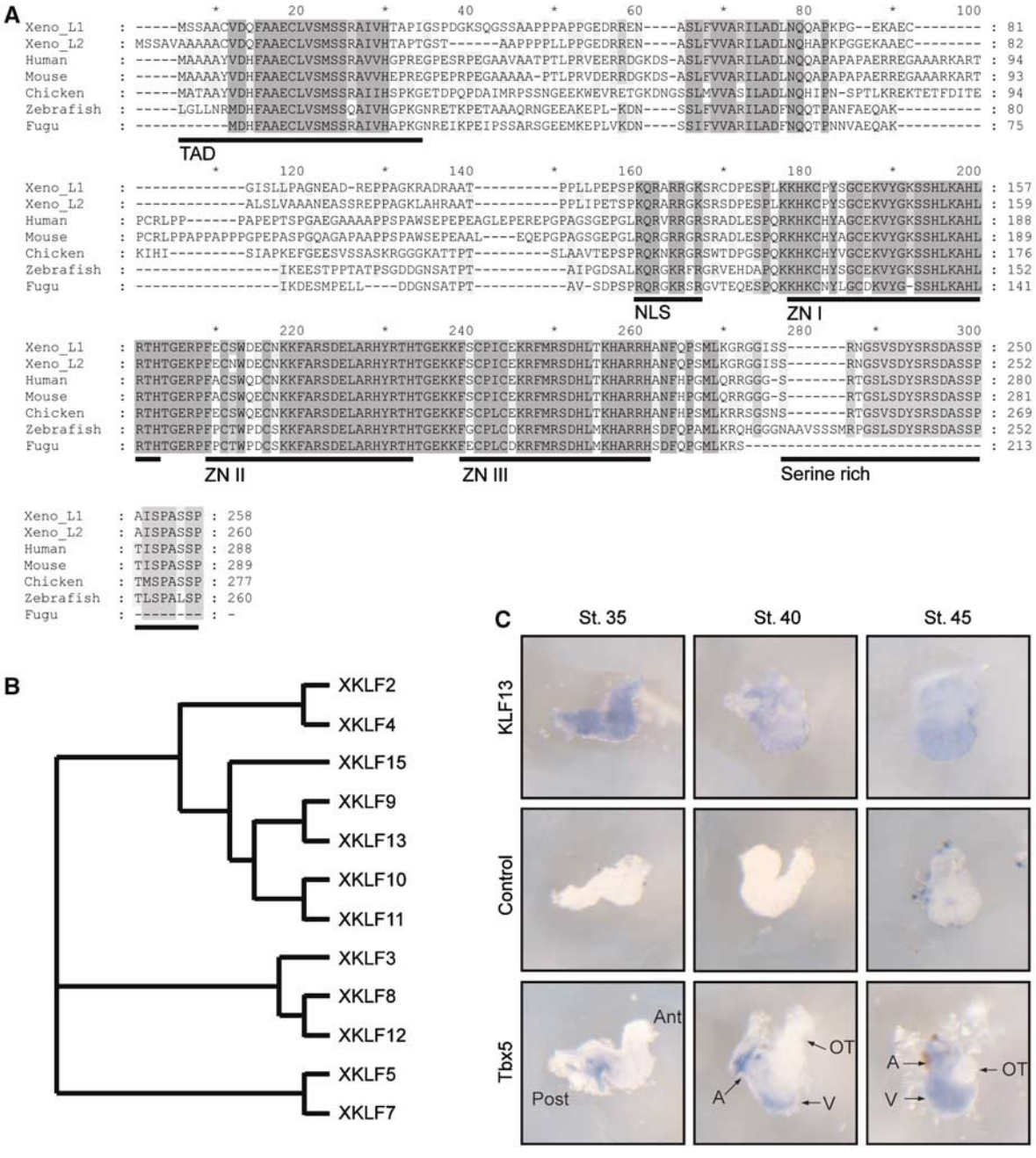

Figure 5 Evolutionary conservation of KLF13. (**A**) Alignment of the deduced *Xenopus* KLF13 protein with KLF13 orthologues. Xeno-L1 is the isoform cloned in the lab. Xeno-L2 is the one from the *X. leavis* database. The accession numbers are as follows: Xeno_L1, ENSXETT00000035943; Xeno_L2, Contig032020 from NIBB; human, NM_015995; mouse, NM_021366; chicken, ENSGALT00000006042.1; zebrafish, ENSDART00000047819. The % identity between *Xenopus* KLF13 and KLF13 orthologues is as follows: mouse, 63%; human, 59%; zebrafish, 59%; chicken, 61%. TAD, transactivation domain; NLS, nuclear localization signal; zn, zinc-finger. AD and NLS are based on Song *et al* (2002). (**B**) Phylogenetic tree of *Xenopus* KLF homologues. Note that XKLF13 is most closely related to XKLF9. (**C**) XKLF13 expression during *Xenopus* heart development using whole-mount *in situ* hybridization. The embryonic stages are shown above each panel. The control is hybridization with XKLF13 sense probe. Post, posterior; Ant, anterior; A, atria; V, ventricle; OT, outflow tract.

members, which revealed that XKLF13 is most closely related to XKLF9 (overall identity 45%) than to any other KLF (Figure 5B). This phylogenetic relation is identical to that observed for mammalian KLF13 and KLF9 (Suske *et al*, 2005).

Next, we examined the expression pattern of XKLF13 during heart development using whole-mount *in situ* hybridization. Initial expression of KLF13 is detected at late neurula stages in the eyes and, at lower levels, in the anterior neural tube. At later tail bud stages, expression continues in these regions and at stage 25, expression is detected in the pharyngeal endoderm (data not shown). By stage 35, KLF13 is expressed throughout the heart tube (Figure 5C). At stage 40–42, expression remains throughout the entire heart, including the outflow tract, the two atria and the common ventricle. Starting at stage 45, KLF13 expression is downregulated in the myocardial compartments and by stage 47, expression mainly persists in the atrioventricular valves (Figure 5C and data not shown). This expression pattern resembles the murine pattern, except for the predominant expression in the A-V valve, which is observed only in postnatal mouse hearts.

Knockdown of KLF13 reveals a crucial role in cardiac morphogenesis

To assess the developmental role of KLF13, we designed antisense morpholino oligonucleotides (MOs), XKMO1 and XKMO2, to target both alleles of XKLF13, which have differing 5′ ends. The ability of MOs to specifically inhibit translation of the appropriate XKLF13 allele was confirmed in *in vitro* translation assays (Figure 6A). XKMO1 injection was sufficient to produce tadpoles with disrupted heart development in a dose-dependent manner: with 5, 10 and 20 ng, respectively, 35, 71 and 91% of the embryos had a cardiac phenotype. XKMO2 was slightly less effective with a maximum of 63% tadpoles presenting a cardiac phenotype possibly owing to its lower efficiency at inhibiting translation (Figure 6A, top panel). A combination of both morpholinos (10 ng each) produced similar but more consistent results than a 20 ng dose of XKMO1. Embryos were injected with the morpholinos at either the one-cell stage or into the dorsolateral marginal zone at the four-cell stage with equivalent results. XKMO-injected embryos began development normally and no defects were observed during gastrulation and neurulation. However, by stage 46, they exhibited smaller as well as slower beating hearts and pericardial edema (Figure 6B and Supplementary video). In extreme phenotypes, the heart only developed as a string-like muscle by stage 40, 2 days before appearance of pericardial edema. Histological sections of the tadpole hearts at this stage revealed smaller ventricles with abnormal patterning of the myocardium (Figure 6C). This phenotype was specific and was not observed in embryos injected with control (Figure 6C) or XKLF4 MO (data not shown), consistent with the phenotype of KLF4 null mice that have normal hearts (Segre *et al*, 1999). The cardiac phenotypes, subdivided into normal, mild and severe, were consistently obtained in 10 independent experiments by two different experimenters. Mild phenotypes were defined as conserved morphological structures (atria, ventricle and outflow), which are smaller than normal and with an enlarged pericardial space indicative of pericardial edema. Severe phenotypes were defined as beating tissues without clear morphological demarcations (Figure 6C). Coinjection of mKLF13 rescued the morpholino-induced phenotype in a dose-dependant manner, with 200 pg being the lowest effective and 400 pg being the optimal rescue dose (Figure 6D).

To identify at what stage KLF13 functions in heart development, we examined tadpoles at different developmental stages (Figure 6E). Heart defects were present as early as stage 29, shortly after fusion of the two lateral heart fields and formation of the single linear heart tube. In XKMO-injected embryos, the heart tube had not formed properly and the cardiac mesoderm seemed less organized. At stage 35, the XKLF13-depleted embryos underwent heart looping normally, but had delayed resorption of the cardiac jelly in the myoendocardial space. A striking difference was seen at stage 42 when trabeculation of the ventricles is initiated. In KLF13-depleted embryos, there was complete lack of trabeculation and, in addition, the atrial septum was impaired. By stage 47, XKMO-injected embryos exhibited a smaller, hypotrabeculated ventricle and atrial septal defects. Moreover, atrioventricular cushion formation and maturation of the valves were delayed and coarser in KLF13-depleted embryos (Figure 6E and data not shown).

To determine the molecular changes that occur upon KLF13 depletion, we examined the expression of several cardiac markers using whole-mount *in situ* hybridization and real-time PCR. Examination of stage 25 (during cardiac crescent fusion) and 30 embryos (this is when we first detect histological abnormalities) revealed an almost complete absence of GATA-4, GATA-5 and Tbx5 gene expression (Figure 7A and data not shown). At this stage, these genes are normally expressed in the ventral midline demarcating the initial linear heart. Interestingly, we did detect some GATA-6-expressing cells, although at a much reduced level compared to control. To quantitate these changes temporally, we examined the levels of several heart markers using real-time PCR. GATA-4, GATA-5 and GATA-6 as well as Nkx2.5 and Tbx5 transcripts were reduced in KLF13-depleted embryos starting from stage 20, 14 h before any histological abnormalities (Figure 7B). Expression of other cardiac genes, including ANP and the atrial isoform of myosin light chain (αMLC), was severely reduced and delayed in KLF13-depleted embryos (Figure 7B). Interestingly, by stage 42, expression of αMLC and ANP was upregulated, reflecting cardiac dysfunction. These results point to a role for KLF13 in cardiac cell differentiation. Additionally, the phenotype of embryos in which KLF13 was knocked down—small, hypoplastic hearts—suggested a role for KLF13 in cardiac cell survival and/or proliferation. TUNEL assays were carried out on stage 35–40 embryos, but revealed no significant increase in cardiac cell apoptosis in XKLF13-depleted embryos (data not shown). Although an earlier effect on cardioblast survival cannot be excluded, the results pointed to a role for KLF13 in cell proliferation. Consistent with this, we found that KLF13 was a potent transactivator of the cyclin D1 promoter (Figure 7C), which contains several KLF binding motifs (Zhao *et al*, 2003). Moreover, GATA-4 potentiated KLF13 activation of the cyclin D1 promoter (Figure 7C). Together, the results suggest that KLF13 and GATA-4 act cooperatively to regulate cardiac development and morphogenesis. Consistent with the hypothesis that KLF13 and GATA-4 are mutual cofactors, injection of GATA-4 RNA rescued the

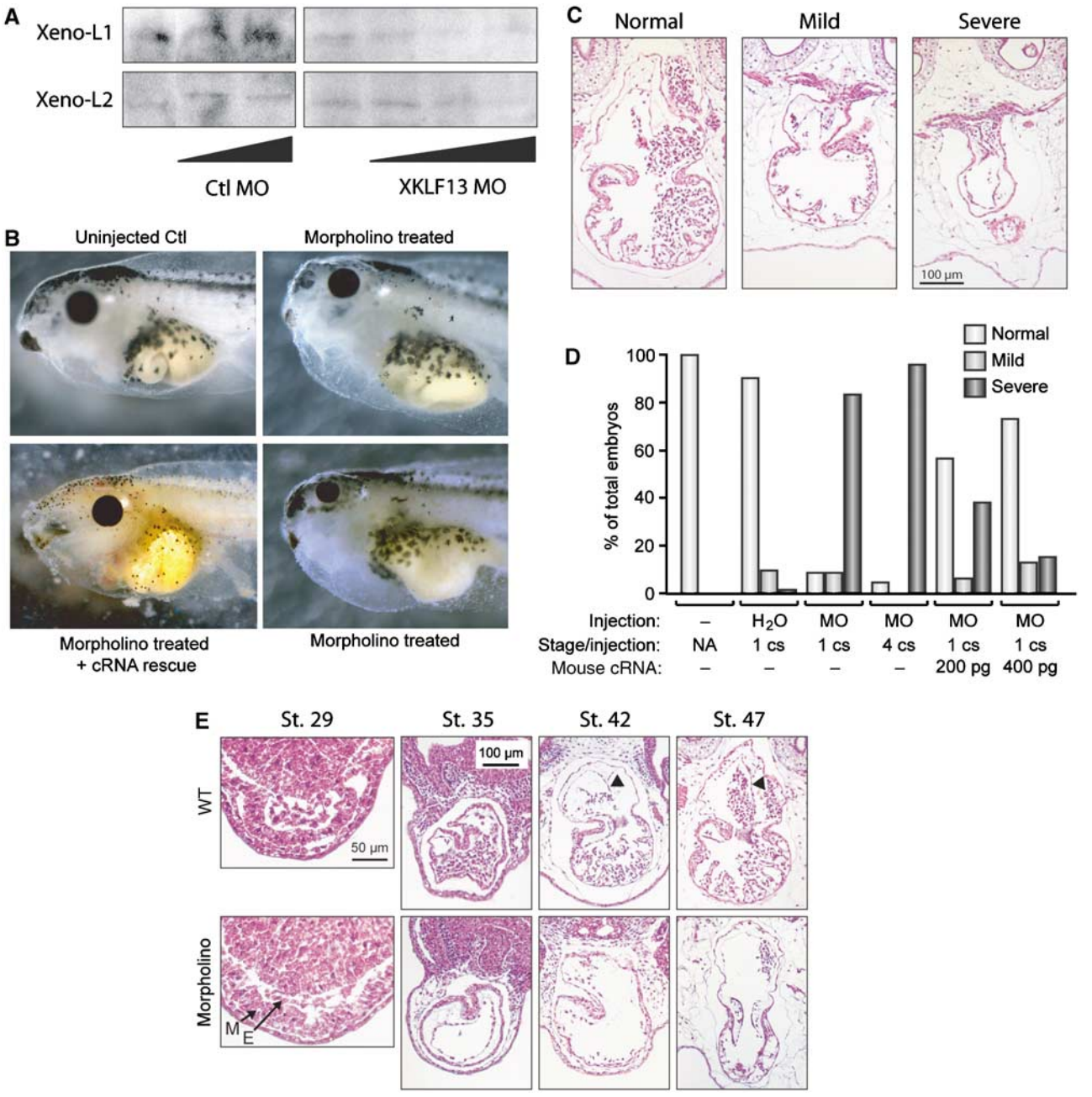

Figure 6 Functional analysis of XKLF13 in *Xenopus* heart development. (**A**) *In vitro* translation of the two XKLF13 alleles in the presence of increasing concentrations of control (100 and 200 ng) or the corresponding MO (50, 100 and 200 ng). (**B–E**) Morpholino knockdown of XKLF13 disrupts normal heart development. Note the absence of discernable cardiac structure and pericardial edema in morpholino-treated embryos (B). (C) Histological section of stage-47 hearts from control and morpholino-treated embryos. Note the absence of the atrial septum and ventricular trabeculation in XKLF13 morpholino-treated embryos. In severe cases, the heart is also smaller. (D) mKLF13 is able to rescue the KLF13 morpholino phenotype. (E) Histology of control and XKLF13-depleted embryos at different developmental stages. Note the thin myocardium and hypotrabeculation in XKLF13 morpholino-injected embryos. The arrowhead points to the atrial septum, which is absent in morpholino-treated embryos. E, endocardium; M, myocardium.

cardiac defects of XKMO-treated embryos in a dose-dependent manner (Figure 7D).

Discussion

In this paper, we show that KLF13, a cardiac-enriched member of a new family of transcriptional regulators, is a novel modulator of cardiac growth and differentiation. KLF13 is detected in the early forming heart and knockdown of KLF13 levels in *Xenopus* causes heart malformations. KLF13 physically interacts with GATA-4 and enhances its transactivating properties. Thus, KLF13 is part of the early regulatory network involved in heart development.

The KLF family of transcriptional regulators

KLFs are a family of DNA binding proteins containing three Cys_2-His_2 zinc-fingers with homology to the *Drosophila Krüppel* transcription factor. Initially discovered in mammals, the KLF family has 16 members that are related to the SP1-like family (Suske *et al*, 2005). Following isolation of KLF1

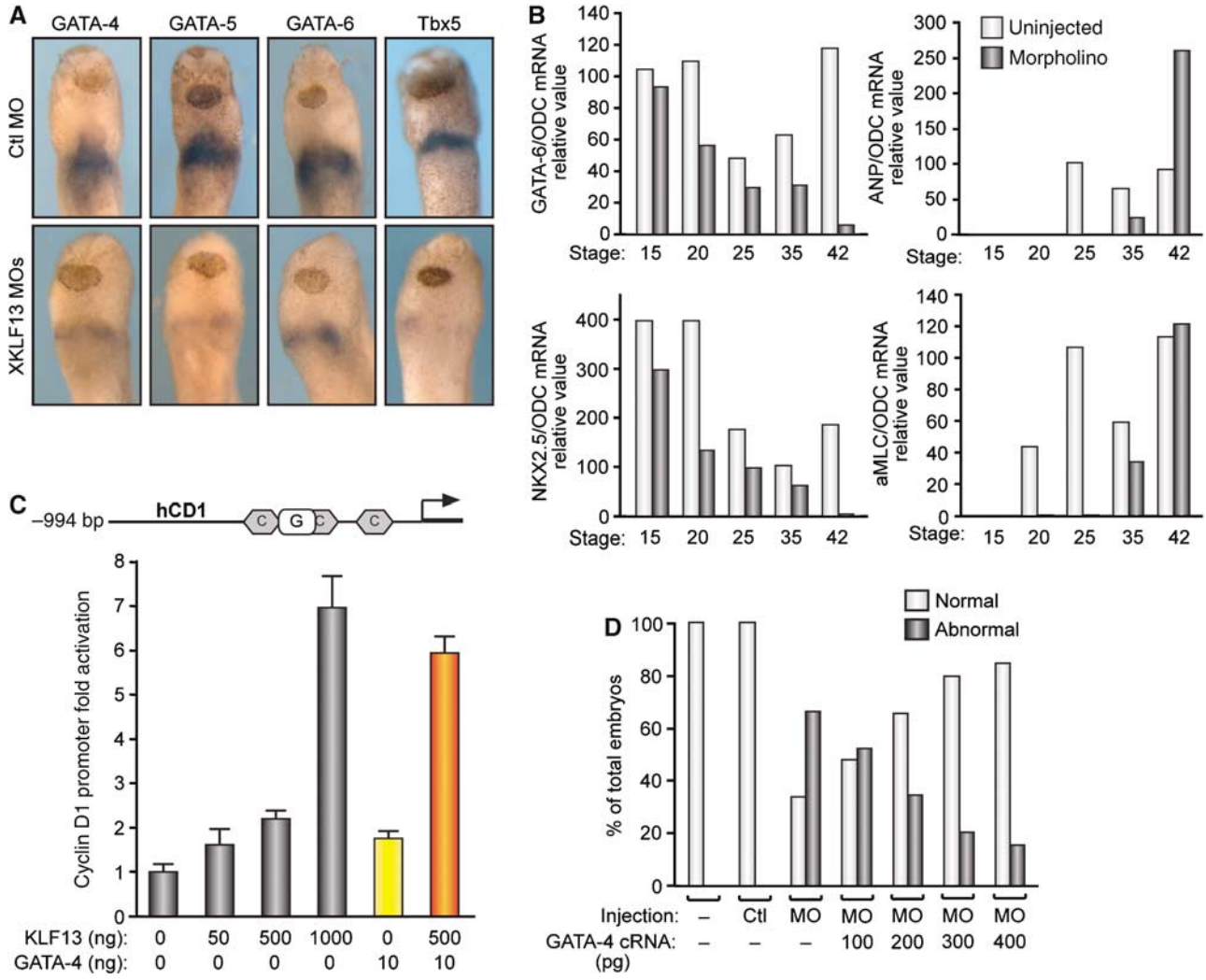

Figure 7 Characterization of the XKLF13 loss-of-function phenotype. (**A**) Whole-mount *in situ* hybridization of stage-30 embryos to the indicated probes. (**B**) Quantitative RT–PCR analysis at different developmental stages in control and XKLF13-depleted embryos. Late upregulation of ANP and aMLC is indicative of cardiac dysfunction. (**C**) Cotransfection of a human cyclin D1 (hCD1) promoter-driven luciferase reporter and KLF13 expression vectors in NIH3T3 cells. (**D**) Rescue of the cardiac phenotype in KLF13-depleted embryos by GATA-4.

(EKLF), other KLFs were identified in erythroid and non-erythroid cells, and several of them, including KLF1–5, have been shown to play important roles in differentiation and proliferation of hematopoietic, vascular and skin cells (reviewed by Bieker, 2001; Suske *et al*, 2005). A role for members of the KLF family in the cardiovascular system was first suggested when gene disruption studies revealed the importance of KLF2 for blood vessel organization (Kuo *et al*, 1997). Interestingly, another KLF member, KLF5, is abundantly expressed in developing blood vessels and its disruption leads to early embryonic lethality (Shindo *et al*, 2002). Moreover, KLF5 expression in smooth muscle cells is upregulated following vascular injury and KLF5 +/− mice have reduced arterial-wall thickening and cardiac remodeling in response to external stress (Shindo *et al*, 2002). Thus, although more than one KLF protein is often present in a given cell type, they appear to play important non-redundant functions therein. The work presented in this paper extends the role of KLF proteins in the cardiovascular system to the heart and reveals, for the first time, a role for a member of this family in heart morphogenesis. Thus, KLFs may have broad significance in cardiovascular development and disease.

CACCC boxes and cardiac transcription

The genetic identification over the past few years of a growing number of transcription factors that participate in heart development has underscored the complexity of the transcriptional networks governing cardiogenesis. In parallel, analysis of cardiac promoters revealed unexpected complexities, as transcription of individual genes appears to be often controlled by multiple distinct spatio-temporal active regulatory domains (Argentin *et al*, 1994; Olson and Schneider, 2003; Chi *et al*, 2005). Unfortunately, relatively few regulatory *cis*-elements have been characterized on cardiac promoters and this constitutes a major impediment to furthering molecular understanding of heart development (Bruneau, 2002). More exhaustive promoter analyses in other systems have led to spectacular insights culminating in the identification of 'molecular codes' (Cantor and Orkin, 2002; Senger *et al*, 2004). In erythroid cells, the CACCC/KLF1 pathway has long been recognized as an essential component for the

developmental switch of globin genes and for proper erythropoiesis (Cantor and Orkin, 2002). The data presented here suggest that CACCC elements are also important for cardiac transcription and identify a new transcription pathway in the heart. A review of cardiac promoters revealed the presence of CACCC boxes within the regulatory domains of many of them. Other than the BNP, ANP, cardiac actin and β-myosin heavy chain promoters contain CACCC motifs within previously described enhancer regions and which were activated by KLF13 (Figure 4F and data not shown). The cardiac-specific element of the cardiac troponin C (cTnC) promoter, CEF2 (Parmacek *et al*, 1992), is composed of juxtaposed CACCC and GATA motifs. It will be interesting to determine whether KLF13 binds CEF-2 and cooperates with GATA-4 in transcriptional activation of cTnC.

Finally, the HF-1 element of the MLC-2 promoter, which targets transgenes to the heart (Zhu *et al*, 1993; Ross *et al*, 1996), contains a Mef2 site juxtaposed to a TGGG motif, the core sequence required for KLF binding. It will be worthwhile to test whether KLF13 contributes to cardiac activity of this element.

GATA–KLF interaction: from blood to heart and beyond

From the preceding discussion, the CACCC and GATA motifs appear to be recurrent elements on cardiac promoters. In this paper, we showed that KLF13 physically associates with GATA-4 resulting in cooperative activation of promoters containing these elements. Interestingly, KLF13 contacts the N-terminal zinc-finger of GATA-4. With the exception of FOG1/2, which also contact the N-terminal of GATA factors, most protein–protein interactions involve the C-terminal zinc-finger of GATA-4 (reviewed by Temsah and Nemer, 2005). This suggests that KLF13 may associate with GATA-4 complexes containing other cardiac regulators such as Nkx2.5, Tbx5 and SRF. Moreover, KLF proteins, including KLF13, associate with coactivators/corepressors such as CBP/p300, PCAF and ctBP2 (Bieker, 2001; Song *et al*, 2003). As GATA factors also interact physically with CBP/p300 (Dai *et al*, 2002), co-recruitment by GATA and KLF proteins of these coactivators may be the mechanism underlying transcriptional cooperativity.

In addition to GATA-4, KLF13 can interact physically and functionally with GATA-6 (Figure 4C and F), which is expressed in smooth as well as cardiac muscle cells (Nemer and Nemer, 2003). Together with the reported interaction of KLF1 and GATA-1 in erythroid cells (Cantor and Orkin, 2002), our findings suggest that GATA–KLF interaction may be relevant to transcriptional regulation in other cell types, most notably in smooth muscle cells and in the lung, where GATA-6 as well as members of the KLF family play important roles in differentiation and proliferation (Yang *et al*, 2002; Lepore *et al*, 2005; Suske *et al*, 2005).

Role of KLF13 in the heart and implications for CHD

During heart development, KLF13 is expressed from the heart tube stage and is present in both myocardial and endocardial cells, a pattern that overlaps that of GATA-4 (Nemer and Nemer, 2003). This colocalization together with KLF13 interaction with GATA-4 suggests that KLF13 may be part of the regulatory network required for early stages of cardiogenesis. This possibility is further supported by the phenotype of KLF13 knockdown in *Xenopus*, which produced atrial septal defects, hypotrabeculation and hypoplastic myocardium. Remarkably, these defects could be rescued by addition of GATA-4. Myocardial hypoplasia was consistently observed in mice with reduced GATA-4 levels or activity (Crispino *et al*, 2001; Pu *et al*, 2004; Zeisberg *et al*, 2005). The finding that KLF13 and GATA-4 cooperatively activate the cyclin D1 promoter suggests a role for these proteins in embryonic cardiomyocyte proliferation, which, in turn, may explain the hypoplastic and hypotrabeculated ventricular phenotype observed when KLF13 or GATA-4 levels/activity are reduced. Additionally, the data reveal a function for KLF13 and GATA-4 in cell differentiation and heart morphogenesis, especially atrial septation. Atrial septal defects were found in all patients with mutations in the C-terminal domain of GATA-4 (Garg *et al*, 2003), which is essential for functional synergy with KLF13 (Figure 4A). In this respect, the higher dosage of KLF13 in atrial versus ventricular nuclear extracts and the greater contribution of the CACCC–KLF13 pathway to BNP promoter activation in atrial cells are noteworthy. Thus, KLF13 may be a chamber-specific modifier of GATA-4. As such, KLF13 may be a novel candidate gene for human CHD, including atrial septal and valvular defects.

Materials and methods

Plasmids

BNP-luciferase (BNP) constructs and GATA-4 expression vectors were obtained as described previously (McBride *et al*, 2003). The full-length KLF13 was a generous gift of Dr Asano (GenBank accession number AF251796). The human cyclin D1 promoter-luciferase plasmid was a kind gift of Dr Nathalie Rivard (Herber *et al*, 1994). KLF13 cDNAs were cloned in-frame into pCGN-HA and pMALC to produce HA and MBP fusion proteins. Plasmids were confirmed by sequencing.

Generation of KLF13 antibody

Recombinant MBP-KLF13 6–135 was obtained as described previously (Durocher *et al*, 1997) and purified on amylose columns (New England Biolabs, Beverly, MA). The antibody against KLF13 was generated by immunizing rabbits with the recombinant MBP fusion protein and purified as described previously (Nemer and Nemer, 2003).

Cell culture and transfections

Transfections of P19, 293T, C2C12 and NIH3T3 cells were carried out as described previously (Morin *et al*, 2000). Primary cultures of cardiomyocytes from 4-day-old Sprague–Dawley rats were prepared and transfected as described previously (Aries *et al*, 2004). All experiments were repeated at least three times in duplicate.

Protein analysis

Immunocytochemistry on cells and tissue sections was performed as described (Nemer and Nemer, 2003; Aries *et al*, 2004). To detect endogenous KLF13, the antibody was used at a dilution of 1/5000 (in cell cultures) or 1/200 (on tissue sections) and revealed by an anti-rabbit biotinylated antibody diluted at 1/250 (Dimension, BA-1000) and avidin-D rhodamine diluted at 1/500 (Dimension). The anti-desmin BAY60851 antibody (Accurate chemical and scientific) was used in cardiomyocytes at a dilution of 1/200. The signal was detected with the anti-mouse Avidin-D fluorescein diluted at 1/200 (Vector). For Western blots, the KLF13 antibody was used at a dilution of 1/500 and revealed with an anti-rabbit peroxidase antibody (A-6154, Sigma) at a dilution of 1/10 000. Co-immunoprecipitations, pull-down and gel shift assays were performed as described before (Morin *et al*, 2001). The probe used corresponding to the rBNP promoter is ATAACCCCACCCCTACTC and the mutant is ATAATCCTACTCCTACTC.

Cloning of Xenopus KLF13 and microinjections

Preparation of total RNA from *X. leavis* embryos was carried out using Trizol reagent according to the manufacturer's instructions (Invitrogen). XKLF13 primers were designed based on EST sequences (http://xenopus.nibb.ac.jp/). RT–PCR-amplified gut cDNAs were subcloned in PBS vectors and sequenced. Alignments of protein sequences were obtained using MultAlin. Capped mRNA for injection was synthesized by *in vitro* transcription using the mMessage mMachine kit (Ambion). For knockdown experiments, two KLF13 MOs targeting the sequence of the first eight translated amino acids of each XKLF13 allele were used. A standard control morpholino directed against a mutated human β-globin pre-mRNA was used for control experiments (Gene Tools, Oregon, USA). Capped mRNAs and morpholinos were injected into the dorsolateral marginal zone of one- or four-cell-stage embryos (volumes 4–10 nl). All animal experiments were approved by the IRCM animal care committee.

RNA analysis

Whole-mount *in situ* hybridization on *Xenopus* embryos was carried out as previously described using the appropriate digoxigenin-labeled probes (Horb and Thomsen, 1999). *Xenopus* GATA-4, GATA-5 and GATA-6 probes were kind gifts of Todd Evans (Jiang and Evans, 1996). Real-time PCR was performed using the QuantiTect Sybr Green PCR Kit (Qiagen) and MX4000 (Stratagene).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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References

Argentin S, Ardati A, Tremblay S, Lihrmann I, Robitaille L, Drouin J, Nemer M (1994) Developmental stage-specific regulation of atrial natriuretic factor gene transcription in cardiac cells. *Mol Cell Biol* **14:** 777–790

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 USA* **101:** 6975–6980

Asano H, Li XS, Stamatoyannopoulos G (1999) FKLF, a novel Kruppel-like factor that activates human embryonic and fetal β-like globin genes. *Mol Cell Biol* **19:** 3571–3579

Asano H, Li XS, Stamatoyannopoulos G (2000) FKLF-2: a novel Kruppel-like transcriptional factor that activates globin and other erythroid lineage genes. *Blood* **95:** 3578–3584

Basson CT, Bachinsky DR, Lin RC, Levi T, Elkins JA, Soults J, Grayzel D, Kroumpouzou E, Traill TA, Leblanc-Straceski J, Renault B, Kucherlapati R, Seidman JG, Seidman CE (1997) Mutations in human TBX5 (corrected) cause limb and cardiac malformation in Holt–Oram syndrome. *Nat Genet* **15:** 30–35

Bhalla SS, Robitaille L, Nemer M (2001) Cooperative activation by GATA-4 and YY1 of the cardiac B-type natriuretic peptide promoter. *J Biol Chem* **276:** 11439–11445

Bieker JJ (2001) Kruppel-like factors: three fingers in many pies. *J Biol Chem* **276:** 34355–34358

Bodmer R (1993) The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* **118:** 719–729

Brewer A, Nemer G, Gove C, Rawlins F, Nemer M, Patient R, Pizzey J (2002) Widespread expression of an extended peptide sequence of GATA-6 during murine embryogenesis and non-equivalence of RNA and protein expression domains. *Mech Dev* **119** (Suppl 1)**:** S121–S129

Bruneau BG (2002) Transcriptional regulation of vertebrate cardiac morphogenesis. *Circ Res* **90:** 509–519

Bruneau BG, Nemer G, Schmitt JP, Charron F, Robitaille L, Caron S, Conner DA, Gessler M, Nemer M, Seidman CE, Seidman JG (2001) A murine model of Holt–Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* **106:** 721

Cantor AB, Orkin SH (2002) Transcriptional regulation of erythropoiesis: an affair involving multiple partners. *Oncogene* **21:** 3368–3376

Centers for Disease Control and Prevention (2001) Infant deaths, and infant mortality rates by race of mother and 130 selected causes of infant death http://www.cdc.gov/nchs/data/dvs/LINK01WK45.pdf

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, Tsimiklis G, Arcand M, Robitaille L, Liang Q, Molkentin JD, Meloche S, Nemer M (2001) Tissue-specific GATA factors are transcriptional effectors of the small GTPase RhoA. *Genes Dev* **15:** 2702–2719

Chi X, Chatterjee PK, Wilson III W, Zhang SX, DeMayo FJ, Schwartz RJ (2005) Complex cardiac Nkx2-5 gene expression activated by noggin-sensitive enhancers followed by chamber-specific modules. *Proc Natl Acad Sci USA* **102:** 13490–13495

Clark KL, Yutzey KE, Benson DW (2006) Transcription factors and congenital heart defects. *Annu Rev Physiol* **68:** 97–121

Crispino JD, Lodish MB, Mackay JP, Orkin SH (1999) Use of altered specificity mutants to probe a specific protein–protein interaction in differentiation: the GATA-1:FOG complex. *Mol Cell* **3:** 219–228

Crispino JD, Lodish MB, Thurberg BL, Litovsky SH, Collins T, Molkentin JD, Orkin SH (2001) Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. *Genes Dev* **15:** 839–844

Dai YS, Cserjesi P, Markham BE, Molkentin JD (2002) The transcription factors GATA4 and dHAND physically interact to synergistically activate cardiac gene expression through a p300-dependent mechanism. *J Biol Chem* **277:** 24390–24398

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

Fossett N, Tevosian SG, Gajewski K, Zhang Q, Orkin SH, Schulz RA (2001) The Friend of GATA proteins U-shaped, FOG-1, and FOG-2 function as negative regulators of blood, heart, and eye development in *Drosophila*. *Proc Natl Acad Sci USA* **98:** 7342–7347

Gajewski K, Fossett N, Molkentin JD, Schulz RA (1999) The zinc finger proteins Pannier and GATA4 function as cardiogenic factors in *Drosophila*. *Development* **126:** 5679–5688

Garg V, Kathiriya IS, Barnes R, Schluterman MK, King IN, Butler CA, Rothrock CR, Eapen RS, Hirayama-Yamada K, Joo K, Matsuoka R, Cohen JC, Srivastava D (2003) GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature* **424:** 443–447

Grépin C, Dagnino L, Robitaille L, Haberstroh L, Antakly T, Nemer M (1994) A hormone-encoding gene identifies a pathway for cardiac but not skeletal muscle gene transcription. *Mol Cell Biol* **14:** 3115–3129

Grépin C, Nemer G, Nemer M (1997) Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. *Development* **124:** 2387–2395

Herber B, Truss M, Beato M, Muller R (1994) Inducible regulatory elements in the human cyclin D1 promoter. *Oncogene* **9:** 1295–1304

Horb ME, Thomsen GH (1999) Tbx5 is essential for heart development. *Development* **126:** 1739–1751

Jiang Y, Evans T (1996) The *Xenopus* GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. *Dev Biol* **174:** 258–270

Kariya K, Farrance IK, Simpson PC (1993) Transcriptional enhancer factor-1 in cardiac myocytes interacts with an alpha 1-adrenergic- and beta-protein kinase C-inducible element in the rat beta-myosin heavy chain promoter. *J Biol Chem* **268:** 26658–26662

Klinedinst SL, Bodmer R (2003) Gata factor Pannier is required to establish competence for heart progenitor formation. *Development* **130:** 3027–3038

Komuro I, Izumo S (1993) Csx: a murine homeobox-containing gene specifically expressed in the developing heart. *Proc Natl Acad Sci USA* **90:** 8145–8149

Kuo CT, Morrisey EE, Anandappa R, Sigrist K, Lu MM, Parmacek MS, Soudais C, Leiden JM (1997) GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev* **11:** 1048–1060

Latinkic BV, Kotecha S, Mohun TJ (2003) Induction of cardiomyocytes by GATA4 in *Xenopus* ectodermal explants. *Development* **130:** 3865–3876

Lepore JJ, Cappola TP, Mericko PA, Morrisey EE, Parmacek MS (2005) GATA-6 regulates genes promoting synthetic functions in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* **25:** 309–314

Lien CL, Wu C, Mercer B, Webb R, Richardson JA, Olson EN (1999) Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. *Development* **126:** 75–84

Lints TJ, Parsons LM, Hartley L, Lyons I, Harvey RP (1993) Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development* **119:** 419–431

Martin KM, Ellis PD, Metcalfe JC, Kemp PR (2003) Selective modulation of the SM22alpha promoter by the binding of BTEB3 (basal transcription element-binding protein 3) to TGGG repeats. *Biochem J* **375:** 457–463

Martin KM, Metcalfe JC, Kemp PR (2001) Expression of Klf9 and Klf13 in mouse development. *Mech Dev* **103:** 149–151

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

McBride K, Robitaille L, Tremblay S, Argentin S, Nemer M (1993) Fos/jun repression of cardiac-specific transcription in quiescent and growth stimulated myocytes is targeted at a tissue-specific *cis*-element. *Mol Cell Biol* **13:** 600–612

McFadden DG, Charité J, Richardson JA, Srivastava D, Firulli AB, Olson EN (2000) A GATA-dependent right ventricular enhancer controls dHAND transcription in the developing heart. *Development* **127:** 5331–5341

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

Monzen K, Shiojima I, Hiroi Y, Kudoh S, Oka T, Takimoto E, Hayashi D, Hosoda T, Habara-Ohkubo A, Nakaoka T, Fujita T, Yazaki Y, Komuro I (1999) Bone morphogenetic proteins induce cardiomyocyte differentiation through the mitogen-activated protein kinase TAK1 and cardiac transcription factors Csx/Nkx-2.5 and GATA-4. *Mol Cell Biol* **19:** 7096–7105

Morin S, Charron F, Robitaille L, Nemer M (2000) GATA-dependent recruitment of MEF2 proteins to target promoters. *EMBO J* **19:** 2046–2055

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

Nemer G, Fadlalah F, Usta J, Nemer M, Dbaibo G, Obeid M, Bitar F (2006) A novel mutation in the *GATA4* gene in patients with tetralogy of Fallot. *Hum Mutat* **27:** 293–294

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

Nuez B, Michalovich D, Bygrave A, Ploemacher R, Grosveld F (1995) Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. *Nature* **375:** 316–318

Olson EN, Schneider MD (2003) Sizing up the heart: development redux in disease. *Genes Dev* **17:** 1937–1956

Parmacek MS, Vora AJ, Shen T, Barr E, Jung F, Leiden JM (1992) Identification and characterization of a cardiac-specific transcriptional regulatory element in the slow/cardiac troponin C gene. *Mol Cell Biol* **12:** 1967–1976

Perkins AC, Sharpe AH, Orkin SH (1995) Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. *Nature* **375:** 318–322

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

Ross RS, Navankasattusas S, Harvey RP, Chien KR (1996) An HF-1a/HF1b/MEF-2 combinatorial element confers cardiac ventricular specificity and established an anterior–posterior gradient of expression. *Development* **122:** 1799–1809

Scohy S, Gabant P, Van Reeth T, Hertveldt V, Dreze PL, Van Vooren P, Riviere M, Szpirer J, Szpirer C (2000) Identification of KLF13 and KLF14 (SP6), novel members of the SP/XKLF transcription factor family. *Genomics* **70:** 93–101

Segre JA, Bauer C, Fuchs E (1999) Klf4 is a transcription factor required for establishing the barrier function of the skin. *Nat Genet* **22:** 356–360

Senger K, Armstrong GW, Rowell WJ, Kwan JM, Markstein M, Levine M (2004) Immunity regulatory DNAs share common organizational features in *Drosophila*. *Mol Cell* **13:** 19–32

Serbedzija GN, Chen JN, Fishman MC (1998) Regulation in the heart field of zebrafish. *Development* **125:** 1095–1101

Shindo T, Manabe I, Fukushima Y, Tobe K, Aizawa K, Miyamoto S, Kawai-Kowase K, Moriyama N, Imai Y, Kawakami H, Nishimatsu H, Ishikawa T, Suzuki T, Morita H, Maemura K, Sata M, Hirata Y, Komukai M, Kagechika H, Kadowaki T, Kurabayashi M, Nagai R (2002) Kruppel-like zinc-finger transcription factor KLF5/BTEB2 is a target for angiotensin II signaling and an essential regulator of cardiovascular remodeling. *Nat Med* **8:** 856–863

Song A, Patel A, Thamatrakoln K, Liu C, Feng D, Clayberger C, Krensky AM (2002) Functional domains and DNA-binding sequences of RFLAT-1/KLF13, a Kruppel-like transcription factor of activated T lymphocytes. *J Biol Chem* **277:** 30055–30065

Song CZ, Keller K, Chen Y, Stamatoyannopoulos G (2003) Functional interplay between CBP and PCAF in acetylation and regulation of transcription factor KLF13 activity. *J Mol Biol* **329:** 207–215

Suske G, Bruford E, Philipsen S (2005) Mammalian SP/KLF transcription factors: bring in the family. *Genomics* **85:** 551–556

Temsah R, Nemer M (2005) GATA factors and transcriptional regulation of cardiac natriuretic peptide genes. *Regul Pept* **128:** 177–185

Tevosian SG, Deconinck AE, Tanaka M, Schinke M, Litovsky SH, Izumo S, Fujiwara Y, Orkin SH (2000) FOG-2, a cofactor for GATA transcription factors, is essential for heart morphogenesis and development of coronary vessels from epicardium. *Cell* **101:** 729–739

Walters M, Martin DI (1992) Functional erythroid promoters created by interaction of the transcription factor GATA-1 with CACCC and AP-1/NFE-2 elements. *Proc Natl Acad Sci USA* **89:** 10444–10448

Watt AJ, Battle MA, Li J, Duncan SA (2004) GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. *Proc Natl Acad Sci USA* **101:** 12573–12578

Yang H, Lu MM, Zhang L, Whitsett JA, Morrisey EE (2002) GATA6 regulates differentiation of distal lung epithelium. *Development* **129:** 2233–2246

Zeisberg EM, Ma Q, Juraszek AL, Moses K, Schwartz RJ, Izumo S, Pu WT (2005) Morphogenesis of the right ventricle requires myocardial expression of Gata4. *J Clin Invest* **115:** 1522–1531

Zhao J, Bian ZC, Yee K, Chen BP, Chien S, Guan JL (2003) Identification of transcription factor KLF8 as a downstream target of focal adhesion kinase in its regulation of cyclin D1 and cell cycle progression. *Mol Cell* **11:** 1503–1515

Zhu H, Nguyen VT, Brown AB, Pourhosseini A, Garcia AV, van Bilsen M, Chien KR (1993) A novel, tissue-restricted zinc finger protein (HF-1b) binds to the cardiac regulatory element (HF-1b/MEF-2) in the rat myosin light-chain 2 gene. *Mol Cell Biol* **13:** 4432–4444