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Review

Rieger syndrome: a clinical, molecular, and biochemical analysis

B. A. Amendt[a,*], E. V. Semina[b] and W. L. M. Alward[c]

[a]Department of Biological Sciences, The University of Tulsa, 600 S. College Ave., Tulsa (Oklahoma 74104-3189, USA), Fax + 918 631 2762, e-mail: brad-amendt@utulsa.edu
Departments of [b]Pediatrics, and [c]Ophthalmology and Visual Sciences, University of Iowa, 51 Newton Road, Iowa City (Iowa 52242, USA)

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Abstract. Rieger syndrome (RIEG 1; MIM 180500) is an autosomal dominant disorder of morphogenesis. It is a phenotypically heterogeneous disorder characterized by malformations of the eyes, teeth, and umbilicus. RIEG belongs to the Axenfeld-Rieger group of anomalies, which includes Axenfeld anomaly and Rieger anomaly (or Rieger eye malformation), which display ocular features only. Recently, mutations in the homeodomain transcription factor, PITX2, have been shown to be associated with Rieger syndrome. This review discusses the clinical manifestations of Rieger syndrome and how they correlate with the current molecular and biochemical studies on this human disorder.

Key words. Rieger syndrome; PITX2; homeodomain; human disorder.

Introduction

Rieger syndrome was first defined as a genetic disorder in 1935 [1]. It is an autosomal dominant human disorder characterized by dental hypoplasia, mild craniofacial dysmorphism, ocular anterior chamber anomalies causing glaucoma, and umbilical stump abnormalities. The dental hypoplasia is manifested as missing, small, and/or malformed teeth [2]. Other features associated with Rieger's include abnormal cardiac, limb, and pituitary development. Murray and coworkers [3] reported linkage of Rieger syndrome to chromosome 4q25; another locus for this disorder was more recently reported at chromosome 13q14 [4]. A positional cloning strategy was used to identify a novel homeobox gene at the Rieger locus [2]. This gene, initially called *Rieg*, has since been cloned from mouse and human DNA by several other groups and has been assigned various names (*Rieg*, *Ptx2*, *Otlx2*, *Brx1*, *ARP1*) [5–8]. We now refer to this gene as *PITX2* as the recommended gene symbol [5, 9].

The *PITX2* gene has point mutations in Rieger syndrome patients [2]. In this review, we will discuss the clinical manifestations of Rieger syndrome and the molecular basis of this genetic disorder.

Clinical manifestations

Rieger syndrome is a disease with ocular, facial, dental, and umbilical anomalies as primary manifestations. Sometimes Rieger syndrome is considered to be part of Axenfeld-Rieger syndrome, a spectrum of diseases that share many ocular characteristics [10]. In 1920, Axenfeld [11] first described two of the ocular signs that make up Rieger syndrome. He reported a patient with a

* Corresponding author.

Table 1. The clinical features of the Axenfeld-Rieger family of diseases.

	Prominent Schwalbe's line	Iris adherent to cornea and trabecular meshwork	Iris hypoplasia	Corectopia/ polycoria	Glaucoma	Systemic abnormalities
Axenfeld anomaly	+	+			+	
Rieger anomaly	+	+	+	+	+	
Rieger syndrome	+	+	+	+	+	+

white line in the posterior portion of the peripheral cornea to which iris tissue was adherent. He termed this 'posterior embryotoxon of the cornea.' In the mid-1930s, Rieger [1] described patients who had similar findings with the addition of iris abnormalities. Rieger called this condition 'dysgenesis mesodermalis corneae et iridis' [1]. Recognition followed that patients could have dental and facial anomalies in addition to the ocular manifestations [12, 13]. This family of diseases was noted to be heritable by Rieger [1, 14].

Clinical features

Rieger syndrome is the most extreme member of the family of diseases called Axenfeld-Rieger syndrome. The diseases in Axenfeld-Rieger syndrome have a wide spectrum of clinical features (table 1). One hallmark is a white ring in the peripheral cornea that is said to represent a prominent and anteriorly displaced Schwalbe's line of the trabecular meshwork (fig. 1). This abnormality, called posterior embryotoxon, can occur alone in otherwise normal eyes. Patients with isolated posterior embryotoxon are not at increased risk of developing glaucoma. Up to 15% of the population has some degree of posterior embryotoxon [15].

Some patients have strands of abnormal iris tissue crossing the angle formed between the iris and cornea (iridocorneal angle) and attaching to the trabecular meshwork and the posterior embryotoxon (fig. 2). These patients have Axenfeld anomaly. Rieger anomaly patients have all of the abnormalities seen in Axenfeld anomaly with the addition of iris changes such as hypoplastic stroma (fig. 3A), a displaced pupil (corectopia) (fig. 3A), or extra holes in the iris (polycoria) (fig. 4). A normal eye is shown in figure 3B for comparison and emphasizes the severity of the eye defects seen in Rieger anomaly patients.

The most important ocular feature of the Axenfeld-Rieger family of diseases is glaucoma, which develops in about 50% of affected individuals [10]. The glaucoma can be present at birth or can develop at any time thereafter. The glaucoma associated with Rieger syndrome can be difficult to control and often requires surgical intervention. Glaucoma can lead to blindness in these patients. Patients with glaucoma onset in infancy or early childhood may present with symptoms and signs of congenital glaucoma such as tearing, photophobia, blepharospasm, cloudy corneas, and large eyes. Otherwise, Rieger syndrome may be diagnosed on routine ophthalmic, dental, or pediatric examination.

Rieger syndrome patients have the changes seen in Rieger anomaly with the addition of non-ocular findings. These patients can have a flattened midface (fig. 5), abnormally small teeth (microdontia) (fig. 6), missing teeth (hypodontia) (fig. 6), redundant skin around the umbilicus (fig. 7), and males may have hypospadius. Pituitary abnormalities such as empty sella syndrome and growth hormone deficiency are sometimes associated with Rieger syndrome. There are many other ocular and systemic features that are less consistent.

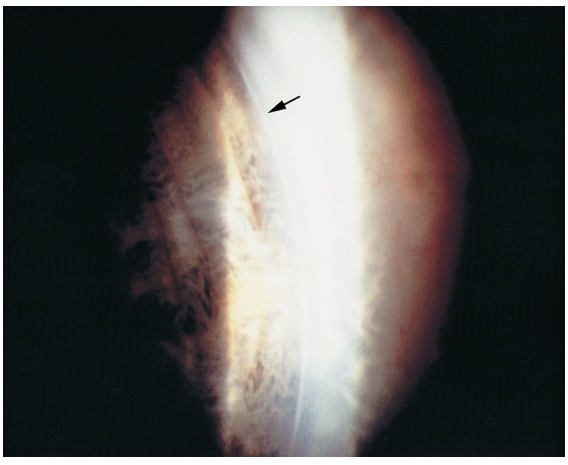


Figure 1. Posterior embryotoxon is an abnormally visible anterior Schwalbe's line of the trabecular meshwork (arrow) [reprinted from ref. 98 with permission].

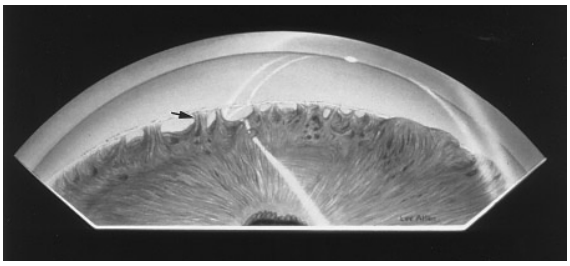


Figure 2. Gonioscopic view of Axenfeld anomaly. There are broad bands of iris adherent to the posterior embryotoxon and the trabecular meshwork (arrow) [reprinted from ref. 98 with permission].

Mechanism

A thorough review of the various proposed mechanisms for the development of the anterior segment ocular changes was written by Shields [10]. Rieger first proposed that the abnormalities were due to cells derived from mesoderm [1]. The affected ocular structures are now known to be derived from neural crest cells [16]. Later, Reese and Ellsworth [17] theorized that the ocular defects seen in Axenfeld-Rieger syndrome were caused by a failure of the anterior segment structures to split properly during development. These disorders have sometimes been called the 'cleavage syndromes.' The cleavage theory is based on a proposed mechanism of embryogenesis that is not totally accurate [10]. Shields [10] has theorized that primordial remnants of neural crest cells remain on the iris and in the iridocorneal angle. These cells and/or their deposited basement membrane can contract, leading to the iris and angle changes seen in these eyes. Recent transgenic mice studies expressing either transforming growth factor α (TGF-α) or epidermal growth factor (EGF) in the ocular lens have resulted in anterior segment dysgenesis [18]. Expression of either factor appears to interfere with corneal endothelial cell differentiation during early ocular morphogenesis resulting in the loss of the anterior chamber. These factors act to alter the differentiation of corneal mesenchymal cells into corneal endothelial cells. This study suggests that formation of the corneal endothelium is required for normal formation of the anterior segment. Thus, interference with corneal endothelial differentiation may result in anterior segment dysgenesis during early stages of ocular development [18].

Molecular characteristics

Molecular genetic studies

Patients in whom Rieger syndrome is one of a host of disorders caused by chromosomal abnormalities have yielded clues to the location of the genetic defect in this syndrome. Clinical features of Rieger syndrome have

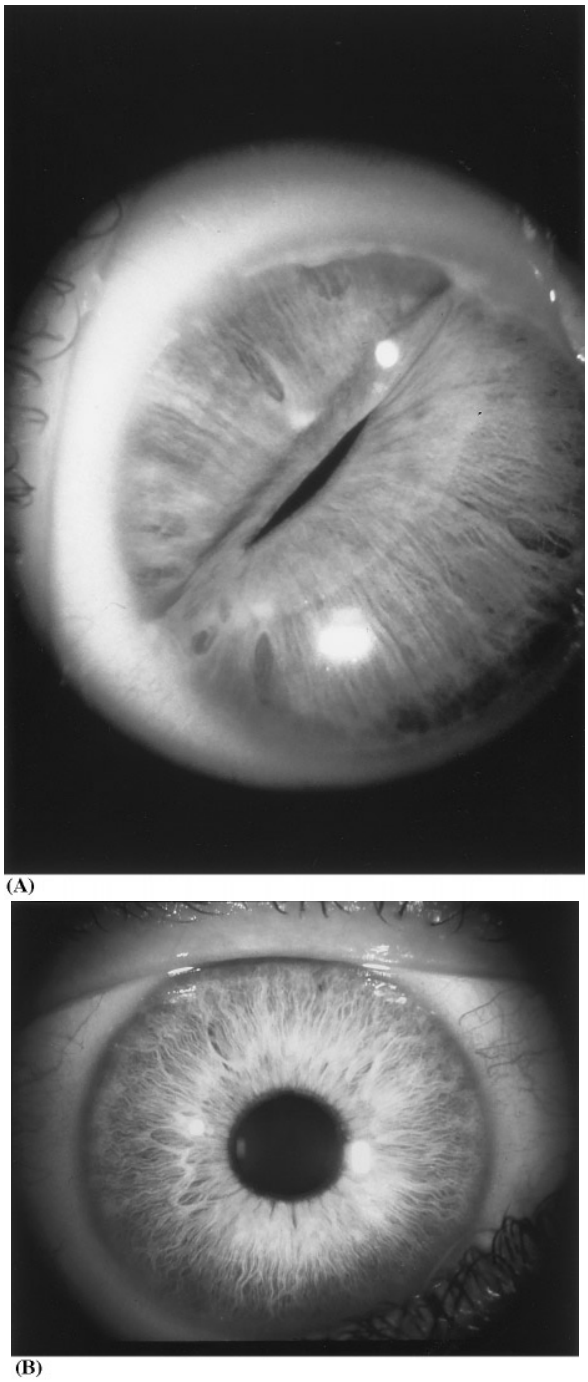


Figure 3. (*A*) Corectopia and iris hypoplasia. The iris stroma is thin, which makes the iris sphincter muscle clearly visible surrounding the pupil. The pupil is stretched [reprinted from ref. 99 with permission]. (*B*) An unaffected eye with a normal pupil and iris.

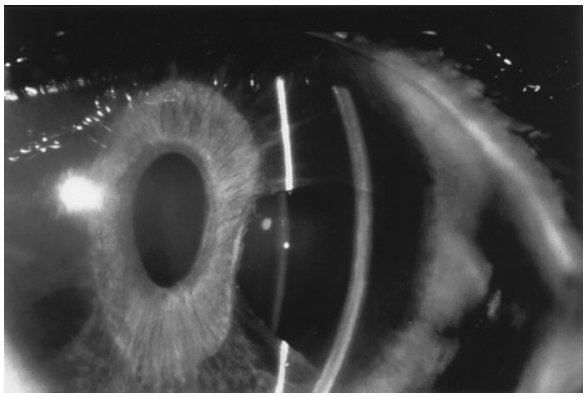


Figure 4. Polycoria. Two holes have developed in the iris (note the prominent iris sphincter surrounding the pupil) [reprinted from ref. 100 with permission].

been associated with abnormalities on chromosomes 4, 6, 10, 13, 16, and 22 [19–29]. The most common site for chromosomal abnormalities associated with Rieger syndrome was the region of chromosome 4q23–26 [26–29]. Linkage studies were performed in the region of 4q25 in families with Rieger syndrome. Given a cytogenetic target, even small families can be used to identify significant genetic linkage. In the initial linkage study, three families with characteristic Rieger syndrome were investigated. In each of these families, at least one individual manifested three cardinal signs of Axenfeld-Rieger syndrome: anterior segment ocular anomalies, hypodontia, and redundant periumbilical skin. Other individuals in the family were considered affected if they had any one of the three cardinal manifestations. Significant linkage was identified to markers D4S193 and D4S191 on chromosome 4q25 [3]. Then, yeast artificial chromosome (YAC) and cosmid contigs were developed across this region and the *PITX2* gene was isolated by CpG island analysis.

Mutations in *PITX2* have been identified in patients with anterior segment abnormalities other than Rieger syndrome. One of these is iris hypoplasia, in which the anterior stoma of the iris is poorly developed giving the iris a characteristic slate-gray or chocolate-brown color with the iris sphincter being abnormally visible (fig. 8) [30]. Like patients with Rieger syndrome, these patients commonly develop glaucoma. They do not have the other anterior segment abnormalities seen in Rieger syndrome and rarely develop non-ocular features [30]. Mutations in the *PITX2* gene have been identified in patients with iris hypoplasia [31]. One patient has been reported with the central corneal opacity typical of Peters anomaly who also had a mutation in *PITX2* [32].

Some Rieger syndrome families have been described that do not link to chromosome 4q25 (table 2) [33]. In 1996, a second locus for Rieger syndrome was identified on chromosome 13q14 in a large four-generation pedigree [4]. The affected gene at this locus has not yet been identified.

Two patients with primary congenital glaucoma had chromosomal abnormalities involving chromosome 6p25 [34]. Study of the breakpoint revealed mutations in the forkhead transcription factor (*FKHL7*) gene in the two patients with primary congenital glaucoma and also patients with Axenfeld anomaly, Rieger anomaly, and iris hypoplasia [34]. Mears et al. [35] subsequently described *FKHL7* mutations in patients with Rieger anomaly. Honkanen and coworkers [36] described a family with a *FKHL7* mutation in which the phenotypes included Axenfeld anomaly, Rieger anomaly, Rieger syndrome, and Peters anomaly (table 2).

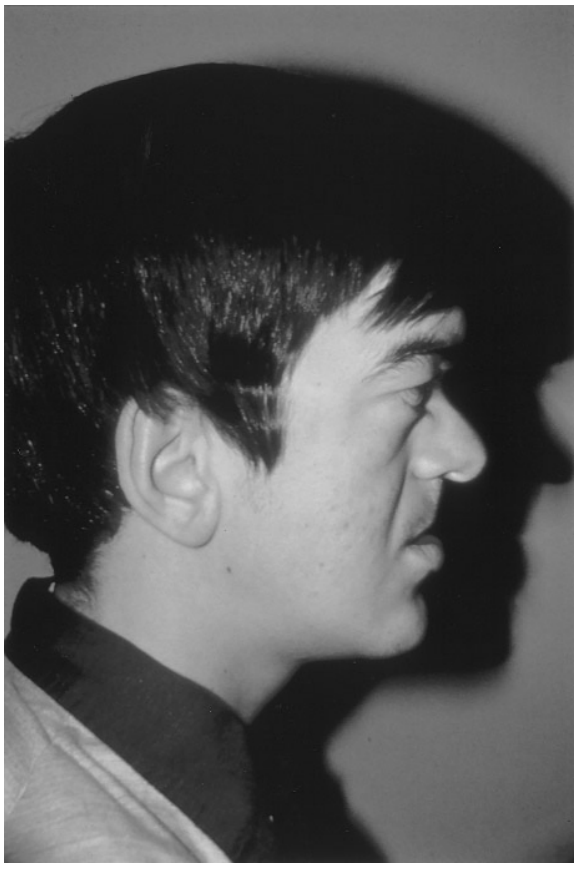


Figure 5. Patient with Rieger syndrome showing poorly developed maxilla with flattened midface [reprinted from ref. 101 with permission].

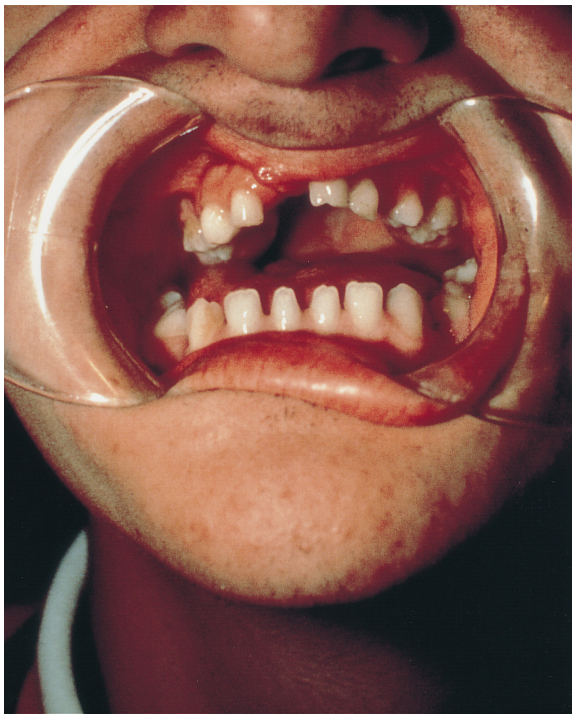


Figure 6. Small teeth (microdontia) and missing teeth (hypodontia) in a patient with Rieger syndrome [reprinted from ref. 101 with permission].

Several studies have shown linkage of anterior segment abnormalities to the same region of chromosome 6. These abnormalities include Axenfeld anomaly, Rieger anomaly, iridogoniodysgenesis anomaly, and familial glaucoma iridogoniodysplasia [37–41]. Iridogoniodysgenesis and familial glaucoma iridogoniodysplasia are described as having iris hypoplasia, iridocorneal angle abnormalities, and glaucoma. These diseases are very similar and differ from Rieger anomaly by the lack of a posterior embryotoxon with attached iris tissue and by the lack of corectopia or polycoria. The locus for these similar disorders has been called *IRID1*.

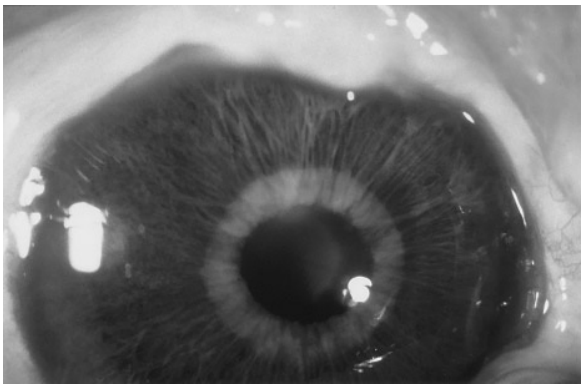


Figure 8. Iris hypoplasia in a person with a *PITX2* mutation. The hypoplastic iris permits easy visualization of the iris sphincter [reprinted from ref. 31 with permission].

Rieger syndrome is a heterogeneous condition. It was first suggested by identification of different chromosomal aberrations in Rieger syndrome patients and later confirmed by linkage studies. Most of the noted cytogenetic abnormalities involve deletions/translocations affecting 4q, 13q, and 6p with occasional reports of trisomy/monosomy 9, 16, 18, 20, and 21. Most cases were characterized only in respect to ocular findings

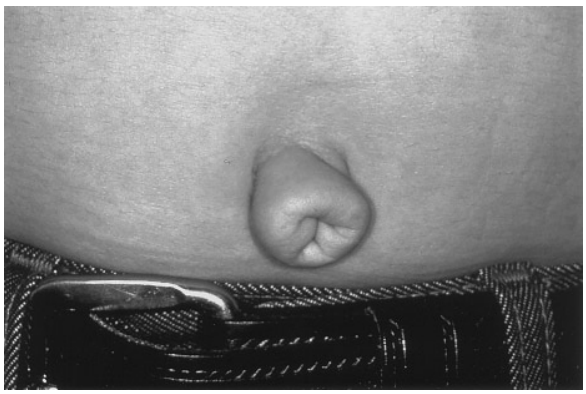


Figure 7. Redundant periumbilical skin in Rieger syndrome. This is often mistaken for an umbilical hernia [reprinted from ref. 101 with permission].

Table 2. The genes involved in Rieger syndrome and related diseases.

Chromosome	Gene	Disease
4p25	*RIEG1*/*PITX2*	Rieger syndrome [2] iridogoniodysgenesis [52] iris hypoplasia [31] Peters anomaly [32]
13q14	not yet identified	Rieger syndrome [4]
6p25	*FKHL7*	Rieger anomaly [34, 37] Axenfeld anomaly [34, 37]] iridogoniodysgenesis [40] familial glaucoma iridogoniodysplasia [39] primary congenital glaucoma [34]
10q25	*PITX3*	anterior segment mesenchymal dysgenesis [43]

Table 3. Summary of *PITX2* mutations.

Mutation	Diagnosis	Exon	Gene region	Effect on protein function	Reference
Leu54Gln	Rieger syndrome	5	homeobox	unstable protein	[2, 9]
Thr68Pro	Rieger syndrome	5	homeobox	reduced DNA binding, transactivation defective	[2, 9]
defective	[2, 9]				
Arg62His	Rieger syndrome	5	homeobox	not determined	E. V. Semina et al., unpublished data
Arg69His	iridogoniodysgenesis syndrome	5	homeobox	not determined	[52]
Arg84Trp	iris hypoplasia	5	homeobox	reduced DNA binding, reduced transactivation activity	[31]; B. A. Amendt et al., unpublished data
G to C	1) Rieger syndrome	5	position +5, ss	frameshift	[2]
	2) Rieger anomaly	-//-	-//-	unstable protein	E. V. Semina et al., unpublished data; B. A. Amendt et al., unpublished data
A to G	Rieger syndrome	6	position −11, ss	frameshift unstable protein	[2] B. A. Amendt et al., unpublished data
Lys88Glu	Rieger syndrome	6	homeobox	not determined	E. V. Semina et al., unpublished data
Arg91Pro	1) Rieger syndrome	6	homeobox	defective DNA binding	[2, 9]
	2) Rieger syndrome	-//-	-//-		
Trp94 STOP	Rieger syndrome	6	homeobox	truncates protein	Semina et al., unpublished data
Trp135 STOP	Rieger syndrome	6	C terminal	truncates protein	[2]
Breakpoint t(4;16)	Rieger syndrome	none	5′ region	changes expression	[2]
Breakpoint t(4;11)	Rieger syndrome, polydactyly, developmental delay	none	5′ region	changes expression	[2]
Breakpoint t(4;12)	Rieger syndrome	none	5′ region	changes expression	[46]
Deletion del(4) (q24q26)	Rieger syndrome, multiple congenital anomalies	all	whole gene	no protein	[45]
Deletion del(4) (q25q27)	Rieger syndrome, dyscrania, dysplastic ears	all	whole gene	no protein	[46]

ss, splice site; -//-, mutation in reading frame caused by use of new splice site.

[reviewed in ref. 42]. There are two genetic loci for Rieger syndrome identified to date: Rieger syndrome I on chromosome 4q25 [3] and Rieger syndrome II on 13q14 [4]. Another closely related condition, Axenfeld-Rieger anomaly characterized mainly by characteristic ocular anomalies, has been mapped to chromosome 6p25 [37, 40].

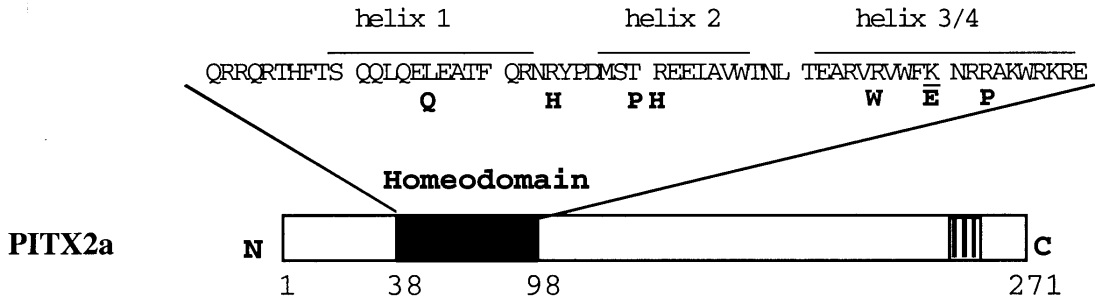
PITX2 is responsible for 4q25-linked cases of Rieger syndrome and was identified in 1996 by positional cloning [2]. The mouse homologue of this gene was identified by other groups searching for homeobox genes expressed in specific tissues, such as pituitary, *Ptx2* [5], or brain, *Otlx2* [6]. The *PITX2* gene encodes a homeodomain-containing transcription factor. This gene was shown to lie in close proximity to chromosomal breakpoints that occurred in two independent patients with the disorder and was also found to be mutated in six unrelated families with Rieger syndrome (table 3, fig. 9).

Once the *PITX2* gene was identified, a search for homologous genes resulted in the discovery of *PITX3*. This homeobox gene resides on chromosome 10q25 [43]. A collection of 80 DNA samples from individuals with various eye anomalies was screened for mutations in the *PITX3* gene. Mutations were found in members of a family with anterior segment mesenchymal dysgenesis, a rare familial disorder that has the changes typically seen in Rieger anomaly with the addition of congenital cataracts [43, 44]. The homolog of *PITX3* in the mouse, *Pitx3*, has recently been shown to have a 650-base pair deletion in the 5′ untranslated region (UTR) in the naturally occurring mouse aphakia mutant [102].

Haploinsufficiency was suggested as a possible mechanism for Rieger syndrome, as deletions of the complete gene were reported in some patients [45, 46]. The mouse homologue, *Pitx2*, was isolated and showed remarkable homology with the human gene at both the nucleotide

and protein level. By in situ hybridization on whole-mount embryos and sections, *Pitx2* mRNA was detected in the mesenchyme around the developing eye, the epithelium and mesenchyme of the maxilla and mandible, dental lamina, umbilical region, pituitary, midbrain region, and limbs [2]. The ocular, dental, and umbilical sites of *Pitx2* expression are in a good agreement with the affected phenotype of Rieger syndrome patients. The *Pitx2* gene was later shown to be expressed asymmetrically on the left side at early stages of mouse development and is involved in left-right determination of internal organs and heart morphogenesis in *Xenopus* [47–51]. The fact that Rieger syndrome patients with mutations in the *PITX2* gene do not manifest any pituitary or limb anomalies may be explained by a differential gene dosage effect: the presence of one normal copy of the gene is enough for proper development of some organs, but not others. Patients with mutations of both copies of the *PITX2* gene have not been reported. The *PITX2* gene was recently shown to be involved in two other disorders, whose differential diagnosis from Rieger syndrome was mostly based on specific ocular features—iris hypoplasia [31] and iridogoniodysgenesis syndrome [52]. Screening of 128 unrelated patients with different anterior segment malformations including 30 samples with Rieger syndrome phenotype resulted in a frequency of *PITX2* mutations of 37% for Rieger syndrome and 1% for isolated anterior chamber anomalies [E. V. Semina et al., unpublished data]. Most mutations affect the homeobox region of the gene, which plays a major role in target DNA motif recognition and binding. There is no obvious correlation between the position of the mutation in the gene and the severity of the phenotype. In

A



B

PITX2 MUTATION	*REGION*	*FREQUENCY*	*DIAGNOSIS*
L54Q	homeodomain	1/30	Rieger
T68P	homeodomain	1/30	Rieger
R84W	homeodomain	1/30	Rieger
g(+6)c	5' ss, intron3/homeo	1/30	Rieger
a(-11)g	3' ss, intron3/homeo	1/30	Rieger
K88E	homeodomain	1/30	Rieger
R91P	homeodomain	1/15	Rieger
W94 STOP	homeodomain	1/30	Rieger
W133 STOP	C-terminal	1/30	Rieger

Figure 9. (*A*) Schematic of PITX2 protein with the amino acid sequence of the wild-type homeodomain (HD) shown. Point mutations associated with Rieger syndrome are indicated in bold below the wild-type residues. The striped box indicates the conserved 14-amino acid OAR domain. The lysine residue (K), important for recognition of the *bicoid* sequence is underlined. (*B*) *PITX2* mutations and the splice mutations (ss). The splice site mutations change the homeodomain reading frame. Data from Semina et al. [2] and J. Murray (personal communication).

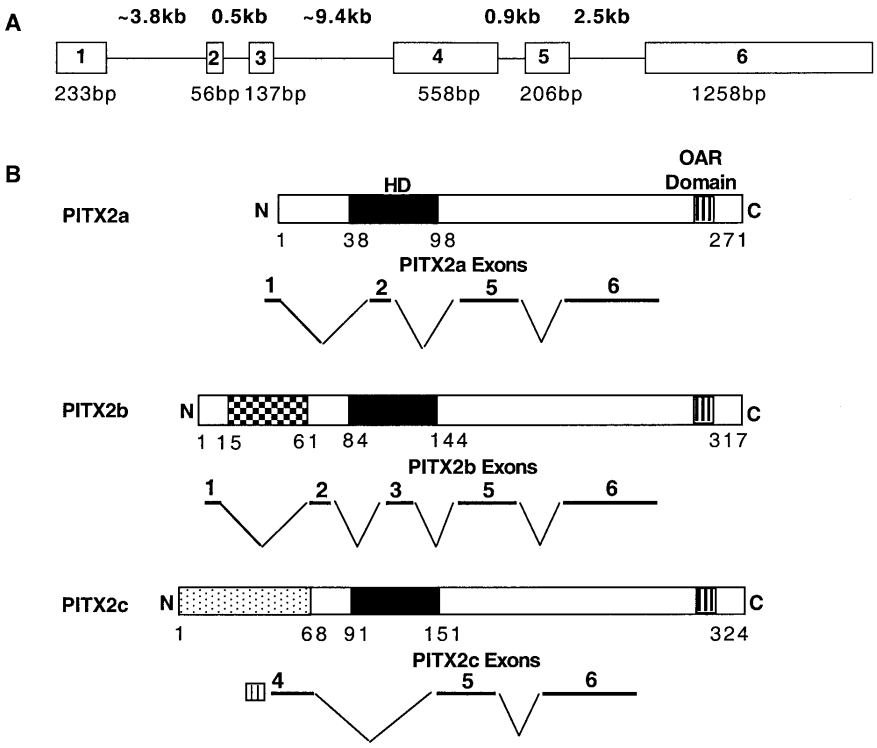


Figure 10. PITX2 major isoforms found in humans. (*A*) Genomic organization of the *PITX2* gene; intron sizes are shown at the top and exon sizes at the bottom; exons are numbered. (*B*) Protein structure shown with the location of the homeodomain (HD) and 14-amino acid conserved OAR domain. The differences in the N-terminal region of the isoforms are denoted by checkered and stippled boxes. The exons that code for the respective proteins are shown below each isoform. *PITX2c* RNA is transcribed using an internal promoter shown as a striped box flanking exon 4 [67; E. U. Semina, unpublished data].

fact, the same mutation was found to have different phenotypic expression: it was associated with Rieger syndrome in one family but with the ocular anomaly only, and without systemic defects in another. Analysis of patient histories revealed Meckel's diverticulum and omphalocele as features frequently associated with the 4q25-linked cases of Rieger syndrome. Other genes involved in the pathogenesis of conditions similar to Rieger syndrome include *PAX6* (aniridia, Peters anomaly, iris hypoplasia [53]), *FREAC3/FKHL7* (Axenfeld-Rieger anomaly, iridogoniodysgenesis, iris hypoplasia [34, 35]), *PITX3* (anterior segment mesenchymal dysgenesis [43, 54]), and *JAG1* (Alagille syndrome [55]).

Biochemical analysis

Human PITX2 is a member of the *bicoid*-like homeobox transcription factor family [2, 9]. The homeobox gene family members play fundamental roles in the genetic control of development, including pattern formation and determination of cell fate [for reviews see refs 56–58]. The homeodomain of PITX2 has a high degree of homology to another Bicoid-like homeodomain protein, P-OTX/Ptx1/Pitx1 [59, 60] and to Pitx3 [54], and to a lesser extent to unc-30, Otx-1, Otx-2, otd, and goosecoid [see ref. 2]. The homeobox proteins contain a 60-amino acid homeodomain that binds DNA. PITX2 contains a lysine at position 50 in the third helix of the homeodomain that is characteristic of the Bicoid-related proteins (fig. 9) [61–63]. This lysine residue selectively recognizes the 3′CC dinucleotide adjacent to the TAAT core [57, 64]. PITX2 can bind the DNA sequence 5′TAATCC3′ [9], which is also recognized by Bicoid protein [65]. The molecular and biochemical properties of the newly discovered human homeodomain transcription factor, PITX2, are being studied by several laboratories [2, 5, 9, 51, 66]. Several PITX2 isoforms have been identified [67; V. E. Semina, unpublished observations]. We have compared the activities of the wild-type PITX2a isoform to homeodomain mutations in PITX2 that cause Rieger syndrome. The major PITX2 isoforms are illustrated in figure 10. The activity

of the PITX2a isoform will be reviewed here. All isoforms contain identical homeodomains and C-terminal tails.

Biochemical analysis of PITX2 and six PITX2 point mutations associated with Rieger syndrome

The specificity of PITX2 binding to the *bicoid* element was determined by competition analyses using non-specific oligonucleotides and oligonucleotides matching known homeobox binding sites in electrophoretic mobility shift assays (EMSAs) [9, 68]. PITX2 also binds to the paired class P3 element (5′ TAAT CTG ATTA 3′), however, at reduced levels compared to the *bicoid* element [B. A. Amendt, unpublished data]. An apparent K_d of 50 nM was calculated, demonstrating that PITX2 binds the DNA *bicoid* sequence with a reasonable affinity that compares well with but is somewhat lower than the reported K_ds of 10^{-8} to 10^{-9} for other homeodomain proteins [57, 69–72].

Four PITX2 mutations associated with Rieger syndrome do not bind the *bicoid* (5′TAATCC3′) element (see fig. 9 for location of mutations). The PITX2 L54Q, g(+6)c and a(−11)g mutants were apparently unstable and mostly degraded in bacterial preparations. Binding could not be detected, although the cause is most likely protein instability. The PITX2 R91P mutant did produce a stable protein but did not bind the *bicoid* site. However, the Rieger mutants, PITX2 T68P and R84W bound to the *bicoid* element, albeit more weakly than the wild type [9; and unpublished data]. Thus, two of the Rieger syndrome mutations, PITX2 T68P and R84W, appear to bind the correct DNA sequence and another mechanism must be responsible for producing the Rieger syndrome phenotype.

In contrast to wild type, the mutant PITX2 T68P protein demonstrated reduced binding specificity [9]. The Scatchard plot of PITX2 T68P binding to the *bicoid* element demonstrates that the binding capacity (Bmax) of PITX2 T68P is about twofold lower than wild type. However, the dissociation constant of the mutant protein was not significantly altered compared to wild type ($K_d = 58$ nM). Thus, the threonine to proline mutant retains its ability to bind the *bicoid* element, although with a quantitative decrease in binding specificity and capacity compared to wild type.

PITX2 T68P, L54Q, and R91P mutants are transactivation deficient

The PITX2 T68P, L54Q, and R91P mutants did not transactivate a *bicoid*-TK reporter plasmid in COS-7 cells [9]. However, similar to findings with the bacterially expressed PITX2 L54Q mutant, this mutant was apparently unstable in COS-7 cells and was undetectable by Western blot analysis [9]. As expected, the R91P mutant protein, which did not bind to the *bicoid* probe also did not transactivate promoters containing the *bicoid* site. Since PITX2 is expressed in the pituitary among other tissues, the prolactin promoter was used to determine if PITX2 could transactivate a pituitary-specific gene. PITX2 transactivated the prolactin promoter reporter in COS-7 cells [9]. The Rieger mutant proteins, PITX2 T68P and R91P, also failed to transactivate this naturally occurring promoter. However, the PITX2 R84W mutant transactivated both promoters at approximately one-third of wild-type levels [B. A. Amendt, unpublished observations].

PITX2 acts synergistically with the pituitary-specific protein Pit-1 to transactivate the prolactin promoter

PITX2 expression in Rathke's pouch suggests that this new family also plays an important role in anterior pituitary gland development. Both PITX2 and P-OTX/Pitx1, a family member of PITX2, were shown to interact with the POU homeodomain protein Pit-1 [9, 60]. Pit-1 is an important transcription factor that regulates pituitary cell differentiation and expression of thyroid-stimulating hormone, growth hormone, and prolactin [73, 74]. The C terminus of P-OTX/Pitx1 was further shown to bind the family of LIM domain-associated cofactors, P-Lim and CLIM 1a [75]. These results suggest that protein-protein interactions may also occur in the corresponding region of PITX2. While most of the C-terminal sequence of P-OTX/Pitx1 and PITX2 are divergent, PITX2 has a 14-amino acid conserved sequence found in P-OTX/Pitx1. This sequence was identified in other homeodomain proteins and speculated to be involved in protein-protein interactions [2]. We will use the term OAR domain for this conserved 14 amino acid sequence. The combination of PITX2 and Pit-1 in a transient transfection assay yielded a synergistic 65-fold activation of luciferase activity [68]. The PITX2 T68P mutant did not synergize with Pit-1 to stimulate transcription activity. Thus, PITX2 and Pit-1 act in vivo to synergistically transactivate the prolactin gene promoter, and Pit-1 was unable to rescue the PITX2 T68P mutant transcriptional activity.

Addition of Pit-1 in an EMSA increased the amount of PITX2-DNA bound complex approximately two- to three-fold [9]. Interestingly, Pit-1 caused the formation of apparent PITX2 homodimers [9, 68]. Furthermore, Pit-1 had a comparable two- to three-fold stimulatory effect on the binding of the mutant PITX2 T68P protein. Pit-1 physically interacts with PITX2 to facilitate DNA binding and apparently to increase transcriptional activity [9, 68]. The T68P mutation did not affect the ability of this mutant protein to interact with Pit-1. Thus, the loss of transcriptional activity by this muta-

tion was not caused by its inability to interact with other factors such as Pit-1.

The multifunctional role of the PITX2 C-terminal tail

To determine if the 14-amino acid sequence in the C-terminal end of PITX2 affected DNA binding, a 39-amino acid deletion was engineered to remove the conserved 14-amino acid OAR domain from PITX2 (PITX2CD39) (fig. 11). Deletion of the C-terminal 39 residues did not affect the binding specificity of PITX2, but did increase binding of monomers and initiated dimer formation [68]. The apparent K_d of PITX2CD39 binding to the *bicoid* sequence was similar at 58 nM with a twofold increase in the Bmax [68]. PITX2HD (homeodomain only) bound the *bicoid* site with the same specificity as PITX2 and PITX2CΔ39 [68]. The binding of PITX2HD similarly resulted in the formation of homodimers suggesting that dimerization occurs through the homeodomain of PITX2. Deletion of either the N or C terminus flanking the homeodomain acts to increase PITX2 DNA-binding activity. However, deletion of either the complete N terminus or C terminus resulted in decreased transcriptional activity [68].

The PITX2 C terminus is required for synergistic transactivation of the prolactin promoter with Pit-1. We have demonstrated that the PITX2 C-terminal 39 residues are required for synergistic transactivation of the prolactin promoter by Pit-1 [68]. Our data suggest that the PITX2 N-terminal 16–38 residues and a C-terminal region upstream of the last 39 amino acids may contain transactivation domains. Most importantly, these data demonstrate that the C-terminal 39 residues are required for synergism between PITX2 and Pit-1. In vitro solution binding assays revealed that the C-terminal 39 residues were required and sufficient for Pit-1 binding [68]. Thus, Pit-1 physically interacts with the C-terminal 39 amino acids of PITX2. Interestingly, no mutations have been identified in the N terminus or C-terminal 39 residues of PITX2 associated with Rieger syndrome.

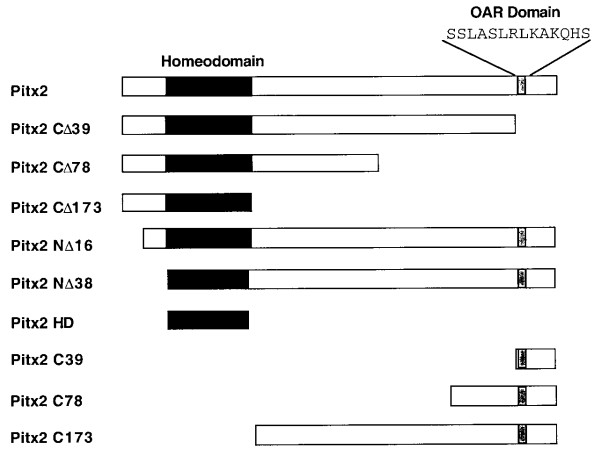


Figure 11. The constructs used to determine the regulatory regions of PITX2. The homeodomain (black box) and OAR elements are shown.

The PITX2 C-terminal 39 amino acids inhibit PITX2 transactivation

Cotransfection of an expression vector encoding the C-terminal 39 amino acids inhibited PITX2 transactivation and decreased synergistic transactivation by PITX2 and Pit-1 [68]. Since the C39 peptide did not affect CMV β-galactosidase, *bicoid*, or prolactin reporter luciferase activity, these data suggested that it was not binding a general transcription factor. The C39 peptide inhibited wild-type, PITX2NΔ16, and all of the C-terminal deletion constructs. In contrast, C39 peptide did not affect PITX2NΔ38 transactivation of the *bicoid* reporter [68]. These data suggest that the N-terminal 16–38 residues are required for inhibition by the C39 peptide. The C-terminal 39 amino acids appear to interact with the N terminus to attenuate PITX2 transcription activity. Addition of C39 peptide to an EMSA binding reaction increased the level of PITX2 binding approximately three-fold compared to PITX2 binding without C39, and resulted in homodimer formation. Although transactivation of the C-terminal truncations were repressed by the C39 peptide, the DNA-binding activities were not affected. These results suggest that the C39 peptide binds to the N-terminal 16–38 amino acids. Therefore, binding of the C39 peptide to the N terminus disrupts the intrinsic interaction of the C-terminal tail of PITX2 with the N terminus to allow increased DNA binding in vitro. We have proposed a model for the regulation of PITX2 activity (fig. 12).

Summary

The *PITX2* gene was originally identified as a gene responsible for Rieger syndrome in humans. Later, it was shown to be responsible for other related conditions (see above). Mouse *Pitx2* was identified by several laboratories and shown to be expressed in all sites that affected Rieger syndrome patients. The expression pattern of *Pitx2* in Rathke's pouch suggests that this new transcription factor may play an important role in pituitary gland development. The POU homeodomain protein Pit-1 also binds to specific sites on the prolactin promoter to activate prolactin transcription. Co-expression of *Pit-1* and *PITX2* resulted in synergistic transac-

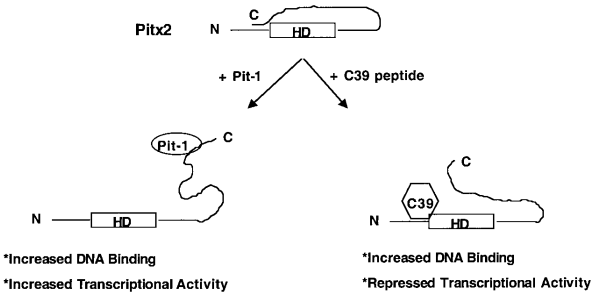


Figure 12. Model for the multifunctional role of the Pitx2 C-terminal tail. The Pitx2 protein is shown as an intramolecular folded species. The folding interferes with DNA binding of Pitx2. Pit-1 binds to the C-terminal tail of Pitx2 and disrupts the inhibitory function of the C terminus. This allows for a more efficient homeodomain interaction with the target DNA and transactivation. Pitx2 C39 peptide interaction with the N terminus of Pitx2 displaces the C-terminal tail and increases its binding activity. However, the C39 peptide masks an N-terminal transactivation domain that results in repressed transcriptional transactivation. N, N-terminal end; C, C-terminal end; HD, homeodomain [reprinted from ref. 68 with permission].

tivation of the prolactin promoter. Consistent with this synergism, a direct interaction was observed between PITX2 and Pit-1 in vitro; furthermore, PITX2 binding to the *bicoid* element is enhanced upon interacting with Pit-1. These results establish that PITX2 transactivation activity is enhanced by interaction with another transcription factor. PITX2 and P-Lim/Lhx3 have recently been reported to synergistically activate the αGSU promoter in transfection assays [76]. There is precedence for proteins interacting together to increase their binding activity [77–83].

Recent reports using genetic and epigenetic studies and *Pitx2* knockout mice have demonstrated that this gene product is required for the proper development of the embryo [5, 47–51, 54, 66, 76, 84, 85]. Several laboratories have shown that Pitx2 is a mediator of left-right signaling in vertebrates. Epigenetic studies suggested a role for Pitx2 in the determination of vertebrate heart and gut looping [47–51, 66]. Analysis of $Pitx2^{-/-}$ homozygous knockout mice reveals that Pitx2 is required for normal heart morphogenesis, development of the mandibular and maxillary facial prominences, and normal tooth and pituitary development [76, 84, 85]. However, $Pitx2^{-/+}$ heterozygous mice display certain defects in embryogenesis seen in the homozygous mice [76, 84]. A small fraction of heterozygous mice exhibit anterior chamber defects of the eye, and heart defects [84]. These mice also failed to close the ventral body wall, consistent with omphalocele found in Rieger patients [84]. Rieger syndrome has been postulated to result from haploinsufficiency [45, 46, 84, 86]. However, the overall consistent lack of developmental defects in the *Pitx2* heterozygous mice suggests that this may not be the case in humans. We have shown that PITX2 forms homodimers, both in vitro and in vivo, and that at least two mutations, PITX2 T68P and R84W, are stable proteins that can bind DNA. These mutations may act in a dominant negative fashion by dimerizing with the protein expressed from the normal allele. Thus, these dimers would probably be transactivation defective or have reduced activity and essentially result in a homozygous condition. Alternatively, the lack of developmental defects in the *Pitx2* heterozygous mice may result from or be dependent upon the genetic background of the mice. Interestingly, some Rieger syndrome patients present with pituitary and eye anomalies while others are unaffected. We can further speculate that these defects may also be caused by genetic background differences in these patients. It is interesting that some of the defects reported for the $Pitx2^{-/-}$ homozygous mice are similar to those reported in humans.

In Rieger syndrome, the PITX2 T68P point mutation lies in helix 2 at position 30 of the homeodomain, while the PITX2 L54Q point mutation is in helix 1 at position 16 of the homeodomain. Furthermore, the PITX2 R84W point mutation is in helix 3 at position 46 of the homeodomain. To our knowledge, there are no reports of amino acids in these positions affecting DNA-binding specificity in homeodomain proteins. Comparison of the amino acid sequence of over 300 homeobox proteins reveals that position 30 of the homeodomain is not conserved, and several amino acids can be located at this position [87]. While we have found no other proteins with a proline at position 30, this position can apparently accommodate changes in amino acid identity without affecting DNA-binding activity. In contrast, the amino acid residues at position 16 and 46 of the homeodomain are highly conserved [87]. In the approximately 300 homeobox proteins analyzed, the residue at position 16 is a leucine, except in EgHbx4, ap, and LH-2, which contain a methionine, and in Lmx-1 which has a phenylalanine [87]. This strong conservation suggests that the leucine residue plays a fundamental role in the homeodomain. Our results support this prediction by demonstrating that a mutation of the leucine to a glutamine (L54Q) is detrimental for PITX2 activity. Since this mutant protein could not be detected in transfected mammalian cells, leucine at position 16 is probably important for homeodomain stability. The strong conservation of the argininine residue at position 46 of the homeodomain (R84W) would also suggest that this residue plays a fundamental role in homeodomain activity. However, this mutation is clinically less severe, and binds DNA slightly less than wild-type levels; thus, a change to a tryptophan residue

does not appear to adversely affect its DNA-binding activity.

The binding specificity of homeodomains is dictated mostly by residues in the recognition helix and N-terminal arm [61, 64, 88–90]. While we have shown that the PITX2 T68P mutation binds DNA and is transactivation deficient, the mechanism of this mutation is not yet known. The results with our PITX2 deletion constructs suggest that PITX2 transactivation domains lie outside the homeodomain. Thus, how a mutation in the homeodomain affects transactivation is not entirely clear. In unpublished data, we have confirmed by immunofluorescence that this mutation is correctly localized to the nucleus [T. Hjalt, unpublished data]. A summary of PITX2 mutations associated with Rieger syndrome and their molecular and biochemical activities are shown in table 3.

Recently, the molecular basis of Boston-type craniosynostosis was determined to involve a point mutation in the N-terminal arm of the MSX2 homeodomain [91]. Similar to the PITX2 mutants T68P and R84W, a mutation in the MSX2 homeodomain did not abolish DNA binding as one might have expected. Overexpression of the wild-type *Msx2* gene can also produce craniosynostosis; therefore, enhanced binding by the MSX2 mutant is implicated as the cause for this disorder. PITX2 T68P binds DNA; however, this mutation results in slightly reduced DNA-binding specificity and capacity. This reduction in binding specificity might account for the loss of PITX2 transactivation activity. However, while we cannot rule out this possibility, these changes are unlikely to be sufficient to yield no detectable transacting activity, especially in the presence of Pit-1.

Protein-protein interactions play a major role in modulating the activity of homeodomain proteins during development. Some of these interactions can stimulate [75, 92–94] while others act to inhibit transcriptional activity [77, 82, 95–97]. A mechanism for regulating the transcriptional actions of PITX2 is its interaction with other transcription factors. The C-terminal protein-protein interaction domain of PITX2 regulates DNA binding and transcriptional activities in response to specific factors, such as Pit-1. Interestingly, approximately 50% of Rieger syndrome patients do not present with mutations in *PITX2* and may result from defects in PITX2-interacting factors. Alternatively, other genes may be involved in the pathogenesis of these Rieger syndrome patients and Axenfeld-Rieger anomaly, such as *FKHL7*.

We propose that intramolecular folding of the full-length PITX2 protein brings the C-terminal tail in direct contact with the N-terminal domain (fig. 12). This folding would interfere with DNA binding by the homeodomain. When a specific cofactor such as Pit-1 binds to the C terminus, it relieves this inhibition. Pit-1 binding to PITX2 may cause a conformational change in the C-terminal tail that unmasks the homeodomain and transactivation domain. Our model predicts that PITX2 may not be fully activated until expression of the appropriate cofactors (fig. 12).

In addition to its expression in the pituitary, PITX2 is also required for eye and tooth development, suggesting that *PITX2* is regulated in multiple tissues by a combination of interacting factors [2]. The ability of *PITX2* to be activated during development could be a function of factors interacting with its C terminus to increase DNA binding and transcriptional activity. The PITX2 C-terminal tail contains a 14-amino acid OAR domain that is conserved among the *Pitx* family members and several other homeodomain proteins [2]. Many of these proteins, prx1, prx2, Cart1, Alx4, Alx3, chx10, otp, and Pitx1, are expressed at high levels in the craniofacial region, suggesting an important role for this multifunctional C-terminal regulatory mechanism in craniofacial development.

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