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Transcriptional Control of *SLC26A4* Is Involved in Pendred Syndrome and Nonsyndromic Enlargement of Vestibular Aqueduct (*DFNB4*)

Tao Yang, Hilmar Vidarsson, Sandra Rodrigo-Blomqvist, Sally S. Rosengren, Sven Enerbäck, and Richard J. H. Smith

Although recessive mutations in the anion transporter gene *SLC26A4* are known to be responsible for Pendred syndrome (PS) and nonsyndromic hearing loss associated with enlarged vestibular aqueduct (EVA), also known as "*DFNB4,*" a large percentage of patients with this phenotype lack mutations in the *SLC26A4* coding region in one or both alleles. We have identified and characterized a key transcriptional regulatory element in the *SLC26A4* promoter that binds FOXI1, a transcriptional activator of *SLC26A4*. In nine patients with PS or nonsyndromic EVA, a novel c.−103T→C mutation in this regulatory element interferes with FOXI1 binding and completely abolishes FOXI1-mediated transcriptional activation. We have also identified six patients with mutations in *FOXI1* that compromise its ability to activate *SLC26A4* transcription. In one family, the EVA phenotype segregates in a double-heterozygous mode in the affected individual who carries single mutations in both *SLC26A4* and *FOXI1*. This finding is consistent with our observation that EVA occurs in the *Slc26a4*$^{+/-}$; *Foxi1*$^{+/-}$ double-heterozygous mouse mutant. These results support a novel dosage-dependent model for the molecular pathogenesis of PS and nonsyndromic EVA that involves *SLC26A4* and its transcriptional regulatory machinery.

Pendred syndrome (PS [MIM 274600]) accounts for up to 10% of hereditary hearing loss in humans, making it the most common form of syndromic deafness.[1] It is inherited in an autosomal recessive manner and has been causally linked to mutations in the coding sequence of *SLC26A4* (MIM 605646; GenBank accession number NC_000007.12).[2,3] The disease phenotype is characterized by sensorineural hearing loss; structural malformation of the inner ear, such as enlargement of vestibular aqueduct (EVA [MIM 603545]) with or without cochlear dysplasia; and defects in iodide transport that can lead to goitrous changes of the thyroid gland. Consistent with this phenotype, *SLC26A4* mRNA and the encoded protein pendrin are found in the inner ear and thyroid. In the inner ear, pendrin is expressed in external sulcal cells and the endolymphatic duct and sac—regions involved in endolymph homeostasis[4]—and, in the thyroid, pendrin is expressed in the apical membrane of thyrocytes, where it regulates iodide flux into thyroid follicles.[5,6] In addition to PS, mutations in *SLC26A4* also cause nonsyndromic hearing loss with EVA in the absence of a thyroid phenotype (*DFNB4* [MIM 600791]).[7,8]

Familial cases of both PS and nonsyndromic EVA support the recessive inheritance of this disease spectrum. In previous reports,[7,9,10] as well as in our current study (table 1), however, many patients segregate only one or no mutations in the *SLC26A4* coding region, suggesting that environmental or other genetic factors may contribute to disease expression. The transcription-factor gene *FOXI1* (MIM 601093) has been proposed as a candidate that may contribute to the genetic cause of PS. Mice homozygous for the targeted disruption of *Foxi1* are deaf and have EVA.[11] Furthermore, there is complete absence of *Slc26a4* (GenBank accession number NC_000078.4) expression in the epithelial cells of endolymphatic duct and sac in these mice, where normally both *Foxi1* and *Slc26a4* messages are present. These data suggest that Foxi1 (GenBank accession number NP_076396.2) is an upstream regulator of *Slc26a4*.[12] In this study, we show that transcriptional control of *SLC26A4* expression involves a key *cis*-regulatory element in the *SLC26A4* promoter and its associated transcription factor FOXI1 (GenBank accession number NP_036320.2), both of which play a role in the pathogenesis of PS and nonsyndromic EVA.

Subjects and Methods

Subjects

Persons with EVA or Mondini dysplasia were ascertained clinically on the basis of the presence of hearing loss and findings of temporal bone CT or magnetic resonance imaging. In addition to these studies, their evaluation included a complete history and physical examination. Thyroid defects were recorded but were not used as classification criteria. For their condition to be classified as EVA, enlargement of the vestibular aqueduct had to be

From the Department of Otolaryngology–Head and Neck Surgery (T.Y.; R.J.H.S.) and Interdepartmental PhD Program in Genetics (T.Y.; R.J.H.S.), University of Iowa, Iowa City; Department of Medical Biochemistry, Göteborg University, Göteborg, Sweden (H.V.; S.R.-B.; S.E.); and University of Connecticut Health Partners, West Hartford (S.S.R.)

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Address for correspondence and reprints: Dr. Richard J. H. Smith, Department of Otolaryngology–Head and Neck, 200 Hawkins Drive, 21151-PFP, University of Iowa, Iowa City, Iowa 52242. E-mail: richard-smith@uiowa.edu

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Table 1. Summary of *SLC26A4* Mutation-Screening Results in Patients with PS and Nonsyndromic EVA

Families	*n*	No. (%) of Families by No. of *SLC26A4* Mutations		
		Zero	One	Two
Multiplex	31	13 (42)	6 (19)	12 (39)
Simplex	398	284 (71)	69 (17)	45 (11)
Total	429	297 (69)	75 (17)	57 (13)

>1.5 mm at a point midway between the endolymphatic sac and the vestibule; to be classified as Mondini dysplasia, the cochlea also had to be abnormal, with incomplete partition and a scala communis. All procedures were approved by the institutional review board at the University of Iowa, and informed consent was obtained from all subjects.

Mutation Screening

Mutation screening was performed by denaturing high-performance liquid chromatography and bidirectional sequencing on DNA extracted from whole blood, as we have described elsewhere.[13,14] Mutation screening of *SLC26A4* promoter and *FOXI1* was completed in all patients with (1) one mutation in *SLC26A4* or (2) no mutations in *SLC26A4* but with either an affected sibling or a classic PS phenotype (Mondini dysplasia with or without goiter).

Luciferase Assay

FOXI1 was PCR amplified and cloned into the pcDNA3.1/zeo(+) vector (Invitrogen). The 5-kb genomic region upstream of the *SLC26A4* start codon was amplified from control genomic DNA by long-range PCR and was cloned into the luciferase reporter vector pGL3.0 Basic (Promega). Mutant *FOXI1* and *SLC26A4* promoter constructs were generated by site-directed mutagenesis, by use of the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The *SLC26A4* promoter-reporter assay was performed using the Luciferase Assay System (Promega). In brief, COS-7 cells grown in 6-well plates were transfected with 750 ng *FOXI1* construct and 250 ng luciferase reporter construct, with use of FUGENE 6 Transfection reagent (Roche). In place of *FOXI1* constructs, 750 ng pcDNA3.1/zeo(+) empty vector was used in controls. Before each luciferase assay, cell growth medium was carefully removed. Cells were washed with PBS once, were mixed with 100 μl of 1 × lysis buffer, and were frozen at −20°C for 20 min. Cells and all lysates were scraped and transferred to microcentrifuge tubes, were vortexed for 15 s, and were centrifuged at 12,000 *g* for 2 min at 4°C. From each sample, 20 μl of the supernatant was transferred to a 96-well plate for automatic luciferase activity assay. Luciferase activities were determined as fold induction compared with activities of corresponding control cells transfected with 750 ng empty pcDNA3.1/zeo(+) vector and were normalized to the total protein level measured by Bicinchoninic Acid Protein Assay Kit (Sigma). Experiments were performed in at least triplicate.

Electrophoretic Mobility Shift Assay

FOXI1 protein was transcribed in vitro and was translated using the TnT Quick Coupled Transcription/Translation System (Promega). As the DNA template, 1 μg pSP64 Poly(A) expression plasmid with the full FOXI1 coding sequence was used. Mobility shift reactions were performed in 1 × binding buffer (5 mM HEPES, 26% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 100 mM KCl [pH 7.9]) supplemented with 1 μl poly d(I-C) in a 15-μl reaction. Double-stranded oligonucleotide probes were 5′-end labeled with use of α^{32}P-CTP and Klenow polymerase. Binding reactions contained 5×10^4 cpm of labeled probe, the indicated amount of unlabeled competitor probe, and 3 μl protein lysate. Binding reactions were incubated for 15 min at room temperature and were resolved using a nondenaturing 4% polyacrylamide gel. Electrophoresis was performed at 4°C at 200 V for 1.5 h, and the gel was subjected to radioautography. Band intensity was quantified using the AlphaEaseFC imaging system and software (Alpha Innotech).

Histology

The morning that vaginal plugs were detected was designated embryonic day 0.5 (E0.5). Tails from embryos were collected for genotyping by use of PCR as described above. Embryos were fixed overnight in PBS with 4% paraformaldehyde at 4°C. For histology, embryos were dehydrated and embedded in paraffin wax, and 6-μm sections were cut. Tissue sections were stained with hematoxylin and eosin for histological analysis. At least four mice of each genotype were analyzed at E16.5.

Results

Identification of an SLC26A4 *Promoter Mutation Associated with PS and Nonsyndromic EVA*

We completed mutation screening of the *SLC26A4* coding region in 429 deaf probands given a diagnosis of EVA with or without cochlear dysplasia (table 1). In 31 multiplex families with two or more affected siblings, only 12 (39%) had sibships that segregated two disease-causing allele variants of *SLC26A4*; in the remaining 398 simplex families, the percentage was much lower (11%, or 45 families). Interestingly, one disease-causing mutation was identified in 6 multiplex and 69 simplex families (17% of all families). This finding suggested that additional genetic factors are involved in PS and nonsyndromic EVA.

To study additional genetic causes of this disease, we first searched for mutations in the noncoding region of *SLC26A4* by screening its promoter. Three highly homologous regions in the 5-kb upstream sequence of *SLC26A4* were found by comparing human and mouse genomes. Mutation screening of these regions revealed a T→C single-nucleotide change 103 bp upstream of the *SLC26A4* translation start in nine families with PS or nonsyndromic EVA. This c.−103T→C mutation is within an evolutionarily conserved nucleotide among many mammalian species (fig. 1*A*) and was not seen in 100 unrelated controls with normal hearing.

Functional Characterization of the Promoter Mutation and the Embracing Regulatory Cis*-Element*

On the basis of comparison with the TRTTKRY FOXI1 consensus binding sequence (R = A/G; K = G/T; Y = C/T)[15,16] the c.−103T→C promoter mutation lies within

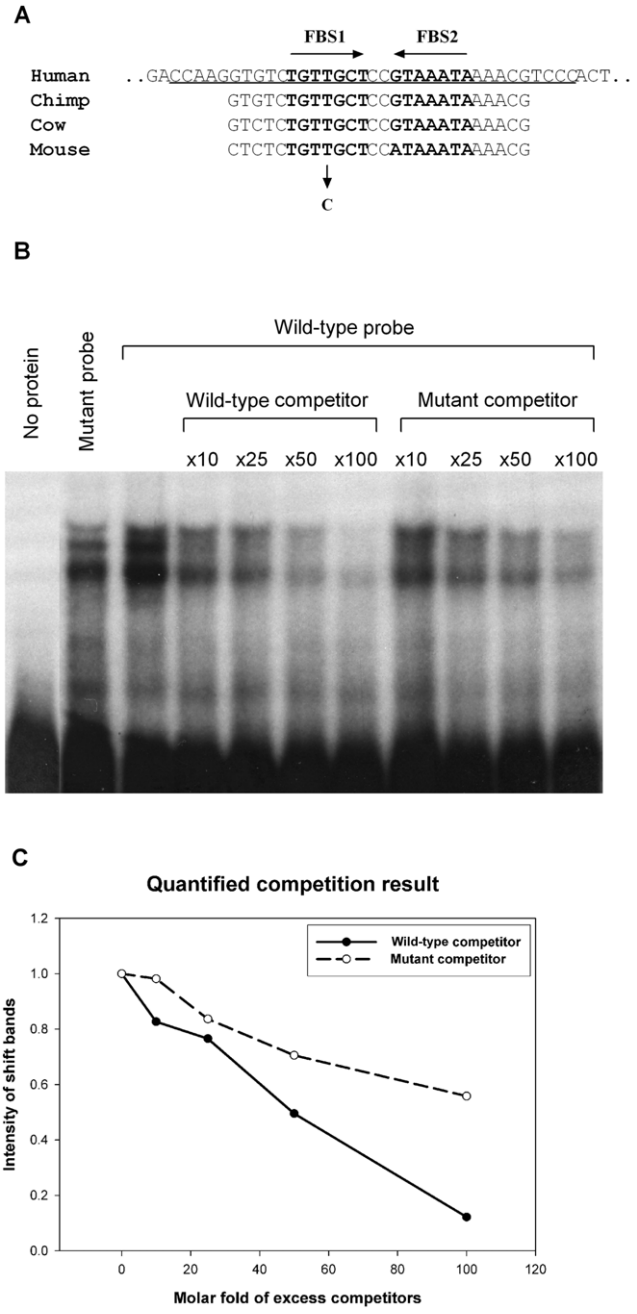

Figure 1. FOXI1-binding affinity to wild-type and mutant (c.−103T→C) FBS1. *A,* Conservation of FBS1 and FBS2. FBS1 and FBS2 are shown in bold, arrows indicate orientation, the c.−103T→C mutation is indicated by the vertical arrow, and the oligonucleotide EMSA probe is underlined. *B,* ^{32}P-labeled double-stranded oligonucleotide probes containing FBS1 and FBS2 bind to FOXI1 protein as shift bands. Protein binding to the mutant probe (*lane 2*) is significantly weaker than that to the wild-type probe (*lane 3*). Protein binding to the wild-type labeled probe was competed with either wild-type (*lanes 4–7*) or mutant (*lanes 8–11*) unlabeled competitor (10–100 molar fold excess). Mutant competitor competed significantly less efficiently than wild-type competitor. *C,* Quantification of EMSA competition result: intensity of the top shift band of lanes 3–11 relative to the intensity of the top shift band without competition. At 100 × molar excess of competitor, the intensity of the top shift band drops to 0.12 fold for the wild-type competitor but to only 0.56 fold for the mutant competitor.

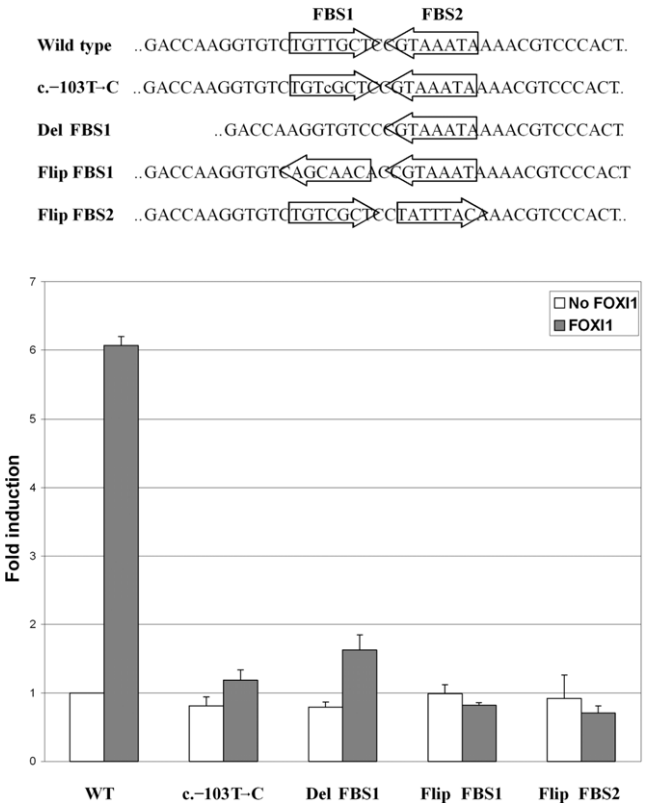

Figure 2. Luciferase assay showing complete loss of FOXI1 transcriptional activation in mutant and modified promoter-reporter constructs. *Top panel,* Sequence of the FBS1-FBS2 promoter site in various promoter-reporter constructs (*from top to bottom*): wild type, c.−103T→C mutant, FBS1 deletion, FBS1 inversion (Flip FBS1), and FBS2 inversion (Flip FBS2). The −103C nucleotide is marked with lowercase letters. *Bottom panel,* COS-7 cells were transfected with 250 ng promoter-reporter construct and 750 ng empty vector (*white bars*) or FOXI1 construct (*gray bars*). Reporter activity is shown as "fold induction" compared with activity of the wild-type promoter-reporter construct without exogenous expression of FOXI1. Results from three independent experiments are shown (mean ± SD).

a predicted FOXI1-binding site highly conserved across mammalian species, which we called "FBS1" (fig. 1*A*). We confirmed binding of FOXI1 to FBS1 by electrophoresis mobility shift assay (EMSA), which showed that the c.−103T→C mutation significantly reduces FOXI1-binding affinity (fig. 1*B*).

To determine whether this promoter mutation directly affects the transcription of *SLC26A4,* we used the luciferase promoter-reporter expression assay. Five kilobases of the upstream sequence of *SLC26A4* were fused to the luciferase reporter gene in the promoter-reporter construct, which was transfected into COS-7 cells with *FOXI1*-expressing constructs (see the "Subjects and Methods" section). Compared with controls transfected with empty vector, wild-type FOXI1 induced a sixfold increase in luciferase activity. However, when the c.−103T→C mutation was introduced into the *SLC26A4* promoter-reporter construct, FOXI1 transactivation of the luciferase reporter was completely abolished (fig. 2). The effect was identical if FBS1 was deleted, implying that this binding site is a major transcriptional regulatory element of *SLC26A4* and is required for FOXI1-induced transcriptional activation of *SLC26A4.*

We identified a second FOXI1-binding site, called "FBS2," 2 nt downstream of FBS1. FBS2 is also highly conserved across species and is a perfect match to the TRTTKRY consensus binding sequence, although in the reverse orientation to FBS1 (fig. 1*A*). Flipping either FBS1 or FBS2 in the *SLC26A4* promoter-reporter construct completely abolishes any response to FOXI1 transcriptional activation (fig. 2), suggesting that the unique head-to-head orientation of these tandem binding sites is necessary for FOXI1 to form a functional transcriptional-activation complex.

Table 2. List of Families with PS and Nonsyndromic EVA with *FOXI1* Mutations

Family	Goiter	*SLC26A4* Mutation	*FOXI1* Mutation
82230	Not noted	E29Q/+	G258E/+
7980	Not noted	+/+	161DelN/+
8280	Not noted	+/+	G258R/+
518350	Not noted	+/+	R267Q/+
7390	Yes	+/+	R267Q/+
81720	Yes	+/+	G335V/+

NOTE.—All families have nonsyndromic EVA. + = Wild-type allele.

Identification and Characterization of FOXI1 *Mutations*

We further asked whether mutations in *FOXI1* contribute to the PS-EVA disease spectrum. Mutation screening of FOXI1 revealed five nonsynonymous variants of *FOXI1* in six patients (table 2). Three of these mutations—161DelN, R267Q, and G335V—are conserved between human and mouse, and the remaining two involve Gly258, changing this small noncharged amino acid into either arginine or glutamate, both of which are substantially more bulky (fig. 3). One mutation, 161DelN, is also unique, in that it lies within the conserved forkhead DNA-binding domain. None of the five mutations was seen in 250 unrelated controls with normal hearing screened for *FOXI1.*

To study the possible effects of these *FOXI1* mutations, we first completed immunofluorescence assays of FOXI1 in mammalian cells transfected with mutant and wild-type *FOXI1* constructs and found that nuclear translocation of the mutant protein was not altered (data not shown). We then used the same promoter-reporter assay system described in the promoter mutation study, to test whether the *FOXI1* mutations affect transcriptional activation. Compared with the sixfold induction of luciferase expression by wild-type FOXI1, all five FOXI1 variants showed significantly decreased luciferase activation (fig. 4). These results suggest that the variants in these patients compromise FOXI1 transactivation ability of *SLC26A4*

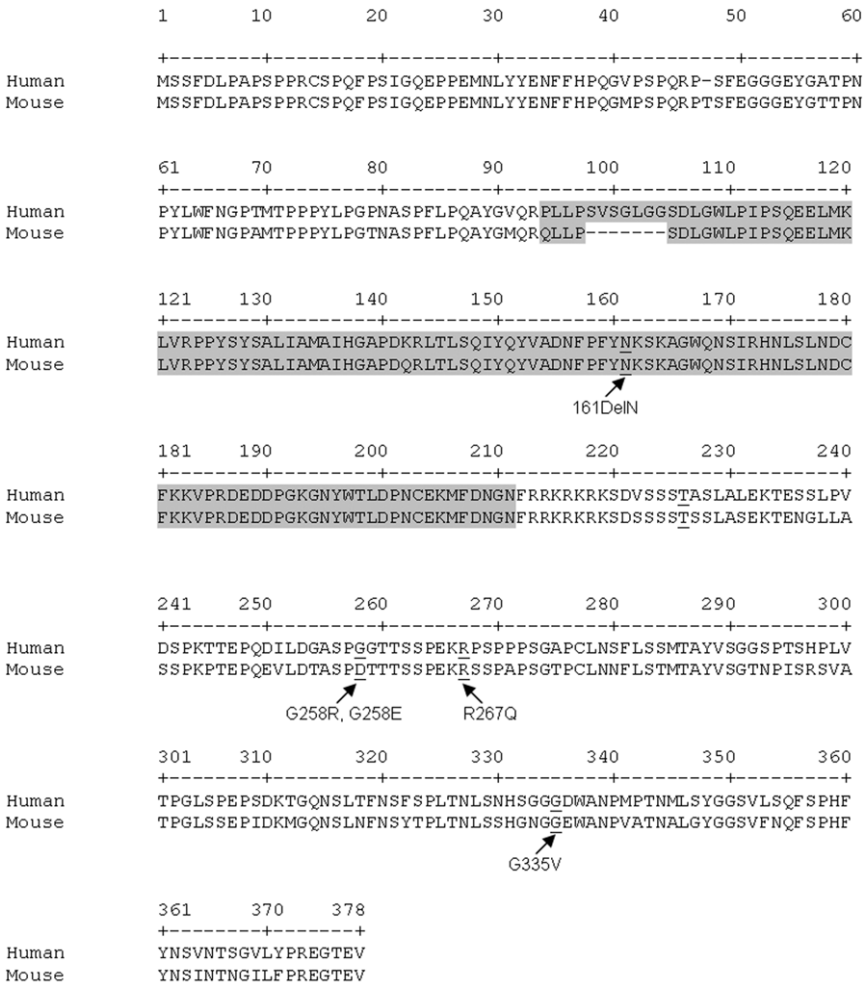

Figure 3. Conservation of human and mouse FOXI1 protein. The amino acid changes identified in patients with PS and nonsyndromic EVA are underlined and marked by arrows. The conserved forkhead DNA-binding domain is shaded in gray.

expression and are causally related to disease in these patients.

Double-Heterozygosity Model of Nonsyndromic EVA

In one family (82210) with hearing loss and nonsyndromic EVA, the disease phenotype segregates in a double-heterozygous mode, with the mother carrying the SLC26A4 E29Q mutation, the father carrying the FOXI1 G258E mutation, the affected child carrying both mutations, and the unaffected child carrying the SLC26A4 E29Q mutation only. Allele segregation in this family also supports double heterozygosity, in that both siblings inherited the same two *SLC26A4* alleles from their parents, indicating that the difference in the phenotypes between those two siblings is caused by mutations at another locus (fig. 5).

We crossed *Slc26a4*$^{+/-}$ and *Foxi1*$^{+/-}$ mice to determine whether this animal model supports double heterozygosity. Both *Slc26a4*$^{-/-}$ and *Foxi1*$^{-/-}$ mutants develop EVA, whereas their heterozygous counterparts are phenotypically indistinguishable from wild-type controls. In the *Slc26a4*$^{+/-}$; *Foxi1*$^{+/-}$ double-heterozygous mutant, however, temporal bone development is abnormal, and EVA is also observed (fig. 6).

Discussion

Our work shows that the transcriptional machinery that controls *SLC26A4* expression is involved in the PS-EVA disease spectrum. This finding is consistent with reports by several investigators noting that many patients with a PS-EVA phenotype lack mutations in either one or both alleles of *SLC26A4*.[7,9,10] Our database of 429 deaf probands given clinical diagnoses of PS or nonsyndromic EVA represents the largest study to date to confirm this observation (table 1). In only 39% of multiplex families and 11% of simplex families did we identify two *SLC26A4* mutations; in 42% and 71% of multiplex and simplex families, respectively, no mutations were identified. These differences between groups suggest the involvement of envi-

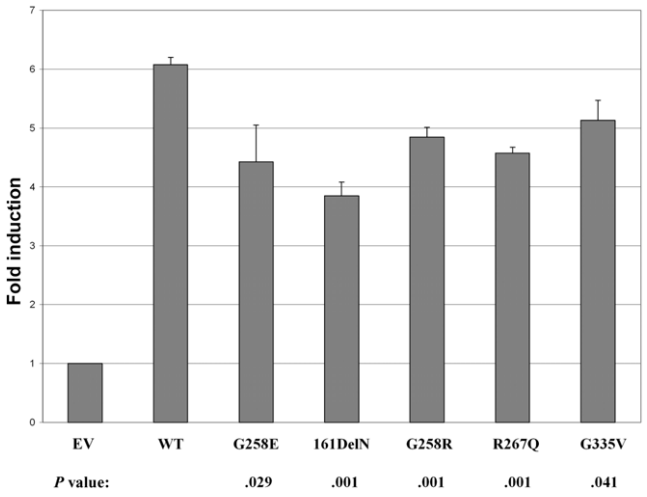

Figure 4. The transcriptional activation ability of *FOXI1* variants, as measured using a luciferase assay. Together with 750 ng of empty vector (EV), *FOXI1* wild-type construct (WT), or one of the five *FOXI1* mutant constructs, 250 ng of promoter-reporter construct was transfected into COS-7 cells. Reporter-gene activity was quantitated as "fold induction" relative to the empty vector control. Results from three independent experiments are shown (mean ± SD); the activity of each mutant *FOXI1* construct has been compared with that of the wild-type construct (*P* values calculated by two-tailed Student's *t* test).

ronmental factors but are also consistent with other possibilities, including (1) the presence of large deletions in *SLC26A4* that are not recognized by our screening methodology; (2) mutations in the noncoding regions of *SLC26A4,* such as intronic cryptic splicing or promoter mutations; or (3) mutations in other genes that contribute in *trans* to cause a PS-EVA phenotype.

Our current PCR-based mutation-screening method does not distinguish hemizygosity from homozygosity at the genomic DNA level, making detection of large deletions challenging. To address this problem, we completed real-time PCR or SNP genotyping in a group of patients ($n = 35$) and failed to identify any large deletions (data not shown). To address the second hypothesis, we completed a detailed study of the promoter region of *SLC26A4* and identified a *cis*-regulatory element critical for *SLC26A4* expression. This element is a binding target for the transcription factor FOXI1 and is required for FOXI1-mediated transcriptional activation of *SLC26A4*. A mutation within this *cis*-element, c.−103T→C, completely abolishes this activation and was present in 9 of 429 patients, consistent with this mutation having a disease-causing role in the PS-EVA phenotype (figs. 1 and 2). Although we failed to identify a second *SLC26A4* mutation in these families, it is not uncommon to detect a single disease-causing *SLC26A4* mutation presumably in combination with a yet-to-be-identified mutation either in *cis* or in *trans* (table 3).

Although other studies have described large genomic deletions 5′ to *POU3F4* (MIM 300039) and *GJB2* (MIM 121011) that may contain regulatory elements causally associated with nonsyndromic deafness at the *DFN3* and *DFNB1* loci, respectively,[17,18] our work is the first to identify a specific *cis*-regulatory element of a deafness-related gene and to reveal its role in the pathogenesis of nonsyndromic hearing loss.

The FOXI1-binding *cis*-element we discovered has a unique head-to-head structure, with FBS1 and FBS2 orientated in opposite directions. Both binding sites and this specific orientation are required for FOXI1-mediated transcriptional activation (fig. 2). Two recent promoter-reporter studies have shown that Foxi1 also activates transcription of two other ion transporter genes—*Slc4a9* (MIM 610207; GenBank accession number NC_000084.4) encoding AE4, an HCO_3^-/Cl^- exchanger in the type B intercalated cells of the renal collecting-duct epithelium,[19] and *Atp6v1b1* (MIM 192132; GenBank accession number NC_000072.4) encoding the B1 subunit of the vacuolar H^+-ATPase proton pump in the apical pole of narrow and clear cells of the epididymis.[20] Interestingly, adjacent to the reported Foxi1-binding *cis*-elements of both genes, we observed a second putative Foxi1-binding site that matches the FOXI1 consensus binding sequence and is in the opposite orientation (fig. 7). We hypothesize that this unique head-to-head arrangement is a conserved FOXI1-

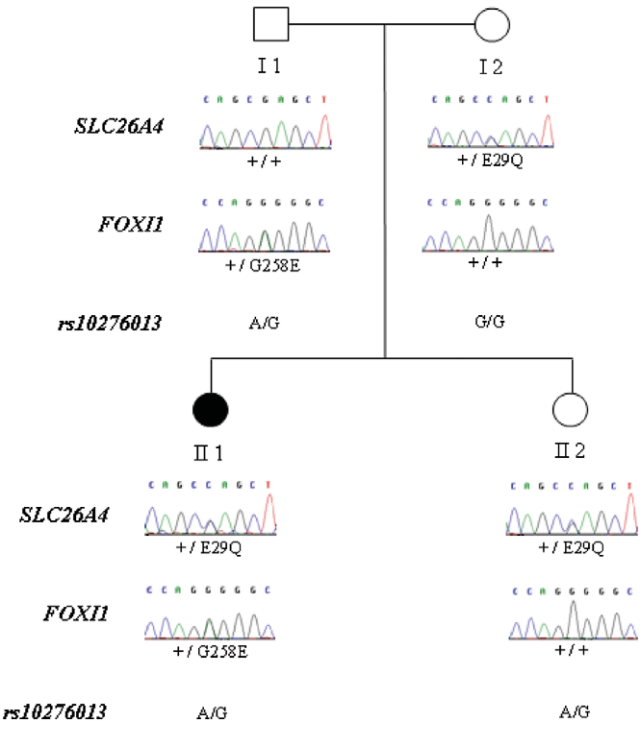

Figure 5. Segregation of *SLC26A4* and *FOXI1* mutations in family 82230. Parents carry a single mutation in either *SLC26A4* or *FOXI1,* the affected child carries both mutations, and her unaffected sister carries the *SLC26A4* mutation only. Both siblings inherited the same wild-type *SLC26A4* allele from their father.

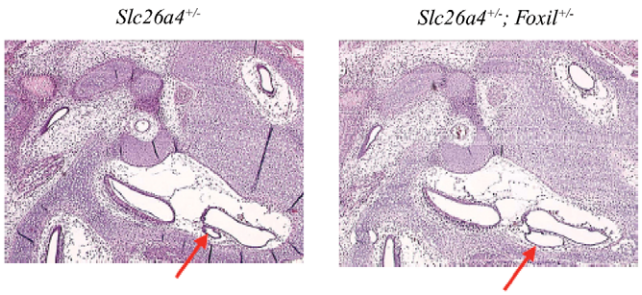

Figure 6. Temporal bone histology from *Slc26a4*$^{+/-}$ embryos and *Slc26a4*$^{+/-}$; *Foxi*$^{+/-}$ double-heterozygous embryos at the E16.5 stage. Red arrows mark selective enlargement of the endolymphatic duct in *Slc26a4*$^{+/-}$; *Foxi*$^{+/-}$ double mutants, as compared with its normal morphology in *Slc26a4*$^{+/-}$ mice. The endolymphatic duct is also normal in the *Foxi1*$^{+/-}$ mice.[12]

binding motif to enable FOXI1 dimers to interact in a specific protein-DNA complex structure. In support of this hypothesis, high-resolution structural studies show that FOXP2 (MIM 605317), another forkhead box-containing transcription factor, dimerizes though subdomain-swapping interfaces within the forkhead DNA-binding domain to bind to multiple DNA sites.[21]

Consistent with our third hypothesis, that mutations in other genes may contribute in *trans* to a PS-EVA phenotype, we identified five *FOXI1* mutations in six patients that compromise the transcriptional-activation ability of FOXI1 (table 2 and figs. 3 and 4). These data are the first to link mutations in *FOXI1* to PS-EVA in humans, although mice homozygous for the targeted deletion of *Foxi1* have a phenotype that includes cochlear dysplasia and EVA.[12] Also included in the *Foxi1*$^{-/-}$ mouse phenotype are male infertility and distal renal tubular acidosis,[20,22] two abnormalities not yet reported in humans with PS or EVA.

On the basis of our results, we propose a dosage-dependent model for the pathogenesis of PS and nonsyndromic EVA that involves not only *SLC26A4* but also its transcriptional control machinery. Mutations in the *SLC26A4* promoter in *cis* and the transcription factor *FOXI1* in *trans,* when combined with conventional *SLC26A4* mutations or possibly other yet-to-be-identified mutations, reduce gene expression levels to below the threshold required for normal inner ear and/or thyroid function, leading to a disease phenotype. Notably, in none of the six patients with *FOXI1* mutations was there a family history of hearing impairment, which supports the recessive or double-heterozygous nature of these mutations.

Although mutations in many transcription factors—including *EYA1* (MIM 601653) and *SIX1* (MIM 601205) in branchio-oto-renal syndrome (BOR [MIM 113650]), *EYA4* (MIM 603550) in *DFNA10* (MIM 601316), *POU4F3* (MIM 602460) in *DFNA15* (MIM 602459), *POU3F4* in *DFN3* (MIM 304400), *GRHL2* (MIM 608576) in *DFNA28* (MIM 608641), and *PAX3* (MIM 606597), *MITF* (MIM 156845), *SNAI2* (MIM 602150), and *SOX10* (MIM 602229) in Waardenburg syndrome types I–IV (WS1 [MIM 277580], WS2A [MIM 193510], WS3 [MIM 148820], and WS4 [MIM 277580])—lead to nonsyndromic or syndromic hearing impairment, to date, no specific downstream target genes have been found among those pathogenic pathways (Hereditary Hearing Loss Homepage). Our work is the first to link a transcription factor to its specific downstream gene in the pathogenesis of deafness.

Another interesting discovery resulting from this study is a family in which the disease phenotype shows a double-heterozygous inheritance pattern, with the affected child carrying single *SLC26A4* and *FOXI1* mutations (fig. 5). Although other inheritance patterns, such as *FOXI1* compound heterozygosity with a second yet-to-be-identified *FOXI1* mutation, cannot be completely excluded, the pathogenicity of the double-heterozygous genotype is supported by several facts. First, the *Slc26a4*$^{+/-}$; *Foxi1*$^{+/-}$ mouse mutant has a similar phenotype (fig. 6); second, the FOXI1 G258E mutation reduces transcription of *SLC26A4* in vitro (fig. 4); third, the SLC26A4 E29Q mutation has been reported previously in families segregating PS-EVA in association with other *SLC26A4* mutations[7,23]; fourth, both the affected and the unaffected child have identical *SLC26A4* genotypes, which is consistent with the presence of additional genetic mutations in the affected child (fig. 5); and, fifth, neither of these mutations has been reported in screens of 500 chromosomes.

To our knowledge, this example of digenic inheritance is the first to be verified as a cause of human deafness. Other studies have proposed a possible digenic role for two adjacent gap-junction genes, *GJB2* and *GJB6,* in nonsyndromic deafness at the *DFNB1* locus, on the basis of reports of two large deletions that include the 5′ end of *GJB6* and segregate with hearing loss when present in *trans* with a recessive mutation in *GJB2*.[24–26] A recent study, however, shows that these deletions prevent expression of both *GJB2* and *GJB6,*[18] suggesting that the deafness is caused by loss of a yet-to-be-identified upstream *cis*-reg-

Table 3. *SLC26A4* Mutation Frequency in Patients with PS-EVA[a]

Mutation[b]	No. of Patients with Mutation[b] and	
	Second Mutation Unidentified	Second Mutation Identified
1001+1G→A	8	15
L236P	4	11
T416P	6	8
G209V	3	9
L597S	8	3
E384G	3	7
F335L	10	0
V138F	6	4
V609G	4	4
E29Q	3	3
L445W	3	3

[a] $n = 429$.
[b] Mutations were detected more than five times.

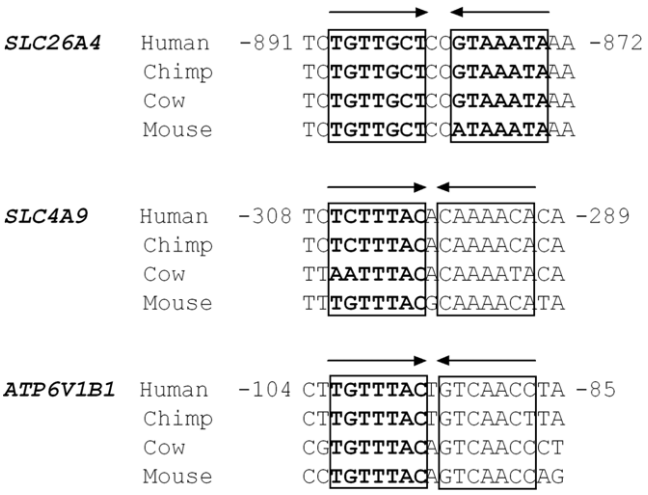

Figure 7. Sequence alignment in multiple species shows the head-to-head FOXI1-binding motif in the promoter region of *SLC26A4, SLC4A9* (GenBank accession number NC_000005.8), and *ATP6V1B1* (GenBank accession number NC_000002.10) (UCSC Genome Browser). The two adjacent FOXI1-binding sites are shown in boxes; arrows indicate orientation. The binding sites experimentally confirmed to be required for gene transcriptional activation are in bold. Numbers indicate the upstream position of the nucleotides relative to the translational start site.

ulatory element of *GJB2,* as opposed to loss of *GJB6* expression.

As a model of double heterozygosity in which mutations in a transcription factor and its downstream target gene combine to produce to a disease phenotype, this finding implies that PS-EVA is a complex disease. Most genetic disorders for which causative genes have been identified are monogenic, and little is known about how genetic factors interact to lead to polygenic disorders. This bigenic disease model at the transcriptional-control level presents a novel oligogenic disease mechanism, which may guide gene-discovery efforts targeting complex diseases after one or a few genes have been implicated. A dosage-dependent model based on transcriptional control also may explain some of the phenotypic variability that is common in complex diseases, especially those diseases in which multiple tissues and organs are affected. Finally, our finding may provide new therapeutic targets by which to manipulate gene expression and the disease phenotype.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://ncbi.nlm.nih.gov/Genbank/ (for *SLC26A4* [accession number NC_000007.12], *Slc26a4* [accession number NC_000078.4], Foxi1 [accession number NP_076396.2], FOXI1 [accession number NP_036320.2], *Slc4a9* [accession number NC_000084.4], *Atp6v1b1* [accession number NC_000072.4], *SLC4A9* [accession number NC_000005.8], and *ATP6V1B1* [accession number NC_000002.10])

Hereditary Hearing Loss Homepage, http://webh01.ua.ac.be/hhh/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for PS, *SLC26A4,* EVA, *DFNB4, FOXI1, POU3F4, GJB2, Slc4a9, Atp6v1b1,* FOXP2, *EYA1, SIX1,* BOR, *EYA4, DFNA10, POU4F3, DFNA15, DFN3, GRHL2, DFNA28, PAX3, MITF, SNAI2, SOX10,* WS1, WS2A, WS3, and WS4)

UCSC Genome Browser, http://genome.ucsc.edu/cgi-bin/hgGateway/ (for the genomic sequence alignment)

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