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Screening and Biochemical Analysis of GATA4 Sequence Variations Identified in Patients with Congenital Heart Disease

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Full Title: Screening and Biochemical Analysis of *GATA4* Sequence Variations Identified in Patients with Congenital Heart Disease

Running Title: *GATA4* Mutations in Congenital Heart Disease

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ABSTRACT

Few known monogenic causes of non-syndromic congenital heart disease (CHD) have been identified. Mutations in *NKX2.5* were initially implicated in familial cases of cardiac septal defects and subsequently, functionally significant *NKX2.5* mutations were found in diverse forms of non-syndromic CHD. Similarly, mutations in *GATA4*, which encodes a cardiac transcription factor, were first identified in familial cases of cardiac septal defects. We hypothesize that individuals with non-syndromic CHD may harbor *GATA4* mutations and that these mutations alter the biochemical properties of the protein. The coding region encompassing the six exons of *GATA4* was screened in a study population of 157 patients with CHD. We identified several sequence variations in *GATA4*. We tested these novel sequence variations that altered evolutionarily conserved amino acids and other previously reported *GATA4* mutations in various biochemical assays. The novel sequence variations had no biochemical deficits while a previously reported, but unstudied, missense mutation in *GATA4* (S52F) functioned as a hypomorph in transactivation assays. We did not identify any novel *GATA4* mutations in our patient population with non-syndromic CHD. Consistent with previous findings, *GATA4* mutations that result in deficits in transactivation ability are consistently associated with CHD suggesting that normal transactivation properties of GATA4 are required for proper cardiac development.

Key Words: congenital heart defects; cardiovascular malformations; genetics; transcription factor; GATA4; NKX2.5

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INTRODUCTION

Congenital heart disease (CHD) is the most common type of birth defect with an estimated incidence of nearly 1%, affecting nearly 36,000 children annually [Hoffman and Kaplan, 2002; AHA Statistics, 2006]. Although CHD may occur in association with other birth defects as part of a syndrome, it is most often found as an isolated birth defect [Bosi et al., 2003]. The etiology of non-syndromic cases of CHD is multifactorial, but recent reports studying large autosomal dominant families have identified mutations in the cardiac transcription factors, *NKX2.5* and *GATA4*, as a cause of non-syndromic CHD in a subset of individuals [Schott et al., 1998; Garg et al., 2003]. We described two mutations in *GATA4*, G296S and E359del, as the genetic etiology of familial cardiac septal defects and pulmonary valve stenoses [Garg et al., 2003]. The cardiac septal defects were ostium secundum atrial septal defects and perimembranous ventricular septal defects but one family member did have an atrioventricular septal defect. Subsequently, other investigators have reported additional *GATA4* mutations, S52F, E216D and E358del, in individuals with non-syndromic CHD [Okubo et al., 2004; Hirayama-Yamada et al., 2005; Sarkozy et al., 2005; Nemer et al., 2006]. We hypothesize that mutations in *GATA4* are present in diverse types of non-syndromic CHD similar to *NKX2.5*, and that these mutations might result in alterations of Gata4 function in biochemical assays thereby accounting for genotype-phenotype differences [Benson et al., 1999; Goldmuntz et al., 2001; Kasahara et al., 2000; Kasahara et al., 2004]. To determine the frequency of *GATA4* mutations in patients with non-syndromic CHD, we studied 157 individuals with sporadic and familial cases of CHD and tested

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novel and previously unstudied *GATA4* mutations in biochemical assays to identify in vitro functional deficits.

MATERIALS AND METHODS

Study Population

The subjects were comprised of 157 unrelated individuals (90 males and 67 females) who received their cardiovascular care at Children's Medical Center Dallas. The individuals were of variable ethnicity and were composed of 76 European Americans, 53 Hispanics, 21 African Americans, and 7 Asians. All subjects had CHD (see Table I) and in 22 cases, an additional affected family member was identified. The phenotype of the subjects with a familial recurrence were 5 with ostium secundum atrial septal defect, 3 with perimembranous ventricular septal defect, 4 with valve or subvalvar aortic stenosis, 1 with dysplastic mitral valve, 1 with coarctation, 2 with total anomalous pulmonary venous return, 3 with complex CHD, 1 with tetralogy of Fallot, 1 with d-transposed great vessels, and 1 with pulmonic valve stenosis. Between December 2001 and February 2003, subjects were prospectively recruited for genetic testing and informed consent obtained according to protocol as approved by the Institutional Review Board at the University of Texas Southwestern Medical Center. The cohort was randomly selected from this population and included all subjects with a familial recurrence. Patients with chromosomal abnormalities such as Trisomy 21 or 22q11 deletion were excluded from the study. Patients underwent complete cardiac evaluation at Children's Medical Center Dallas and echocardiogram, cardiac catheterization and operative reports were reviewed, when available. Venous blood samples were

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collected and genomic DNA isolated using the PUREGENE kit (Gentra Systems) from affected subjects.

Genomic DNA was obtained for the control subject population from the Dallas Heart Study [Cohen et al., 2005]. The control population consisted of 159 individuals of variable ethnicity, including 78 European Americans, 33 Hispanics and 48 African Americans. In addition, 192 control individuals of Asian ethnicity were screened for the C1180A sequence variation using a previously described allelic discrimination assay [Cohen et al., 2005].

Sequencing of human *GATA4* gene

All 6 exons of GATA4 were sequenced bidirectionally and all sequence variations were identified in the affected and control subjects. The sequencing primers have been reported and are available on request [Garg et al., 2003]. PCR amplification was performed using the BD Biosciences Advantage GC Genomic PCR kit following the manufacturer's instructions, with an annealing temperature of 60°C.

Plasmid Construction and Site-Directed Mutagenesis

Expression constructs were generated for the *GATA4* S52F, E359del, S377G and P394T recombinant proteins. Point mutations were introduced into the orthologous mouse Gata4 cDNA (Accession number AF179424) by generating overlapping fragments by PCR and were verified by sequencing (Gata4 mS52F, mE360del, S377G and mP394T, respectively). The mutant Gata4 cDNAs were cloned into pcDNA3.1-N-Myc vector (Invitrogen).

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Luciferase Assays

HeLa cells were transfected using Fugene 6 (Roche) with 300ng of either alpha myosin heavy chain or atrial natriuetic factor luciferase reporter, 100-300ng of wildtype Gata4 or 300ng of Gata4 mS52F, S377G, and P394T mutant plasmids [Molkentin et al., 1994; Sprenkle et al., 1995]. Immunoblots were used to verify appropriate protein expression. Gata4 mS52F protein was consistently expressed at lower levels than wildtype necessitating transfection with three fold greater plasmid to achieve similar protein levels. Luciferase activity was measured 48h after transient transfection as previously described [Garg et al., 2003]. Three independent experiments were performed in duplicate with the alpha myosin heavy chain and atrial natriuetic factor luciferase reporters. Luciferase data for the Gata4 mutant proteins are shown as a percentage of wildtype Gata4 activation (normalized to 100% for each experiment). Statistical comparisons were performed using Student's t test. P value < 0.05 was considered significant.

Cellular Localization

Forty eight hours after transient transfection of Gata4 wildtype and mutant protein expression plasmids with Fugene6 (Roche), HeLa cells were grown on slides that 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).

Electromobility Shift Assays

Annealed oligonucleotides (5'-TCGAGGTAATTAAC<u>TGATA</u>ATGGTGC-3') with a GGG overhang representing the GATA *cis* element upstream of *Hand2* [McFadden et

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al., 2000] were labeled with [^{32}P]dCTP using Klenow enzyme and incubated with 3λ of wild type and/or 2λ of mS52F or mE360del which were synthesized using the TNT Quick Coupled Transcription/Translation System (Promega) in binding buffer (20mM Hepes, pH7.9, 60mM KCl, 1mM $MgCl_2$, 0.5mM DTT and 10% glycerol) for 20 min at RT and separated on a 6% polyacrylamide gel. [^{35}S]-labeled protein products were verified by SDS-PAGE.

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RESULTS

Identification of *GATA4* Sequence Variations

The *GATA4* gene was sequenced in 157 individuals with diverse forms of non-syndromic CHD with the predominant diagnoses being cardiac septal defects and left-sided obstructive malformations (details are shown in Table I). Our analysis resulted in the identification of one sequence variation (C1180A) that predicted an amino acid substitution at codon 394 (P394T). This nucleotide change was not found in the original control population of 159 individuals, but it had been identified in an individual of Asian ethnicity. Therefore, we undertook screening of 192 control individuals of Asian origin and found this sequence variation to have an allele frequency of 3.1% consistent with it representing a single nucleotide polymorphism (SNP) in the Asian subpopulation (Table IIB). In addition, we identified 3 sequence variations (A700G, C732T, C786T) that were not found in the ethnically-diverse control population and predicted synonymous amino acid changes (T233T, Y244Y, S262S, respectively) in 4 individuals. The phenotypes and ethnicities of these individuals are summarized in Table IIA. The A700G variation has been reported by other investigators [Poirier et al., 2003].

In addition to the C1180A polymorphism, several sequence variations were found in both the affected and control populations and likely represented common SNPs. Two predicted a non-synonymous amino acid change (S377G and V380M) while three did not alter the amino acid (N352N, S371S and P407P) (Table IIB). The N352N, S377G, and P407P alterations are known sequence variations in *GATA4* as they have been described in the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html), while the valine residue at codon 380 is not conserved in the murine ortholog of *GATA4* . Sequencing of the control population resulted in the identification of three sequence variations that result in non-synonymous amino acid changes (Q3P, A32P and R151H) and two that did not predict an amino acid substitution (P342P and S379S) (Table III). In sum, we identified six sequence variations that predicted an amino acid substitution, the majority of which altered a conserved residue in the GATA4 protein (see Supplementary Figure), but none occurred at a significantly higher frequency in the affected population.

Biochemical Analysis of Identified *GATA4* Sequence Variations

Previous reports by us and other investigators have led to the identification of a total of 3 missense and 2 frameshift *GATA4* mutations in subjects with CHD, which are summarized along with the associated phenotype in Figure 1A [Garg et al., 2003; Okubo et al., 2004; Hirayama-Yamada et al., 2005; Sarkozy et al., 2005; Nemer et al., 2006]. These mutations are located in different regions of the GATA4 protein suggesting that each mutation may result in unique deficits in GATA4 function (Figure 1B). We had previously reported that the *GATA4* G296S (mG295S) mutation had normal nuclear localization but decreased DNA binding affinity and this likely resulted in

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its hypomorphic transactivation ability as measured by luciferase reporter assays. In addition, we found that the Gata4 mG295S protein was unable to interact with TBX5, the genetic cause of Holt-Oram syndrome characterized by CHD, but maintained its interaction with NKX2.5 [Garg et al., 2003; Basson et al., 1997; Li et al., 1997; Bruneau et al., 2001]. The E359del mutation in *GATA4* was reported to have decreased transactivation ability [Garg et al., 2003]. This deficit was not due to defective DNA binding or nuclear localization (data not shown) similar to the E216D mutation [Nemer et al., 2006]. Recently, a mutation in the first transactivation domain, S52F, was found to segregate with affected family members with secundum atrial septal defects (Hirayama-Yamada et al., 2005]. We generated this mutant Gata4 protein and examined its ability to activate transcription of downstream target genes in vitro, using several Gata-dependent cardiac enhancers upstream of a luciferase reporter. In transcriptional assays using an alpha-myosin heavy chain (α-MHC) luciferase reporter, we found that the mS52F mutant protein had significantly decreased transcriptional activity (Figure 2A). A similar hypomorphic pattern of transactivation was seen when an atrial natriuetic factor (ANF) enhancer was utilized for the mS52F mutant protein (Figure 2A). This decreased activation ability was not explained by abnormal subcellular localization or DNA binding affinity (Figure 2B, C). To determine if these transcriptional deficits were unique, we tested two sequence variations that altered conserved amino acids, S377G and P394T, in similar assays and found that they had normal, or possibly increased, activation ability when compared to wildtype consistent with them not being associated with CHD (Figure 2D). These findings suggested that the dosage of *GATA4* was critical

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for normal cardiac development and that *GATA4* mutations resulting in decreased transactivation ability likely contribute to the human disease phenotype.

DISCUSSION

In summary, we did not identify any novel mutations in *GATA4* in our cohort that are responsible for non-syndromic human CHD. Our study population was diverse and therefore composed of relatively small numbers for each subtype of cardiac malformation. As such, we cannot rule out the possibility that mutations in *GATA4* have a higher frequency in certain sporadic cases of CHD such as septal defects, tetralogy of Fallot or pulmonary valve stenosis. We did identify 3 sequence variations that resulted in "silent" mutations (or synonymous amino acid changes) in an additional 4 individuals, one with a cardiac septation defect, two with tetralogy of Fallot, and one with left ventricular outflow tract obstruction. The significance of these "silent" genetic variations is unclear but recent studies have identified a synonymous SNP, which is associated with psoriasis and leads to increased stability of the mRNA transcript of the causative gene [Capon et al., 2004]. Another possibility is that these nucleotide variations may lie within exonic splicing enhancers or silencers and interfere with pre-mRNA splicing resulting in the production of abnormal protein products [Modrek and Lee, 2002; Modrek and Lee, 2003]. Gata4 is exquisitely dosage sensitive as mice hypomorphic for *Gata4*, expressing 30% of normal Gata4 protein levels, exhibit severe embryonic cardiac defects while mice heterozygous for *Gata4* (i.e. 50% of Gata4 protein) are normal [Pu et al., 2004; Molkentin et al., 1997; Kuo et al., 1997; V.G., unpublished observations].

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Additional functional studies on these “silent” mutations in *GATA4* are necessary in order to determine if they may be contributing to human CHD.

The identified *GATA4* missense mutations found in cases of CHD consistently exhibit functional differences in biochemical assays in vitro. Similar to the other reported mutations, the S52F mutant protein had decreased transactivation ability. The mutation at position 52 altered a serine residue within in a known activation domain and may be disrupting this region by altering its structure or possibly affecting phosphorylation. The identification that *GATA4* mutations associated with CHD have hypomorphic transactivation ability not always explained by DNA binding deficits is consistent with the dosage sensitivity of *GATA4*. These findings are different from those reported for *NKX2-5* mutations which usually exhibit decreased transactivation ability and associated reduced DNA binding affinity [Kasahara et al, 2004]. In summary, the results of in vitro biochemical studies are consistent with reports of human patients who have similar types of CHD and harbor deletions of chromosome 8p that lead to haploinsufficiency of *GATA4* [Pehlivan et al., 1999].

In conclusion, *GATA4* is one of the known monogenic contributors to non-syndromic CHD along with *NKX2.5*, *MYH6*, *NOTCH1* and others [Ching et al., 2005; Garg et al., 2005; Garg et al., 2006]. Continued sequencing of *GATA4* in larger populations of specific cardiac malformations is needed to further define the impact of *GATA4* mutations in CHD. Ultimately, increased knowledge of the structure-function relationships of GATA4 protein may lead to better genotype-phenotype correlations. Finally, it is becoming more evident that a threshold level of *GATA4* is necessary for normal cardiac development in humans.

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COMPETING INTERESTS

The authors declare no competing interests.

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Table I. Diagnoses of screened study population with congenital heart disease.

Cardiac Diagnosis	Number of Patients
Septation Defects	**(32)**
ASD	14
Ostium secundum (12)	
Sinus venosus (2)	
VSD	18
Perimembranous (16)*	
Muscular (1)	
Supracristal (1)	
Left-sided Defects	**(45)**
Dysplastic MV	3
AS or sub AS	18
CoA	13
HLHS	11
Right-sided Defects	**(18)**
TV Atresia	6
PS	5
PA (without VSD)	7
Conotruncal defects	**(20)**
TOF	18
Truncus Arteriosus	2
Complex	**(19)**
DILV, DORV, or single ventricle	19
Endocardial Cushion Defects	**(7)**
AVSD	6
ASD – ostium primum	1
Other	**(16)**
D-TGA	6
TAPVR	4
PDA	3
Abnormal Coronary	2
Ebstein's Anomaly	1

ASD, atrial septal defect; VSD, ventricular septal defect; MV, mitral valve; AS, aortic stenosis; CoA, coarctation; HLHS, hypoplastic left heart syndrome; TV, tricuspid valve; PS, pulmonary valve stenosis; PA, pulmonary valve atresia; TOF; tetralogy of Fallot; DILV, double inlet left ventricle; DORV, double outlet right ventricle; AVSD, atrioventricular septal defect; D-TGA, d-transposition of the great arteries; TAPVR, total anomalous pulmonary venous return; PDA, patent ductus arteriosus. * 3 children with perimembranous ventricular septal defects had additional small muscular ventricular septal defects.

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Table II. Sequence variations identified in congenital heart disease (CHD) population

A	AMINO ACID CHANGE	NUCLEOTIDE CHANGE	CARDIAC PHENOTYPE	ETHNICITY
	T233T	A700G	sub- and supra-valvar AS	African-American
	Y244Y	C732T	AVSD	African-American
			TOF with PA, LSVC	African-American
	S262S	C786T	TOF with PA	European-American

B	AMINO ACID CHANGE	NUCLEOTIDE CHANGE	MINOR ALLELE FREQUENCY	
			Affected	*Controls*
	N352N	C1056T	0.006 (2/314)	0.038(12/318)
	S371S	A1113G	0.006 (2/314)	0.013 (4/318)
	S377G	A1129G	0.067(21/314)	0.072(23/318)
	V380M	G1138A	0.006 (2/314)	0.009 (3/318)
	P394T	C1180A	0.003 (1/314)	0.031(12/384)*
	P407P	A1221C	0.006 (2/314)	0.003 (1/318)

A. Sequence variations identified in only affected CHD population. AS, aortic stenosis; AVSD, atrioventricular septal defect; TOF, tetralogy of Fallot; PA, pulmonary atresia; LSVC, left superior vena cava. **B. Sequence variations identified in both the CHD and control populations.** Only 3 of these sequence variations has been reported in the dbSNP database [N352N (rs#3729855), S377G (rs#3729856), and P407P (rs#7830178)]. In a North-American population the frequency of the minor allele was 8.9% and 8.3% for the N352N and S377G alterations, respectively, while the P407P sequence variation was not identified in a mixed population of Europeans, Asians and African-Americans. The nucleotide position is based upon the human

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GATA4 mRNA sequence (Accession number NM002052). * frequency in Asian subpopulation.

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Table III. Sequence variations identified in only control population.

AMINO ACID CHANGE	NUCLEOTIDE CHANGE	ALLELE FREQUENCY
Q3P	A8C	1/318 (0.003)
A32P	G94C	1/318 (0.003)
R151H	G452A	1/318 (0.003)
P342P	C1026T	11/318 (0.035)
S379S	C1137T	1/318 (0.003)

The A8C, G94C, G452A, and C1137T sequence variations were identified in African-Americans while the C1026T was found in 9 African-Americans, 1 European-American and 1 Hispanic. None of these sequence variations has been previously reported in the dbSNP database.

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FIGURE LEGENDS

Figure 1. Summary of previously reported disease-associated *GATA4* mutations. (A) Human *GATA4* mutations and associated cardiac phenotype. All of these mutations were identified in patients with familial congenital heart defects except for the E216D. ASD, atrial septal defect; VSD, ventricular septal defect; AVSD, atrioventricular septal defect; PS, pulmonary valve stenosis. (B) Schematic of GATA4 protein indicates location of mutations (*). TAD1, transactivation domain 1, TAD2, transactivation domain 2; ZF, zinc finger; NLS, nuclear localization signal.

Figure 2. Functional analysis of Gata4 sequence variations. (A) Decreased luciferase activity in HeLa cells transfected Gata4 mS52F expression plasmid (mean and ± S.D. are shown in graph). Mean transcriptional activity of Gata4 mS52F was 38.0 ± 11.9% with α-MHC luciferase reporter (p value < 0.0001) and 15.7 ± 7.6% with ANF luciferase reporter (p value < 0.0001) when compared to wildtype (WT). Transfection experiments were performed using 100ng of Gata4 WT and 300ng of Gata4 mS52F plasmids. (B) Nuclear localization of Myc-tagged Gata4 mS52F mutant protein was similar to wildtype Gata4 (WT) when expressed in HeLa cells. (C) Gata4 mS52F mutant proteins demonstrated normal DNA-binding affinity compared to wildtype Gata4 by electromobility shift assay of ^{32}P-labelled GATA cis-element. 100x cold wildtype oligo (WT) competed for Gata4 binding, while 100x cold mutant oligo (MT) failed to compete demonstrating sequence-specific binding of the Gata4 protein. (D) Relative luciferase activity of Gata4, Gata4 mS377G, and mP394T in HeLa cells co-transfected with α-MHC luciferase and ANF luciferase reporters. Mean and ± S.D. are shown in graph. S377G mutant plasmid had 183 ± 58% and 152 ± 64% activity compared to

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wildtype with α-MHC and ANF luciferase reporters (p value <0.01 and NS, respectively). P394T mutant plasmid had 202 ± 44% and 166 ± 82% activity compared to wildtype with α-MHC and ANF luciferase reporters (p value <0.001 and NS, respectively). Equal amounts (300ng) of Gata4, mS377G, mP394T expression plasmid were used. Protein levels were kept equivalent for reporter assays, as indicated by immunoblot (IB) of total cell lysate (TCL) for (A) and (D). NS, not significant.

Supplementary Figure

Figure. Cross-species alignment of Gata4 protein. Six sequence variations are predicted to alter amino acid residues in the Gata4 protein. The amino acid changes are marked numerically **(1)**Q3P, **(2)**A32P, **(3)**R151H, **(4)**S377G, **(5)**V380M, and **(6)**P394T in this alignment of human, pig, rat, mouse and zebrafish Gata4 protein sequences. Only the R151H amino acid change is predicted to be “possibly damaging” by PolyPhen (http://genetics.bwh.harvard.edu/pph/), a bioinformatics tool that predicts the possible impact of an amino acid substitution. All other amino acid alterations are predicted to be “benign”. Identical residues are highlighted in black.

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Mutation	Cardiac Phenotype	References
S52F	ASD	Yamada et al., 2005
E216D	TOF	Nemer et al., 2006
G296S	ASD, VSD, AVSD, PS	Garg et al., 2003; Sarkozy et al., 2005
S358del	ASD	Okubo et al., 2004
E359del	ASD, dextrocardia	Garg et al., 2003

B

Schluterman et al, Figure 1

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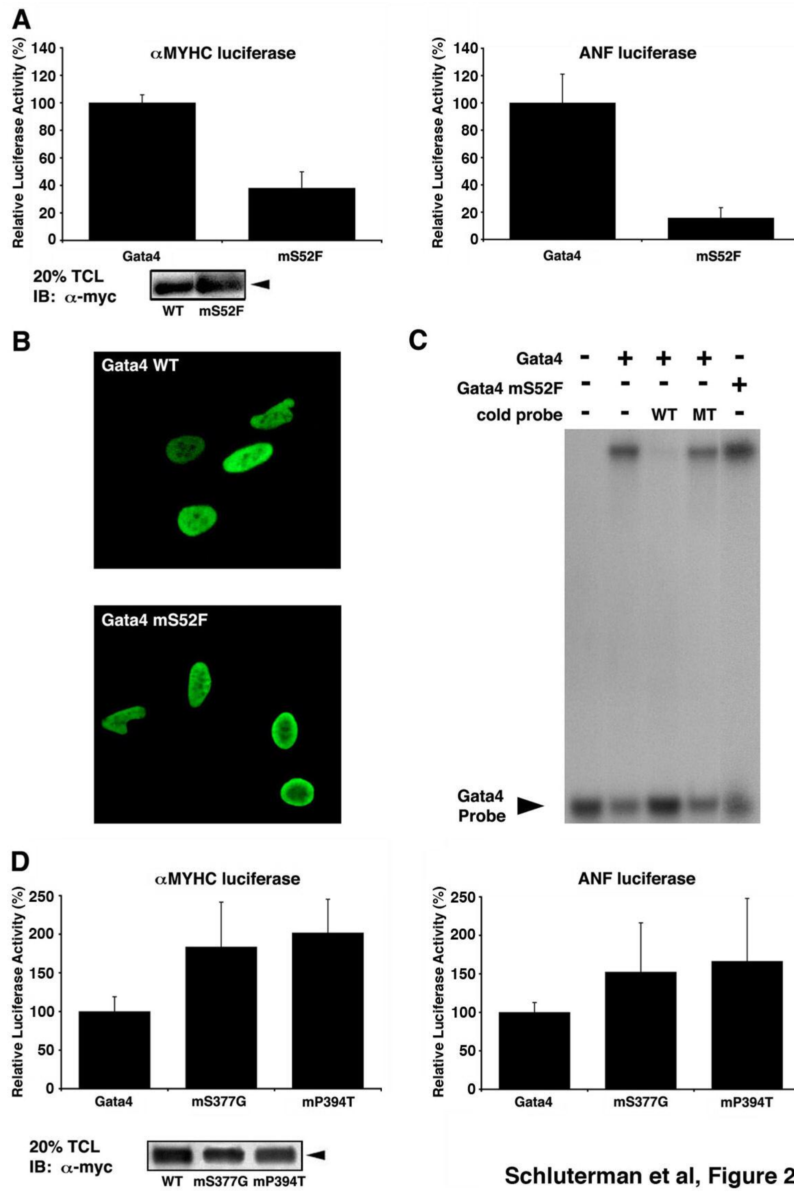
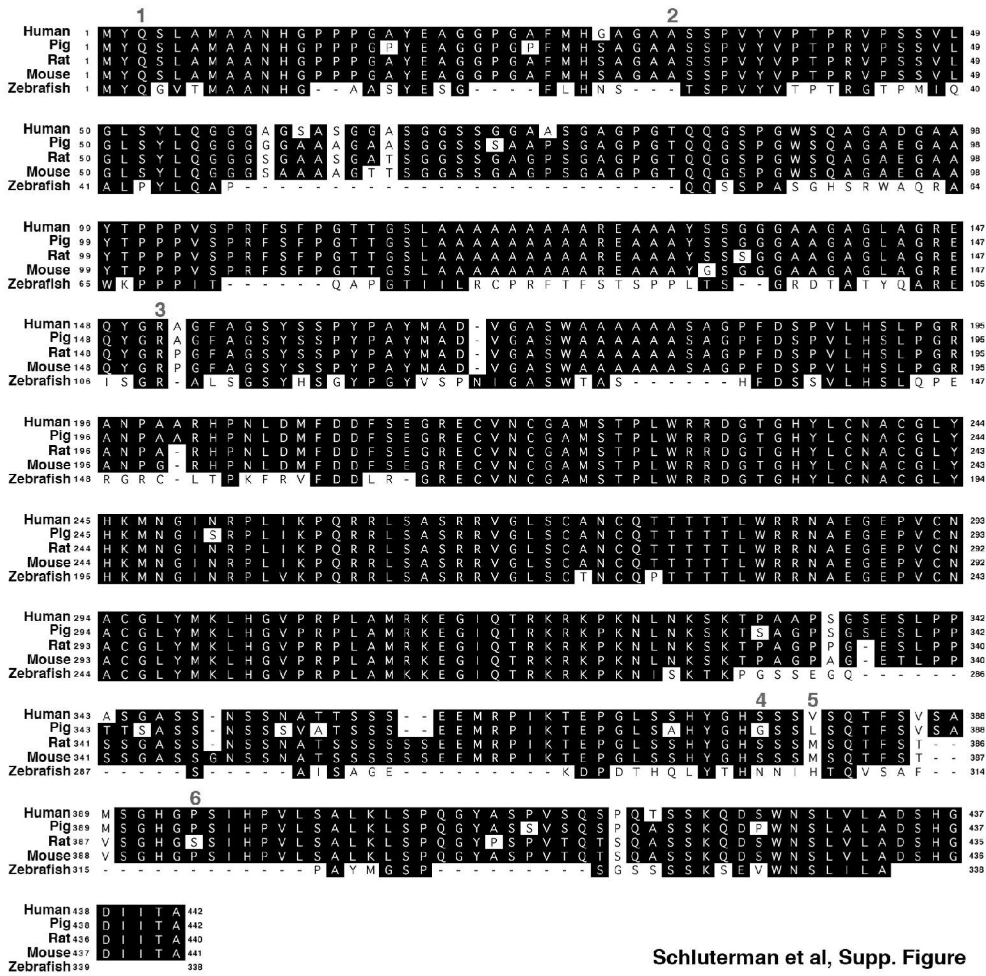


Schluterman et al, Figure 2



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