Potential Novel Mechanism for Axenfeld-Rieger Syndrome: Deletion of a Distant Region Containing Regulatory Elements of *PITX2*

Bethany A. Volkmann,[1,2,3] *Natalya S. Zinkevich,*[1,3] *Aki Mustonen,*[4] *Kala F. Schilter,*[1,2] *Dmitry V. Bosenko,*[1] *Linda M. Reis,*[1] *Ulrich Broeckel,*[1] *Brian A. Link,*[2] *and Elena V. Semina*[1,2]

Purpose. Mutations in *PITX2* are associated with Axenfeld-Rieger syndrome (ARS), which involves ocular, dental, and umbilical abnormalities. Identification of *cis*-regulatory elements of *PITX2* is important to better understand the mechanisms of disease.

Methods. Conserved noncoding elements surrounding *PITX2/pitx2* were identified and examined through transgenic analysis in zebrafish; expression pattern was studied by in situ hybridization. Patient samples were screened for deletion/duplication of the *PITX2* upstream region using arrays and probes.

Results. Zebrafish *pitx2* demonstrates conserved expression during ocular and craniofacial development. Thirteen conserved noncoding sequences positioned within a gene desert as far as 1.1 Mb upstream of the human *PITX2* gene were identified; 11 have enhancer activities consistent with *pitx2* expression. Ten elements mediated expression in the developing brain, four regions were active during eye formation, and two sequences were associated with craniofacial expression. One region, CE4, located approximately 111 kb upstream of *PITX2,* directed a complex pattern including expression in the developing eye and craniofacial region, the classic sites affected in ARS. Screening of ARS patients identified an approximately 7600-kb deletion that began 106 to 108 kb upstream of the *PITX2* gene, leaving *PITX2* intact while removing regulatory elements CE4 to CE13.

Conclusions. These data suggest the presence of a complex distant regulatory matrix within the gene desert located upstream of *PITX2* with an essential role in its activity and provides a possible mechanism for the previous reports of ARS in patients with balanced translocations involving the 4q25 region upstream of *PITX2* and the current patient with an upstream deletion. (*Invest Ophthalmol Vis Sci.* 2011;52: 1450–1459) DOI:10.1167/iovs.10-6060

Axenfeld-Rieger syndrome (ARS) is a relatively rare developmental disorder characterized by maxillary hypoplasia, anterior segment defects of the eye (with glaucoma in ~50%), and dental and umbilical abnormalities. The *PITX2* homeodomain-containing transcription factor gene plays a major role in this condition, explaining approximately 40% of classic ARS.[1–5] In addition, *PITX2* mutations were shown to cause isolated ocular conditions,[6–8] to be associated with additional brain anomalies,[9,10] and possibly to contribute to other phenotypes such as omphalocele and VATER-like association.[11]

The function of *Pitx2* is conserved in vertebrates. Total or conditional knockouts of *Pitx2* result in severe developmental phenotypes in mice that include craniofacial, ocular, dental, brain, heart, lung, and other systemic defects consistent with the expression sites of *Pitx2*. With respect to Axenfeld-Rieger syndrome, *Pitx2*-deficient mice exhibit arrest of eye and tooth organogenesis, defective body wall closure and brain abnormalities.[12–17] The defects observed in mice are associated with a complete loss of *Pitx2* activity in the corresponding structures and, therefore, are noticeably more severe that what is reported in humans with heterozygous *PITX2* mutations, whereas heterozygous mice are generally described as normal. This suggests a variable requirement of *PITX2/Pitx2* for normal human/mouse embryogenesis, with human development more sensitive to correct *PITX2* dosage.

Human *PITX2* mutations mainly result in a complete or partial loss of function, with mutations retaining some wild-type activity producing milder phenotypes.[18–20] Some mutations identified in patients with Axenfeld-Rieger syndrome displayed very minor defects in DNA-binding and transactivation activities, again suggesting that normal human development is highly susceptible to alterations of PITX2 function.[5] In addition, several human mutations associated with gain-of-function have been reported,[21] consistent with the dramatic ocular and limb phenotypes seen in mice with *Pitx2* overexpression during eye and forelimb development.[22,23] Therefore, correct dosage of *PITX2* is critical for normal development, which further underscores the importance of precise transcriptional regulation of its expression.

Regulation of *Pitx2* expression has been studied by several groups, but only a few *cis*-regulatory elements have been identified and examined in animal models. In mouse, *Pitx2* appears to be a part of the Wnt/β-catenin/TCF/LEF and cAMP-CREB signaling pathways.[24,25] Transcriptional activation of *Pitx2* during eye development has been shown to be dependent on retinoic acid signaling[26–28]; the nature of this interaction, direct or indirect, has not yet been established. A few studies

From the [1]Department of Pediatrics and Children's Research Institute and the [2]Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin; and the [4]Department of Clinical Genetics, Oulu University Hospital, OYS, Finland.

[3]These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Corresponding author: Elena V. Semina, 8701 Watertown Plank Road, Milwaukee, WI 53226-0509; esemina@mcw.edu.

reported in vivo identification of *Pitx2* enhancer elements, which facilitated upstream factor analysis. Asymmetric *Pitx2* expression in the developing visceral organs was shown to require an intronic enhancer located in the proximity of the last exon,[29,30] whereas a 7-kb fragment located 4.2 kb downstream of the last exon of *Pitx2* was found to direct reporter expression in the oral ectoderm and Rathke's pouch.[31] Finally, several regulatory elements associated with *pitx2* expression in the stomodeum territory have been isolated in ascidians; the *cis*-sequences were either intronic or located within 2.5 kb of the 5′ or 3′ end of the gene.[32]

PITX2 is flanked by glutamyl aminopeptidase (aminopeptidase A) (*ENPEP*) located approximately 54 kb downstream and chromosome 4 open reading frame 32 (*C4orf32*) expressed sequence positioned approximately 1.5 Mb upstream of *PITX2*; thus, the 1.5-Mb sequence upstream of the gene is considered a gene desert. Gene deserts often contain conserved sequences involved in transcriptional regulation of nearby genes.[33] The presence of *PITX2* regulatory elements in this region were consistent with the previous reports of patients with Axenfeld-Rieger syndrome with translocation breakpoints that occurred within the distant upstream region and did not disrupt the coding region of *PITX2*.[1,2,34,35]

We describe identification of several novel regulatory sequences in the *PITX2/pitx2* region, including several distant elements located within the gene desert upstream of *PITX2*. The activities of these elements appear to be consistent with *PITX2/pitx2* expression, suggesting that they play a role in this gene's function. In addition, we report the identification of a de novo deletion located 106 to 108 kb upstream of *PITX2* in a patient with Axenfeld-Rieger syndrome, thus providing additional support for the essential role of the identified regulatory elements in *PITX2* function.

METHODS

Animals

Zebrafish (*Danio rerio*) maintenance and developmental staging were performed as previously described.[36]All experiments were conducted in accordance with the guidelines set forth by the animal care and use committees at the Medical College of Wisconsin and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

In Situ Hybridization

To prepare probes for in situ hybridization, a plasmid containing the final *pitx2* exon, which is common to both isoforms *pitx2a* and *pitx2c*, and a pG1 plasmid-containing GFP sequence (obtained from Chi-Bin Chien, University of Utah) were used. The *pitx2* (1345 bp) and *GFP* (750 bp) digoxigenin-labeled antisense riboprobe probes were synthesized from the corresponding plasmids and used in in situ hybridization, as previously described.[36] Sections measuring 5 to 10 μm were cut on a cryostat (Richard Allan Scientific Microm HM 550; Thermo Fisher Scientific Inc., Waltham, MA) and mounted on gelatin-coated glass slides. The samples were examined with a microscope (AxioImager [Z1]; Carl Zeiss MicroImaging, Inc., Thornwood, NY), and photographs were taken with a digital color camera (AxioCam MRc5; Carl Zeiss MicroImaging, Inc.).

Sequence Analyses and Construction of Reporter Plasmids

The *PITX2* genomic region and 1650 kb of surrounding sequence were analyzed for conserved noncoding elements using the multiple species alignment tool at UCSC Genome Browser (http://genome.ucsc.edu) and BLAST (http://blast.ncbi.nlm.nih.gov) with a focus on regions conserved between human and zebrafish (July_2007 zebrafish and March_2006 human genome assemblies were initially used and then verified using NW_001838915.1, *Homo sapiens* chromosome 4 genomic reference (HuRef) and NW_001877464.1, *D. rerio* chromosome 14 genomic reference assembly (Zv7_scaffold1394)).

To generate the *Tg(-2.6pitx2:GFP)* promoter construct, a 2627-bp *pitx2c* zebrafish promoter (that included conserved element [CE1]) was amplified using forward (GAGCATCAAAATGCAAACAG) and reverse (AGACAAGCGATTTACCGACG) primers and inserted upstream of the GFP coding region into promoterless pG1 plasmid (obtained from Chi-Bin Chien, University of Utah). An additional promoter construct, *Tg(-1.9pitx2:GFP)*, was generated that contained 1.9 kb *pitx2c* promoter sequence but lacked CE1 using the same reverse primer and an alternative forward primer, GAGGTCACATCTAAAACACC. The *Tg(-2.6pitx2:GFP)* promoter plasmid was then used to generate additional constructs containing the CE2 to CE13 noncoding conserved sequences, *Tg(-2.6pitx2-CE2:GFP)* through *Tg(-2.6pitx2-CE13:GFP)*. The CE2 to CE13 zebrafish regions (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6060/-/DCSupplemental) were PCR amplified using *PfuUltra* high-fidelity DNA polymerase (Stratagene, La Jolla, CA) and standard conditions, cloned into pCRII-TOPO vector (Invitrogen), and subcloned downstream of the GFP coding region in the *Tg(-2.6pitx2:GFP)* promoter plasmid. All constructs were named consistent with ZFIN nomenclature (http://zfin.org/zf_info).

Generation and Analyses of Transgenic Fish

Reporter plasmid DNA was prepared using a midi-kit (Qiagen, Valencia, CA) and suspended at 25 ng/μL in injection buffer [0.1% (wt/vol) phenol red (Sigma-Aldrich, St. Louis, MO) in 0.3× Danieau buffer (17 mM NaCl, 2 mM KCl, 0.12 mM $MgSO_4$, 1.8 mM $Ca(NO_3)_2$ and 1.5 mM HEPES, pH 7.6]. Microinjections of plasmid DNA into zebrafish embryos were performed immediately after fertilization at the one- to two-cell stage. Microinjections were performed with an injector (MM33 Micromanipulator [Stoelting Co., Wood Dale, IL] or Nanoject II [Drummond Scientific, Broomall, PA]). Embryos were incubated at 28.5°C and maintained in 0.2 mM 1-phenyl-2-thiourea to inhibit pigment formation and were anesthetized with 0.05% tricaine before imaging. Developing embryos were inspected for GFP expression until 5 days after fertilization, and the *cis*-regulatory activity of each construct was documented; at least 100 transient transgenics were examined for each construct. Fluorescence microscopes (SMZ 1500 [Nikon Instruments, Inc., Melville, NY] and AxioImager [Carl Zeiss MicroImaging, Inc.]) and in situ hybridization with GFP-specific probe were used in this process. To generate permanent lines, approximately 200 embryos were injected with linearized plasmid DNA and raised to maturity, and their progeny were examined for germline transmission of the transgene using the same techniques as described. Transgenic fish were raised and inbred to establish a permanent line.

Human Sample Analysis

This human study research adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of the Children's Hospital of Wisconsin, with informed consent obtained for every subject. Eight patients were screened for deletions or duplications of the *PITX2* upstream region: three patients had classic Axenfeld-Rieger syndrome, three had syndromic Axenfeld-Rieger anomaly without dental or umbilical features, and two had isolated Axenfeld-Rieger syndrome. Previous screening of the *PITX2* coding sequence did not identify a causative mutation in these patients. The screening for the coding sequence mutations was performed by direct bidirectional DNA sequencing of PCR products encompassing all coding exons of *PITX2A, PITX2B,* and *PITX2C* isoforms using previously described gene-specific primers.[11] Six patients with classic Axenfeld-Rieger syndrome and previously identified *PITX2* mutations (four patients) or whole-gene deletion (two patients) were excluded from this analysis. Patients were screened using Affymetrix Genome-Wide Human SNP Array 6.0, as previously described[37], and/or TaqMan assays

(Applied Biosystems, Carlsbad, CA) for the *PITX2* region. The following *PITX2* probes were used for TaqMan assays: Hs00452261_cn (P1, located in the last exon of *PITX2*), Hs00958157_cn (P2, *PITX2C* promoter), Hs01402614_cn (P3, most 5′ end of *PITX2*, exon 1), Hs04822300_cn (P4, located 110,366 kb 5′ of *PITX2*), Hs04838001_cn (P5, located 284,481kb 5′ of *PITX2*), and Hs04811562_cn (P6, located 649,476 upstream of *PITX2*). Assays were carried out in accordance with manufacturer recommendations on a real-time PCR cycler (Corbett Rotor-Gene; Qiagen); the values were normalized to amplification of a reference gene (*RNase P*) with both region-specific (P1-P6) and reference gene probes analyzed simultaneously in one reaction. Each experiment was performed in triplicate. Relative fold change in copy number in patient genome was determined using the ΔΔ CT quantification method and compared with the same measurement in an unaffected control sample.

Results

pitx2 Is Expressed during Ocular and Craniofacial Development in Zebrafish

The main human *PITX2* transcripts, *PITX2A, PITX2B,* and *PITX2C*, are produced by alternative splicing (*A* and *B*) or use of different promoters (*A/B* vs. *C*); *pitx2a* and *pitx2c* in zebrafish are orthologs of the corresponding mammalian isoforms, whereas a *pitx2b* isoform has not been identified; there is no evidence of *pitx2* duplication in zebrafish (Fig. 1). We performed in situ hybridization in zebrafish embryos ranging from 10 to 120 hpf with a probe that recognizes both isoforms, *pitx2* (Fig. 1).

The first expression of *pitx2* in the periocular mesenchyme became evident at 20 to 24 hpf (Figs. 2A–C); additional sites of expression at this stage included the developing brain, hatching gland, and oral region. At 48 hpf, *pitx2* transcripts were observed in the developing diencephalon and midbrain, the oral cavity, periocular mesenchyme, and the anterior segment of the eye (Figs. 2D–F, 2M). At 72 to 120 hpf, *pitx2* transcripts continued to be detected around the oral cavity, in the region of pharyngeal arches, brain, and the anterior segment of the eye with a strong presence in the ventral canal (Figs. 2G–L, 2N, 2O).

In summary, expression of zebrafish *pitx2* during development demonstrated conservation with other vertebrate species and, most importantly, with sites affected by human *PITX2* mutations. Evolutionary conservation of human and zebrafish *PITX2/pitx2* expression profiles indicated likely preservation of regulatory elements mediating this expression.

Identification of Conserved Noncoding Elements in Human and Zebrafish *PITX2/pitx2*

Human *PITX2* and zebrafish *pitx2* genes are located at chromosomes 4q25 and 14, respectively. To identify regulatory sequences responsible for the expression patterns of *PITX2/pitx2* during development, we scanned a 1.6-Mb sequence around the human *PITX2* gene for noncoding genomic elements conserved between human and zebrafish genomes. Thirteen major conserved elements, CE1 to CE13, were identified that ranged in size from approximately 50 to 300 bp, demonstrated approximately 80% to 90% sequence identity at the nucleotide level, and were positioned in the same order between human and zebrafish genomes (Fig. 1; Supplementary Table S1 and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6060/-/DCSupplemental). In addition to this, BLAST comparison of the CE1 to CE13 sequences against mouse, chicken, and fugu genomes revealed similarly strong conservation of all elements: 94% to 100% nucleotide identity (mouse), 71% to 98% (chicken; except for the CE1 region, which showed no significant homology), and 50% to 92% (fugu). All conserved elements are located upstream of the *PITX2C/pitx2c* isoform; with respect to the *PITX2A/pitx2a* isoform, CE1 and CE2 are positioned internally whereas CE3 to CE13 are upstream of the gene at a distance of approximately 2.4 to 1111 kb (human) or approximately 1 to 260 kb (zebrafish) (Fig. 1; Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6060/-/DCSupplemental).

The probable association of these CEs with *PITX2/pitx2* function and not another gene in the region is supported by the fact that *PITX2/pitx2* is flanked by different genes in the human and zebrafish genomes. The human *PITX2* gene is bordered by chromosome 4 open reading frame 32 (*C4ORF32*) at the 5′ end and glutamyl aminopeptidase (aminopeptidase A) (*ENPEP*) at the 3′ end (GRCh37/hg19; UCSC genome browser http://genome.ucsc.edu). Analysis of the zebrafish genome using cross-species megaBLAST identified no significant homology for the human *C4ORF32* and several regions of low homology (corresponding to only ~3%-11% of the human sequence) for the *ENPEP* gene involving zebrafish chromosomes 21, 25, and 13. The zebrafish *pitx2* gene is flanked by the hypothetical protein *LOC402939* and the *elovl* family member 6, elongation of long chain fatty acids (*elovl6*) genes (Zv8/danRer6; UCSC genome browser http://genome.ucsc.edu). Cross-species megablast comparison identified that zebrafish *LOC402939* is highly homologous to several human genomic regions, including *ADH4* and *AHD5* located 11.5- and 11.6-Mb up-

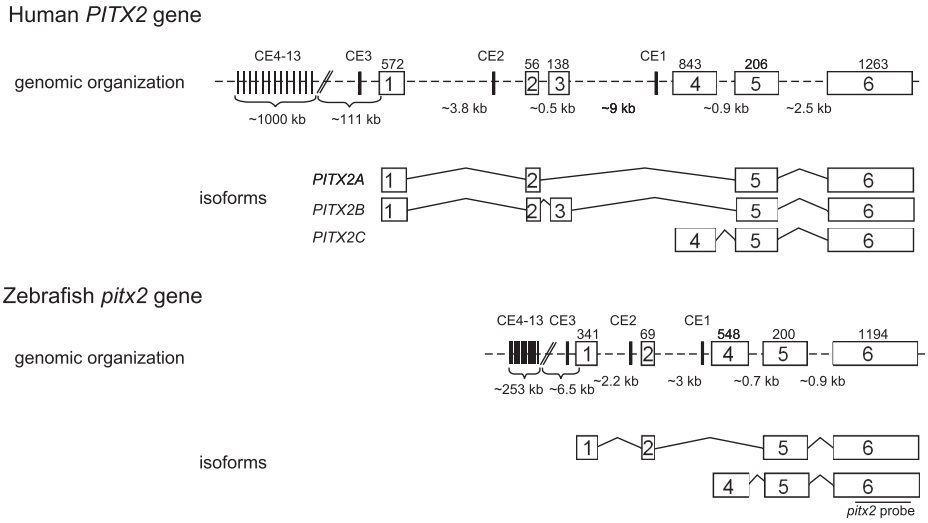

Figure 1. Schematic drawing of the genomic organization/isoforms of the (**A**) human *PITX2* and (**B**) zebrafish *pitx2* genes. *PITX2/pitx2* exons are indicated as *numbered boxes*. Conserved elements are shown as *black boxes/lines* and are marked CE1, CE2, CE3, and CE4-CE13. Exonic sizes are indicated at the *top* intronic sizes are shown at the *bottom* of the *numbered boxes* and Distance to the distant element CE4 from the first exon and size of the genomic region containing CE4 to CE13 elements are indicated next to the *brackets* encompassing the corresponding regions (see Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6060/-/DCSupplemental, for more details). Position of the *pitx2* probe used in in situ hybridization experiments is indicated.

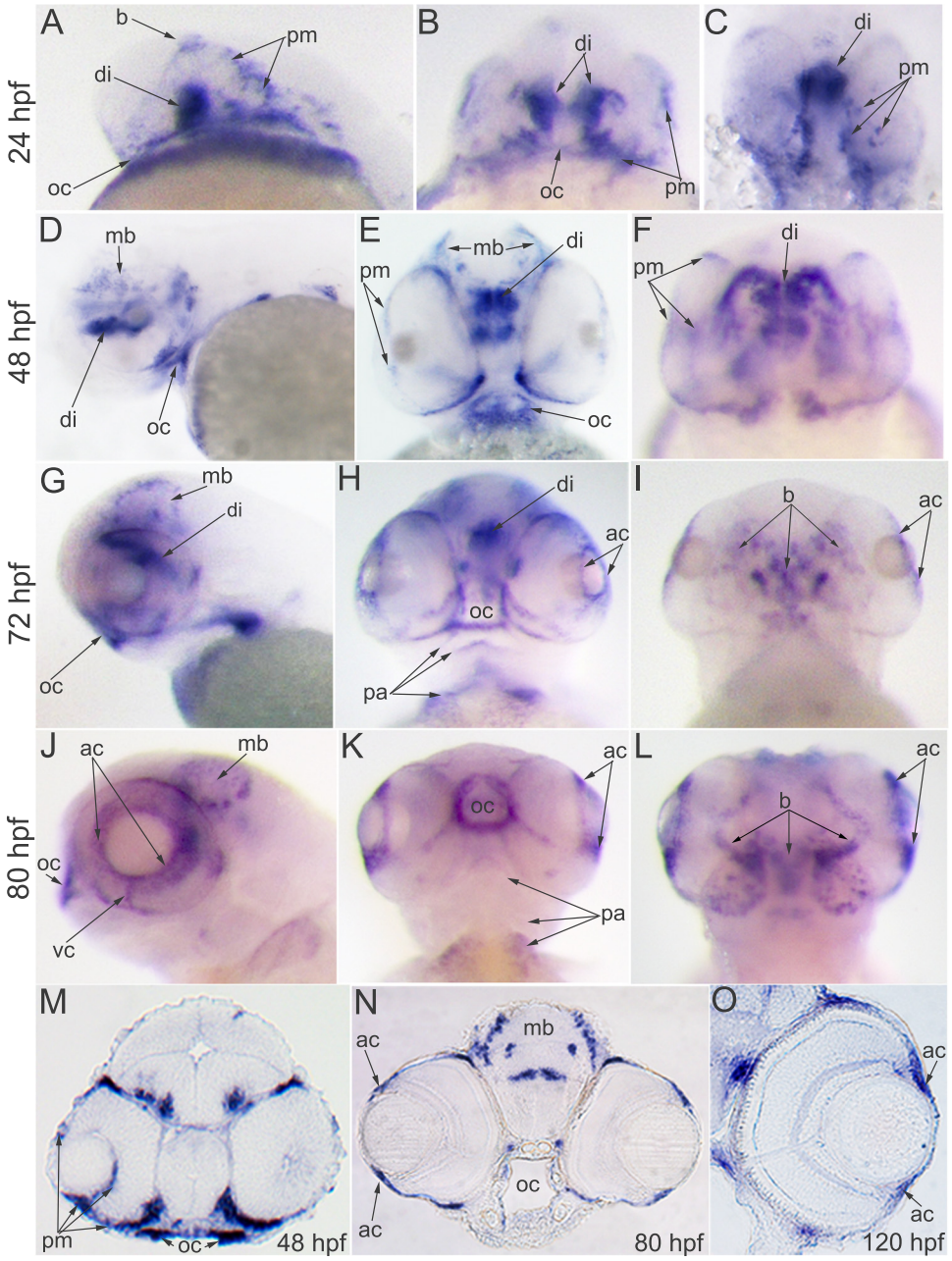

FIGURE 2. Expression of the zebrafish *pitx2* gene during development. Images of whole mount embryos (**A–L**) and cryosections (**M–O**) are presented. Lateral (**A**, **D**, **G**, **J**), ventral (**B**, **E**, **H**, **K**), and dorsal (**C**, **F**, **I**, **L**) views are shown for every stage. Developmental stages are indicated on the *left* (whole mount) or in the *lower right* (cryosections). Note the expression in the periocular mesenchymal (pm) cells of neural crest origin that migrated into the anterior segment of the eye in 24- to 48-hpf embryos (**A–F**, **M**) and continuing strong expression in the developing anterior segment structures (ac), specifically the ventral canal, in 72- to 120-hpf embryos (**G–L**, **N**, **O**). Expression in the developing brain (b, brain; di, diencephalon; mb, midbrain), around the oral cavity (oc), and in the pharyngeal arches (pa) was also observed at the examined stages.

stream of *PITX2*, whereas zebrafish *elovl6* is homologous to the human *ELOVL6* sequence located 567,786-bp downstream of *PITX2* (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6060/-/DCSupplemental). Therefore, even though the *ADH4/5-PITX2-ELOVL6* order has been maintained in both zebrafish and human genomes, the human genome apparently acquired additional genes upstream and downstream of *PITX2*, suggesting that the CEs are not involved in regulating the flanking genes.

Finally, the identified conserved sequences did not appear to be part of any zebrafish or human transcript based on database records. In addition to this, RT-PCR analysis using CE-specific primers and RNA extracted from zebrafish embryos and human cell lines failed to amplify any transcripts (data not shown). Therefore, we propose that the high conservation of these regions is likely to be due to their function in transcriptional regulation of *pitx2/PITX2.*

Conserved Elements Mediate Expression during Brain, Eye, and Craniofacial Development

To test the identified conserved noncoding elements for enhancer activity, we developed a series of reporter constructs based on the pG1 promoterless plasmid containing the coding region of green fluorescent protein (GFP). The *Tg(-2.6pitx2:GFP)* construct contains 2.6 kb zebrafish *pitx2c* promoter sequence (that includes the CE1 region), the *Tg(-1.9pitx2:GFP)* construct contains a 1.9-kb *pitx2* promoter region lacking the CE1 conserved element, and *Tg(-2.6pitx2-CE2:GFP)* through *Tg(-2.6pitx2-CE13:GFP)* plasmids contain 2.6 kb promoter along with the CE2 through CE13 elements, respectively, inserted downstream of GFP. These constructs were tested for *cis*-regulatory activity by transient transgene expression in zebrafish embryos.

Zebrafish embryos injected with promoterless pG1 plasmid displayed either weak sporadic GFP fluorescence or none at all

(data not shown). In contrast, transient transgenic embryos generated with the various *pitx2* reporter constructs demonstrated strong and specific expression patterns that were largely consistent with endogenous expression of *pitx2*. Specifically, embryos injected with *Tg(-2.6pitx2:GFP)* reporter exhibited robust expression in the developing trunk muscles but lacked fluorescence in the head region (Fig. 3A). Embryos injected with the *Tg(-1.9pitx2:GFP)* construct lacking CE1 displayed expression in the developing muscles similar to the *Tg(-2.6pitx2:GFP)* construct but with reduced overall signal intensity (data not shown). Expression patterns in transient transgenic fish for reporters containing conserved elements *Tg(-2.6pitx2-CE2:GFP)* through *Tg(-2.6pitx2-CE13:GFP),* were as follows: CE2-, CE-3, CE6-, and CE12-containing plasmids showed strong and consistent expression in the developing midbrain or diencephalic neurons (Figs. 3B–G, 3J); the CE9 region demonstrated a dynamic pattern with strong brain expression in 48-hpf embryos that weakened at 80 hpf when heart expression became evident (Figs. 3H, 3I); CE13 was also associated with heart expression in addition to strong craniofacial and some brain expression (Figs. 3K, 3L); CE5, CE7, and CE10 directed expression in the periocular mesenchyme (Figs. 3M–O) with brain expression also observed for CE5 and CE7 (data not shown). When these three ocular elements (CE5, CE7, CE10) were consolidated in the same reporter, stronger ocular expression was observed, suggesting an additive effect (Fig. 3P). Weak transgene expression during brain development was observed for the CE11-containing reporter, and no specific activity was detected for the CE8 region (data not shown). Finally, CE4 was associated with the most complex pattern of expression, including the developing eye, brain, and craniofacial region (Fig. 4).

Since *Tg(-2.6pitx2-CE4:GFP)* transgenic animals demonstrated a complex pattern of expression that involved the developing brain, jaw, and eye consistent with the multiple sites affected in Axenfeld-Rieger syndrome patients, a permanent transgenic line carrying this transgene was generated to further study the expression mediated by the CE4 conserved region (Fig. 4). In situ hybridization was performed to better visualize the expression and to confirm specificity. In the 20–24-hpf *Tg(-2.6pitx2-CE4:GFP)* permanent transgenic embryos, strong GFP expression was detected in the periocular mesenchymal cells migrating into the anterior segment of the eye (Figs. 4A, 4B) and in the developing somites and brain. At 48 hpf, some expression around the oral cavity could also be observed (Figs. 4C, 4D, 4F). At 72 to 120 hpf, expression remained in the developing brain, jaw, pharyngeal arches, and anterior segment of the eye (iridocorneal angle) (Figs. 4E, 4G, 4H). Overall, the observed transgene expression demonstrated significant overlap with the endogenous *pitx2* pattern; some specific sites were not present or demonstrated noticeably weaker expression (oral cavity, for example), and ectopic expression was detected in some tissues (strong hindbrain and retinal expression in some embryos; data not shown).

These data support the possibility that the identified conserved elements are involved in regulation of *pitx2/PITX2* expression and thus play an important role in *pitx2/PITX2* function.

Mutation Search Identifies a Deletion in a Patient with Axenfeld-Rieger Syndrome Located 106 to 108 kb Upstream of *PITX2*, Thus Removing Distant Conserved Elements CE4 to CE13

DNA samples from eight patients with Axenfeld-Rieger syndrome (three patients), syndromic Axenfeld-Rieger anomaly without dental or umbilical findings (three patients), and isolated Axenfeld-Rieger anomaly (two patients) were screened for deletions and duplications of the upstream sequence. Copy number variation analysis was performed (Affymetrix Genome-Wide Human SNP Array 6.0 and/or TaqMan assays) for the *PITX2* region. Patient 1, affected with Axenfeld-Rieger syndrome (Fig. 5), was found to carry a 7645-kb deletion involving the 4q25-q26 region (Fig. 6A); the proximal end of the deletion is located between probes at approximately 105.6 kb (diploid

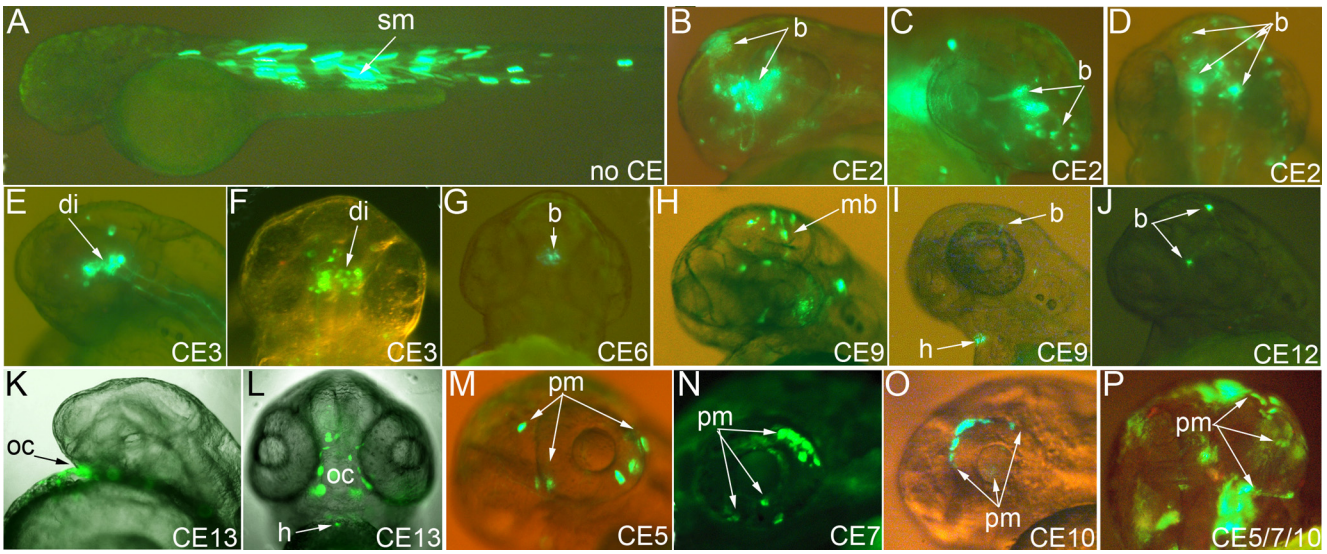

Figure 3. Reporter expression mediated by conserved elements identified in the *PITX2* region. GFP expression associated with *pitx2* promoter region only (**A**), and *pitx2* promoter region with additional elements CE2 (**B–D**), CE3 (**E**, **F**), CE6 (**G**), CE9 (**H**, **I**), CE12 (**J**), CE13 (**K**, **L**), CE5 (**M**), CE7 (**N**), CE10 (**O**), and a combination of CE5, CE7, and CE10 (P) in 36- to 80-hpf embryos. Note the strong expression in the developing brain in 36-hpf (**B**) and 48-hpf embryos for CE2 (**C**, **D**) and 48-hpf embryos for CE3 (**E**, **F**), CE6 (**G**), CE9 (**H**), and CE12 (**J**). For CE9, expression in the developing brain weakened by 80 hpf, whereas robust expression in the heart region became evident (**I**). CE13 was associated primarily with expression during heart and craniofacial development in 24-hpf (**K**) and 48-hpf (**L**) embryos. CE5, CE7, and CE10 regions appeared to be associated with ocular expression that could be easily observed in 48-hpf embryos (**M–P**). b, brain; di, diencephalon; h, heart; mb, midbrain; oc, oral cavity; pm, periocular mesenchyme; sm, skeletal muscles (trunk).

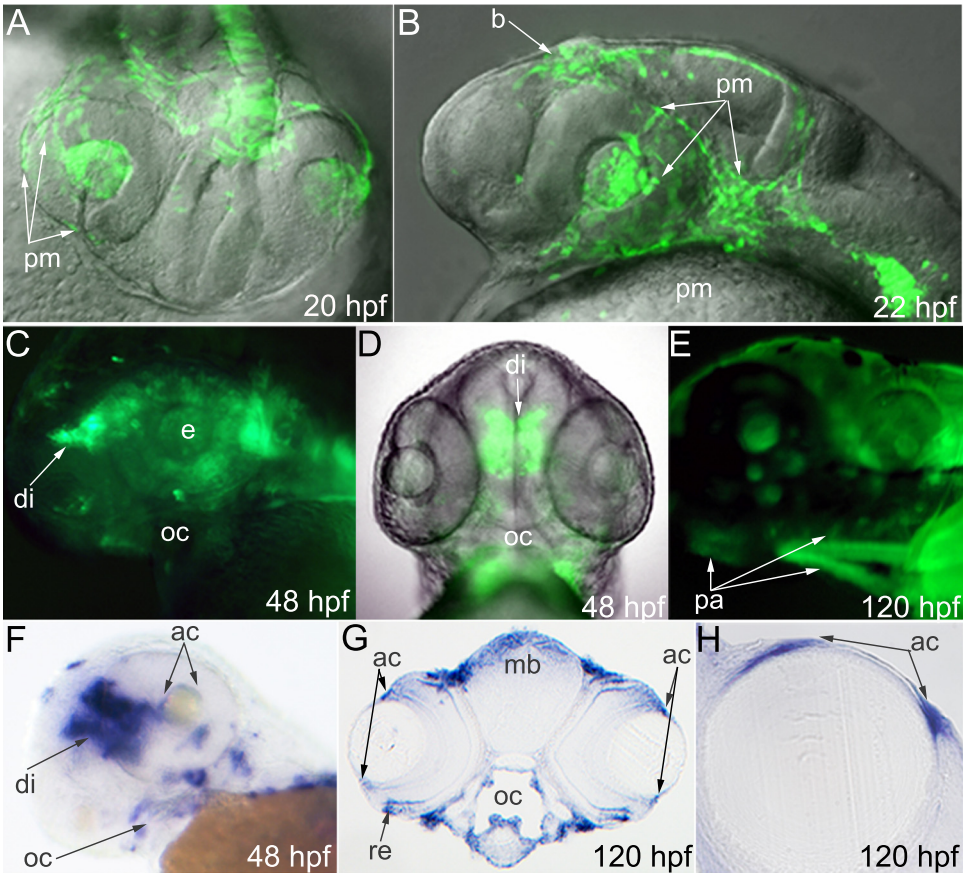

FIGURE 4. Reporter expression in *Tg(-2.6pitx2-CE4:GFP)* permanent transgenic line. (**A**–**E**) GFP fluorescence images. (**F**–**H**) Images of in situ hybridization performed using *Tg(-2.6pitx2-CE4:GFP)* embryos and GFP antisense riboprobe. Developmental stages are indicated in the *lower right* of every image. Note the strong expression in the periocular mesenchymal (pm) cells of neural crest origin that migrated to the anterior segment region (**A**, **B**) and expression in the developing brain (**A**–**G**) and around the oral cavity (**C**–**G**), pharyngeal arches (**E**), and the anterior segment of the eye (**G**, **H**). ac, anterior segment of the eye; b, brain; di, diencephalon; e, eye; mb, midbrain; oc, oral cavity; pa, pharyngeal arches; pm, periocular mesenchyme; re, retina.

state) and approximately 108.1 kb (haploid state) upstream of *PITX2*, and its distal end is positioned between probes at approximately 7753 kb (haploid state) and approximately 7759 kb (diploid state) upstream of *PITX2*; 4768 of 4777 (99.8%) probes (Affymetrix Array 6.0) in this region showed a copy number of 1 (Figs. 6A, 6B). Analysis of 22 unaffected persons included in our study and genomic variation reported in the Database of Genomic Variants (http://projects.tcag.ca/variation/?source=hg18) revealed an absence of similar deletions in controls.

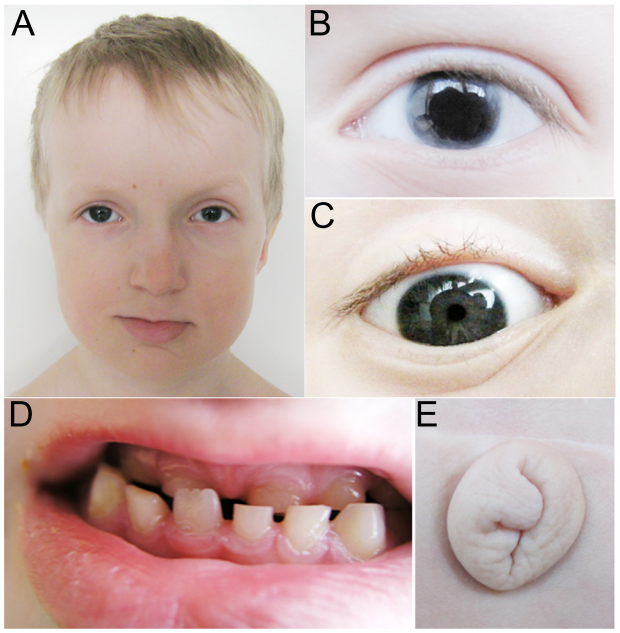

FIGURE 5. Images of patient 1 with Axenfeld-Rieger syndrome. (**A**) Facial photograph showing maxillary hypoplasia, thin upper lip, and broad nasal bridge. (**B**) Left eye with corectopia. (**C**) Right eye with posterior embryotoxon. (**D**) Dental anomalies, including maxillary hypodontia. (**E**) Redundant periumbilical skin.

This deletion, in addition to the removal of the *PITX2* distant upstream region, results in deletion of one copy of the following genes *C4ORF32, C4ORF16, TIFA, ALPK1, NEUROG2, LOC91431, C4ORF21, LARP7, ANK2, CAMK2D, ARSJ, UGT8, NDST4, TRAM1L1, NDST3,* and *PRSS12* but leaves *PITX2* intact (Figs. 6A, 6B). Quantitative PCR data obtained with TaqMan probes located within the *PITX2* gene and the upstream region were consistent with the Affymetrix array results. Results for *PITX2*_CNV probes 1, 2, and 3, which correspond to sequences within *PITX2* exons including the most 5′ exon 1, were consistent with the diploid state, whereas data obtained for *PITX2*_CNV probes 4, 5, and 6, corresponding to the distant 5′ region upstream of *PITX2* (~110, 284, and 649 kb from the gene), confirmed the haploid state in patient 1 (Fig. 6C). Analysis of parental samples showed a normal diploid state for all six probes (Fig. 6C), consistent with their unaffected status.

The phenotype of the patient is consistent with Axenfeld-Rieger syndrome associated with *PITX2* haploinsufficiency. Patient 1 is a Caucasian (Finland) male referred at age 7 months with bilateral iris hypoplasia and iridocorneal adhesions, left corectopia, maxillary hypoplasia, broad nasal bridge, redundant periumbilical skin, and Meckel's diverticulum. Height was in the low-normal range at −1.5 SD. Chromosomal analysis showed a normal 46,XY karyotype with no evidence of translocations (400 band resolution,

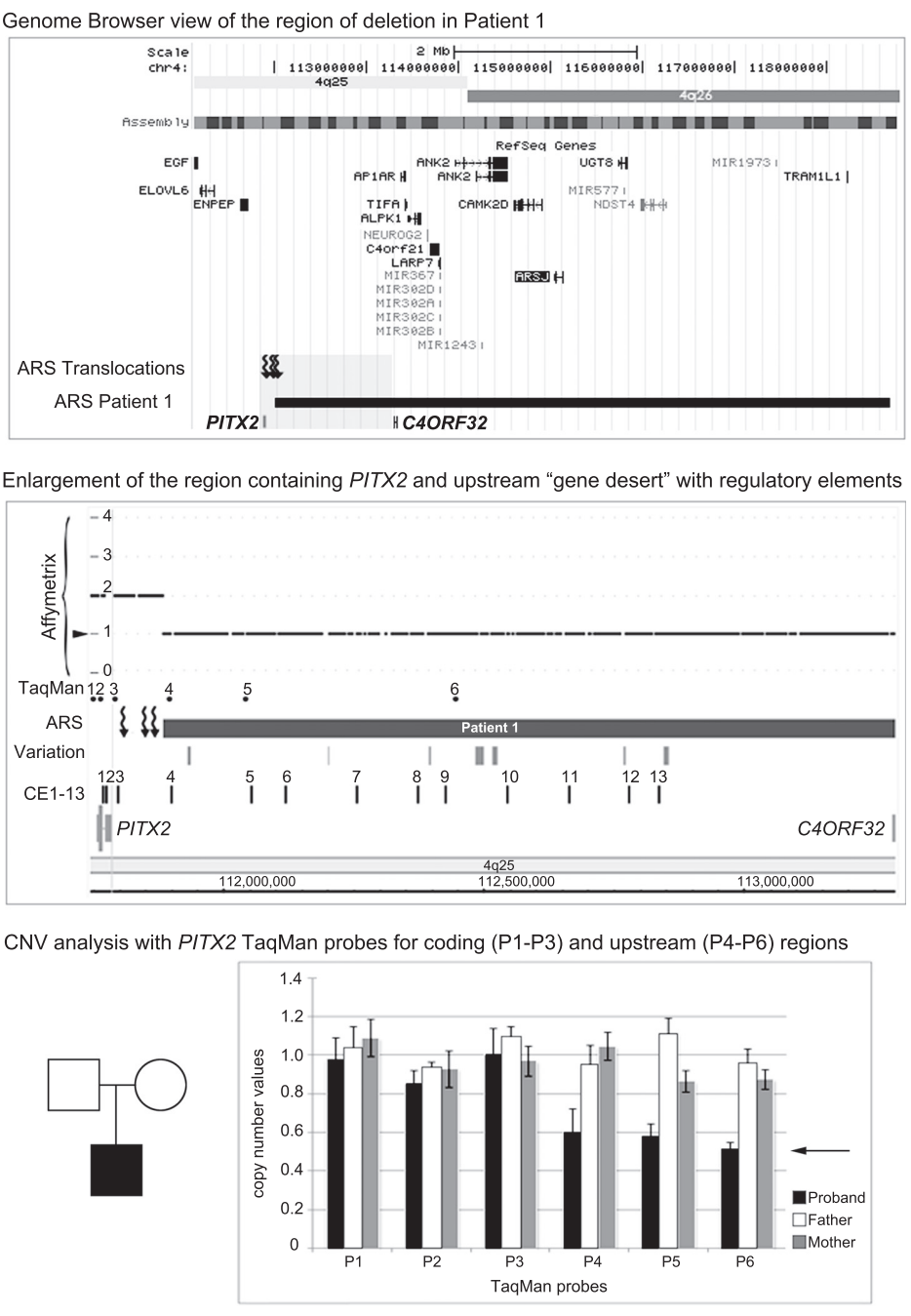

FIGURE 6. Identification of 4q25–26 deletion in a patient with Axenfeld-Rieger syndrome. (**A**) Genome Browser view of the region of deletion in patient 1. The deleted region is indicated by a *black rectangle*, and the previously reported ARS translocations are indicated by with *wavy arrows*. The *shaded area* is shown in more detail in **B**. (**B**) Enlargement of the region containing *PITX2* and the upstream gene desert with regulatory elements (*shaded area*). Affymetrix Genotyping Console (version 3.0.2.) representation of the array data for patient 1 is shown. *Top*: 0 to 4 indicate copy number, with 2 corresponding to diploid and 1 (*arrow*) to haploid states. Positions of TaqMan CNV probes used to confirm Affymetrix array data are shown as *black circles* and numbered 1 to 6. Position of the patient 1 deletion and translocation breakpoints previously reported in ARS patients are indicated with a *dark gray rectangular box* and *wavy arrows*, correspondingly. Regions of genomic variation (Database of Genomic Variants) in this region, as appear in the Genotyping Console, are shown as *gray boxes*, whereas conserved elements (CE1-CE13) are indicated as *black boxes* and are numbered 1 to 13; positions of *PITX2* and *C4ORF32* and chromosomal band q25 are shown as they appear in Genotyping Console. The image clearly demonstrates that no deletions similar in size to the one identified in patient 1 or involving conserved elements have been reported as normal variation. The region between these two transcribed units, *PITX2* and *C4ORF32*, is devoid of any genes and therefore represents a gene desert. (**C**) Copy number variation analysis with TaqMan probes within *PITX2* (P1–P3) and upstream region (P4–P6) in a family with Axenfeld-Rieger syndrome. Pedigree is shown on the *left*. Note proband with ARS and unaffected parents. Summary of qPCR analysis (*right*) shows normal diploid state for all six probes in unaffected parents and diploid state for probes P1 to P3 but haploid state for probes P4 to P6 for the proband (*arrow*).

Giemsa staining). In follow-up at 6 years of age, ocular, dental, and umbilical anomalies were again noted. Specifically, posterior embryotoxon was seen in the right eye, and amblyopia and iridocorneal adhesions were noted in the left eye. Intraocular pressure was normal (18 mm Hg bilaterally), there were no signs of glaucoma, and lenses, papillae, and maculae were normal. Dental anomalies included several missing permanent teeth (especially in the maxilla), general teething delay, and retrognathia of the maxilla and mandibula (more pronounced in the maxilla). He has mild mental retardation (IQ scores not available), short stature (102.5 cm at 5 years 5 months [−1.9 SD]), and mildly delayed bone age. Patient 1 is the only child born to his parents. Both parents are unaffected; his mother has posterior embryotoxon (reported in healthy persons, including in 30% of one isolated Finnish population[38–41]) with no other signs of anterior segment dysgenesis and normal teeth/umbilicus.

DISCUSSION

The PITX family of transcriptional factors is involved in a number of human disorders and displays unique expression patterns during development that are conserved in vertebrates. Regions that are responsible for the specific expression of *PITX* factors remain largely unknown. The identification of regulatory sequences provides an opportunity to isolate upstream factors and to investigate the contribution of these regions and their interacting proteins to human disease.

Regulatory regions contain elements that control gene expression (*cis*-regulatory regions: promoters, enhancers, and

silencers) or function in chromatin organization (insulators and scaffold/matrix attachment regions). Identification of regulatory regions represents the new challenge of gene characterization. Interspecies analysis has been successfully used to identify genomic regions containing regulatory sequences, in particular promoters and enhancers.[42] Given that genomes of closely related species share a larger degree of conservation that may not be functionally important, comparison of distantly related organisms is normally used to give additional strength to the analysis. Another criterion for species selection is based on the presence or absence of an expression domain of interest in the organisms included in the analysis. Absence of an expression domain in some species may be associated with an evolutionary loss of a corresponding enhancer as was demonstrated, for example, for the *Sox2* late lens enhancer in human and chicken genomes but with no counterpart in the mouse genome, consistent with the absence of *Sox2* expression in the mouse lens after the lens vesicle stage.[43]

To study regulatory regions of *PITX2/pitx2* that demonstrate conservation across vertebrate species (zebrafish-human), we first performed careful analysis of the expression pattern of the zebrafish *pitx2* gene. Although mouse *Pitx2* is known to be expressed in the developing eye, pituitary, forebrain and midbrain, maxillary and mandibular epithelia, umbilicus, limb, heart, and some other internal organs,[2,12–17] zebrafish *pitx2* expression had been described only for earlier developmental stages (up to 28 hpf) and with a focus on left-right patterning.[44] During later stages of development, we detected *pitx2* expression in the developing forebrain and midbrain, around the oral cavity, and in the pharyngeal arches, periocular mesenchyme, and developing anterior segment structures. This further demonstrates conservation of *pitx2* expression patterns in vertebrates and correlates with the sites affected in patients with Axenfeld-Rieger syndrome.

We then identified 13 genomic regions that are conserved between human and zebrafish genomes and are located in the approximately 1.6-Mb region surrounding the human *PITX2* gene and tested them for their potential role in this gene's expression. Eleven of these regions were found to be associated with specific expression patterns consistent with endogenous expression of *pitx2* in the developing brain, eye, and craniofacial region. Nine of these enhancers, CE4 to CE13, were located at a significant distance from the *PITX2* gene (111–1111 kb), within a so-called gene desert.[33] Conserved elements within gene deserts were shown to have regulatory activities consistent with expression of nearby genes and were, therefore, likely to be involved in their transcriptional activation.[33,45] The significance of gene deserts remains unclear because megabase deletions of some regions in mice resulted in a normal phenotype.[46]

The potential role of the identified regulatory elements/gene desert region in *PITX2* function is supported by our discovery of a novel deletion that removes CE4 to CE13 in a patient with Axenfeld-Rieger syndrome. Although there are a number of other genes in the region, only two have been previously associated with human phenotypes: heterozygous missense mutations in *ANK2* are associated with autosomal dominant cardiac arrhythmia/Long QT syndrome (MIM 600919) in several families,[47,48] and a homozygous 4-bp deletion in *PRSS12* has been associated with autosomal, recessive, nonsyndromic mental retardation (MIM 249500) in two Algerian families.[49] Microscopic and submicroscopic deletions of *PITX2* are a common mechanism of Axenfeld-Rieger syndrome,[3,50–53] but a patient reported with a 4q26 deletion that did not affect 4q25 did not show signs of Axenfeld-Rieger syndrome,[54] suggesting that the additional deleted genes in patient 1 do not contribute to his Axenfeld-Rieger syndrome phenotype. Thus, we propose that his phenotype is caused by the deletion of regulatory elements with the gene desert upstream of *PITX2*.

Furthermore, the occurrence of Axenfeld-Rieger syndrome in previously described families with balanced translocations with breakpoints upstream of the *PITX2* gene[1,2,34,35,55] supported the notion that removal of this upstream region from the *PITX2* gene disrupts *PITX2* expression and leads to disease. The CE4 to CE13 distant regulatory sequences described here are located upstream of the 4q25 breakpoints identified in three independent families with balanced translocations and Axenfeld-Rieger syndrome[1,2,34,35,55]; the breakpoints in all three families are located between CE3 and CE4. The t(4;16)(q26:q22) breakpoint was found in a mother and daughter exhibiting ocular and dental features diagnostic of Axenfeld-Rieger syndrome.[1,2,55] The t(4;11)(q27:q21) breakpoint was found in a patient with a de novo balanced translocation and all three cardinal features of Axenfeld-Rieger syndrome (anterior segment abnormalities, dental hypoplasia, and umbilical outpouching), along with developmental delay and polydactyly.[1,2,55] The coding region of *PITX2* was normal in all three affected persons. The t(4;12)(q25;q15) breakpoint was described in a mother and son with ocular, dental, and umbilical features consistent with Axenfeld-Rieger syndrome[34,35]; the translocation is reported to be balanced,[34] and no additional *PITX2*/4q25 anomalies were observed in these patients. Previously, introduction of negative regulatory elements from another chromosome leading to partial silencing of *PITX2* was suggested as a potential mechanism for Axenfeld-Rieger syndrome in the t(4;16)(q26:q22) and t(4;11)(q27:q21) patients.[55] Our data suggest another strong possibility: the phenotype observed in these patients may be attributed to the downregulation of *PITX2* expression as a result of displacement of the CE4 to CE13 enhancers to another chromosome. The presence of additional regulatory elements not identified by our studies within the gene desert region is also a likely possibility. Overall, our data suggest that gene deserts can be essential for normal functioning of neighboring genes and that deletion or misplacement of these regions can be associated with human disease.

Although a few *cis*-regulatory elements of *pitx2* have been identified, no elements associated with expression in the periocular mesenchyme or the developing anterior segment, highly important sites of *PITX2* activity, are known. The identification of regulatory sequences mediating various aspects of *PITX2/pitx2* activity presented here allows for analysis of the previously reported upstream proteins and pathways for direct interaction with these *PITX2 cis*-acting regions and facilitates isolation of novel factors involved in the *PITX2* pathway and human embryonic development.

In conclusion, *PITX2* makes use of an extensive and conserved regulatory network to mediate its expression pattern. Eleven new conserved regulatory regions involved in different aspects of *PITX2/pitx2* expression have been identified; nine are located within the upstream gene desert region. The existence of element(s) essential for *PITX2* transcriptional activity in the remote upstream region of the gene provides a probable explanation for the occurrence of Axenfeld-Rieger syndrome in the patient reported here to have a deletion of this region. This statement is supported by the presence of the identified deletion in the affected patient but not in his unaffected parents, the absence of the deletion in healthy controls, the presence of the classic Axenfeld-Rieger syndrome features consistent with haploinsufficiency for *PITX2* in the reported patient, an apparent presence of *PITX2/pitx2* regulatory elements in the deleted region, and previous reports of balanced translocations in Axenfeld-Rieger syndrome patients that, similar to the deletion in patient 1, removed the *PITX2* upstream region by displacing it to another chromosome without affecting the coding part of

PITX2. At the same time, even though the deletion of the *PITX2* upstream regulatory region in patient 1 represents a likely explanation of the observed phenotype, a mutation in a yet to be identified gene cannot be completely ruled out. Our finding suggests that analysis of the *PITX2* distant upstream region, in addition to exonic sequencing/deletion analysis, must be performed in patients with Axenfeld-Rieger syndrome and may provide further support for this new mechanism. Discovery of regulatory elements is essential to the understanding of *PITX2* developmental regulation and mechanisms of human disease.

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