THE KRUPPEL-LIKE TRANSCRIPTION FACTOR KLF13 IS A NOVEL REGULATOR OF HEART DEVELOPMENT

Short title: KLF13 is a novel cardiac regulator

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ABSTRACT

In human, 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 lead to atrial septal defects and hypotrabeculation similar to those observed in human 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.

INTRODUCTION

Congenital heart malformations represent the single largest class of birth defects in human and are the leading cause of mortality in infants under one 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 for genetic determinants in congenital heart disease (CHD). Despite remarkable progress over 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 congenital heart disease.

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 (Lints et al., 1993;Bodmer, 1993;Komuro and Izumo, 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 G et al., 2006).

Initially identified as one of the key transcription factors required for expression of the cardiac natriuretic peptide genes ANP and BNP (Grépin et al., 1994;Charron et al., 1999), GATA-4 has turned out to be a critical regulator of various aspects of embryonic and post-natal heart development (Grépin et al., 1997;Molkentin et al., 1997;Crispino et al., 2001;Charron 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 for 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 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 post-natal cardiomyocyte survival and homeostasis (Charron *et al.*, 1999;Charron *et al.*, 2001;Aries et al., 2004). Mice

with 70% reduction in GATA-4 display cardiac malformations and reduced cardiomyocyte proliferation (Pu et al., 2004) while 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 ortholog 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 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 B-type natriuretic peptide (BNP) is sufficient for maximal cardiac transcription (Grépin *et al.*, 1994). Other than the GATA elements, sequence alignment revealed the presence of an

evolutionary conserved CACCC box centered at -75 bp (Fig. 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 (Fig. 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 (Fig. 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 either asymmetrically expressed in heart chambers or that it functionally interacts with other regulators in a chamber-specific manner.

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 (Perkins et al., 1995;Nuez 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 (Fig. 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 (Fig. 1D and data not shown). The antibody also detected endogenous KLF13 in nuclear cardiomyocyte extracts and showed higher abundance in atrial versus ventricular cells (Fig. 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 (Fig. 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 (Fig 1E).

Spatiotemporal expression of KLF13 was analyzed using histological sections of staged murine embryos (Fig. 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 (Fig. 2A). By E12.5, staining was evident in atria and ventricles (Fig. 2B) but the signal remained stronger in the atria (Fig. 2B, middle panel). Within the ventricle, KLF13 positive cells were present predominantly in the trabeculae (Fig. 2B, right panel) and this pattern persisted at later stages (Fig. 2C). In addition, KLF13 immunoreactivity was evident in the cardiac cushions of the atrioventricular region (Fig 2A right panel and Fig. 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 (Fig. 2E).

As development progressed, KLF13 was also found outside the heart. Apart from epithelial cells (Fig. 2H), KLF13 was present in brain mesenchyme (Fig. 2F), dorsal ganglions (Fig. 2I) and skeletal muscles (Fig. 2J). At E14.5, the endothelial cells of the vascular vessels in the liver started showing KLF13 immunoreactivity (Fig. 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 addition of cold self but not mutated probe (Fig. 3A). No specific DNA-protein complex could be detected on the probe harboring mutation in the CACCC box (Fig 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 (Fig. 3B, right panel).

Next, the ability of KLF13 to act as a transcriptional activator of BNP was investigated using transient co-transfection assays. KLF13 dose-dependently activated the BNP promoter up to 15-fold in several non-cardiac cell types including myoblasts C2C12 cells and NIH3T3 fibroblasts (Fig. 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 (Fig. 3C). Interestingly, co-transfection 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 (Fig. 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. Co-transfection of a rat BNP promoter construct into P19 cells with KLF13, GATA-4 or both resulted in synergistic transcriptional activation (Fig. 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 (Fig. 4A). This cooperativity reflected physical interaction between both proteins as evidenced by co-immunoprecipitation (Fig. 4B) and pull-down (Fig. 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;Crispino *et al.*, 2001). This was evidenced by the ability of 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 (Fig. 4C). To determine whether KLF13 and FOG2 recognize similar residues within the GATA-4 N-zinc finger, we tested the ability of

KLF13 to functionally interact with GATA-4 mutants defective in FOG interaction. Residues E215 and 217 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 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 were 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 (Fig 4D) whereas KLF13 synergy with the V217G mutant was insensitive to FOG2 addition (Fig 4E). We conclude that KLF13 and FOG2 contact distinct residues within the N-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 evolutionary 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 as well as 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 co-transfection assays, KLF13 alone activated transcription from the ANP, α-cardiac actin and β-myosin heavy chain promoters by 8- to 12-fold (Fig. 4F and data not shown). The simultaneous addition of KLF13 and GATA-4 lead to superactivation of all three promoters (Fig. 4F and data not shown). Interestingly, KLF13 was also able to physically (Fig. 4C) and functionally (Fig. 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 search the *X. tropicalis* genome assembly for a KLF13 homologue and the information was used to isolate a X *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 XKLF13 alleles shares 61% overall identity with murine KLF13 (Fig. 5A). Chromosomal synteny and intraspecies phylogeny were consistent with 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 members which revealed that XKLF13 is most closely related to XKLF9 (overall identity 45%) than to any other KLF (Fig 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 (Fig. 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 down-regulated in the myocardial compartments and by stage 47, expression mainly persists in the atrioventricular valves (Fig. 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.

Knock-down of KLF13 reveals a crucial role in cardiac morphogenesis

To assess the developmental role of KLF13 we designed antisense morpholino oligonucleotides (MO), 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 (Fig 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 due to its lower efficiency at inhibiting translation (Fig 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 1 cell stage or into the dorsolateral marginal zone at the 4 cell stage with equivalent results. XKMOs

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 (Fig. 6B and video in supplemental data). In extreme phenotypes, the heart only developed as a string-like muscle by stage 40, 2 days prior to appearance of pericardial edema. Histological sections of the tadpole hearts at this stage revealed smaller ventricles with abnormal patterning of the myocardium (Fig. 6C). This phenotype was specific and was not observed in embryos injected with control (Fig. 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, subdivide into normal, mild and severe, were consistently obtained in 10 independent experiments by two different experimentators. 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 (Fig 6C). Co-injection 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 (Fig. 6D).

To identify at what stage KLF13 functions in heart development, we examined tadpoles at different developmental stages (Fig.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 XKMOs 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 XKMOs 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 (Fig. 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 (Fig. 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, albeit 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, -5 and -6 as well as Nkx2.5 and Tbx5 transcripts were reduced in KLF13 depleted embryos starting from stage 20, fourteen hours prior to any histological abnormalities (Fig. 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 (Fig. 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 (Fig 7C) which contains several KLF binding motifs (Zhao et al., 2003). Moreover, GATA-4 potentiated KLF13 activation of the cyclin D1 promoter (Fig 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 co-factors, injection of GATA-4 RNA rescued the cardiac defects of XKMO treated embryos in a dose dependent manner (Fig 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

Krüppel-like factors (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 (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 in (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 *et al.*, 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 *et al.*, 2002). The

data presented here suggest that CACCC elements are also important for cardiac transcription of 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 which contain CACCC motifs within previously described enhancer regions and which were activated by KLF13 (Fig. 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 may contribute 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 in (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 co-activators/co-repressors such as CBP/p300, PCAF and ctBP2 (Bieker, 2001;Song et al., 2003).

Since GATA factors also interact physically with CBP/p300 (Dai et al., 2002), co-recruitment by GATA and KLF proteins of these co-activators may be the mechanism underlying transcriptional cooperativity.

In addition to GATA-4, KLF13 can interact physically and functionally with GATA-6 (Fig. 4C, F) which is expressed in smooth as well as cardiac muscle cells (Nemer *et al.*, 2003). Together with the reported interaction of KLF1 and GATA-1 in erythroid cells (Cantor *et al.*, 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 congenital heart disease

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 *et al.*, 2003). This co-localization 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 suggest 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 (Fig. 4A). In this respect, the higher dosage of KLF13 in atrial vs 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 congenital heart disease, including atrial septal and valvular defects.

MATERIAL AND METHODS

Plasmids

BNP-luciferase (BNP) constructs and GATA-4 expression vectors were obtained as previously described (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 maltose binding protein (MBP) fusion proteins. Plasmids were confirmed by sequencing.

Generation of KLF13 antibody

Recombinant MBP-KLF13 6-135 was obtained as previously described (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 previously described (Nemer *et al.*, 2003).

Cell culture and transfections

Transfections of P19, 293T, C2C12 and NIH3T3 cells were carried out as previously described (Morin et al., 2000). Primary cultures of cardiomyocytes from 4 day-old Sprague-Dawley rats were prepared and transfected as described before (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 *et al.*, 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 ATAATCCTACTCCTAC TC.

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 mMessage mMachine kit (Ambion). For knockdown experiments, two KLF13 morpholino oligonucleotides targeting the sequence of the first 8 translated aa of each XKLF13 allele were used. A standard control morpholino directed against a mutated human beta-globin pre-mRNA was used for control experiments (Gene Tools, Oregon USA). Capped mRNAs and morpholinos were injected into the dorsolateral marginal zone of 1- or 4-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, -5 and -6 probes were kind gifts of Todd Evans (Jiang and Evans, 1996). Real-time PCR was performed using QuantiTect Sybr Green PCR Kit (Qiagen) and MX4000 (Stratagene).

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FIGURE LEGENDS

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 ± SD of at least 4 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 cardiomyocytes extracts (right panel). **E)** Immunohistochemical co-localization of KLF13 and desmin in primary cardiomyocyte cultures.

Figure 2. Spatiotemporal expression of KLF13 in mouse embryos. Immunohistochemical localization of KLF13 in staged murine embryos using immunoperoxidase staining. Panels A to E represent heart sections; F is brain, G is liver, H shows the epithelial layer of the dermis, I is a dorsal ganglion, J is 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 addition of 100 fold excess of unlabelled 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) or endogenous (right panel) complex formation. **C)** Co-transfection 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. Co-transfections 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 ± SD of two independent duplicate experiments carried out simultaneously on cultures prepared from the same neonate rat hearts.

Figure 4. KLF13 is a GATA-4 interacting partner. A) Co-transfections 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 ± SD. 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 -6 (G6) proteins. The two bands observed with GATA-6 correspond to alternate ATG usage (Brewer et al., 2002). Note how GATA-$4_{1\text{-}266}$ which contains the N- but not the C-terminal zinc finger is retained on the MBP-KLF13 column. The results shown were reproduced on 3 different occasions. **D)** and **E)** KLF13 and FOG2 recognize distinct domains within the N-terminal zinc finger of GATA-4. Cotransfections were carried out as in A above. **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.

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: 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: 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 (Song *et al.*, 2002). **B)** Phylogenetic tree of *Xenopus* KLF homologues. Notice 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.

Figure 6. Functional analysis of XKLF13 in *Xenopus* heart development. A) *In vitro* translation of the two XKLF13 alleles in presence of increasing concentrations of control (100 and 200 ng) or the corresponding morpholino oligonucleotide (50, 100 and 200ng). **B) to 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 arrow head points to the atrial septum which is absent in morpholino treated embryos. E, endocardium; M, myocardium

Figure 7. Characterization of the XKLF13 loss-of-function phenotype. **A)** Wholemount *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.

REFERENCES

Argentin,S., Ardati,A., Tremblay,S., Lihrmann,I., Robitaille,L., Drouin,J., and 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,R.J., and 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,X.S., and Stamatoyannopoulos,G. (1999). FKLF, a novel Kruppel-like factor that activates human embryonic and fetal beta-like globin genes. *Mol. Cell Biol,* **19,** 3571-3579.

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

Basson,C.T., Bachinsky,D.R., Lin,R.C., Levi,T., Elkins,J.A., Soults,J., Grayzel,D., Kroumpouzou,E., Traill,T.A., Leblanc-Straceski,J., Renault,B., Kucherlapati,R., Seidman,J.G., and Seidman,C.E. (1997). Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome. *Nat. Genet.,* **15,** 30-35.

Bhalla,S.S., Robitaille,L., and 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,J.J. (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., and 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,B.G. (2002). Transcriptional regulation of vertebrate cardiac morphogenesis. *Circ. Res.,* **90,** 509-519.

Bruneau,B.G., Nemer,G., Schmitt, J.P., Charron,F., Robitaille,L., Caron,S., Conner,D.A., Gessler,M., Nemer,M., Seidman,C.E., and Seidman,J.G. (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,A.B. and Orkin,S.H. (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., and 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,J.D., Meloche,S., and Nemer,M. (2001). Tissue-specific GATA factors are transcriptional effectors of the small GTPase RhoA. *Genes Dev.,* **15,** 2702-2719.

Chi,X., Chatterjee,P.K., Wilson,W., III, Zhang,S.X., DeMayo,F.J., and Schwartz,R.J. (2005). Complex cardiac Nkx2-5 gene expression activated by noggin-sensitive enhancers followed by chamber-specific modules. *Proc. Natl. Acad. Sci. U. S. A,* **102,** 13490-13495.

Clark,K.L., Yutzey,K.E., and Benson,D.W. (2006). Transcription factors and congenital heart defects. *Annu. Rev. Physiol,* **68,** 97-121.

Crispino,J.D., Lodish,M.B., Mackay,J.P., and Orkin,S.H. (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,J.D., Lodish,M.B., Thurberg,B.L., Litovsky,S.H., Collins,T., Molkentin,J.D., and Orkin,S.H. (2001). Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. *Genes Dev.,* **15,** 839-844.

Dai,Y.S., Cserjesi,P., Markham,B.E., and Molkentin,J.D. (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,R.J., and Nemer,M. (1997). The cardiac transcription factors Nkx2-5 and GATA-4 are mutual cofactors. *EMBO J.,* **16,** 5687-5696.

Fossett,N., Tevosian,S.G., Gajewski,K., Zhang,Q., Orkin,S.H., and Schulz,R.A. (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,J.D., and Schulz,R.A. (1999). The zinc finger proteins Pannier and GATA4 function as cardiogenic factors in *Drosophila*. *Development,* **126,** 5679-5688.

Garg,V., Kathiriya,I.S., Barnes,R., Schluterman,M.K., King,I.N., Butler,C.A., Rothrock,C.R., Eapen,R.S., Hirayama-Yamada,K., Joo,K., Matsuoka,R., Cohen,J.C., and 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., and 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., and 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., and Muller,R. (1994). Inducible regulatory elements in the human cyclin D1 promoter. *Oncogene,* **9,** 1295-1304.

Horb,M.E. and Thomsen,G.H. (1999). Tbx5 is essential for heart development. *Development,* **126,** 1739-1751.

Jiang,Y. and 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,I.K., and Simpson,P.C. (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,S.L. and Bodmer,R. (2003). Gata factor Pannier is required to establish competence for heart progenitor formation. *Development,* **130,** 3027-3038.

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

Kuo,C.T., Morrisey,E.E., Anandappa,R., Sigrist,K., Lu,M.M., Parmacek,M.S., Soudais,C., and Leiden,J.M. (1997). GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. *Genes Dev.,* **11,** 1048-1060.

Latinkic,B.V., Kotecha,S., and Mohun,T.J. (2003). Induction of cardiomyocytes by GATA4 in Xenopus ectodermal explants. *Development,* **130,** 3865-3876.

Lepore,J.J., Cappola,T.P., Mericko,P.A., Morrisey,E.E., and Parmacek,M.S. (2005). GATA-6 regulates genes promoting synthetic functions in vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.,* **25,** 309-314.

Lien,C.L., Wu,C., Mercer,B., Webb,R., Richardson,J.A., and Olson,E.N. (1999). Control of early cardiac-specific transcription of Nkx2-5 by a GATA-dependent enhancer. *Development,* **126,** 75-84.

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

Martin,K.M., Ellis,P.D., Metcalfe,J.C., and Kemp,P.R. (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,K.M., Metcalfe,J.C., and Kemp,P.R. (2001). Expression of Klf9 and Klf13 in mouse development. *Mech. Dev.,* **103,** 149-151.

McBride,K., Charron,F., Lefebvre,C., and 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., and 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,D.G., Charité,J., Richardson,J.A., Srivastava,D., Firulli,A.B., and Olson,E.N. (2000). A GATA-dependent right ventricular enhancer controls dHAND transcription in the developing heart. *Development,* **127,** 5331-5341.

Molkentin,J.D., Lin,Q., Duncan,S.A., and Olson,E.N. (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., and 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., and Nemer,M. (2000). GATA-dependent recruitment of MEF2 proteins to target promoters. *EMBO J.,* **19,** 2046-2055.

Morin,S., Paradis,P., Aries,A., and 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., and Bitar,F. (2006). A novel mutation in the *GATA4* gene in patients with tetralogy of Fallot. *Hum. Mutat.,* **27,** 293-294.

Nemer,G. and 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., and Grosveld,F. (1995). Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. *Nature,* **375,** 316-318.

Olson,E.N. and Schneider,M.D. (2003). Sizing up the heart: development redux in disease. *Genes Dev.,* **17,** 1937-1956.

Parmacek,M.S., Vora,A.J., Shen,T., Barr,E., Jung,F., and Leiden,J.M. (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,A.C., Sharpe,A.H., and Orkin,S.H. (1995). Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. *Nature,* **375,** 318-322.

Pu,W.T., Ishiwata,T., Juraszek,A.L., Ma,Q., and Izumo,S. (2004). GATA4 is a dosage-sensitive regulator of cardiac morphogenesis. *Dev. Biol,* **275,** 235-244.

Ross,R.S., Navankasattusas,S., Harvey,R.P., and Chien,K.R. (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,P.L., Van Vooren,P., Riviere,M., Szpirer,J., and Szpirer,C. (2000). Identification of KLF13 and KLF14 (SP6), novel members of the SP/XKLF transcription factor family. *Genomics,* **70,** 93-101.

Segre,J.A., Bauer,C., and 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,G.W., Rowell,W.J., Kwan,J.M., Markstein,M., and Levine,M. (2004). Immunity Regulatory DNAs Share Common Organizational Features in *Drosophila*. *Mol. Cell,* **13,** 19-32.

Serbedzija,G.N., Chen,J.N., and Fishman,M.C. (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., and 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., and Krensky,A.M. (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,C.Z., Keller,K., Chen,Y., and 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., and Philipsen,S. (2005). Mammalian SP/KLF transcription factors: bring in the family. *Genomics,* **85,** 551-556.

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

Tevosian,S.G., Deconinck,A.E., Tanaka,M., Schinke,M., Litovsky, SH, Izumo,S., Fujiwara,Y., and Orkin,S.H. (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. and Martin,D.I. (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.

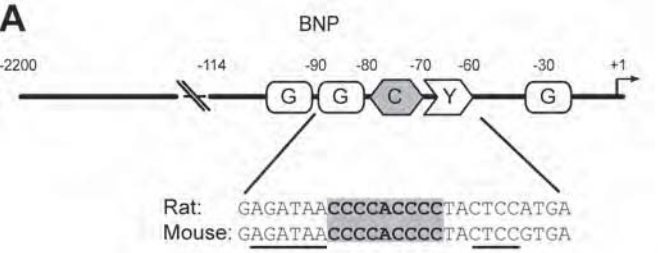
Watt,A.J., Battle,M.A., Li,J., and Duncan,S.A. (2004). GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. *Proc. Natl. Acad. Sci. U. S. A,* **101,** 12573-12578.

Yang,H., Lu,M.M., Zhang,L., Whitsett,J.A., and Morrisey,E.E. (2002). GATA6 regulates differentiation of distal lung epithelium. *Development,* **129,** 2233-2246.

Zeisberg,E.M., Ma,Q., Juraszek,A.L., Moses,K., Schwartz,R.J., Izumo,S., and Pu,W.T. (2005). Morphogenesis of the right ventricle requires myocardial expression of Gata4. *J Clin. Invest,* **115,** 1522-1531.

Zhao,J., Bian,Z.C., Yee,K., Chen,B.P., Chien,S., and Guan,J.L. (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,V.T., Brown,A.B., Pourhosseini,A., Garcia,A.V., van Bilsen,M., and Chien,K.R. (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.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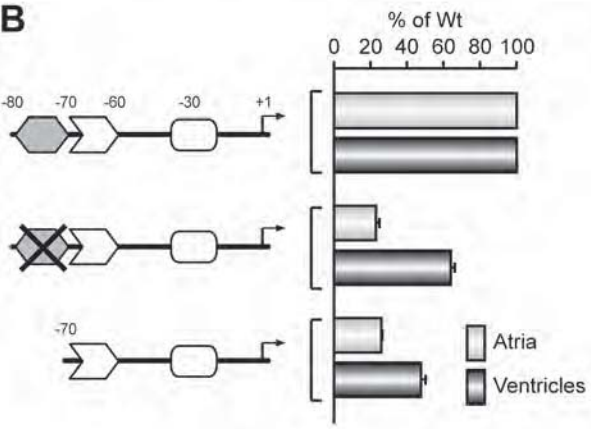
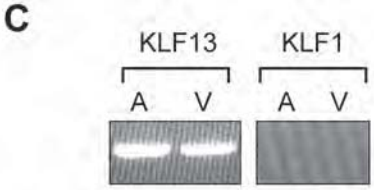
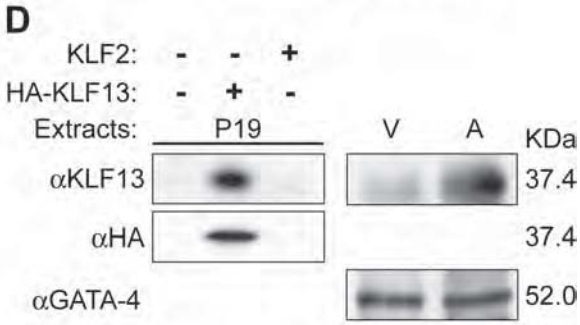
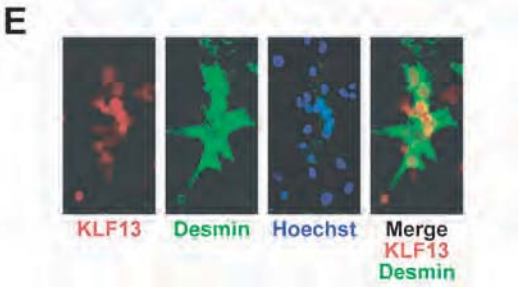
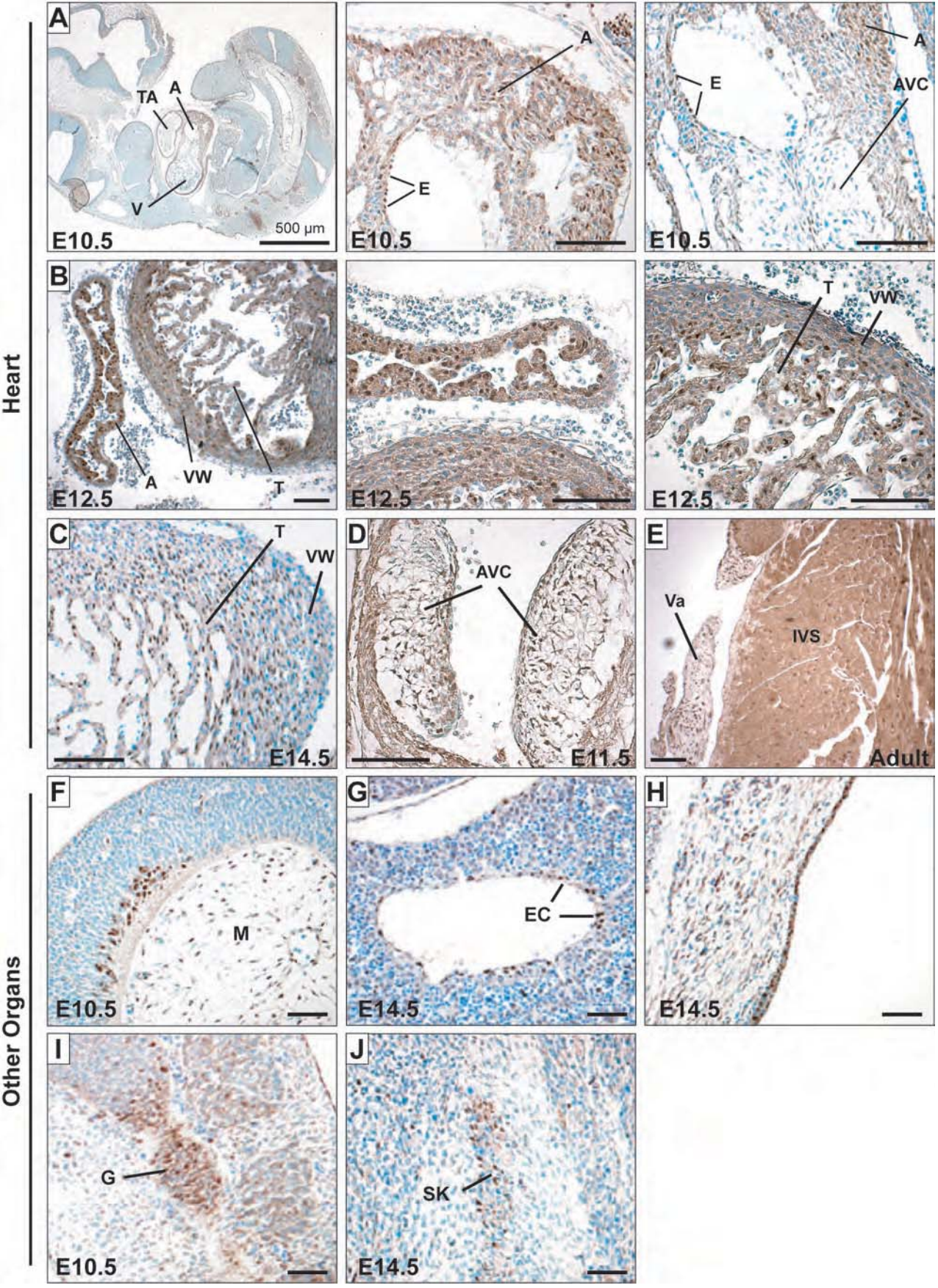
Rat: GAGATAACCCCACCCCTACTCCATGA
Mouse: GAGATAACCCCACCCCTACTCCGTGA

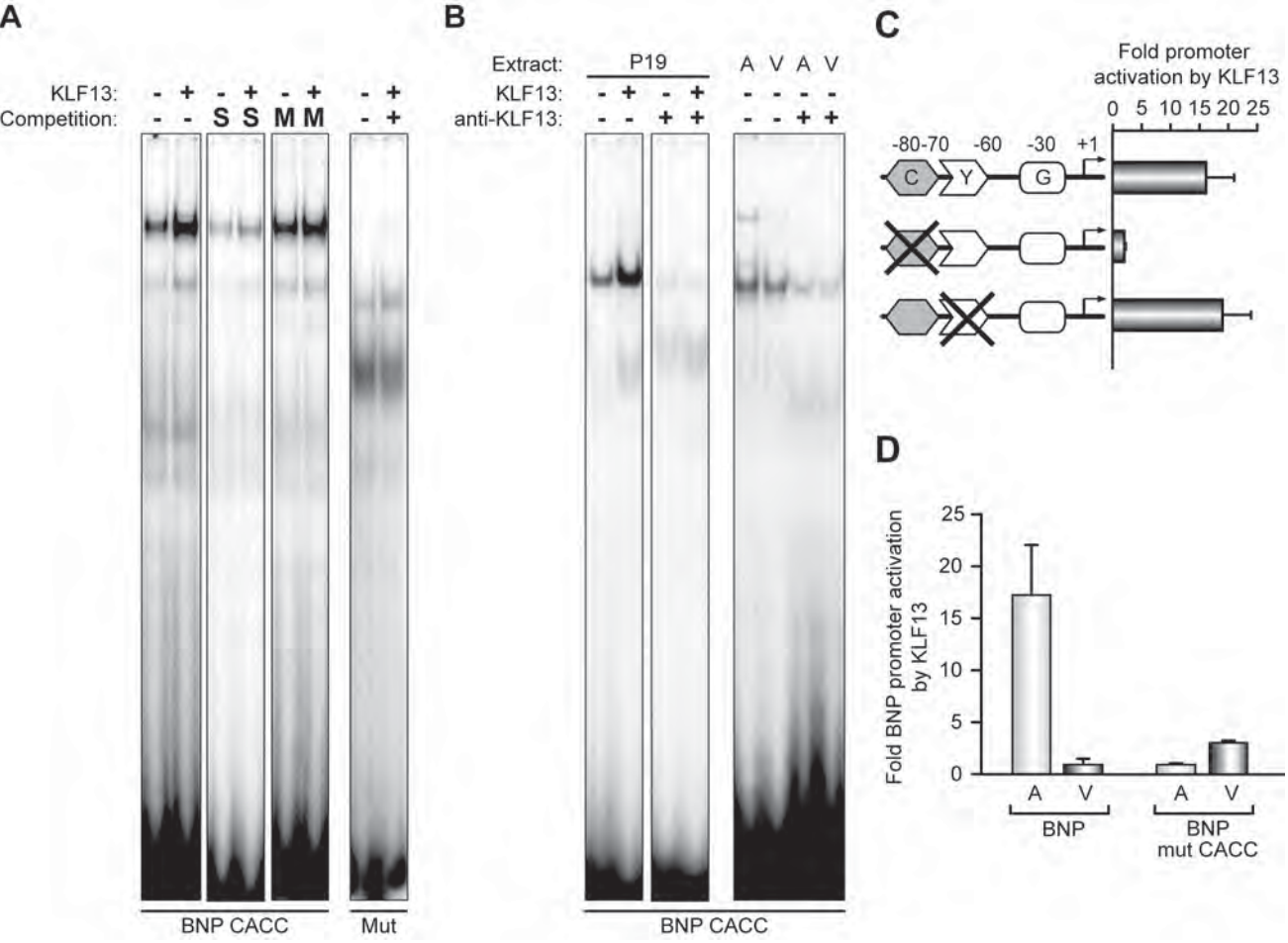

B
% of Wt
0 20 40 60 80 100
-80
-70
-60
-30
+1
-70
Atria
Ventricles

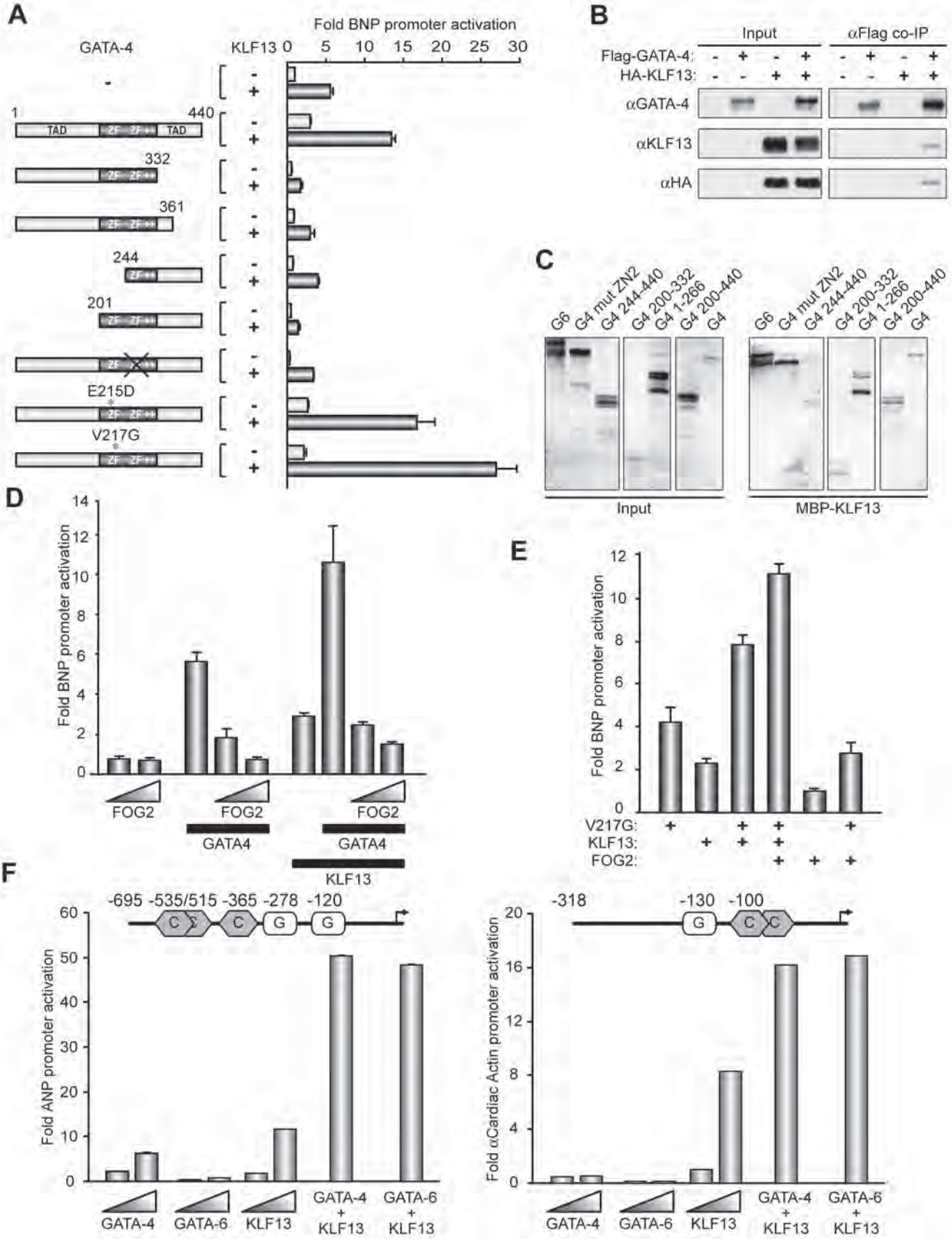

C
KLF13
KLF1
A
V
A
V

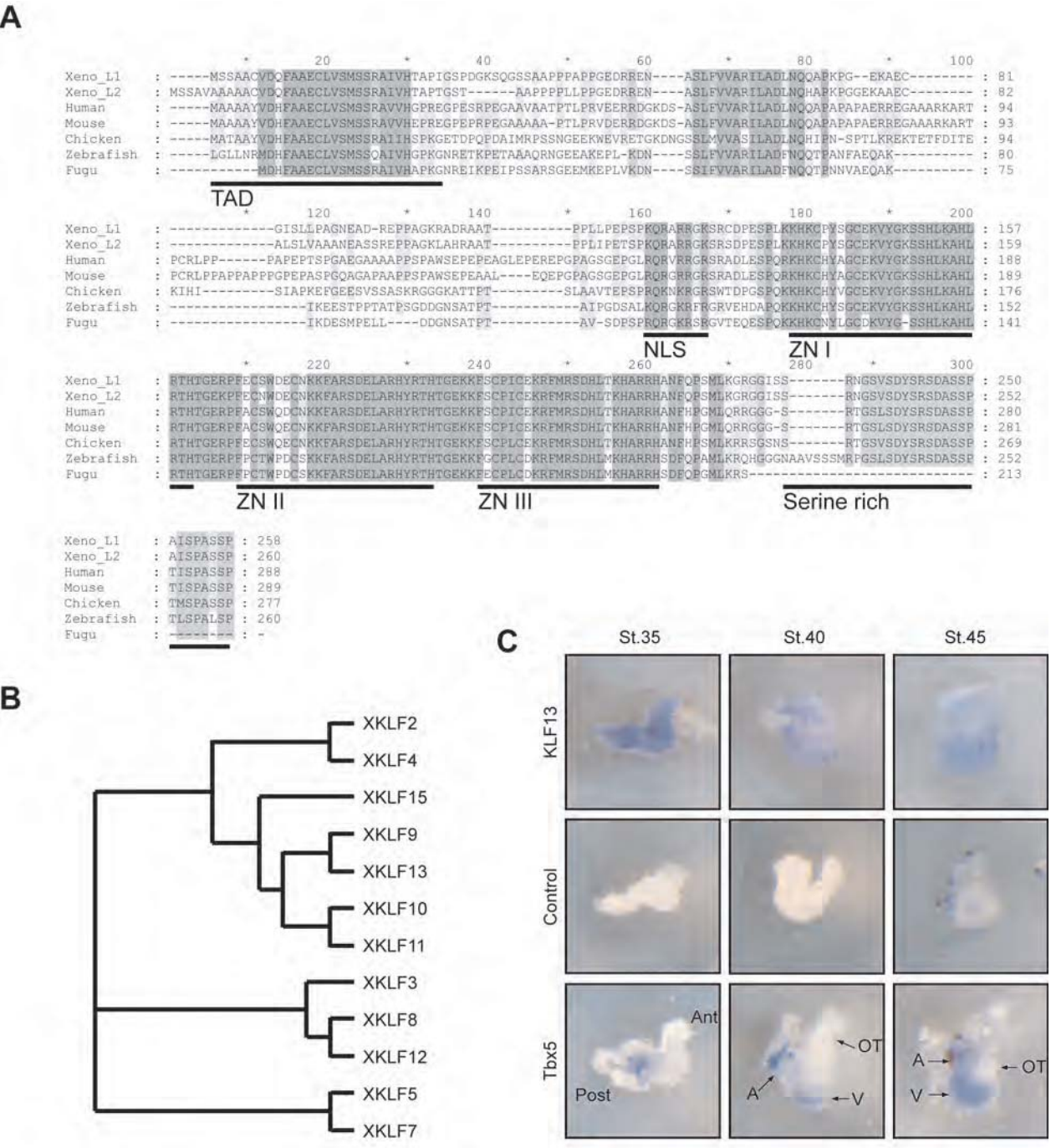

D
KLF2: - - +
HA-KLF13: - + -
Extracts: P19
V
A
KDa
αKLF13
37.4
αHA
37.4
αGATA-4
52.0

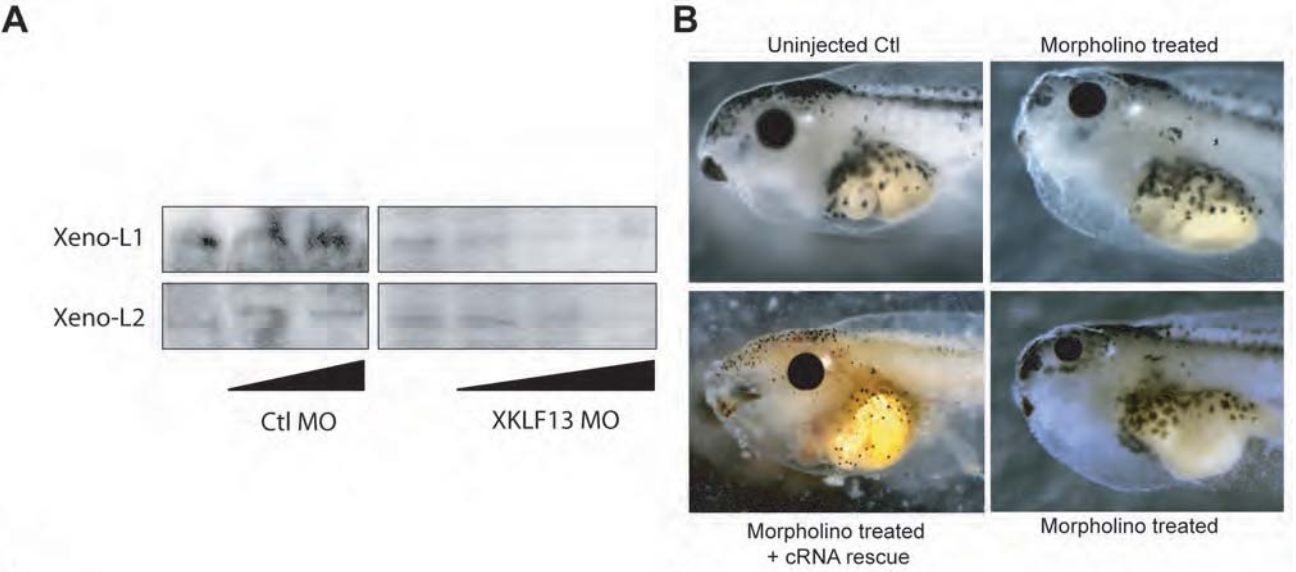

E
KLF13
Desmin
Hoechst
Merge
KLF13
Desmin

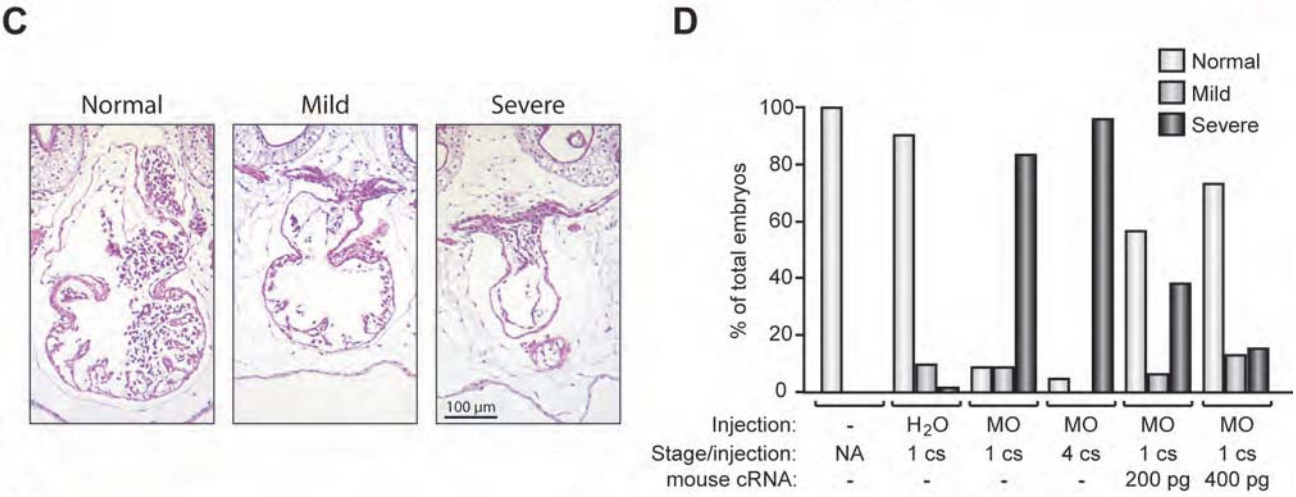

Heart
Other Organs
A
TA
A
V
E10.5
500 µm
A
E
E10.5
A
E
AVC
E10.5
B
A
VW
T
E12.5
E12.5
T
VW
E12.5
C
T
VW
E14.5
D
AVC
E11.5
E
Va
IVS
Adult
F
M
E10.5
G
EC
E14.5
H
E14.5
I
G
E10.5
J
SK
E14.5

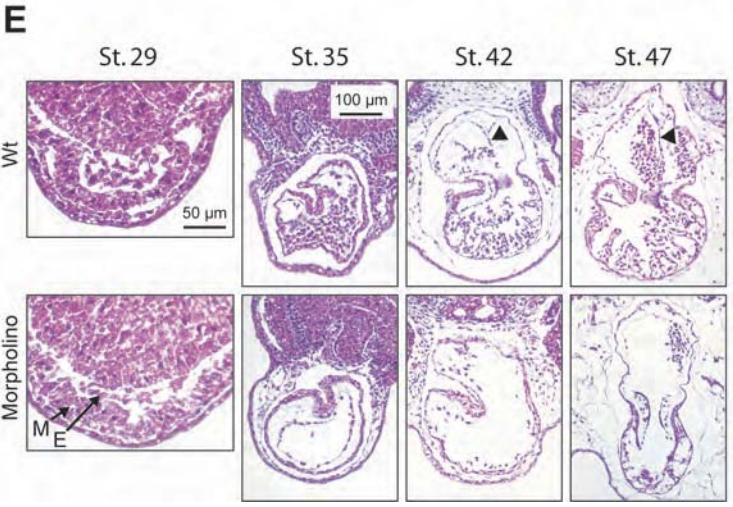

A
KLF13: - + - + - + - +
Competition: - - S S M M - +
BNP CACC
Mut
B
Extract: P19 A V A V
KLF13: - + - + - - - -
anti-KLF13: - - + + - - + +
BNP CACC
C
Fold promoter activation by KLF13
0 5 10 15 20 25
-80-70 -60 -30 +1
C Y G
D
Fold BNP promoter activation by KLF13
25 20 15 10 5 0
A V A V
BNP
BNP mut CACC

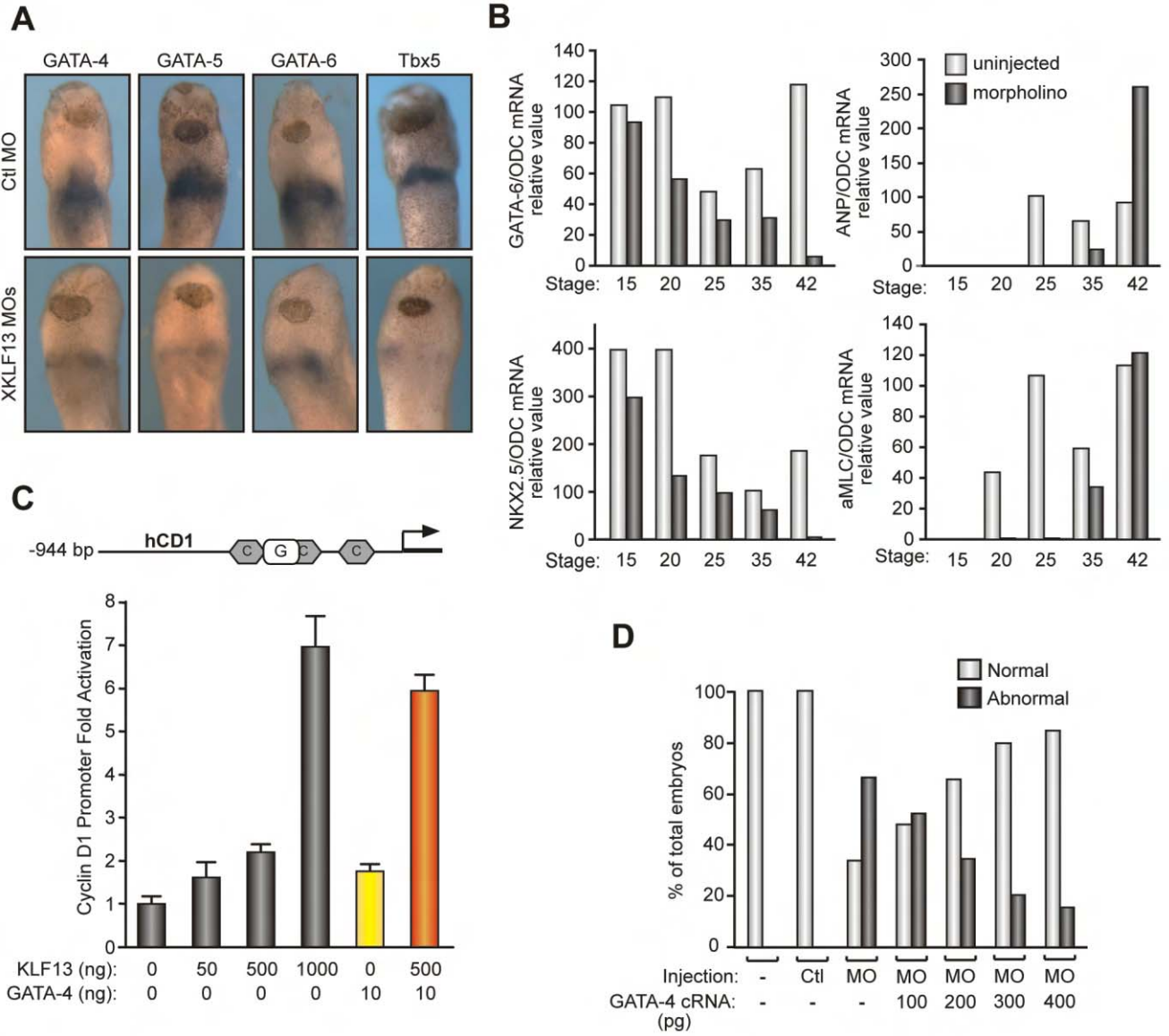

A
Fold BNP promoter activation
GATA-4
KLF13
0 5 10 15 20 25 30
1
440
TAD
ZF ZF++
TAD
332
361
244
201
E215D
V217G
B
Input
αFlag co-IP
Flag-GATA-4:
HA-KLF13:
αGATA-4
αKLF13
αHA
C
G6
G4 mut ZN2
G4 244-440
G4 200-332
G4 1-266
G4 200-440
G4
Input
MBP-KLF13
D
Fold BNP promoter activation
FOG2
GATA4
KLF13
E
Fold BNP promoter activation
V217G:
KLF13:
FOG2:
F
-695 -535/515 -365 -278 -120
Fold ANP promoter activation
-318 -130 -100
Fold αCardiac Actin promoter activation
GATA-4
GATA-6
KLF13
GATA-4 + KLF13
GATA-6 + KLF13


A
TAD
NLS
ZN I
ZN II
ZN III
Serine rich
B
XKLF2
XKLF4
XKLF15
XKLF9
XKLF13
XKLF10
XKLF11
XKLF3
XKLF8
XKLF12
XKLF5
XKLF7
C
St.35
St.40
St.45
KLF13
Control
Tbx5
Ant
Post
A
V
OT


A
Xeno-L1
Xeno-L2
Ctl MO
XKLF13 MO
B
Uninjected Ctl
Morpholino treated
Morpholino treated
+ cRNA rescue
Morpholino treated


C
Normal
Mild
Severe
100 µm
D
Normal
Mild
Severe
% of total embryos
100
80
60
40
20
0
Injection: - H2O MO MO MO MO
Stage/injection: NA 1 cs 1 cs 4 cs 1 cs 1 cs
mouse cRNA: - - - - 200 pg 400 pg


E
St. 29
St. 35
St. 42
St. 47
Wt
Morpholino
100 µm
50 µm
M
E


A
GATA-4
GATA-5
GATA-6
Tbx5
Ctl MO
XKLF13 MOs
B
GATA-6/ODC mRNA relative value
ANP/ODC mRNA relative value
uninjected
morpholino
NKX2.5/ODC mRNA relative value
aMLC/ODC mRNA relative value
Stage: 15 20 25 35 42
C
-944 bp
hCD1
C G C C
Cyclin D1 Promoter Fold Activation
KLF13 (ng): 0 50 500 1000 0 500
GATA-4 (ng): 0 0 0 0 10 10
D
Normal
Abnormal
% of total embryos
Injection: - Ctl MO MO MO MO MO
GATA-4 cRNA (pg): - - - 100 200 300 400