protein in *Escherichia coli* BL21(DE3) and purified the RS2 fusion protein by Ni-NTA affinity chromatography (Qiagen). The fusion protein was separated by SDS–PAGE, and gel slices containing about 300 ng of protein were pulverized and used directly to immunize rabbits. The $His_{10}$–RS2 fusion protein was coupled to Affi-Gel 10 beads (Bio-Rad) to generate an affinity column. Polyclonal antiserum was loaded onto the affinity column (serum, 8 ml; bed volume, 2 ml) and the column was washed extensively with TBS (20 mM Tris-HCl (pH 7.4) and 0.15 M NaCl) and 20 mM Tris-HCl (pH 7.4), 0.5 M NaCl and 0.2% Triton X-100. Antibodies were eluted in 0.2 M glycine-HCl (pH 2.0) and 0.15 M NaCl, and the collected fractions were immediately neutralized with 60 μl of 2 M Tris-HCl (pH 8.5). Each fraction was dialysed against TBS overnight at 4 °C. We determined wild-type and *rs2* mutant apices. Scanning electron microscopy was done as described[19].

### Phylogenetic analysis

Nine *PHAN* orthologues were obtained from GenBank (accession numbers: rice, AB071600; Medicago, AF308453; tobacco, AJ006181; *Antirrhinum*, AJ005586; tomato, AF148934; maize, AF143447; *Arabidopsis*, NM_129319; pea, AF299140; *Ipomoea batatas*, BM878751). Additional *PHAN* orthologues from pinnate and palmate compound-leafed species (*A. formosa*, *V. cannabifolia*, *S. actinophylla*, *A. hindisii*, *P. aquatica* and *F. americana*) were cloned by PCR with the following degenerate primers, designed on the basis of available *PHAN* orthologue sequences: DePHAN1, 5′-CACGGNAACAARTGG AARAA-3′; DePHAN2, 5′-GCTTCRATYTCCTCCATYTT-3′. We aligned nucleotide and amino acid sequences by ClustalX (Supplementary Fig. 2c) and carried out parsimony analyses by PAUP4 and McClade.

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**Correspondence** and requests for materials should be addressed to N.S. (nrsinha@ucdavis.edu). The sequences are deposited in GenBank under accession codes AY180131, *A. formosa*; AY180132, *V. cannabifolia*; AY180133, *S. actinophylla*; AY180134, *A. hindisii*; AY180135, *P. aquatica*; AY180136, *F. americana*.

# GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5

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**Congenital heart defects (CHDs) are the most common developmental anomaly and are the leading non-infectious cause of mortality in newborns[1]. Only one causative gene, *NKX2-5*, has been identified through genetic linkage analysis of pedigrees with non-syndromic CHDs[2,3]. Here, we show that isolated cardiac septal defects in a large pedigree were linked to chromosome 8p22-23. A heterozygous G296S missense mutation of GATA4, a transcription factor essential for heart formation[4–7], was found in all available affected family members but not in any control individuals. This mutation resulted in diminished DNA-binding affinity and transcriptional activity of Gata4. Furthermore, the Gata4 mutation abrogated a physical interaction between Gata4 and TBX5, a T-box protein responsible for a subset of syndromic cardiac septal defects[8,9]. Conversely, interaction of Gata4 and TBX5 was disrupted by specific human *TBX5* missense mutations that cause similar cardiac septal defects. In a second family, we identified a frame-shift mutation of *GATA4* (E359del) that was transcriptionally inactive and segregated with cardiac septal defects. These results implicate *GATA4* as a genetic cause of human cardiac septal defects, perhaps through its interaction with TBX5.**

Division of a common cardiac atrium and ventricle into right- and left-sided chambers represents an essential evolutionary milestone in development of the four-chambered heart and is necessary for separation of oxygenated and deoxygenated blood. In humans, failure of atrial or ventricular septation accounts for nearly 50% of CHDs and requires open-heart surgery to restore normal circulation[1]. Although cardiac septal defects (CSDs) are common, the precise molecular mechanisms for cardiac septal closure in humans remain to be elucidated. Mutations in *NKX2-5* have been identified in individuals with CSDs and conduction abnormalities, whereas individuals with Holt–Oram syndrome (HOS)—characterized by CSDs, conduction abnormalities and limb anomalies—have point mutations in *TBX5* (refs 2, 8, 9).

We identified a large kindred spanning five generations in which 16 individuals had CHDs (Fig. 1a). Detailed clinical evaluations were reviewed for all available family members, and demonstrated an autosomal dominant pattern of inheritance. All affected members had atrial septal defects. Eight individuals had additional forms of CHDs, including ventricular septal defects, atrioventricular septal defects, pulmonary valve thickening, or insufficiency of

cardiac valves (Fig. 1b, c; see also Supplementary Fig. 1). Ten of the sixteen affected members required surgical repair of the CHD (shaded red in Fig. 1a). Unlike the familial cases of CSDs associated with mutations of *NKX2-5* and *TBX5*, neither the cardiac conduction system nor other organs were affected in this kindred, suggesting that the CHDs were isolated and not associated with syndromic disease.

Direct sequencing of *NKX2-5* and *TBX5* in this family failed to reveal mutations, suggesting an alternative genetic aetiology. We performed a genome-wide scan of all family members that revealed linkage of the CHD phenotype to a single locus on chromosome 8p22-23 (LOD score = 5.7, $\theta = 0$) between D8S264 and D8S1827, spanning approximately 30 cM (about 12.7 megabases). Examination of genes in this interval revealed the presence of *GATA4*, which encodes a zinc-finger transcription factor essential for cardiogenesis in flies, fish and mice[4–7]. Direct sequencing of *GATA4* in an affected family member identified a G-to-A transition of nucleotide 886 (Fig. 1d) that predicted a glycine to serine substitution at codon 296 (hG296S) (Fig. 2b). All affected individuals that were clinically evaluated had the hG296S mutation, suggesting complete penetrance of the disease phenotype (Fig. 1d). The mutant allele was not detected in unaffected family members nor in 3,000 unrelated individuals of diverse ethnicity, making it unlikely that hG296S represented a rare polymorphism. In the proband, we sequenced 100 additional regulatory genes essential for, or expressed during, cardiac development but failed to identify other linked mutations, consistent with a monogenic aetiology (V.G. and D.S., unpublished data).

By direct sequencing of *GATA4*, we identified a second family with autosomal dominant transmission of atrial septal defects in which a mutation of *GATA4* (hE359del) was found in all available affected members spanning four generations (Fig. 1e, f; see also Supplementary Fig. 2). Similar to family A, neither the cardiac conduction system nor other organs were affected in this kindred. The hE359del mutation in this pedigree resulted in a frame shift past amino acid 359, thus severely altering the encoded GATA4 protein (Fig. 1g). A premature stop codon is predicted by the frame shift and may result in a truncated protein or nonsense-mediated decay of the transcript[10]. This nucleotide deletion was not found in unaffected family members or in 300 other individuals. The segregation of *GATA4* mutations with cardiac septal defects in two large unrelated families provides strong evidence that mutations in *GATA4* are the underlying cause of a subset of familial, non-syndromic cardiac septal defects. Consistent with this, deletion of the terminal end of chromosome 8p that contains *GATA4*, among many other genes, is characterized by cardiac septal defects[11].

GATA4 belongs to a family of transcription factors that binds a consensus HGATAR DNA motif and contains two class IV zinc-finger domains[12–14]. Gata1, Gata2 and Gata3 are expressed predominantly in haematopoietic cells, and heterozygous mutations of *GATA1* and *GATA3* cause human blood disorders and organ malformations, respectively[15,16]. In contrast, Gata4, Gata5 and

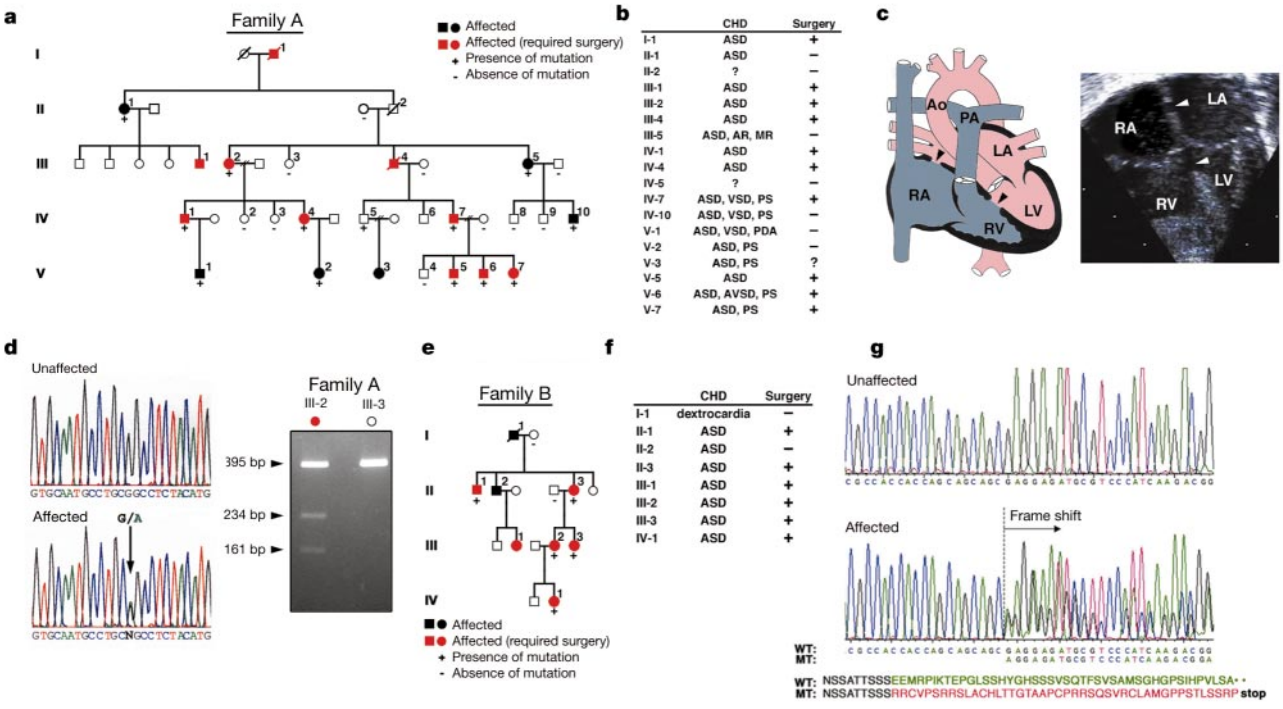


**Figure 1** *GATA4* mutations segregate with familial cardiac septal defects. **a**, Kindred with five generations (indicated in Roman numerals) affected by congenital heart defects (CHDs). Participating members of each generation are indicated numerically. III-1, V-3 and deceased family members were not available for mutation analysis. **b**, Types of CHDs and need for surgical repair in affected family members. Echocardiography or operative data were available for all subjects except for I-1, II-2 and IV-5. **c**, Atrial and ventricular septal defects, indicated by arrowheads, in schematic and echocardiogram of a representative family member. Ao, aorta; AR, aortic regurgitation; ASD, atrial septal defect; AVSD, atrioventricular septal defect; LA, left atrium; LV, left ventricle; MR, mitral regurgitation; PA, pulmonary artery; PDA, patent ductus arteriosus; PS, pulmonary stenosis; RA, right atrium; RV, right ventricle; VSD, ventricular septal defect. **d**, Sequence chromatogram displaying G to A transition of nucleotide 886, generating a new *Pst*I restriction enzyme site in exon 3 of *GATA4* that resulted in the appearance of 234-bp and 161-bp fragments of a 395-bp PCR product in affected members of family A. **e**, Second pedigree affected by CHDs, as described in **f**. **g**, Sequencing revealed a single nucleotide deletion that was linked to disease and altered the GATA4 sequence after amino acid 359, resulting in a premature stop codon. The wild type (WT) and predicted mutant (MT) protein sequences are shown. Two (II-2 and III-1) affected members were not available for the mutation study.

Gata6 are expressed in the developing heart and in several endodermal lineages, but have not been implicated in human disease[14,17]. However, null mutations in the *Drosophila Gata4* orthologue, *pannier*[6], zebrafish *gata5* (ref. 7) or mouse *Gata4* (refs 4, 5) result in early defects in cardiogenesis. Although mice heterozygous for *Gata4* do not have obvious cardiac anomalies, further reduction in Gata4 dosage from a hypomorphic *Gata4* allele causes cardiac septal and other congenital heart defects (W. Pu and S. Izumo, personal communication).

The hG296S mutation affects a residue highly conserved across species and lies adjacent to the nuclear localization signal (NLS) and the carboxy-terminal zinc finger, whereas the hE359del mutation results in truncation of the last forty amino acids (Fig. 2a, b). We generated both mutations in the highly conserved mouse orthologue (mG295S or mE360del) and tested their abilities to activate transcription of downstream genes *in vitro*, using several Gata-dependent cardiac enhancers upstream of a luciferase reporter. When equivalent amounts of protein were expressed, mG295S displayed less transcriptional activation of the alpha myosin heavy chain (*Myhc*)[18] and atrial natriuretic factor (*ANF*)[19] enhancers compared to wild type, suggesting mildly reduced activity in this overexpression system (Fig. 2c and data not shown). In contrast, the truncated protein generated from recombinant mE360del was unable to activate transcription of either reporter (Fig. 2c). Although it cannot be determined whether transcripts encoding hE359del undergo nonsense-mediated decay *in vivo*, the inability of

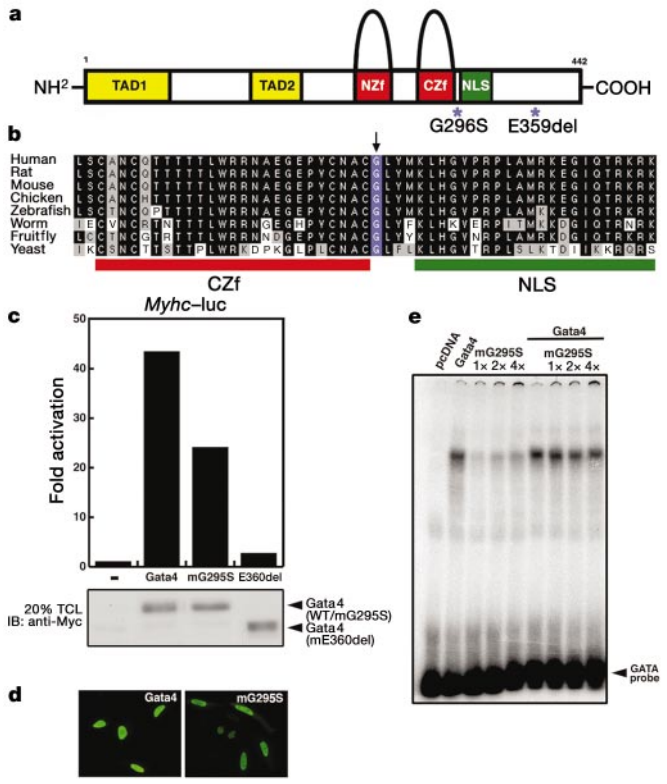


**Figure 2** Functional deficits associated with Gata4 mutations. **a**, Schematic of GATA4 protein domains indicates transactivation domains (TAD), N-terminal zinc finger (NZf), C-terminal zinc finger (CZf) and nuclear localization signal (NLS). The location of hG296S and hE359del mutations is indicated. **b**, Cross-species alignment of amino acids in the region of the hG296S mutation. Conserved glycine residues are highlighted in blue with identical residues shaded in black; the CZf or NLS is indicated in red or green, respectively. **c**, Activation of *Myhc*-luciferase reporter in HeLa cells by Gata4 wild type (WT), mG295S or mE360del demonstrated decreased transactivation by mutant proteins, with protein levels indicated by immunoblot (IB) of total cell lysate (TCL). **d**, Nuclear localization of Myc-tagged Gata4 wild type or mG295S, represented in green, was similar when expressed in HeLa cells. **e**, Electromobility shift assay of $^{32}$P-labelled GATA *cis*-element with Gata4 wild type or mG295S reveals decreased DNA-binding affinity of the mutant, even at four times concentration. The presence of increasing amounts of mutant protein did not affect the ability of wild-type protein to bind DNA.

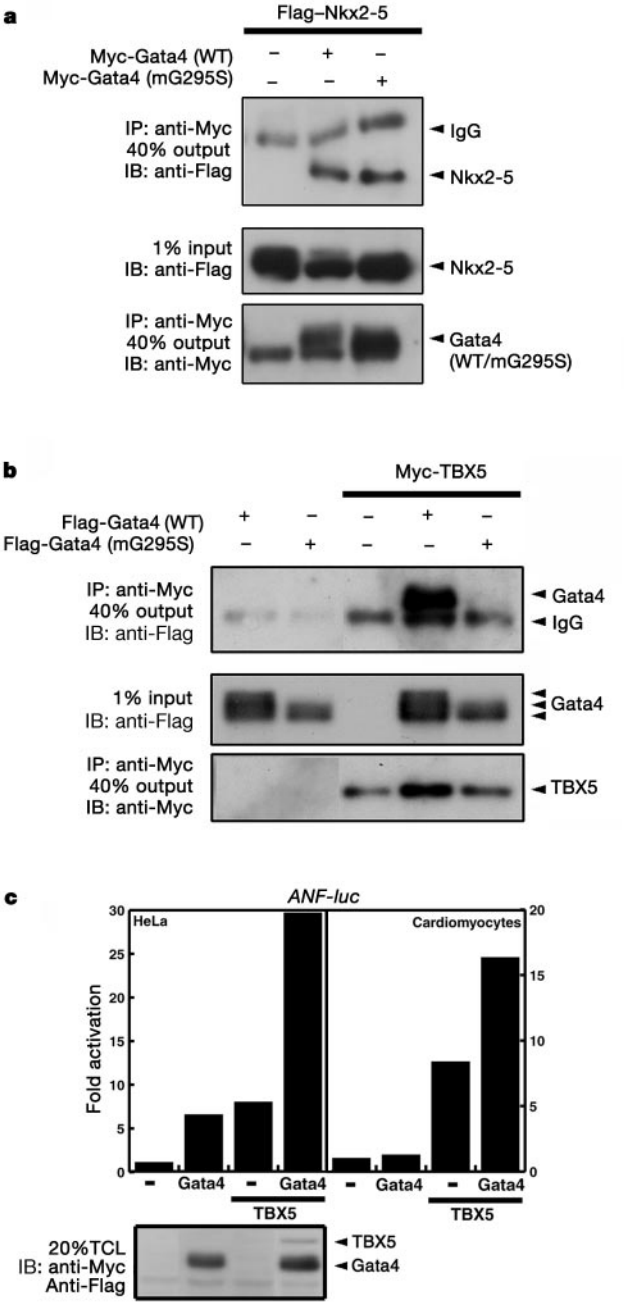


**Figure 3** The Gata4 interaction with TBX5 is specifically disrupted by the Gata4 mG295S mutation. **a**, Immunoprecipitation (IP) with Myc-tagged wild-type or mutant Gata4 (mG295S) revealed an association of each with Flag-Nkx2-5 on immunoblot (IB) with anti-Flag antibody. IgG, immunoglobulin band. **b**, Co-immunoprecipitation of Myc-TBX5 demonstrated an association with Flag-Gata4 wild type but not mG295S. **c**, Luciferase (luc) activity directed by the *ANF* promoter in the presence of Gata4 and TBX5 in HeLa or neonatal rat ventricular cardiomyocytes displayed cooperative activation. Protein levels of Gata4 and TBX5 are indicated by IB. TCL, total cell lysate.

mE360del to activate transcription suggests that GATA4 is probably haploinsufficient.

Because mG295S functioned as a hypomorph, we investigated the mechanisms through which this mutation affected Gata4 function. Although mG295S is near the NLS, the protein localized to the nucleus in HeLa and COS1 cells, suggesting that the NLS was not disrupted (Fig. 2d and data not shown). mG295S is also immediately adjacent to the C-terminal zinc finger, which is essential for DNA-binding and interaction with co-factors[20] (Fig. 2a, b). In electromobility shift assays (EMSA) to test DNA-binding affinity, wild-type Gata4 efficiently retarded gel migration of a $^{32}$P-labelled GATA *cis*-element corresponding to the *Hand2* enhancer[21]. In contrast, equivalent amounts of mG295S displayed limited affinity for the GATA element (Fig. 2e). With two- or fourfold excess of mG295S, we observed only weak DNA binding. When wild-type Gata4 was co-expressed in the presence of increasing amounts of mG295S, no alteration of wild-type DNA-binding affinity was detected (Fig. 2e). Together, these data suggest that the impaired DNA-binding of Gata4 mG295S may contribute to the reduced transcriptional activity of the mutant, although forced overexpression of the hypomorphic protein in tissue culture may compensate for the decreased DNA-binding affinity.

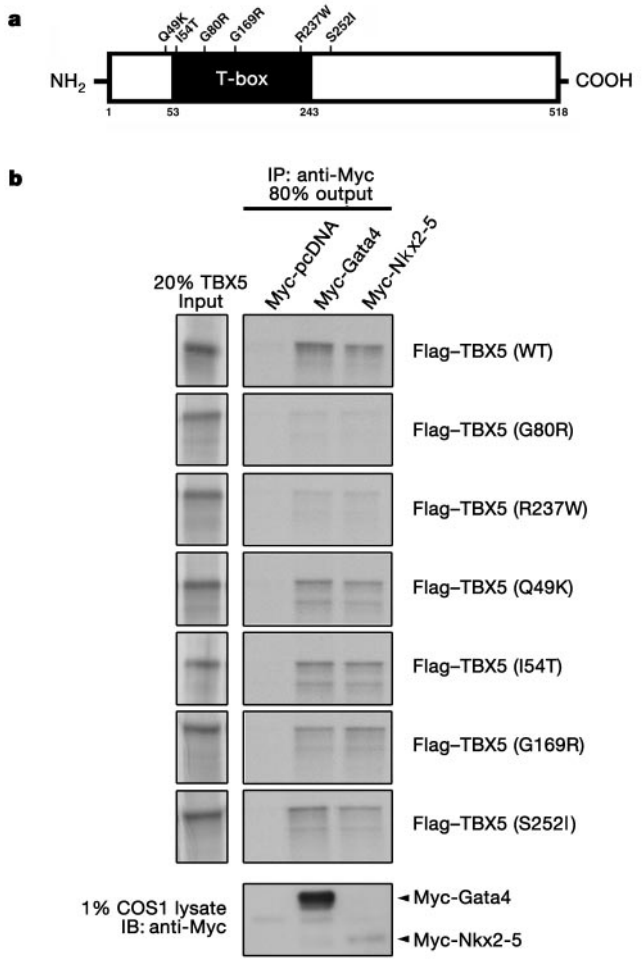


**Figure 4** Disrupted interaction of human TBX5 mutant proteins with Gata4 and Nkx2-5. **a**, Schematic of mutations of *TBX5* in humans with Holt-Oram syndrome. **b**, All $^{35}$S-labelled TBX5 mutants co-immunoprecipitated with Myc-Gata4 and Myc-Nkx2-5 with similar affinity to wild-type Flag–TBX5, except for the G80R and R237W mutants, when immunoprecipitated with an anti-Myc antibody and visualized by autoradiography. TBX5 input controls are shown on the left; immunoblot (IB) of Myc-tagged control proteins are shown at the bottom.

The zinc-finger domains of Gata4 also mediate numerous protein–protein interactions that often dictate DNA-binding specificity and therefore subsets of target gene activation. Hence, we tested whether mG295S affected the ability of Gata4 to interact with the two other transcriptional regulators implicated in human cardiac septal formation; that is, NKX2-5 and TBX5. By immunoprecipitation, mG295S was able to interact with Nkx2-5 through the C-terminal zinc finger, similar to wild-type Gata4 (ref. 22), suggesting that this motif was intact (Fig. 3a). In addition, we discovered a new interaction between Gata4 and TBX5, as both wild-type proteins could immunoprecipitate with one another (Fig. 3b). However, mG295S could not interact with wild-type TBX5, suggesting specific disruption of the Gata4–TBX5 interaction by the Gata4 mutation (Fig. 3b).

To determine whether Gata4 cooperates with TBX5 to activate transcription, we co-transfected the *ANF* reporter with TBX5 and Gata4 in HeLa cells and primary cardiomyocytes. Similar to Tbx5–Nkx2-5 interactions[23,24], co-expression of TBX5 and Gata4 resulted in cooperative activation of the reporter (Fig. 3c). Consistent with this observation, when we co-expressed Gata4 and TBX5, we were unable to detect an increase in transactivation of a luciferase reporter downstream of six tandem GATA sites (data not shown). We were also unable to detect a tertiary complex of TBX5 and Gata4 with the GATA *cis*-element by EMSA (data not shown), suggesting that additive transactivation of the complex may require DNA-binding of both Gata4 and TBX5.

The similarity between cardiac septal defects observed in the setting of human *GATA4* mutations and human *TBX5* mutations is consistent with the Gata4–TBX5 complex described here. Human *NKX2-5* mutations also cause similar septal defects, and NKX2-5 can physically interact with TBX5 (refs 23, 24). We therefore asked whether reported human *TBX5* mutations affect the ability of TBX5 to interact with GATA4 or NKX2-5. Six unique *TBX5* missense mutations from families that display HOS[25–27] (Fig. 4a) were examined for their ability to interact with wild-type Gata4 or Nkx2-5. All TBX5 mutants co-immunoprecipitated with Gata4 or Nkx2-5 except for the G80R and R237W mutants (Fig. 4b). In contrast, G80R and R237W were able to interact with several other cardiac transcription factors (I.S.K. and D.S., unpublished data), suggesting specific disruption of TBX5–Gata4/Nkx2-5 complexes. The reciprocal evidence that human *GATA4* and *TBX5* mutations affect interaction with one another in the setting of similar CHDs provides support that GATA4 and TBX5 may cooperate during cardiogenesis.

The combination of human genetics and biochemical analyses used here reveal a previously unrecognized genetic aetiology for CHDs and potential mechanisms through which certain cardiac defects may occur. The mutations in *GATA4* suggest that haploinsufficiency of *GATA4* in humans can result in the most common types of cardiac malformations and that GATA4 is essential for functional separation of the four cardiac chambers. In particular, the G296S mutation of GATA4 demonstrated not only an effect on DNA-binding affinity and transactivation of downstream targets, but also revealed an interaction between GATA4 and TBX5 that is disrupted in the setting of CHD. The similar effect of TBX5 mutations on the interaction with GATA4 and NKX2-5 raises the possibility that TBX5, NKX2-5 and GATA4 function in a complex to regulate a subset of genes required for cardiac septal formation. Whereas haploinsufficiency of *TBX5* has been linked to cardiac septal malformation in HOS, the disruption of a GATA4–TBX5 complex by some *TBX5* point mutations provides a potential mechanistic understanding of how disruption of combinatorial interactions of transcription factors can lead to specific birth defects. Broad screening for *GATA4* mutations in humans with heart disease may provide new avenues for understanding the

development of CHDs, leading to future therapeutic or preventive interventions. □

## Methods

### Clinical evaluation and DNA collection

CHD families were ascertained for genetic analyses at Children's Medical Center of Dallas, University of Texas Southwestern Medical Center (family A), and Tokyo Women's Medical University (family B). Clinical evaluations and genetic studies were performed in accordance with human subject guidelines after informed consent according to the protocol approved by the individual Institutional Review Boards. Family members were studied by history, physical examination, 12-lead electrocardiogram, and echocardiography. Medical records were reviewed of individuals who had died. Genomic DNA was extracted from peripheral lymphocytes for genetic analyses.

### Genetic linkage analysis

A whole-genome linkage analysis was performed using 358 polymorphic DNA markers at about 11 cM intervals (Cooperative Human Linkage Center/Weber Human Screening Set Version 8, Research Genetics, Inc.). Markers were genotyped in all family members as previously described[28]. Linkage analysis was performed using GENEHUNTER[29].

### Identification of *GATA4* mutations

All exons of the *GATA4* gene were sequenced in both directions to search for mutations in a proband from both pedigrees. The third or fifth exon of the human *GATA4* gene was amplified from family A or B, respectively, by PCR (primers and conditions available on request). The 395-bp (exon 3) or 411-bp (exon 5) PCR product was sequenced in both directions. The 395-bp fragment was digested with *Pst*I, resulting in the generation of two bands of 234 bp and 161 bp in subjects harbouring the mutant G296S *GATA4* allele; the 411-bp fragment was digested with *Bse*RI resulting in 142-bp and 269-bp fragments in subjects without the E359del mutation.

### Plasmid construction and site-directed mutagenesis

*Myhc*-luc[18], *ANF*-luc[19] and Flag-TBX5 (ref. 23) have been described previously; 6 × GATA site-luc was provided by S. R. Grant. For additional expression constructs, cassettes flanked by unique restriction sites were generated by PCR and cloned into pcDNA3.1-N-Myc and pcDNA3.1-N-Flag vectors (Invitrogen). Point mutations in Gata4 or TBX5 were introduced by generating two overlapping fragments by PCR and were verified by sequencing.

### Luciferase assays

HeLa cells were transfected using Fugene 6 (Roche) with 300 ng reporter, 300 ng Gata4, 600 ng Gata4 mG295S, 300 ng Gata4 mE360del or 300 ng TBX5. Immunoblots were used to verify appropriate protein expression. mG295S was consistently expressed at lower levels than wild type, necessitating transfection of twofold greater plasmid to achieve similar protein levels. Neonatal rat ventricular cardiomyocytes were collected and transfected with 150 ng reporter, 200 ng Gata4 and 400 ng TBX5, using Lipofectamine Plus reagent (Invitrogen). Luciferase activity was measured 48 h after transient transfection as previously described[30]. At least three independent experiments were performed in duplicate, with representative data shown.

### Immunocytochemistry

Forty-eight hours after transient transfection with Fugene 6 (Roche), HeLa and COS1 cells grown on slides were fixed with 3.7% formaldehyde/PBS, permeabilized with 0.1% TritonX-100/PBS, incubated with monoclonal anti-Myc antibody (Santa Cruz) and detected using anti-mouse conjugated-FITC antibody (Jackson Immunoresearch).

### Electrophoretic mobility shift assay

Annealed oligonucleotides (5′-TCGAGGTAATTAAC*TGATA*ATGGTGC-3′) with a GGG overhang representing the GATA *cis*-element upstream of *Hand2* (ref. 21) were labelled with [$^{32}$P]dCTP using Klenow enzyme and incubated with 2λ of wild-type and/or 2–8λ of mG295S Gata4 synthesized using the TNT Quick Coupled Transcription/Translation System (Promega) in binding buffer (20 mM HEPES, pH 7.9, 60 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT and 10% glycerol) for 20 min at room temperature and separated on a 6% polyacrylamide gel. [$^{35}$S]-labelled protein products were verified by SDS–PAGE.

### Co-immunoprecipitation assays

COS1 cells were transiently transfected with Fugene 6 (Roche), collected after 48 h in lysis buffer (PBS, 1 mM EDTA, pH 8.0, 0.5% Triton X-100), incubated with polyclonal anti-Myc antibody (Santa Cruz) and protein A Sepharose beads (Amersham Pharmacia), immunoblotted with monoclonal anti-Myc (Santa Cruz) or anti-Flag M2 (Sigma) antibodies and detected by the AP-conjugated CDP-Star system (Perkin Elmer). For overexpression of TBX5 point mutations, $^{35}$S-labelled products were synthesized using the TNT Quick Coupled Transcription/Translation System (Promega), incubated with COS1 lysates transfected with Myc-Gata4 or Myc-Nkx2-5, immunoprecipated with polyclonal anti-Myc, and visualized by autoradiography.

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