ARTICLE

Reduced NODAL Signaling Strength via Mutation of Several Pathway Members Including FOXH1 Is Linked to Human Heart Defects and Holoprosencephaly

Erich Roessler,[1] Maia V. Ouspenskaia,[1] Jayaprakash D. Karkera,[1] Jorge I. Vélez,[1] Amy Kantipong,[1] Felicitas Lacbawan,[1] Peter Bowers,[2] John W. Belmont,[3] Jeffrey A. Towbin,[3] Elizabeth Goldmuntz,[4] Benjamin Feldman,[1] and Maximilian Muenke[1,*]

Abnormalities of embryonic patterning are hypothesized to underlie many common congenital malformations in humans including congenital heart defects (CHDs), left-right disturbances (L-R) or laterality, and holoprosencephaly (HPE). Studies in model organisms suggest that Nodal-like factors provide instructions for key aspects of body axis and germ layer patterning; however, the complex genetics of pathogenic gene variant(s) in humans are poorly understood. Here we report our studies of *FOXH1, CFC1*, and *SMAD2* and summarize our mutational analysis of three additional components in the human NODAL-signaling pathway: *NODAL, GDF1*, and *TDGF1*. We identify functionally abnormal gene products throughout the pathway that are clearly associated with CHD, laterality, and HPE. Abnormal gene products are most commonly detected in patients within a narrow spectrum of isolated conotruncal heart defects (minimum 5%–10% of subjects), and far less commonly in isolated laterality or HPE patients (~1% for each). The difference in the mutation incidence between these groups is highly significant. We show that apparent gene dosage discrepancies between humans and model organisms can be reconciled by considering a broader combination of sequence variants. Our studies confirm that (1) the genetic vulnerabilities inferred from model organisms with defects in Nodal signaling are indeed analogous to humans; (2) the molecular analysis of an entire signaling pathway is more complete and robust than that of individual genes and presages future studies by whole-genome analysis; and (3) a functional genomics approach is essential to fully appreciate the complex genetic interactions necessary to produce these effects in humans.

Introduction

Holoprosencephaly (HPE) is the most common structural malformation of the embryonic brain (observed in 1 in 250 human conceptuses).[1–4] Its etiology rests in the failure to appropriately specify ventral or dorsal prosencephalon midline structures that, in turn, leads to varying degrees of incomplete separation of the forebrain into discrete hemispheres.[1,2,5–8] A very broad range of malformations of the brain and face comprise the HPE clinical spectrum. In contrast, most examples of targeted disruption of genes in mice or other model systems produce only the single-eye (i.e., cyclopic) extreme in homozygous null genotypes, whereas animals heterozygous for these null alleles are typically normal (reviewed in [9]). Unlike this cyclopic extreme, HPE in humans is generally considered to be multifactorial, with numerous genes and environmental factors thought to interact in its causation.[5,7,8] Empirical evidence for such a multifactorial etiology has been difficult to establish because of the multiplicity of causative agents and limitations of our understanding of precisely how the genes and environmental causes can interact[5,7,8] (E.R. and M.M., unpublished data). Nevertheless, rare examples of mutations in more than one gene have been described and tend to support this multiple-hit hypothesis[5] (reviewed in [7]).

Central to our present understanding of HPE pathogenesis is a failure in the patterning of the midline resulting from either (1) defects in the formation or positioning of the midline per se (e.g., features that are dependent on NODAL[10] [MIM 601265] factors), (2) defects in the signaling from the ventral-most aspect of the axial midline (the prechordal plate signals, such as secreted SHH [MIM 600725], culminating in the activation of its transcription factor GLI2 [MIM 165230]), or (3) the inability of the future forebrain cells to appropriately respond to such signals (e.g., neuroectodermal SIX3 [MIM 603714], ZIC2 [MIM 603073], and TGIF [MIM 602630] haploinsufficiency). Although there is excellent evidence for the roles of each of these genes in producing HPE,[1,2,5,7–9] there is limited evidence for more than one gene contributing to HPE in any individual clinical case.

Most cases of HPE appear to be sporadic, because parents of affected individuals are usually phenotypically normal. In addition, genetic counseling is typically extremely difficult given that only 20%–25% of cases can be attributed to a detectable abnormality in any one of the known HPE genes.[7,8] Studies of HPE families, although less common, usually support apparent autosomal-dominant transmission of a principal susceptibility gene. However, it has long been recognized that the penetrance is reduced and a wide range of expressivity can be seen even within

[1]Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892, USA; [2]Department of Pediatrics, Yale University School of Medicine, New Haven, CT 06510, USA; [3]Division of Cardiology, Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA; [4]Division of Cardiology, The Children's Hospital of Philadelphia, Philadelphia, PA 19104, USA
*Correspondence: mmuenke@nhgri.nih.gov
DOI 10.1016/j.ajhg.2008.05.012.

a single family transmitting a known HPE gene variant.[1,5,7,8] A complete understanding of HPE etiology is elusive; many of these known gene mutations are likely to be necessary (yet not entirely sufficient) to produce an effect. The multiple-hit hypothesis[5] argues that the readily detectable mutations in HPE genes observed in patients represent only one of several discrete steps needed to produce these pathological disturbances. However, it has been difficult to demonstrate the gene-gene or gene-environmental interactions considered essential to produce this complex syndrome given the limitations imposed by its inherent genetic heterogeneity and low frequency of any given private mutation. For most HPE patients, only a single heterozygous change is detectable through routine molecular screening, and systematic attempts have only rarely identified mutations in more than one HPE gene in a single patient[5] (E.R. and M.M., unpublished data).

Heterotaxy disorders (such as discordant or mirror-image development of intrinsically asymmetric internal organs[11,12] and/or isolated congenital heart defects[13–15]) are also thought to be multifactorial conditions ultimately linked to the integrity of the axial midline and/or L-R patterning signals. These conditions also comprise a clinical spectrum and are considered equally complex in the interplay of genetic and environmental factors underlying their causation. For example, X-linked heterotaxy (HTX [MIM 306955]) occurs in males resulting from hemizygosity for a loss-of-function allele of the zinc-finger transcription factor ZIC3 (MIM 300265).[16] Interestingly, males can manifest the discordant internal organ positioning typical for the heterotaxy syndrome or can instead display isolated congenital heart defects as the sole clinical manifestation.[17] Both phenotypes are associated with similar degrees of functional impairment of the ZIC3 gene product,[18] suggesting that genetic or environmental modifiers act in concert with these gene lesions. Like HPE, these malformations are due to heterogeneous causes, most of which are presently unknown. However, in order for asymmetric organogenesis to proceed, there must be an elaboration of signals that distinguish the left and right sides of the embryo; this link with Nodal signaling is shared, along with the requirement for the axial midline, for preventing cyclopia.[19,20]

We selected the NODAL signaling pathway as a test case for study because the principal genetic components of this pathway are well understood (Figure 1) and have been clearly implicated in clinically relevant dysmorphologies in model systems. To gain insight into the frequency and types of combinations of alterations evident in our patient groups, we performed a detailed molecular survey of NODAL pathway genes. Our current and previous studies are summarized in Figure 2. To assess the multiple-hit hypothesis[5,21,22] in the context of NODAL signaling, we examined genomes of individuals with CHD, laterality, and HPE, as well as controls, for mutations in the following core NODAL signaling components: *NODAL, GDF1* (MIM 602880), *CFC1* (MIM 605194; GenBank NM_032545), *TDGF1* (MIM 187395; GenBank NM_003212), *FOXH1*

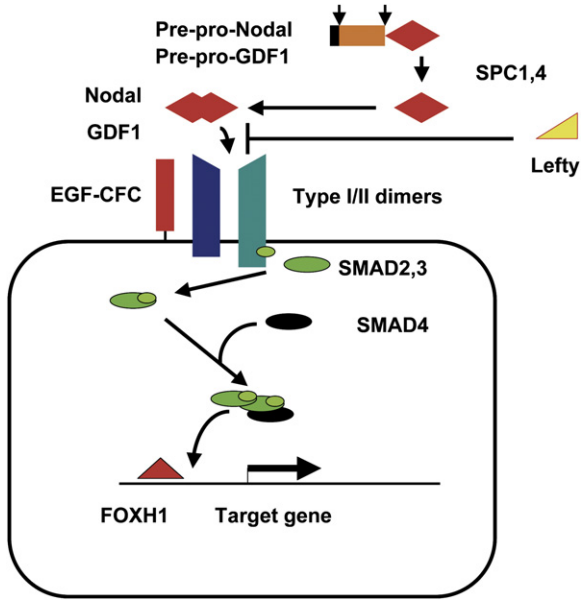

Figure 1. The Essential Components of Nodal Signaling
TGF-β proteins are synthesized as pre-pro-proteins requiring cleavage of the signal peptide (black) and prodomain (orange) prior to dimerization of active ligand (red diamonds). Cells responsive to Nodal-like ligands display Type I/II Activin receptors on their cell surface with coreceptors of the EGF-CFC family required for certain ligands, such as Nodal and Gdf1. The Lefty proteins (yellow) interfere with mature ligand binding to receptors. Ligand binding triggers transphosphorlyation of type I receptors by the type II receptor serine-threonine kinase domain. Receptor-regulated R-Smads (green) become activated on the inner surface of the cell membrane through phosphorylation by type I receptors. Activated Smad complexes include two R-Smads and the common Smad4 (black) that translocate into the nucleus to affect gene expression. Target genes typically contain binding sites for Smads and cofactors such as FoxH1 (red triangle) to achieve tissue-specific gene expression. Human mutations have been previously described in *LEFTYA* (MIM +601877), *LEFTYB* (MIM *603037), *ACVR2B* (MIM +602730) (see reference [12] for review), *CFC1* (MIM *605194),[27,28] *TDGF1* (MIM +187395),[25] and *GDF1* (MIM +602880).[39]

(MIM 603621; GenBank NM_003923), and *SMAD2* (MIM 601366; GenBank NM_001003652). We then developed strategies to assess the functional consequences of these sequence variations, exploiting the zebrafish as a biosensor amenable to rapid functional analysis.

Our studies now demonstrate that (1) although mutations in individual *NODAL* pathway genes are uncommon, when considered collectively these pathway lesions are not rare and are slightly more common than well-described genetic changes such as chromosome 22q11.2 deletion syndrome (MIM 611867);[23,24] (2) combinations of gene lesions are likely the determinative factor of phenotypic outcome, with specific phenotypes being more prevalent than others; (3) modifier genes within a signaling pathway can account for a substantial proportion of reduced pathway activity and are quite common in human populations (E.R. and M.M., unpublished data); and (4) common

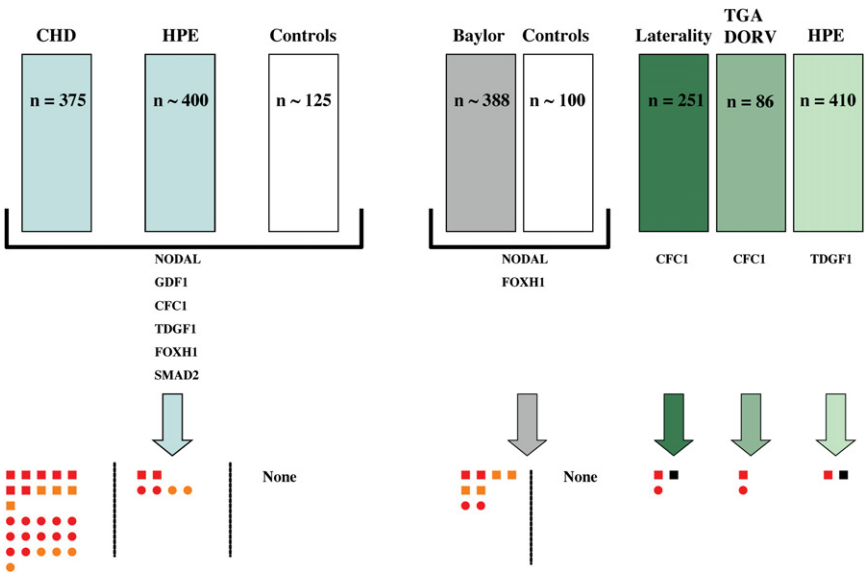

Figure 2. A Summary of Previously Studied Genes and Patient Groups Are Presented to Help Clarify the Presentation and Analysis

Only "typical mutations" (i.e., disease variant alleles, circle; or unique alleles, square) are shown for each study. The common polymorphisms and synonymous coding region changes (omitted here) are not likely to have a distortion in allele frequency among groups; however, this needs to be rigorously tested. Furthermore, only significant (red = <50% function) or mild (orange = 50%–99%) functional impairments are considered. Previously determined published mutations are shown, but do not contribute to the tabulations in Table 1. The Baylor study yields similar conclusions but are tabulated separately because we were blinded by the study design to their phenotypes.

modifiers can be overlooked unless functional studies are systematically performed.

Material and Methods

Study Population

Our pilot consortium study consists of 375 unrelated individuals prospectively ascertained at several urban centers with a wide spectrum of congenital cardiovascular malformations (essentially as diagrammed in Figure 2). CHD cases including transposition of the great arteries (TGA), tetralogy of Fallot (TOF), double outlet right ventricle (DORV), atrial septal defect (ASD), common atrioventricular canal (CAVC), and interrupted aortic arch (IAA) were studied. Coded affected proband DNA samples were obtained from three consortium centers (Children's Hospital of Philadelphia, Philadelphia, PA; Baylor College of Medicine, Houston, TX; and Yale University School of Medicine, New Haven, CT) according to the guidelines of the Institutional Review Boards (IRBs) of each institution and with the supervisory approval of the IRB of the NHGRI/NIH for patients with cardiovascular anomalies or HPE. All patient samples had been collected purely for research purposes and no consent had been obtained to return results of unknown significance to the subjects. None of the patients with cardiovascular anomalies were seen at the NIH. Therefore, parents and siblings were not consented to participate in our NIH consortium study because of the preliminary nature of the research, to maintain confidentiality, and because the links to patient identifiers were retained by the host institutions where their care was provided. We routinely screened between 300 and 500 unrelated patients with HPE (depending on the gene). These patients and their families participated in a separate research protocol approved by the IRB of the NHGRI/NIH. In addition, we studied at least 125 unrelated individual normal controls obtained as ethnically matched anonymous samples from the Coriell Institute for Medical Research. A separate set of samples was studied for *NODAL* and *FOXH1* variations by independent methods (with funding by the research team at Baylor College of Medicine; J.A.T. and J.W.B., unpublished data). In these cases (cited in Figures 2–5), only information regarding the nature of the mutation and general phenotype was shared in order to allow functional analysis in zebrafish to complement their cell-based assays.

PCR Amplification, Mutation Screening, and DNA Sequencing

Oligo 4.1 was used to design primers for the relevant exons or regulatory elements (see Table S2 available online). Amplification of human genomic DNA and screening was performed essentially as previously described in reference [25].

Site-Directed Mutagenesis and Construct Design

The cDNA-derived coding regions of the *NODAL* (H165 and E203 variants) or *FOXH1* genes were directionally cloned into pcDNA3.1 by standard methods. Site-directed mutagenesis was performed either with a minor modification of the Transformer kit (Promega, WI) by Transponics (York, PA) or by using the GeneTailer (InVitrogen) kit to introduce the human sequence variations.

Zebrafish *FOXH1* Rescue Assays

Human *FOXH1* alleles were prepared in two sets of pcDNA3.1-derived vectors: OV and NV. OV lacked the expected translational stop, such that the predicted protein product included an additional COOH-terminal sequence (DYKDDDKWNSQKPNSA DIQHSGGRSTGPV) that includes a FLAG-tag (underlined) and an additional 22 amino acid residues derived from the pcDNA3.1 vector. The X366Q allele includes two additional codons (GS) before terminating within the 3′UTR of the genomic sequence. Plasmids were linearized with EcoRI and the sense strand was synthesized with the SP6 Message Machine kit (Ambion) followed by the synthetic addition of polyA. Wild-type (wt) or variant *FOXH1* mRNAs were comicroinjected into zebrafish embryos with 4 ng of an antisense morpholino oligonucleotide [MO] targeting the translational start site of zebrafish *foxh1*, as previously described (ATG1 MO[26]). Injected embryos were allowed to develop to the pharyngula stage (28–32 hr after fertilization) and scored as wt, dead, or belonging to one of the following classes. Class I were mildly affected with crooked tails; Class II had more severe defects including tails shorter than trunks; Class III had even shorter tails and/or were immotile; and Class IV were highly deformed and confined to the tops

Figure 3. Summary of Mutational Data for *CFC1*

Disease	Class	Variation(s)	Activity	Domain	NODAL 165	Reference
TOF	dv	G174del1 (522delC)	L.O.F.	frameshift	R/R	this study
CHD	dv	F162L (484T→C)	N.D.	COOH terminus		this study
CHD	dv	F162L (484T→C)[a]	N.D.	COOH terminus	H/H	this study
	uniq	P217R (650C→G)	N.D.	COOH terminus		this study
TOF	dv	F162L (484T→C)	N.D.	COOH terminus		this study
TOF	uniq	R211Q (632G→A)[b]	N.D.	COOH terminus		this study
IAA	uniq	P193L (578C→T)	N.D.	COOH terminus	H/H	this study
	cp rs2721405	(P75P) R78W (225C→A:232C→T)	reduced	NH2 terminus		this study
CHD	dv	F162L (484T→C)	N.D.	COOH terminus		this study
TOF	dv	IVS4+2T→C[c]	N.D.	invariant splice junction	H/H	this study
	uniq	IVS5+6G→A	N.D.	intron		this study
TA	dv	IVS4+2T→C	N.D.	invariant splice junction		this study
TOF	dv	IVS4+2T→C	N.D.	invariant splice junction	R/R	this study
AVC	dv	IVS4+2T→C	N.D.	invariant splice junction	R/R	this study
TOF	dv	A145T (433G→A)	N.D.	CFC domain		this study and [30]
AVC	dv	A145T (433G→A)	N.D.	CFC domain		this study and [30]
AVC	dv	A145T (433G→A)	N.D.	CFC domain		this study and [30]
AVC	dv	A145T (433G→A)	N.D.	CFC domain		this study and [30]
TOF	cp rs2721405	(P75P) R78W (225C→A:232C→T)	reduced	NH2 terminus		this study
TGA	cp rs2721405	(P75P) R78W (225C→A:232C→T)	reduced	NH2 terminus		this study
CHD	cp rs2721405	(P75P) R78W (225C→A:232C→T)	reduced	NH2 terminus		this study
TOF	dv	5′UTR-7A→G	N.D.	5′UTR		this study
AVC	dv	5′UTR-7A→G	N.D.	5′UTR		this study
AVC	uniq	IVS1+6G→C	N.D.	intron		this study
CHD	uniq	E210K (628G→A)	N.D.	COOH terminus		this study
	syn	P196P (588C→A)	N.D.	COOH terminus		this study

Mutations are annotated by amino acid position and color: red, less than 50%; orange, 50%–99%; blue, not adequately tested; black, normal activity.
[a] Also GDF1 R68H (203G→A; N.D.).
[b] Also FOXH1 S113T;S346G (338G→C; 1036A→G).
[c] Also FOXH1 S339G;P354P (1015A→G; 1062T→C).

of yolks. In this way, a signature phenotypic spectrum was assigned to each group of treated embryos. The activity of a given allele was determined from its ability to ameliorate the phenotypic spectrum associated with loss of endogenous zebrafish FoxH1 (MO alone), compared to the amelioration resulting from coinjection of wild-type human *FOXH1* mRNA (MO + *FOXH1*). Activity was quantified as follows: a phenotypic index (PI) was determined for each group of injected embryos by assigning a value to each embryo of each phenotype (wt = 4; Class I = 3; Class II = 2; Class III = 1; Class IV = 0), multiplying the number of embryos in each class by the corresponding assigned value, and dividing the sum by the total number of embryos. Activity relative to wt FOXH1 was determined as follows. First, PIs were background adjusted by subtracting the PI associated with a negative control injection (MO only) that was performed in parallel for each data point, averaged (for multiple experiments), and divided by the average of similarly adjusted PIs for wt FOXH1. A mathematical description of these steps is: wild-type-adjusted activity (WTAA) of $FOXH1_{EXP}$ = Ave ($PI_{EXP} - PI_{MO}$)/Ave ($PI_{WTFOXH1} - PI_{MO}$). For photography, embryos were mounted in 2% methyl-cellulose and imaged on a Leica MZ16 dissecting microscope.

Results

Heterozygous Mutations Can Be Detected in Most Nodal Pathway Components

To assess the distribution of Nodal pathway mutations among affected patients and controls, we screened for

Figure 4. Summary of Mutational Data for *TDGF1* and *SMAD2*

Gene	Disease	Class	Variation(s)	Activity	Domain	NODAL 165	Reference
TDGF1[a]	TOF	dv	P125L (374C→T)	L.O.F.	CFC domain	R/R	this study
	TOF	uniq	V110L (329T→C)	N.D.	EGF domain	H/R	this study
	common	cp	R111G (331C→G)	N.D.	EGF domain		this study
SMAD2[b]	HPE	syn	S276S (678G→A)	N.D.			this study
	HPE	uniq	IVS9-31to32delTT	N.D.	common site of intronic length variation in vertebrates		this study

Mutations are annotated by amino acid position and color: red, less than 50%; orange, 50%–99%; blue, not adequately tested; black, normal activity.
[a] Common polymorphisms in normal controls: ***TDGF1*** V22A (65T→C), Y43D (127T→G), E56E (168A→G), R111G (331C→G).
[b] HPE samples only and not CHD.

Figure 5. Summary of Mutational Data for *FOXH1*

Disease	Class	Variation(s)	Activity	Domain	NODAL 165	Reference
CHD	cp	S16L (48C→T)	8, 65%			Baylor
CHD	uniq	P35L (104C→T)	30, 130%	forkhead domain		this study; Baylor
TOF	uniq	V112M[b] (334G→A)	−0.06, −0.065%	forkhead domain (FHD)	H/R	this study
HPE	cp	S113T (338G→C)	80%	forkhead domain	R/R	this study; Baylor
HPE	cp	S113T (338G→C)	80%	forkhead domain		this study
HPE	cp	S113T (338G→C)	80%	forkhead domain	H/R	this study
TGA	cp	S113T (338G→C)	80%	forkhead domain	H/R	this study
TOF	cp	S113T (338G→C) and	80%	forkhead domain		this study
	uniq	S346G (1036A→G)	27%	COOH terminus		
		S113T/S346G[c] (338G→C; 1036A→G)	50,75%	compound variant		
common	syn	R121R (363G→C) and	N.D.	linker domain		this study; Baylor
	cp	T125S (374C→G)	N.D.	forkhead domain		
HPE	syn	R121R (363G→C) and	N.D	linker domain		this study
	cp	T125S (374C→G) and	N.D.	forkhead domain		
	dv	A352V (1055C→T) and	N.D.	COOH terminus		
	dv	A352S (1054G→T) and	75%	COOH terminus		
	uniq	Stop→Q (1096T→C)	119%	COOH terminus frameshift		
		A352V and Stop>Q (1055C→T; 1096T→C)	68,121%	compound variant		
CHD	uniq	G195V (584G→T)	109%	linker domain		this study; Baylor
CHD	uniq	T206I (617C→T)	80,101%	linker domain		this study; Baylor
HPE	uniq	P218L (653C→T)	42%	linker domain	R/R	this study
CHD	uniq	G267R (799G→A)	34,94%	linker domain		this study; Baylor
CHD	uniq	T242I (725C→T)	66,73%	linker domain		Baylor
HPE	uniq	A271D (812C→A) and	121%			this study
	syn	P261P (783T→C) and	N.D.	linker region		
	syn	A352A (1056G→A)	N.D.	COOH terminus		
HPE	dv	L282S (845T→C) and	68%	FM1		this study
	dv	L282S (845T→C) and	68%	FM1		
	dv	L365M (1093C→A) and	100%	COOH terminus		
	dv	L365M (1093C→A)	100%	COOH terminus		
		L282S/L365M (845T→C; 1093C→A)	48,49%	compound variant		
CHD	dv	D328E (984C→G)	8,76%	SIM		this study; Baylor
CHD	dv	D328E (984C→G)	8,76%	SIM		this study; Baylor
TGA	uniq	P304T (910C→A) and	78,108%	SID	H/R	this study
	syn	L359L (1077G→A)	N.D.	COOH terminus		
HPE	uniq	F331V (991T→G) and	45,69%	SIM		this study
	cp	A352S (1054G→T) and	100%	COOH terminus		
	cp	A353V (1058C→T)	100%	COOH terminus		
HPE	uniq	H269N (805C→A) and	104%			this study
	uniq	P278T (832C→A) and	−0.11%	FM1		
	dv	S364I (1091G→T)	62%	COOH terminus		
		H269N/P278T/S364I (805C→A:832C→A:1091G→T)	−0.15, 0.03%	compound variant		
TOF	uniq	P336L (1004C→T)	−0.4, 0.07%	SIM		this study
	syn	A353A (1059C→A,G,T)	N.D.	COOH terminus		
	cp	P354T (1060C→A)	N.D.	COOH terminus		
TOF	uniq	S339G[d] (1015A→G) and	21,61%		H/H	this study
	syn	P354P (1062T→C)	N.D.	COOH terminus		
HPE	dv	D350G (1049A→G) and	21,87%	COOH terminus	R/R	this study
	dv	D350G (1049A→G)	21,87%	COOH terminus		
HPE	dv	D350G (1049A→G) and	21,87%	COOH terminus		this study
	syn	A352A (1056G→A)	N.D.	COOH terminus		
TOF	dv	D350G (1049A→G) and	21,87%	COOH terminus		this study
	syn	A353A (1059C→A,G,T)	N.D.	COOH terminus		
	syn	P354P (1062T→C)	N.D.	COOH terminus		
TGA	dv	A352E (1055C→A)	N.D.	COOH terminus	H/R	this study
HPE	dv	A352S (1054G→T)	75%	COOH terminus		this study
TGA	dv	A353D (1058C→A)	107%	COOH terminus	H/H	this study
HPE	dv	S364N (1091G→A) and	160%	COOH terminus		this study
	syn	T306T (918C→T,A,G) and	N.D.	linker domain		
	syn	A352A (1056G→A) and	N.D.	COOH terminus		
	uniq	P354S (1060C→T)	N.D.	COOH terminus		this study
		P354S and S364N (1091G→A) (1060C→T); 1091G→A)	62%	compound variant		this study

TGA	dv	S364I (1091G→T)	62%	COOH terminus	this study
HPE	dv	S364N (1091G→A) and	160%	COOH terminus	this study
	syn	L359L (1077G→A) and	N.D.	COOH terminus	
	syn	T306T (918C→T,A,G)	N.D.	linker region	
HPE	uniq	Del1056G	72%	COOH terminus frameshift	this study
HPE	uniq	Del1062T	52%	COOH terminus frameshift	this study

Mutations are annotated by amino acid position and color: red, less than 50%; orange, 50%–99%; blue, not adequately tested; black, normal activity.
[a] Common polymorphisms in normal controls (synonymous changes): ***FOXH1*** intron 3 (13 bp deletion), R121R (363G→C), H150H (450T→C) [dbSNP/rs2721177], T226T (678G→T), G257G (771C→A), P261P (783T→C), D328D (984C→T), P348P (1044T→A,G,C), A352A (1056G→A), P354P (1062T→C), L359L (1077G→>A) [dbSNP/rs7833404], C363C (1089C→T).
[b] Also CFC1 (P75P) R78W (225C→A; 232C→T).
[c] Also CFC1 R211Q (632G→A).
[d] Also CFC1 (IVS4+2T→C).

mutations in the *NODAL*, *GDF1*, *CFC1*, *TDGF1*, *FOXH1*, and *SMAD2* genes in patients with CHD, laterality, HPE, and controls. A tabulation of the sequence variations detected in this summary analysis is shown in Table 1 (for a more complete tabulation from this study, see Figures 3–5; the description of *NODAL* mutations will be presented elsewhere; E.R. and M.M., unpublished data). Except for *SMAD2*, we could find potentially disruptive sequence alterations in each of the pathway components examined, the most being within *NODAL, CFC1,* and *FOXH1*. Assays to test the significance of these variants were developed and performed.

Table 1. Summary of Abnormal Alleles by Phenotype

Phenotypic Distribution[a]	Severe (0%–50%)	Mild (50%–99%)
CHD[b]		
TGA	3	0
DORV	2	2
TOF	13	4
AVC	1	1
IAA	0	0
TA	1	0
Unspecified	0	2
	total: 20 percent: (5.3%)	total: 9 percent: (2.4%)
Laterality[c]	total: 4 percent: (~1%)	total: 4 percent: (~1%)
HPE[d]	total: 4 percent (~1%)	total: 2 percent (<1%)

[a] CHD, congenital heart defects; TGA, transposition of the great arteries (n = 62); DORV, double outlet right ventricle (n = 23); TOF, tetralogy of Fallot (n = 121); AVC, atrioventricular canal (n = 27); IAA, interrupted aortic arch (n = 19); TA, truncus arteriosus (n = 15); unspecified (n = 108); HPE, holoprosencephaly (n = ~400 ± 100). These figures represent minimum estimates and include only unpublished prototypical "mutations" among the 375 patients in this study (i.e., disease variations or unique mutations) where interpretable functional studies have been performed. Synonymous changes, noncoding variations, and common polymorphisms are not included; these syn and cp variants are not known to be more highly enriched among patient versus control groups. Note the percentage of cases with defective NODAL pathway factors by particular anatomic type is even higher (e.g., 17/121 = 0.14 for defective function in the TOF subgroup, or 4/23 = 0.17 for the DORV subgroup, or 3/62 = 0.048 for the TGA subgroup; compared with 0/125 for the controls).
[b] n = 375 (as outlined in Figure 2).
[c] n = 388 cases studied at Baylor (J.W.B. and J.A.T., unpublished data).
[d] n = 400 (approximate).

Because we were interested in potential gene-gene interactions, the same patients (derived from multiple cohorts and controls) were typically screened for sequence variations in the entire gene set. Sequence variations were further annotated as significant loss of function (<50% of normal activity, indicated in red in Figures 3–5 and related figures), mild loss of function (50%–99% normal activity, indicated in orange), normal activity (black), or not yet reliably tested (blue). Contrary to our initial expectations of multifactorial or typical digenic inheritance, each proband can be usually assigned to a principal change in a single gene. There are, however, some notable exceptions to this pattern, especially with respect to putative NODAL modifiers (see below). Our functional analysis of NODAL H165R suggested to us that this common variant has less than 50% normal activity in various assay systems (E.R. and M.M., unpublished data). Our working hypothesis related to this finding is that such variants are neutral to selection pressure because they are still well above the threshold level of 50% of Nodal activity seen in healthy normal mice with a single Nodal null allele (see Tables S3 and S4). Instead, they serve as modifiers of mutations that occur in other NODAL pathway components with which they normally must interact. And being so common, they become essential in the estimation of the impact of mutations among these or related genes.

As can be seen in Figures 3–5, some of the more common changes were synonymous coding region variations. Functional studies were not undertaken for these sequence variants because the ultimate protein structure would not be affected. Similarly, nonsynonymous coding region changes seen in normal controls were generally disregarded as common polymorphisms, a common practice in human genetic studies. In retrospect, this initial bias deserved re-examination (see below). Nonsynonymous coding region changes observed in multiple probands (but provisionally not normal controls) and yet potentially implicated in different phenotypes among affected individuals comprised a third class we have termed disease variant alleles. Finally, we observed very rare variations seen uniquely in a single proband, but not normal control

individuals, which we shall refer to as unique alleles. In our previous studies with HPE, virtually all of the hundreds of disease-associated variants have been unique alleles. Searches of public databases (e.g., dbSNP) were performed to identify any known polymorphisms within the genomic regions examined; these are indicated by their reference numbers in Figures 3–5.

Functionally Abnormal Alleles Occur Most Frequently in Conotruncal Defects

Deleterious sequence variations were seen across most genes and all phenotypes examined with no evidence of an absolute genotype-phenotype correlation. However, mutations were detected far more frequently in patients with congenital heart defects (especially tetralogy of Fallot and related conotruncal malformations) than in laterality patients or those with HPE (Table 1). The population groups were of similar size and ethnicity, so this finding is highly significant ($p < 0.001$). This spectrum of malformation may reflect the cumulative genetic impairment required to produce each phenotype and also might reflect only those select genetic combinations compatible with postnatal survival (see Discussion).

Sequence Variation in the EGF-CFC Proteins

Loss-of-function mutations in *CFC1* (G174 del1 and R112C) were originally detected in patients with laterality defects[27] and subsequently also identified in patients with conotruncal defects (G174 del1 and a unique splice site duplication).[28] In addition, we identified a common polymorphism (P75P) R78W that was more prevalent in subjects of African-American origin and which was hypomorphic in functional studies.[27–29] Similarly, we had detected a single functionally abnormal allele (TDGF1 P125L) in HPE patients.[25] Here we present the results of screening for *CFC1* and *TDGF1* alterations in additional patients with isolated congenital heart defects. These newly detected sequence variations are summarized in Figure 6. Although we did not perform any new functional assays for these *CFC1* and *TDGF1* alleles, functional data is available for some of the disease variant alleles and common polymorphisms that we re-isolated. For instance, G174 del1 is a loss-of-function disease variant allele detected here in a TOF proband as well as previously in DORV and three different probands with laterality. We also observed another disease variant allele, a mutation in the invariant splice donor sequence (IVS4+2T→C), seen in four unrelated subjects and predicting a truncation of the CFC1 protein with an incomplete CFC domain. Additional intronic and 5′UTR variations were detected in this new population; however, these have not yet been tested for their potential effects. Several more disease variant alleles (such as F162L and A145T) and unique alleles (P193L, E210K, R211Q, and P217R) were also detected, but their significance is presently unknown. However, the four unique alleles might not affect bioactivity, because they alter only human-specific amino acids in the carboxyl terminus that are dispensable in other organisms. Finally, we note that Ozcelick et al. also detected the A145T variant, both in normal controls and in patients with cardiac malformations, and concluded that this variant was unlikely to contribute to disease.[30] However, because the distinction between common polymorphisms and disease variant alleles is empiric and given our own documented examples of functionally abnormal common polymorphisms (e.g., (P75P) R78W above), a definitive assignment cannot be made without further studies. For example, we observed a patient that has the hypomorphic common polymorphism (P75P) R78W in *CFC1* as well as a unique *FOXH1* allele (V112M, described below) with diminished function, suggesting that genetic interactions might occur in some individuals with variations in more than one gene (see Figures 3–5).

We observed limited sequence variation in the human *TDGF1* gene in the cardiac samples in this study. Nevertheless, it is noteworthy that the functionally abnormal P125L variant we found in a TOF patient was previously detected in an individual with HPE.[25] These phenotypes are sufficiently distinct, so this observation suggests that either loss of function of a single allele of *TDGF1* is an incidental finding, or, as we consider to be more likely, that there are genetic or environmental modifiers that determine the expressivity of this alteration. Although the significance of TDGF1 V110L is also unknown, it is reasonable to consider that functional studies would be informative and, furthermore, that even common polymorphisms such as the highly conserved R111G variant seen in normal controls should be examined.

Functional Analysis of Variants in FOXH1

FoxH1 depends on the presence of phosphorylated Smad proteins to exhibit the DNA binding associated with biological activity. Interestingly, we observed that overexpression of human *FOXH1* mRNA in zebrafish could partially rescue phenotypic dysmorphologies associated with the loss of endogenous zebrafish Foxh1 that we depleted by injection of an antisense morpholino oligonucleotide targeting translation of the zebrafish mRNA (but not human *FOXH1* constructs).[26] The degree of bioactivity of human FOXH1 variants could then be estimated by morphological scoring of clutches of embryos coinjected with the antisense morpholino oligonucleotide. Four phenotypic classes could be reliably distinguished from each other and from uninjected control embryos (Figure 7) that served to estimate the bioactivity of each variant compared to the wild-type gene (Figures S1A–S1D and Table S1).

FOXH1 is a transcription factor that has two principal domains, namely, the forkhead domain necessary for binding DNA and a C-terminal domain that interacts with Smad proteins. This Smad interaction domain (SID) is further defined by submotifs FM1 and FM2 and a core Smad interaction motif shared by other R-Smad cofactors[31,32] (Figure 8). The extreme C terminus following the SIM tolerates extensive genetic variation without affecting

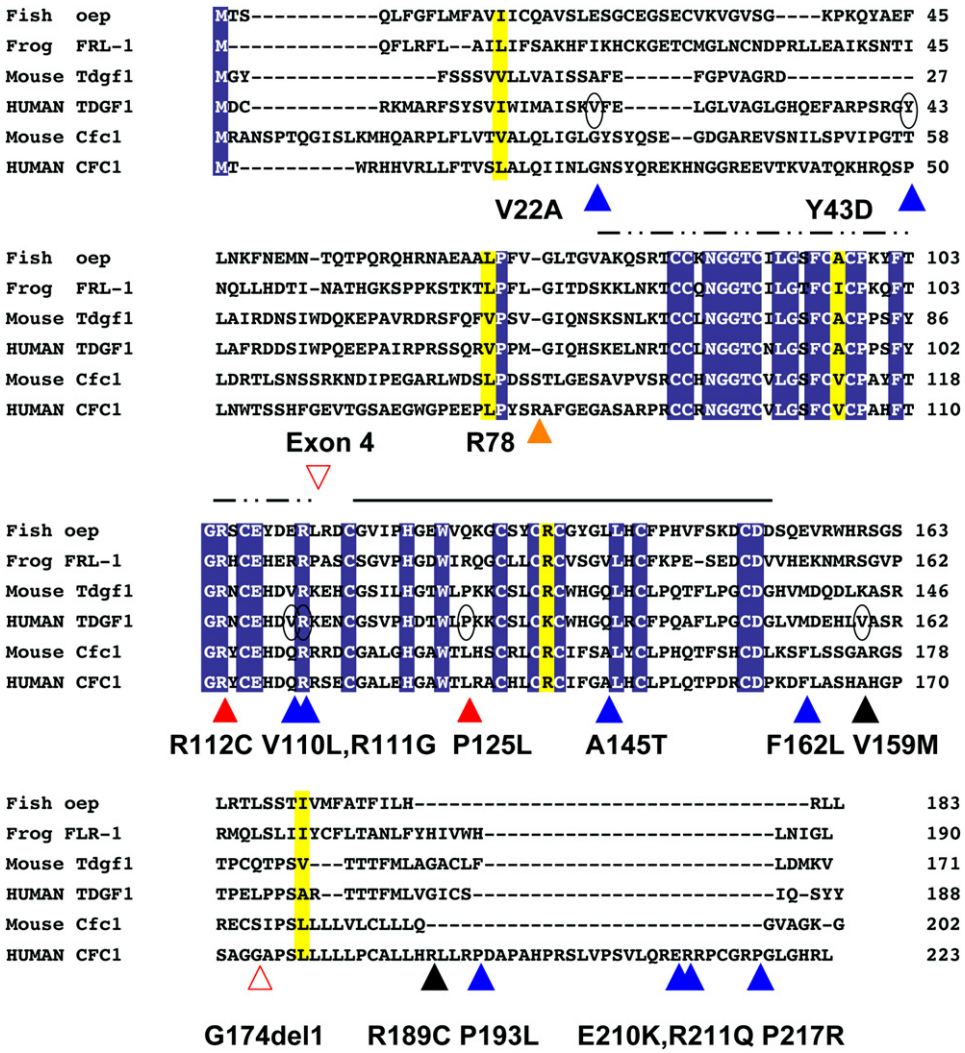

Figure 6. Multiple Sequence Alignment of EGF-CFC Proteins

Missense changes detected in human TDGF1 and CFC1 are shown within the context of an alignment of related proteins. Loss-of-function changes are in red (missense, solid;[25,27,28] coding region frameshift,[27,28] triangle without fill; splice alteration frameshift, inverted triangle without fill), hypomorphic changes are in orange,[29] residues not analyzed are in blue,[30] and those with normal activity are in black.

function in our assays. Three frameshift variants in this segment had little effect on biological activity (residues 352 to 365). This preliminary analysis of the extreme C terminus following the SIM suggests that most of the genetic variability seen in this segment of the protein can probably be disregarded.

We detected three variations in the forkhead domain that appear to affect biological function: P35L, V112M, and the common variant S113T. The latter change is yet another interesting example of a common polymorphism that is seen in HPE as well as TGA and TOF. Although our preliminary finding that S113T reduces activity by 20% needs to be confirmed, we note the case where S113T/S346G in FOXH1 is detected in the same TOF patient with the nonsynonymous R211Q variant in CFC1. Indeed, it seems likely that common polymorphisms are capable of interacting with a variety of other genes. We chose not to examine the T125S variant because it was frequently present in normal controls, which biased us against it at the time of the study. However, this amino acid is conserved and could indeed be functionally relevant (e.g., another example of a deleterious common polymorphism).

There is a striking distribution of variants with diminished function found in the key FM1 and SIM motifs. P278T occurs in the core FM1 motif and completely eliminates detectable activity; this HPE patient also has additional variations including H269Q and S364I. A compound allele with all three changes lacks all detectable activity. The variant L282S is only mildly abnormal and was identified in a proband with HPE in the homozygous state in *cis* with the ostensibly benign L365M change. Significantly, when this compound allele was tested, we detected synergy between these changes with a reduction of bioactivity to 50% (i.e., mild to severe shift in activity).

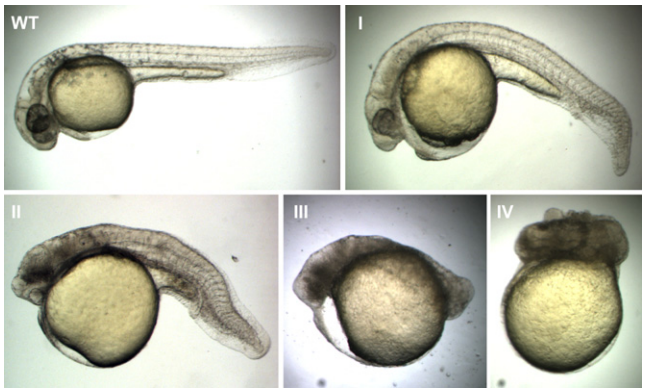

Figure 7. Human *FOXH1* Rescue Assay Phenotypes
Embryos were classified into four degrees of phenotypic manifestations ranging from mild ventral curvature (Class I) to severe embryonic arrest (Class IV). Experimental clutches of embryos were individually scored and a phenotypic index calculated for each experiment.

P304T is a normal allele, whereas the patient with the hypomorphic S339G change in the SIM domain has TOF and a second change in CFC1 (IVS4+2T→C). The core SIM is defined by a consensus sequence (PPNK) and adjacent residues, and mutations in this region lead to significant loss of activity in cases of CHD (D328E), HPE (F331V), and TOF (P336L). Although additional detailed studies of the extreme C terminus are indicated, we note that D350G is another example of a disease variant detected in HPE and TOF that is a mild hypomorph.

Taken together with the functional analysis of other genes within the pathway, these variants in *FOXH1* have the potential to interact significantly with variations in other genes both common and uncommon. In the absence of another transcription factor to compensate for reduced FOXH1 activity, even small reductions in biological activity can have significant effects. Furthermore, genes that cooperate with FOXH1 to activate target genes may be influenced by diminished FOXH1 function. In fact, NKX2.5 (MIM 600584), whose murine ortholog has been shown to act interact with murine Foxh1 in heart formation, has been independently identified as a potential cause of TOF and related cardiac malformations by the identification of mutations in this gene.[33,34] It will be interesting to expand the evaluation of the role of FOXH1 in human disease to include additional transcriptional factor networks.

Discussion

One of the central goals of this study was to determine the frequency and composition of sequence variations in the NODAL pathway contributing to human disease(s). We hypothesized that if the conditions under study were due to typical digenic inheritance, then a consistent pattern of mutations in more than one gene would be readily demonstrable. In fact, we detected functionally significant mutations in more than one unique or disease variant gene only rarely; as such, if one limits one's view to functionally compromised alleles in these two classes, our hypothesis does not bear out. But if one considers that common polymorphisms with reduced bioactivity like NODAL H165R and CFC1 (P75)R78W could act as modifiers, a different picture emerges (see Tables S3 and S4). In these cases, genetic interactions among multiple alleles would need to be considered: one or two copies of the functionally impaired common polymorphism and a third, less common pathway variant (or one or two copies of another functionally impaired common polymorphism). Because the degree of functional impairment ranged from severe to mild for any particular gene, we believe that the cumulative impairment of pathway activity may be a more important consideration than the contribution of any individual gene. Even making conservative assumptions regarding how genes within a pathway interact allows for a considerable closure in the gap between the genetic examples extrapolated from animal models to humans (see Tables S3 and S4 for illustrative examples).

In humans, the most common genetic alteration to date affecting conotruncal development is the consequence of the common deletion seen in 22q11.2/DiGeorge syndrome in approximately 5% of cases.[23,24] Collectively, our study suggests that NODAL pathway defects are detectable at a slightly higher frequency (5%–10%). Perhaps even more significantly, plausible biochemical interactions have been proposed between the Nodal pathway and other key genes affecting conotruncal development including TBX1 (implicated in 22q11.2 deletion syndrome), TBX5 (implicated in Holt-Oram syndrome [MIM 142900]) and NKX2.5 (implicated in TOF).[13–15,33–35] As such, meaningful synergies may exist between defective TBX1, TBX5, or NKX2.5 alleles and the defective Nodal pathway alleles we have described here. Therefore, although genetic heterogeneity will likely continue to confound genetic counseling efforts for patients with CHD, there remains a distinct promise of a more fully integrated genomics approach in the future. As technical and cost challenges are being addressed to solve whole-genome sequencing for patients, there are many reasons to argue that this will be essential to detecting the gene-gene interactions of common and rare variations that affect heart and brain development.

Studies in model organisms have suggested that defective Nodal signaling is a major etiological cause of cyclopic phenotypes. Our systematic analysis of the human NODAL signaling components at the mutational level would suggest that defective signaling of this pathway contributes to only a minority of HPE cases. The relative scarcity of prototypical mutations in the NODAL signaling pathway components in HPE suggest that some of the genetic combinations resulting in cyclopia in mice (such as Nodal; Smad2[36]) are not common representative models of human cases of disease, at least in live-born infants. Unlike somatic mutations in *SMAD2* detected in cancer syndromes, inherited defects in *SMAD2* (and potentially

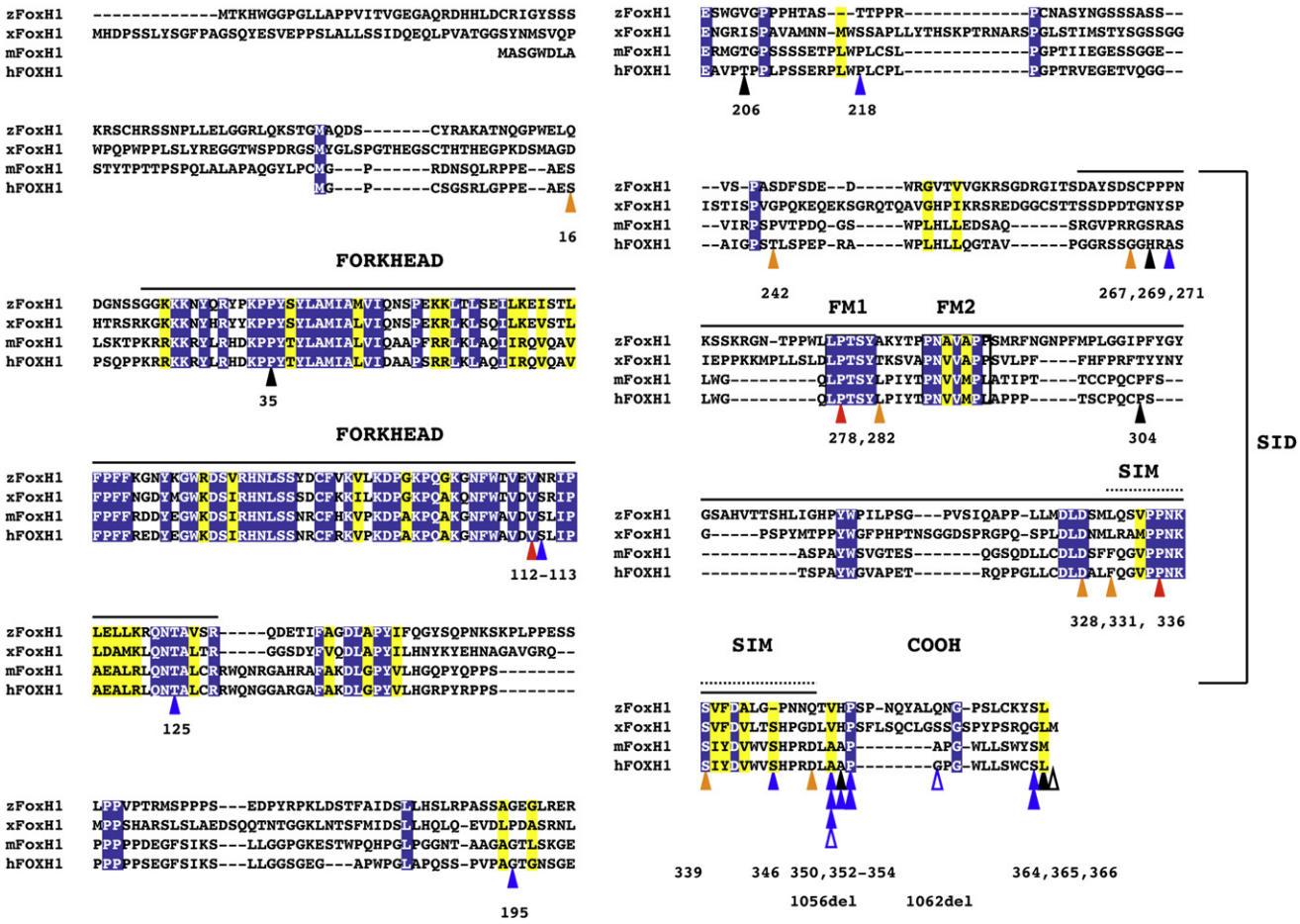

Figure 8. Alignment of Zebrafish, *Xenopus*, Murine, and Human FOXH1 Proteins

Identical residues are shown in blue, and similar residues are shown in yellow. Considerable sequence identity is seen in known functional domains, including the DNA-binding forkhead domain and the SMAD interaction domain. The SMAD interaction domain contains three motifs: FM1, FM2, and the core Smad-interaction motif (SIM).[31,32] Note that the extreme C terminus after the SIM is poorly conserved, but it contains a significant fraction of detectable variations of both patients and controls. Most of these variants are inadequately studied to draw definitive conclusions (scored as blue and, therefore, do not contribute to the tabulations in Table 1). Mutations are annotated by amino acid position and colored as in previous figures (red, less than 50%; orange, 50%–99%; blue, not adequately tested; black, normal activity; frameshift, blue or black with no fill).

SMAD3) might be selected against during fetal development because of the several TGFβ ligands that utilize these transcription factors to control the expression of target genes.

Laboratory strains of mice are particularly powerful for genetic analysis precisely because their inherent genetic diversity has been minimized by deliberate inbreeding. Interesting differences in phenotypes are observed when identical gene-targeted alleles are crossed into different strain backgrounds.[37,38] Such phenotypic differences are commonly attributed to strain-specific modifiers. There is every reason to suspect that gene modifiers play an even more significant role in human populations.[9] We propose that the apparent gulf between mouse and human phenotypes are not nearly as irreconcilable as it seems (because identical gene-gene interaction rules are used to compare mouse models and human patients in Table S3 versus S4). Consideration of the combinations of deleterious alterations in different genes results in estimations of activity that are very close to the levels producing phenotypic effects in the mouse. This would be true even without considering the potential contributions of additional pathway variant alleles in this or parallel pathways.

Clearly, this study does not attempt to incorporate the effects of all known factors relevant to NODAL signaling. Future studies would be needed to systematically examine the extent of sequence variation in an expanded set of genes either directly involved in the processing and generation of ligands, or associated with tissue distribution and receptor binding, partially redundant factors (e.g., *SMAD3* or *GDF3*), downstream target genes, and transcriptional coregulators with the aim of functionally characterizing all types of nonsynonymous variations including those also present in normal control populations. By identifying likely instances of complex interactions contributing to human disease, this study underscores the importance of

functional genomic approaches for understanding these disorders.

Supplemental Data

One figure and three tables are available at http://www.ajhg.org/.

Acknowledgments

The authors wish to thank the patients who participated in this research. We are also grateful to Mahim Jain for statistical analysis, Richard Bamford, and June de la Cruz for initiating this study. This research was supported by the Division of Intramural Research of the National Human Genome Research Institute, National Institutes of Health, and by grants from the NIH to J.A.T. and J.W.B. and by P50 HL74731 to E.G. The authors declare that they have no competing financial interests.

Received: March 14, 2008
Revised: May 15, 2008
Accepted: May 20, 2008
Published online: June 5, 2008

Web Resources

The URLs for data presented herein are as follows:

BLASTN, http://www.ncbi.nlm.nih.gov/blast/
ClustalW, http://align.genome.jp/
GeneBank, http://www.ncbi.nlm.nih.gov/GeneBank/
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/
University of California Santa Cruz Bioinformatics Site, http://genome.ucsc.edu/

References

1. Muenke, M., and Beachy, P.A. (2001). Holoprosencephaly. In The Metabolic & Molecular Bases of Inherited Disease, C.R. Scriver, ed. (New York: McGraw-Hill), pp. 6203–6230.
2. Roessler, E., and Muenke, M. (2001). Midline and laterality defects: left and right meet in the middle. Bioessays *23*, 888–900.
3. Matsunaga, E., and Shiota, K. (1997). Holoprosencephaly in human embryos: epidemiological studies on 150 cases. Teratology *16*, 261–272.
4. Yamada, S., Uwabe, C., Fujii, S., and Shiota, K. (2004). Phenotypic variability in human embryonic holoprosencephaly in the Kyoto Collection. Birth Defects Res. Part A Clin. Mol. Teratol. *70*, 495–508.
5. Ming, J.E., and Muenke, M. (2002). Multiple hits during early embryonic development: digenic disease and holoprosencephaly. Am. J. Hum. Genet. *71*, 1017–1032.
6. Fernandes, M., Gutin, G., Alcorn, H., McConnell, S.K., and Hebert, J.M. (2007). Mutations in the BMP pathway in mice support the existence of two molecular classes of holoprosencephaly. Development *134*, 3789–3794.
7. Cohen, M.M. Jr. (2006). Holoprosencephaly: clinical, anatomic, and molecular dimensions. Birth Defects Res. Part A Clin. Mol. Teratol. *76*, 658–673.
8. Duborg, C., Bendavid, C., Pasquier, L., Henry, C., Odent, S., and David, V. (2007). Holoprosencephaly. Orphant J Rare Dis. *2*, 8.
9. Krauss, R.S. (2007). Holoprosencephaly: new models, new insights. Expert Rev. Mol. Med. *9*, 1–17.
10. Whitman, M. (2001). Nodal signaling in early vertebrate embryos: themes and variations. Dev. Cell *1*, 605–617.
11. Hamada, H., Meno, C., Watanabe, D., and Saijoh, Y. (2003). Establishment of vertebrate left-right asymmetry. Nat. Rev. Genet. *3*, 103–113.
12. Zhu, L., Belmont, J.W., and Ware, S.M. (2006). Genetics of human heterotaxias. Eur. J. Hum. Genet. *14*, 17–25.
13. Brand, T. (2003). Heart development; molecular insights into cardiac specification and early morphogenesis. Dev. Biol. *258*, 1–19.
14. Harvey, R.P. (2004). Patterning the vertebrate heart. Nat. Rev. Genet. *3*, 544–556.
15. Buckingham, M., Meilhac, S., and Zaffran, S. (2005). Building the mammalian heart from two sources of myocardial cells. Nat. Rev. Genet. *6*, 826–835.
16. Gebbia, M., Ferrero, G.B., Pilia, G., Bassi, M.T., Aylsworth, A., Penman-Splitt, M., Bird, L.M., Bamforth, J.S., Burn, J., Schlessinger, D., et al. (1997). X-linked situs abnormalities result from mutations in ZIC3. Nat. Genet. *17*, 305–308.
17. Ware, S.M., Peng, J., Zhu, L., Fernbach, S., Colicos, S., Casey, B., Towbin, J., and Belmont, J.W. (2004). Identification and functional analysis of ZIC3 mutations in heterotaxy and related congenital heart defects. Am. J. Hum. Genet. *74*, 93–105.
18. Zhu, L., Zhou, G., Poole, S., and Belmont, J.W. (2008). Characterization of the interactions of human ZIC3 mutants with GLI3. Hum. Mutat. *29*, 99–105.
19. Schier, A.F. (2003). Nodal signaling in vertebrate development. Annu. Rev. Cell Dev. Biol. *19*, 589–621.
20. Beddington, R.S.P., and Robertson, E.J. (1999). Axis development and early asymmetry in mammals. Cell *96*, 195–209.
21. Dipple, K.M., and McCabe, E.R.B. (2000). Modifier genes convert "simple" mendelian disorders in complex traits. Mol. Genet. Metab. *71*, 43–50.
22. Vockley, J., Rinaldo, P., Bennett, M.J., Matern, D., and Vladutiu, G.D. (2000). Synergistic heterozygosity: disease resulting from multiple defects in one or more metabolic pathways. Mol. Genet. Metab. *71*, 10–18.
23. Baldini, A. (2002). DiGeorge syndrome: the use of model organisms to dissect complex genetics. Hum. Mol. Genet. *11*, 2363–2369.
24. Baldini, A. (2004). DiGeorge syndrome: an update. Curr. Opin. Cardiol. *19*, 201–204.
25. de la Cruz, J., Bamford, R.N., Burdine, R.D., Roessler, E., Donnai, D., Schier, A.F., and Muenke, M. (2002). A loss of function mutation in the CFC domain of *TDGF-1* is associated with human forebrain defects. Hum. Genet. *110*, 422–428.
26. Pei, W., Noushmehr, H., Ouspenskaia, M.V., Elkahloun, A.G., and Feldman, B. (2007). An early requirement for maternal FoxH1 during zebrafish gastrulation. Dev. Biol. *310*, 10–22.
27. Bamford, R.N., Roessler, E., Burdine, R.D., Saplakoglu, U., de la Cruz, J., Splitt, M., Towbin, J., Bowers, P., Marino, B., Schier, A.F., et al. (2000). Loss-of-function mutations in the EGF-CFC gene CFC1 are associated with human left-right laterality defects. Nat. Genet. *26*, 365–369.
28. Goldmuntz, B., Bamford, R.N., Karkera, J.D., de la Cruz, J., Roessler, E., and Muenke, M. (2002). CFC1 mutations in patients with transposition of the great arteries and double outlet right ventricle. Am. J. Hum. Genet. *70*, 776–780.
29. Yan, Y.-T., Liu, J.-J., Luo, Y., Chaosu, E., Haltiwanger, S., Abate-Shen, C., and Shen, M.M. (2002). Dual roles of cripto as

a ligand and coreceptor in the Nodal signaling pathway. Mol. Cell. Biol. *22*, 4439–4449.
30. Ozcelik, C., Bit-Avragim, N., Panek, A., Gaio, U., Geier, C., Lange, P.E., Dietz, R., Posch, M.G., Perrot, A., and Stiller, B. (2006). Mutations in the EGF-CFC gene Cryptic are an infrequent cause of congenital heart disease. Pediatr. Cardiol. *27*, 695–698.
31. Germain, S., Howell, M., Esslemont, G.M., and Hill, C.S. (2000). Homeodomain and winged helix transcription factors recruit activated Smads to distinct promoter elements via a common Smad interaction motif. Genes Dev. *14*, 435–451.
32. Randall, R.A., Howell, M., Page, C.S., Daly, A., Bates, P.A., and Hill, C.S. (2004). Recognition of phosphorylated-Smad2 containing complexes by a novel Smad interaction motif. Mol. Cell. Biol. *24*, 1106–1121.
33. Goldmuntz, E., Geiger, E., and Benson, D.W. (2001). NKX2.5 mutations in patients with tetralogy of Fallot. Circulation *104*, 2565–2568.
34. McElhinney, D.B., Geiger, E., Blinder, J., Benson, D.W., and Goldmuntz, E. (2003). NKX2.5 mutations in patients with congenital heart disease. J. Am. Coll. Cardiol. *42*, 1650–1655.
35. von Booth, I., Silvestri, C., Erdemir, T., Lickert, H., Walls, J.R., Henkelman, R.M., Rossant, J., Harvey, R.P., Attisano, L., and Wrana, J.L. (2004). FoxH1 is essential for development of the anterior heart field. Dev. Cell *7*, 331–345.
36. Nomura, M., and Li, E. (1998). Smad2 role in mesoderm formation, left-right patterning and craniofacial development. Nature *393*, 786–789.
37. Cole, F., and Krauss, R.S. (2003). Microform holoprosencephaly in mice that lack the Ig superfamily member Cdon. Curr. Biol. *13*, 411–415.
38. Zhang, W., Kang, J.S., Cole, F., Yi, M.J., and Krauss, R.S. (2006). Cdo functions at multiple points in the Sonic Hedgehog pathway, and Cdo-deficient mice accurately model human holoprosencephaly. Dev. Cell *10*, 657–665.
39. Karkera, J.D., Lee, J.S., Roessler, E., Banerjee-Basu, S., Ouspenskaia, M.V., Mez, J., Goldmuntz, E., Bowers, P., Towbin, J., Belmont, J.W., et al. (2007). Loss-of-function mutations in *Growth Differentiation Factor-1* (*GDF1*) are associated with congenital heart defects in humans. Am. J. Hum. Genet. *81*, 987–994.