

REPORT

Transcription Factor *SIX5* Is Mutated in Patients with Branchio-Oto-Renal Syndrome

Bethan E. Hoskins, Carl H. Cramer II, Derek Silvius, Dan Zou, Richard M. Raymond Jr., Dana J. Orten, William J. Kimberling, Richard J. H. Smith, Dominique Weil, Christine Petit, Edgar A. Otto, Pin-Xian Xu,* and Friedhelm Hildebrandt*

Branchio-oto-renal syndrome (BOR) is an autosomal dominant developmental disorder characterized by the association of branchial arch defects, hearing loss, and renal anomalies. Mutations in *EYA1* are known to cause BOR. More recently, mutations in *SIX1*, which interacts with *EYA1*, were identified as an additional cause of BOR. A second member of the *SIX* family of proteins, unc-39 (*SIX5*), has also been reported to directly interact with *eya-1* in *Caenorhabditis elegans*. We hypothesized that this interaction would be conserved in humans and that interactors of *EYA1* represent good candidate genes for BOR. We therefore screened a cohort of 95 patients with BOR for mutations in *SIX5*. Four different heterozygous missense mutations were identified in five individuals. Functional analyses of these mutations demonstrated that two mutations affect *EYA1-SIX5* binding and the ability of *SIX5* or the *EYA1-SIX5* complex to activate gene transcription. We thereby identified heterozygous mutations in *SIX5* as a novel cause of BOR.

Branchio-oto-renal syndrome (BOR [MIM 113650]) is a clinically heterogeneous autosomal dominant disorder comprising branchial arch defects, hearing loss, and renal anomalies. The syndrome has an estimated prevalence of 1 in 40,000 and accounts for ~2% of profound deafness.¹ Abnormal pinnae, cervical fistulas, pits, and/or cysts are the most common branchial arch defects seen in patients, whereas hearing loss can be conductive, sensorineural, or mixed and often is associated with preauricular tags or pits.² Renal defects, which are estimated to be severe in only 6% of patients, can include collecting system duplication, hypoplasia, cystic dysplasia, hydronephrosis, and agenesis.³ The penetrance of the syndrome is highly variable both between and within families. Heimler and Lieber⁴ published a report on a large, four-generation kindred containing 16 individuals with confirmed manifestations of the syndrome. Of these, only four had abnormalities of all three systems, whereas seven had branchial arch and/or hearing defects but no reported renal abnormalities. However, because only one of these individuals had undergone a detailed renal evaluation, it is possible that the incidence of renal defects in this kindred is higher than reported.

In addition to being clinically heterogeneous, BOR also displays genetic heterogeneity. Mutations in the *EYA1* gene, the human ortholog of the *eyes absent* gene in *Drosophila*, were the first cause of BOR to be identified after the disease was linked to chromosome 8q13.3.^{5,6} Loss-of-function alleles, including nonsense, frameshift, and

splice-site mutations, were found in 7 of 42 patients screened by sequencing. In addition, deletions estimated to span 6, 7, and 20 kb were identified in 3 of 21 patients screened by Southern blot analysis. Although it was initially predicted that causative mutations would cluster in the *eyes absent* homologous region (*eyaHR*), also known as the *EYA* domain (ED) of *EYA1*,⁷ a large number of alterations distributed throughout the gene have since been reported, including complex rearrangements involving each of the 16 exons of the gene and multiple loss-of-function and missense mutations in all exons, with the exception of exons 1–3.^{8–11}

EYA proteins are characterized by a divergent N-terminal transactivation domain and the conserved C-terminal ED, which mediates interactions with DNA-binding proteins such as *SIX1* and *SIX2* (human orthologs of *Drosophila* “*sine oculis*” [*so*]), thereby forming a potent transactivation complex.^{12,13} To determine whether mutation of residues within the ED affects the ability of *Eya1* to directly interact with *Six1* or *Six2*, Buller et al.¹² performed GST pull-down and yeast two-hybrid assays on five mutations. Three ED constructs, each containing a single substituted residue (E330K, S454P, and L472R), showed a reduction in *Eya1-Six* binding in both assays, suggesting that these residues are essential for direct *Eya1-Six* interaction. Mut-suddi et al.¹⁴ used in vitro biochemical assays, in addition to in vivo studies of *Drosophila* eye, to also test the loss of function due to the S454P and L472R *EYA1* mutations. (Because a different isoform of *EYA1* was used for amino

Departments of Pediatrics and of Human Genetics, University of Michigan, Ann Arbor (B.E.H.; C.H.C.; R.M.R.; E.A.O.; F.H.); McLaughlin Research Institute for Biomedical Sciences, Great Falls, MT (D.S.; D.Z.; P.-X.X.); The Usher Syndrome Center, Boys Town National Research Hospital, Omaha (D.J.O.; W.J.K.); Departments of Otolaryngology, Pediatrics, and Internal Medicine, University of Iowa, Iowa City (R.J.H.S.); Unité de Génétique des Déficiences Sensoriels, INSERM UMR587, Institut Pasteur, Paris (D.W.; C.P.); and Department of Human Genetics, Mount Sinai School of Medicine, New York (P.-X.X.)

Received December 15, 2006; accepted for publication January 25, 2007; electronically published February 22, 2007.

Address for correspondence and reprints: Dr. Friedhelm Hildebrandt, University of Michigan Health System, 8220C MSRB III, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0646. E-mail: fhilde@umich.edu

* These two authors contributed equally to this work.

Am. J. Hum. Genet. 2007;80:800–804. © 2007 by The American Society of Human Genetics. All rights reserved. 0002-9297/2007/8004-0021\$15.00
DOI: 10.1086/513322

Table 1. Clinical and Genetic Data on Five Patients with SIX5 Mutations

Patient	Branchial Defect	Hearing Loss	Renal Defect	Nucleotide Change	Amino Acid Change
A441	PT ^R	-	DK ^B	c.472G→A	A158T
A477	CF ^B , HM ^R , PS ^R , PM ^R	+ ^L , ++ ^R	DK ^B , ↓ function	c.886G→A	A296T
A416	ND	ND	ND	c.1093G→A	G365R
A465	CF ^B	-	HK ^B	c.1655C→T	T552M
A500	CF ^B	++ ^L , + ^R	A ^L , HK ^R	c.1655C→T	T552M

NOTE.— = absent, + = affected, ++ = more affected; ^L = left, ^R = right, ^B = bilateral; PT = preauricular tag, DK = dysplastic kidneys, CF = cervical fistulae, HM = hemifacial microsomia, PS = preauricular sinus, PM = pinna malformation, ↓ function = diminished renal function, HK = hypoplastic kidneys, A = agenesis; ND = no data available.

acid numbering by Mutsuddi et al., S454P and L472R are described as S487P and L505R, respectively.) Both mutations showed an approximately fivefold reduction in the ability of the *eya-so* complex to transactivate. However, coimmunoprecipitation studies showed that this reduction was not due to an inability of the complex to form, as both mutant constructs were able to complex efficiently with so.

Following the identification of *EYA1* as the first BOR gene, a number of reports on the exclusion of the 8q13 locus and mapping of additional BOR loci, such as the *BOS2* (branchiootic syndrome 2 [MIM %120502]) locus on 1q31, were published.^{15–17} Most recently, we used a large BOR kindred with >40 affected individuals to map a third BOR locus (branchiootic syndrome-3 [MIM 608389]) to 14q21.3.¹⁸ Located within the 33-Mb critical interval were three members of the *SIX* family of genes (*SIX1*, *SIX4*, and *SIX6*). Mutation screening of these genes in the family used to map the locus revealed a heterozygous Y129C missense mutation in *SIX1*, with two further mutations (delE133 and R110W) in three additional patients, confirming *SIX1* as a second gene for BOR.¹⁹ Functional analysis of these mutations demonstrated that the R110W mutation, located in the *SIX* domain (SD), interferes with formation of the *Eya1-Six1* complex, whereas the Y129C and delE133 mutations affect not only this protein-protein interaction but also the ability of *Six1* to bind DNA.¹⁹

In 2004, the *Caenorhabditis elegans* interactome was published, containing data on >4,000 direct protein-protein interactions in the nematode identified by high-throughput, yeast two-hybrid analysis.²⁰ At the center of one of the subnetworks made up of >75 interactions was C49A1.4 (*eya-1*), the ortholog of *EYA1*. In addition to the ortholog of *SIX1* (*ceh-33*), a second member of the *SIX* family of proteins, *SIX5* (*unc-39/ceh-35*), was also shown to directly interact with *eya-1* in the nematode. Because *SIX5* has a high degree of homology to *SIX1* and directly interacts with *eya-1* in *C. elegans*, we considered it an excellent candidate for mutation analysis in a cohort of patients with BOR who did not have mutations in either *EYA1* or *SIX1*.

Patients.—The diagnosis of BOR was based on published criteria.¹⁰ All samples were negative for *EYA1* or *SIX1* mutations. This study was approved by the institutional review boards of the University of Freiburg (303102) and

the University of Michigan Medical School (2004-0322, HUM00003330). Patient samples were obtained after informed consent was given.

Mutation analysis.—Primers were designed to amplify each of the three exons and surrounding intronic sequence of *SIX5*. Standard PCR were performed, and sequencing was performed on an ABI capillary sequencer. Sequence traces were analyzed using the Sequencher software program (Gene Codes). Absence of sequence changes in 150 European white control individuals was performed by sequencing or, where possible, by digestion of the PCR product with a restriction enzyme.

Generation of *SIX5* constructs.—The human *SIX5* full-length cDNA was cloned by RT-PCR into either pGBKT7 vector for yeast two-hybrid assays or pcDNA3 vector for cell-culture analysis. To introduce the same amino acid substitutions found in patients with BOR into the human *SIX5* protein, PCR-based mutagenesis was performed using the QuickChange mutagenesis kit (Stratagene). Once mutant colonies were identified, the plasmid DNA was isolated and sequenced through the mutation-containing region. Four mutant cDNAs (A158T, A296T, G365R, and T552M) were constructed in the same manner in either the pGBKT7 or the pcDNA3 vector.

Yeast two-hybrid assay.—The Matchmaker Two-Hybrid System 3 (Clontech) was used for yeast interaction assays. *SIX5* was fused with the GAL4 DNA-binding domain of pGBKT7 (GAL4 BD-*SIX5*). The *Eya1* domain (*Eya1D*) region of *Eya1* was fused with the GAL4 activation domain of pGAD424 (GAL4 AD-*Eya1D*). Small scale LiAc cotransformations of the plasmids into AH109 cells, colony-lift filter assays, and liquid culture β -galactosidase assays were performed as described in the Clontech protocols.

Luciferase assay.—The human embryonic kidney HEK293 cell line and Cos-7 cells were cultured as described elsewhere.¹² The myogenin luciferase reporter pGL3-6 × MEF3 was used as a reporter. One microgram of pGL3-6 × MEF3 plasmid was cotransfected with 1 μ g of pcDNA3-*SIX5* wild type (wt*SIX5*) or its mutant plasmids, or 1 μ g *Eya1* alone, or both *SIX5* and *Eya1* together with use of FuGENE 6 Transfection Reagent (Roche), according to the manufacturer's instructions. Cells were grown in dishes 6 cm in diameter and were additionally cotransfected with 0.25 μ g of the cytomegalovirus (CMV) promoter- β -galactosidase (pCMV β -gal) as an internal control.

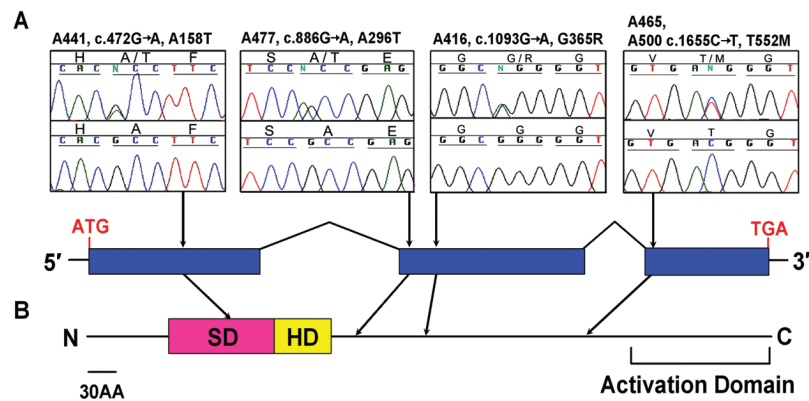


Figure 1. Four missense mutations in *SIX5* identified in patients with BOR. *A*, A158T, A296T, and G365R mutations, present in heterozygous form in a single patient. The T552M mutation was detected in patients A465 and A500. Sequences from the affected individual are shown above sequences from healthy control individuals. Patient identifiers, nucleotide changes, and amino acid changes are shown above the traces. *B*, *SIX5* has an SD and a homeodomain (HD) characteristic of the *SIX* family of proteins at the N-terminus. An activation domain has also been mapped to the C-terminus.²¹ The A158T mutation is located in the *SIX* domain; the remaining three mutations are located between the HD and the activation domain.

Forty-eight hours after transfection, cell extracts were prepared and assayed for luciferase activities.

Mutation screening of *SIX5* in 95 unrelated patients with BOR revealed four different heterozygous missense mutations in five individuals (table 1 and fig. 1). Individual A441, who has bilateral dysplastic kidneys and a right preauricular tag but normal hearing, was found to carry a c.472G→A substitution (amino acid change A158T). Individual A477 has bilateral cervical fistulae; hemifacial microsomia; a preauricular sinus and pinna malformation on the right side; hearing loss in both ears, with the right ear more affected than the left; and bilateral renal dysplasia with diminished renal function. This patient was found to have a c.886G→A transition (amino acid change A296T). In individual A416, a c.1093G→A substitution (amino acid change G365R) was found. In addition, a c.1655C→T transition (amino acid change T552M) was found in two individuals: A465, who has cervical fistulae with hypoplastic kidneys, and A500, who has cervical fistulae, moderate to severe hearing loss in both ears, and left renal agenesis with a hypoplastic right kidney. All sequence changes were absent in 150 healthy control individuals. The phenotypes of the patients presented here show a similarly high degree of variability, as seen in patients with BOR who had mutations in *EYA1* or *SIX1*.^{10,19} Although the cohort of patients is small, there is no evidence of a genotype-phenotype correlation associated with *SIX5* mutations.

To test whether any of the mutations identified in patients affect the ability of *SIX5* to directly interact with *Eya1*, yeast two-hybrid liquid β -galactosidase assays were performed. Cotransformation of yeast with GAL4 BD-*SIX5* and GAL4 AD-*Eya1D* constructs led to strong *lacZ* expression as a result of interaction between the two fusion proteins (fig. 2). Introduction of mutations into the *SIX5* construct showed varying levels of reduction in *lacZ* ex-

pression. A296T and G365R showed only a slight reduction in expression, whereas both A158T and T552M showed a >2-fold reduction, indicating that these two residues of *SIX5* may be required for efficient binding with *EYA1*.

To investigate what effect these mutations may have on the ability of *SIX5* or the *Eya1-SIX5* complex to activate transcription, the myogenin luciferase reporter pGL3-6 × MEF3 was used. This construct contains six MEF3 sites to which *SIX* proteins are known to bind.^{19,21} Transfection

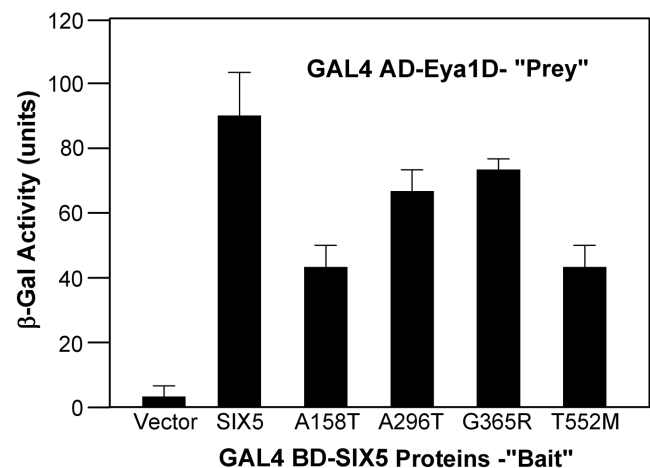


Figure 2. Mutations in *SIX5* affect *SIX5-Eya1* binding. Yeast two-hybrid assays were performed to test the effect of the mutations on the ability of *SIX5* to bind to *Eya1*. Cotransformation of GAL4BD-*SIX5* and GAL4AD-*Eya1D* leads to strong *lacZ* expression. An >2-fold reduction in *lacZ* expression is seen when *SIX5* proteins containing an A158T or a T552M mutation are cotransformed with *Eya1D* fusion proteins.

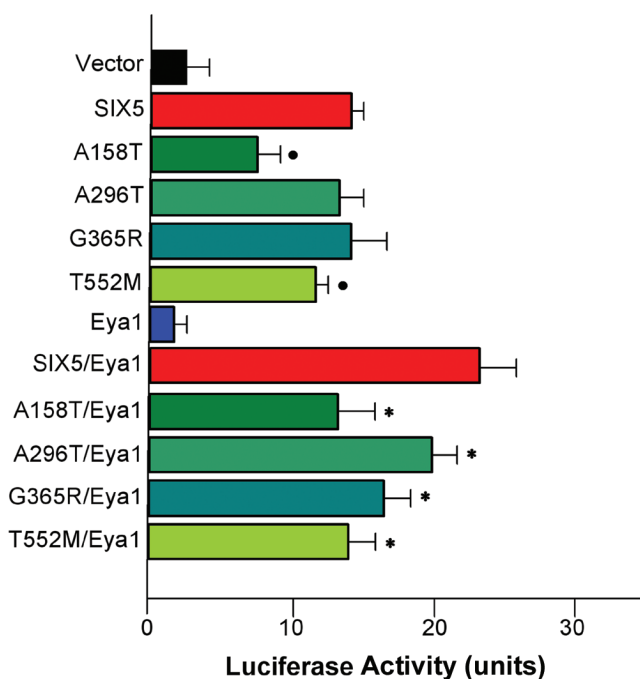


Figure 3. Mutations in *SIX5* reduce its ability to activate transcription of the MEF3 promoter. The myogenin luciferase reporter pGL3-6 × MEF3 was cotransfected with pcDNA3 vector containing *SIX5* or its mutants (A158T, A296T, G365R, and T552M), pFlag-Eya1, or both the *SIX5* and Eya1 plasmids together in HEK293 cells. Luciferase activity in the cell lysate was normalized with β -galactosidase activity of pCMV β -gal as an internal control. The activity at each data point is relative to that obtained by the control pCMV vector. The mean fold activation from three independent experiments (each performed in duplicate) is shown with the standard deviation. A filled circle (•) indicates $P < .002$, compared with *SIX5*; an asterisk (*) indicates $P < .002$, compared with *SIX5*/Eya1 (one-way analysis of variance [ANOVA]).

of HEK293 cells with wt*SIX5* resulted in an ~7-fold increase in luciferase activity compared with vector alone (fig. 3). Transfection of the A296T and G365R mutant constructs alone did not result in a significant decrease in luciferase reporter activity. However, the A158T and T552M mutations resulted in significant decreases in luciferase activity when compared with wt*SIX5*. Decreases were 50% for A158T and 25% for T552M. Coexpression of wt*SIX5* with Eya1 resulted in a 12-fold increase in luciferase activity. However, when each of the mutant *SIX5* proteins was coexpressed with Eya1, the increase in activity was reduced to 52%, 80%, 70%, and 56% of that seen for wt*SIX5* with Eya1 for the A158T, A296T, G365R, and T552M mutants, respectively. Although the reduction in transcriptional activity is small for the A296T and G365R mutants, these reductions are statistically significant when compared with the activity seen on coexpression of wt*SIX5* with Eya1 (fig. 3). The results were reproducible in three independent experiments, each performed in duplicate.

In this study, we have identified heterozygous muta-

tions in *SIX5* as a novel cause of BOR. *SIX5* was considered to be a good candidate gene for BOR, owing to its similarity to *SIX1*, a known BOR gene,¹⁹ and data on *C. elegans* on its direct interaction with *eya-1*,²⁰ mutations of which also cause BOR.⁶ Four different missense mutations were identified in five patients from a cohort of 95 unrelated individuals. Although it was not possible to perform segregation analyses on these mutations, because DNA samples from family members were not available, the absence of each mutation in a cohort of 150 healthy control individuals strongly suggests that these changes are disease-causing mutations rather than nonpathogenic polymorphisms. This is confirmed by the functional studies of Eya1-*SIX5* interaction and reduced function in transcriptional activation assays.

Unlike *SIX1*, *SIX5* possesses an additional activation domain (AD) at the C-terminus, which retains its transactivation function when fused to a GAL4 DNA-binding domain.²² Consistent with this, our data show that expression of *SIX5* alone is able to transactivate MEF3-reporter transcription in cultured cells (fig. 3). *SIX5* is also able to synergistically activate MEF3 reporter gene expression when coexpressed with Eya proteins²¹ (fig. 3). Among the four mutations identified in the *SIX5* protein, the A158T mutation is located in the SD domain, while the other three mutations are located between the HD and AD domains (fig. 1B). Our results show that the SD mutation A158T resulted in a reduction (50%) in reporter activity in a yeast two-hybrid assay (fig. 2) and that this reduction can be explained by a decrease in the Eya1-*SIX5* complex formation. Interestingly, the luciferase assay showed that the SD mutation (A158T) decreased the reporter activity in the absence of Eya1. Because the SD is known to mediate not only protein-protein interaction but also protein-DNA binding affinity through dimerization,^{21,23,24} the reduction in reporter activity observed in cultured cells without addition of Eya1 is likely caused by the SD mutation affecting the formation of the *SIX5*-DNA binding complex.

In contrast to A158T, the two mutations A296T and G365R have no effect on the ability of *SIX5* alone to activate transcription but do affect the transcriptional activity of the Eya1-*SIX5* complex, to varying degrees. The largest effect on coexpression was again seen for A158T and the second largest for the T552M mutation. Because the mutated T552M residue is near the C-terminal activation domain, it may affect *SIX5* transactivation function.

It should be noted that the effect of *SIX1* mutations¹⁹ was greater than that seen with the *SIX5* mutations presented here. This may be in part because *SIX5* itself is transcriptionally active but also because, when transfected together with Eya1, Eya2, or Eya3, the combination of *Six5* with Eya3 showed the highest levels of activity on a myogenin luciferase reporter: 8-fold activation compared with the 4-fold activation seen with *Six5* and Eya1.²¹ Therefore, although EYA1 and *SIX5* do interact, there may be some additional transcriptional activation through EYA3.

We have identified mutations in *SIX5* as a new cause of BOR. In addition, we provided evidence to support the interaction between *Eya1* and *SIX5* and showed that mutations of *SIX5* compromise the interaction between the two proteins, decreasing the transcriptional activity of the *Eya1-SIX5* complex.

Acknowledgments

We thank the patients and their families for their contribution. We also thank Shrawan Kumar, Harold Bass, and Cor Cremers for ascertainment of patient samples. This work was supported by grants from the National Institutes of Health to F.H. (R01-DK045345-01), P-X.X. (R01 DC005824 and DK064640), and C.H.C. (T32DK065551-01). F.H. is the Frederick G. L. Huetwell Professor of the Cure and Prevention of Birth Defects and a Doris Duke Distinguished Clinical Scientist.

Web Resource

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for BOR, *BOS2*, and branchiootic syndrome-3 locus)

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