expert reviews
in molecular medicine

Current molecular understanding of Axenfeld–Rieger syndrome

Tord A. Hjalt and Elena V. Semina

Axenfeld–Rieger syndrome (ARS) is a rare autosomal dominant inherited disorder affecting the development of the eyes, teeth and abdomen. The syndrome is characterised by complete penetrance but variable expressivity. The ocular component of the ARS phenotype has acquired most clinical attention and has been dissected into a spectrum of developmental eye disorders, of which open-angle glaucoma represents the main challenge in terms of treatment. Mutations in several chromosomal loci have been implicated in ARS, including *PITX2*, *FOXC1* and *PAX6*. Full-spectrum ARS is caused primarily by mutations in the *PITX2* gene. The homeobox transcription factor PITX2 is produced as at least four different transcriptional and splicing isoforms, with different biological properties. Intriguingly, PITX2 is also involved in left–right polarity determination, although asymmetry defects are not a feature of ARS. In experimental animal models and in cell culture experiments using *PITX2*, abundant evidence indicates that a narrow window of expression level of this gene is vital for its correct function.

Axenfeld–Rieger syndrome (ARS) encompasses a range of inherited ocular disorders in which the anterior segment (the front half) of the eyes shows structural malformations at birth. In addition, 50% of ARS patients develop glaucoma (neural retinal death), with resulting visual-field loss or blindness (Refs 1, 2; discussed in depth in Refs 3, 4, 5, 6). Defects in other organ systems, typically the teeth and umbilicus, are also often part of ARS. The traits of ARS conditions display a wide range of variability in severity and manifestation (Refs 7, 8, 9, 10, 11): even the same single point mutation can give different manifestations in different patients of the same family. This high degree of variable expressivity can lead to difficulties in disease classification, diagnosis and pathological studies.

The first gene that was shown to be defective in ARS was the homeobox transcription factor *PITX2* ('pituitary homeobox 2'; Ref. 12). Later studies identified additional ARS genes [*FOXC1* ('forkhead box C1'; Refs 13, 14) and *PAX6* ('paired box homeotic gene 6'; Refs 15, 16)], but there is still potential for the discovery of more genes

Tord A. Hjalt (corresponding author)
Assistant Professor, Lund University, Department of Experimental Medical Research, BMC B12, Tornavägen 10, SE-22184 Lund, Sweden. Tel +46 462220814; Fax: +46 462220855; E-mail: tord.hjalt@med.lu.se

Elena V. Semina
Associate Professor, Department of Pediatrics, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, USA. Tel: +1 414 4564996; Fax: +1 414 4566516; E-mail: esemina@mcw.edu

Accession information: DOI: 10.1017/S1462399405010082; Vol. 7; Issue 25; 8 November 2005
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https://doi.org/10.1017/S1462399405010082 Published online by Cambridge University Press

because about 40% of ARS patients lack any of the known chromosomal aberrations or known gene mutations. Despite this, the *PITX2* gene remains the major gene associated with the ARS phenotypes that include both ocular and nonocular features. It is possible that some of the unexplained forms of ARS might be attributable to defects in gene products of the same extended pathway that involves PITX2.

This review outlines past and recent advances in our molecular understanding of ARS, with a focus on *PITX2*: it discusses characterisation of the different biochemical properties of the mutant proteins, the study of PITX2 function in animal models, and the identification of regulatory pathways involving PITX2. Evidence is emerging for a very sensitive regulatory mechanism of gene expression as the main cause for *PITX2*-related ARS, and for *FOXC1*-related ARS.

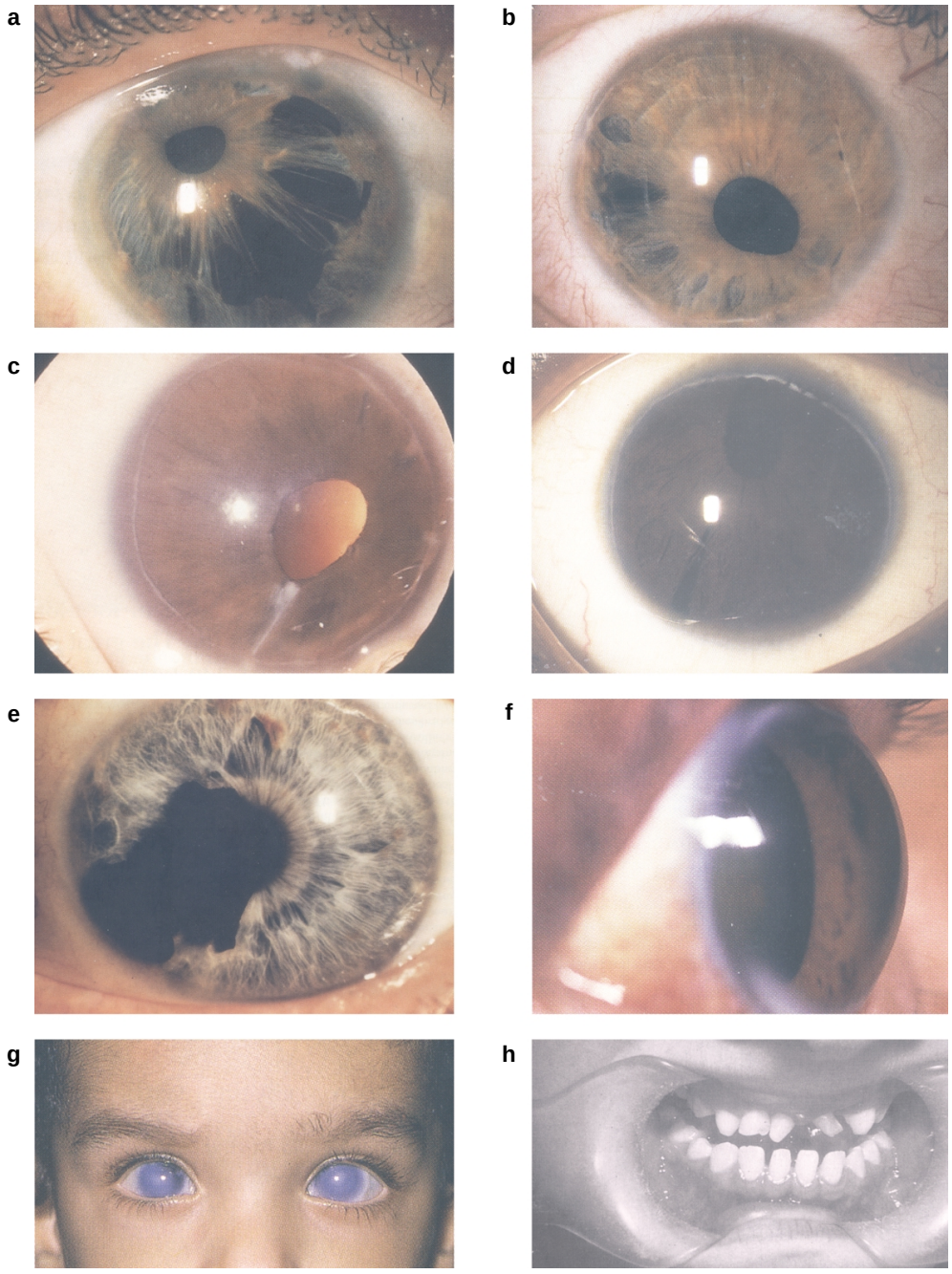
The Axenfeld–Rieger disorders

The Axenfeld–Rieger group of disorders began to be recognised as one entity following an excellent review by Alward (Ref. 1). Before this, separate conditions were diagnosed differentially, such as Rieger syndrome (RS), Axenfeld syndrome (AS), Axenfeld anomaly (AA), Rieger anomaly (RA), iridogoniodysgenesis syndrome (IGDS), iridogoniodysgenesis anomaly (IA), familial glaucoma iridogoniodysplasia (FGI), and iris hypoplasia (IH). It has been proposed that these distinctions have little value, and should all come under the one ARS heading (Refs 1, 17). There are several reasons for grouping these disorders together as ARS: (1) there are overlaps in symptoms between the old subgroups; (2) the variability in severity and range of clinical manifestations is sometimes as large within most of the subgroupings as between the subgroups; (3) defects in either of the same two genes (*PITX2* or *FOXC1*) are prevalent in several of the subgroups; and (4) there is some debate as to whether physicians can reproducibly score an abnormal angle in goniodysgenesis (Ref. 1). For example, AA was originally considered distinct from AS because of the lack of glaucoma (Ref. 18); however, about 50% of the AA patients do develop glaucoma (Ref. 1). Although we support the unified nomenclature of ARS for all of the above, as a matter of interest we describe the typical clinical features of the subgroups below. Regarding genetic transmission of these disorders, most reports find AA and RS to be autosomal dominant (e.g. Refs 12, 19, 20). There are occasional reports of other modes of inheritance, as in noninheritable AA (Ref. 21), or autosomal recessive RS (Ref. 22). Peter's anomaly (PA; see below) occurs either spontaneously (Ref. 23), or as an autosomal recessive trait (Refs 24, 25), or as an autosomal dominant trait (Refs 26, 27).

RS is typically characterised by defects of the eyes, teeth and abdomen (see Fig. 1 and Ref. 2 for clinical photographs). The eyes of an affected individual can appear with a variety of defects (Fig. 1; Fig. 2). Schwalbe's line (the termination of Descemet's membrane – a basement membrane of the trabecular meshwork) is prominently visible in slit-lamp examinations as a white or yellowish ring lining the iris of the eye. This feature is also termed posterior embryotoxon, and milder forms are seen in normal individuals at a rate of about 15% (Ref. 28). In addition, there are several cases of RS without posterior embryotoxon (Refs 17, 29, 30). The angle tissue in these patients is abnormal, and can include iridocorneal adhesions. The iris is hypoplastic, and often disrupted. Some patients may appear to have multiple pupils (polycoria), some have the pupil(s) displaced to one side, and the pupil can also appear thin and elongated, like that of a cat (corectopia). The corneas can be thick and cloudy, the eyes tearing, or the corneas can be large (megalocornea). These signs may be indicative of increased intraocular pressure in the patients, a main risk factor associated with glaucoma development. Glaucoma is degeneration of the optic nerve head, or of retinal ganglion cell layers in other parts of the retina, leading to blindness if left untreated. Glaucoma is present in RS patients at about 50% incidence, with a great variability in age of onset, but usually in the teens (Ref. 17). Dental anomalies represent a second characteristic feature of RS and usually include fewer (hypodontia) or smaller (microdontia) teeth than normal with a complete lack of teeth (anodontia) being the most serious manifestation. There seems to be prevalence for lack of upper incisors (Ref. 31). The third classic mark of RS is redundant periumbilical skin, which is sometimes hyperplastic. The umbilical stump can be abnormally protruding. In serious cases, patients are dead at birth from omphalocele, or failure of the abdominal wall to close. Other gut defects occasionally seen include an anteriorly misplaced anus or imperforate anus. In addition to this, RS patients often demonstrate a flat midface due to maxillary hypoplasia. Other, less frequently present features

Accession information: DOI: 10.1017/S1462399405010082; Vol. 7; Issue 25; 8 November 2005
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https://doi.org/10.1017/S1462399405010082 Published online by Cambridge University Press



Some clinical manifestations of Axenfeld-Rieger syndrome

Published in Expert Reviews in Molecular Medicine 2005 Cambridge University Press

Figure 1. Some clinical manifestations of Axenfeld–Rieger syndrome. (a) Displaced pupil, iris atrophy, polycoria (apparent multiple pupils), prominent and anteriorly displaced Schwalbe's line; (b) irregular pupil and pseudopolycoria; (c) anterior segment dysgenesis with posterior embryotoxon and fibrous band bridging pupil and angle; (d) inferiorly drawn pupil, attachments to prominent and anteriorly displaced Schwalbe's line; (e) congenital ectropion of the iris; (f) megalocornea; (g) bilateral glaucoma, corneal opacification; (h) dental hypoplasia. Umbilical phenotypes are not shown. For more clinical images, see Refs 35, 46. Images reproduced from Genetic Diseases of the Eye by Elias Traboulsi, copyright © 1998 by Oxford University Press, Inc. Used by permission of Oxford University Press, Inc.

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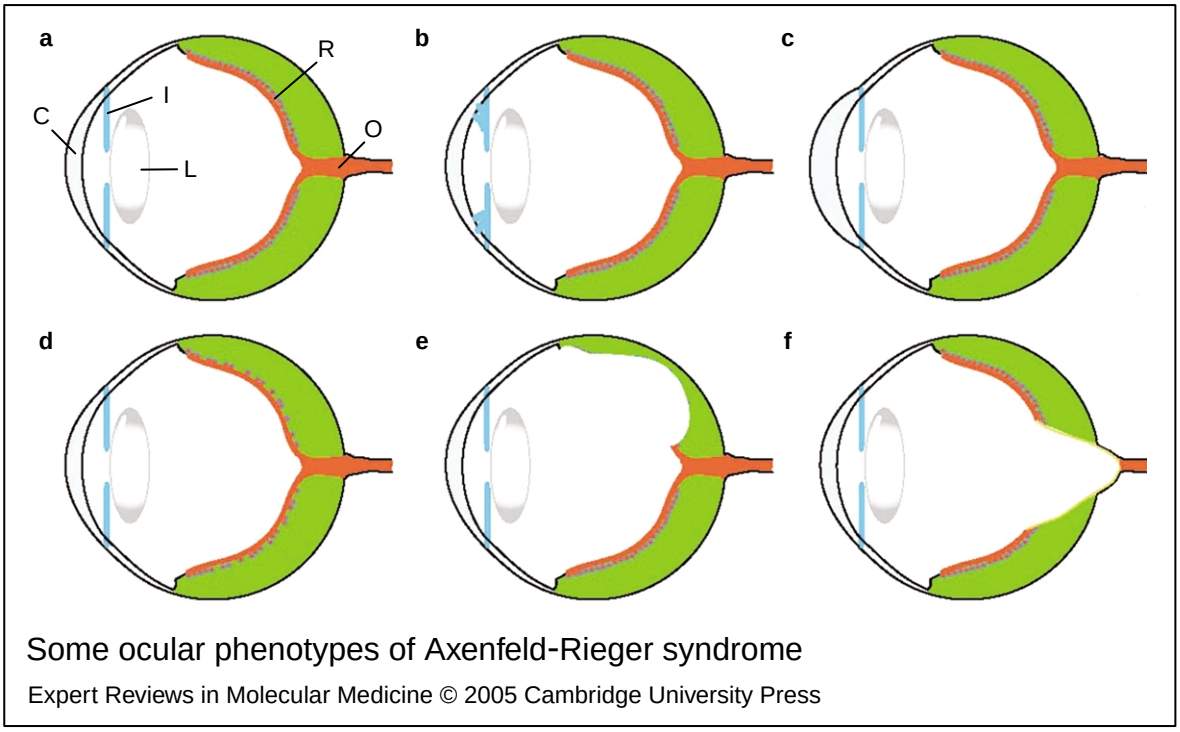


Figure 2. Some ocular phenotypes of Axenfeld–Rieger syndrome. Schematic representations of some of the clinical findings in eyes of Axenfeld–Rieger syndrome (ARS) patients are depicted. Note that some of these can occur in combination, and that there are several other types of findings that resemble these to varying degrees. In order to achieve clarity, many anatomical details are left out, and the drawings are not to scale. (a) Healthy eye; (b) ARS patient with iridocorneal adhesions; (c) ARS patient with megalocornea; (d) ARS patient with loss of retinal ganglion cells; (e) ARS patient with local degradation of retina; (f) ARS patient with completely degenerated optical nervehead. Abbreviations: C, cornea; I, iris; L, lens; R, retina; O, optical nervehead.

include pituitary defects such as empty sella syndrome and growth hormone deficiencies with resulting growth retardation (Refs 32, 33), cardiac defects (Ref. 34), hearing loss (Refs 35, 36), hypospadias (Ref. 31) and mental defects (Refs 37, 38). Even though the pituitary and the heart express *PITX2* strongly throughout development, to date there are no published reports of full-spectrum ARS caused by *PITX2* mutations and manifesting pituitary or heart defects.

RA describes patients who display all the typical ocular features of RS, but without the nonocular (systemic) defects (Ref. 1). AS describes patients with glaucoma, angle tissue defects and posterior embryotoxon, but no iridal or systemic features (Refs 1, 18). AA describes patients with AS but without glaucoma (Ref 18). IGDS has been described as separate from RS because of the absence in IGDS of polycoria, corectopia and posterior embryotoxon (Refs 39, 40), but others have found such defects (Ref. 41). IGDS is now generally considered to be identical to RS (Ref. 1; OMIM 180500). IH has been described as a separate syndrome (Ref. 42) with hypoplastic, discoloured iris, high prevalence of glaucoma, but no posterior embryotoxon, iridocorneal adhesion, corectopia or polycoria. RS-like systemic defects are present, but with lower incidence than normal (Ref. 42). IH can be caused by mutations in *PITX2* (Ref. 43), and is now considered identical to ARS (Ref. 1; OMIM 137600). PA is usually regarded as a separate ocular disorder, but it shares several traits with ARS. Typical for PA is central, or sometimes full, corneal opacity, iridocorneal adhesions, lenticulocorneal adhesions and cataract (opacity of the lens) (Refs 44, 45). Remarkably, there are patients with PA caused by the *PITX2* mutation (Ref. 46). Some PA patients have hypodontia that is quite similar to ARS hypodontia (Ref. 45). In addition, offspring from a parent with mild PA can have mild to severe PA, RA or RS (Ref. 45). PA has not been suggested to be included in the

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ARS spectrum, but we include it in this review to illustrate a functional and aetiological overlap of the two disorders. Future research will determine if indeed PA should be formally included under the ARS umbrella.

Gene mutations implicated in ARS

The first ARS chromosomal locus, 4q25, was initially identified by cytology and linkage (Refs 42, 47, 48, 49). Subsequently, the *PITX2* gene, encoding a homeobox transcription factor, was identified in this region both by positional cloning and by mutation screening in ARS families (Ref. 12). Since then, approximately 30 mutations have been reported to date in the *PITX2* gene, and chromosomal breakage involving 4q has been found in DNA from families affected by ARS (Refs 2, 50). Most of the mutations are point mutations in the homeodomain, and most result in haploinsufficiency (Refs 2, 50).

Other loci have also been implicated in ARS. Several groups initially identified the locus 6p25 as involved in ARS (Refs 39, 51, 52, 53). The culpable gene on 6p25 was subsequently identified as the forkhead transcription factor *FOXC1* (formerly known as *FKHL7*) (Refs 13, 14). Mutations in the *FOXC1* gene are mainly found in patients with isolated ARS ocular anomalies; few cases of ARS with systemic defects have been reported to have *FOXC1* mutations (Ref. 54).

A small deletion in the gene for *PAX6* on 11p13 was identified in the DNA of one ARS patient (Ref. 15). This is the only case of full ARS syndrome (combining ocular, dental and umbilical features) associated with *PAX6* mutation to date. In other reports of this mutation, the *PAX6* mutations were linked with AA and IH phenotypes (Ref. 16).

Some ARS patients have balanced chromosomal translocations involving different regions of the genome, which possibly indicate the involvement of other loci in this condition (Refs 2, 12, 54, 55). Researchers have successfully used the chromosome 4, 13 and 6 malformations in ARS patients as guides to identify gene-containing regions for this condition.

In addition, chromosome 16q has been implicated in disorders within the ARS group (Refs 56, 57). The transcription factor MAF (avian musculoaponeurotic fibrosarcoma oncogene homologue; similar to FOS, JUN, and MYC) is currently the strongest candidate gene in this region (Refs 54, 58). The locus 13q14 has been implicated from the study of two cases with deletions in this region (Refs 59, 60). The region was also implicated by linkage using another large family (Ref. 36). However, this family presented with some non-ARS syndromic defects such as hip, kidney, and hearing defects, in addition to the ARS-typical glaucoma and dental defects, and no umbilical defects were recorded. No gene has yet been identified carrying mutations or rearrangements in this chromosomal region in DNA from such patients.

In Table 1, we summarise recent data on mutations in the genes *PITX2*, *FOXC1*, and the chromosome 13q14 gene. We excluded from this table all deletions, as neighbouring genes might also be affected; therefore, *PAX6* is not listed. In summary, the most common defect in full-spectrum ARS patients seems to be point mutations in the *PITX2* reading frame (Table 1; Refs 2, 50). Mutations in the *FOXC1* gene are about as common, and are more frequent in patients with the ocular ARS symptoms only, but are not excluded from patients

Table 1. Summary of Axenfeld–Rieger syndrome phenotypes associated with nondeletion mutations[a] in specific genes

Gene	Number of families[b]	Ocular defects	Facial defects[c]	Umbilical defects
PITX2	33	33	31	31
FOXC1	28	28	3	0
13q14	1	1	1	0

[a] Deletions are excluded from the table, as neighbouring genes might also be affected; thus *PAX6* is not listed.
[b] Total number of independently reported families with gene mutations (excludes large deletions/duplications).
[c] Number of families with at least one affected person showing nonocular facial abnormalities.

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with the systemic symptoms (Table 1; Refs 2, 61, 62, 63). Approximately 40% of all ARS cases cannot be attributed to any of the loci or genes mentioned above, which leaves room for further discovery (Refs 2, 63).

PITX2 isoforms and biochemistry

The *PITX* genes include *PITX1*, *PITX2* and *PITX3*. The three genes demonstrate partial overlap in their dynamic expression patterns during embryonic development and high similarity in protein and nucleotide sequence (Ref. 64). In mouse models, *Pitx1* was demonstrated to be mostly involved in pituitary and hind leg development (Refs 65, 66, 67); a corresponding human phenotype for this gene has yet to be identified. *PITX3* seems especially important for eye and brain development, and is associated with aphakia in mice and an anterior segment dysgenesis phenotype in humans (Refs 68, 69, 70, 71).

The *PITX2* gene is represented by several different splicing and transcriptional isoforms. The four best characterised are *PITX2A, B, C* (Refs 12, 72, 73, 74, 75) and *D* (Ref. 76) (Fig. 3). Each isoform contains alternatively spliced exons and, in addition to this, isoforms A and B originate from a different promoter than C or D. All four currently characterised human isoforms share the C-terminus that includes a protein–protein interaction domain (Refs 77, 78). PITX2A has a short N-terminus preceding the homeodomain. Isoforms PITX2B and C carry large, different N-termini. The PITX2D isoform has a truncated, nonfunctional homeodomain. It is not known to what degree different isoforms contribute to ARS pathology, but the regions where mutations arise – the homeodomain and the C-terminal region – are shared between the isoforms.

Isoforms A, B, and C are widely expressed in craniofacial and other tissue, such as the pituitary and heart. The D isoform is more restricted, and has only recently been cloned from a human craniofacial cDNA library (Ref. 76). The A–C isoforms have different expression profiles in different model animals, and different isoforms contribute to the left-specific expression characteristic of PITX2 in different animals (Ref. 2). In the mouse, the C isoform is the left-specific Pitx2 in cardiac development (Ref. 74).

In cell culture, isoforms A–C have been shown to transactivate different target gene promoters (prolactin, *PLOD1*, *DLX2*, *ANF*; Table 2) to varying degrees, suggesting that they have diverged functions (Refs 75, 76). The D isoform neither activates promoters nor binds DNA; instead, it serves as a negative regulator of transactivating activity of the other isoforms by protein–protein binding (Ref. 76). This inhibitory function is analogous to that of, for example, the Id ('inhibitor of DNA binding') inhibitory proteins of the basic helix–loop–helix transcription factor superfamily (Ref. 79). Protein kinase C has been found to regulate the transactivating capacity of PITX2, and there is a C-terminal mutation that affects phosphorylation (Ref. 78). Several different downstream transcriptional target genes have been suggested for PITX2 in different organs (Table 2).

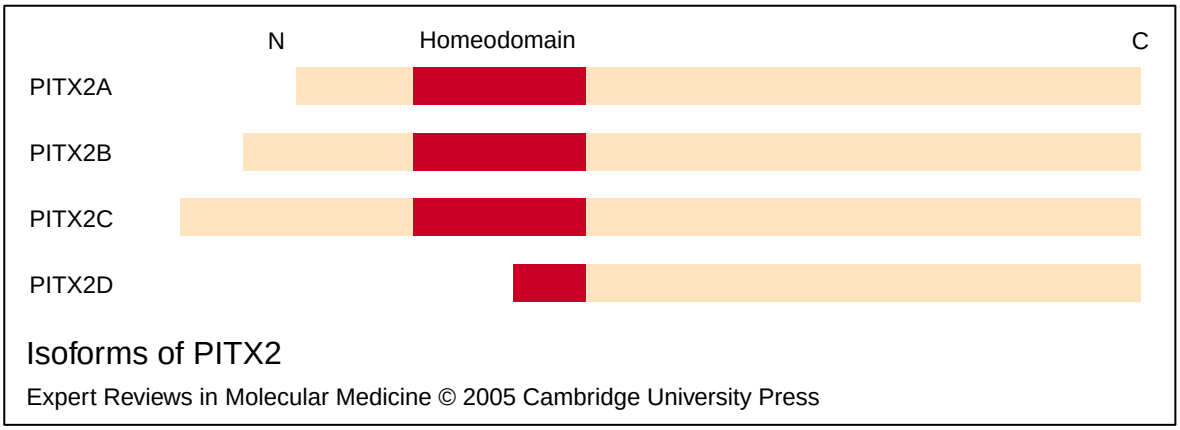


Figure 3. Isoforms of PITX2. The four currently characterised protein isoforms of PITX2 are schematically depicted. The N-termini are to the left, and the C-termini are to the right. The homeodomain that binds DNA is marked in red. Note that the drawing is not to scale, and that several more isoforms are likely to exist. See text for details and references.

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Table 2. Some proposed target genes of PITX2

Gene	Full name	Cell type/organ	Refs
PRL	Prolactin	Pituitary	66
HESX1	Homeobox gene expressed in ESCs	Pituitary	73, 91
PROP1	Prophet of PIT1	Pituitary	73, 91
PLOD1	Pro-collagen lysyl hydroxylase	Eye	121
TRIO	Triple functional domain	HeLa cells	122
DLX2	Distal-less homeobox 2	Tooth	81
CYCD2	Cyclin D2	Pituitary, heart	119
ANF/NPPA	Atrial natriuretic factor	Heart	75
FGF8	Fibroblast growth factor 8	Tooth	94
BMP4	Bone morphogenetic protein 4	Tooth	94
LHX3	Lim-domain homeobox gene 3	Pituitary	123

Abbreviations: ESC, embryonic stem cell; PIT1, pituitary-specific transcription factor 1.

Variability in disease severity: individual mutant strength theory

Mutated PITX2 proteins can be analysed by in vitro binding to radiolabelled oligodeoxyribonucleotides carrying the PITX2 core homeobox target sequence TAATCC. The mutated proteins can also be studied for their capacity to transactivate reporter genes in cell culture. As expected, biochemical analysis of human ARS-causing mutations showed a correlation between the severity of ARS manifestations and the degree of defective DNA binding and decreased transactivating properties. In a study by Kozlowski and Walter of five mutations, the mutation causing mild IH (R46W) still retained 5% DNA-binding affinity and 40% transactivating capacity (Ref. 80). A more-severe, IGDS-causing R31T mutation retained only 0.5% DNA binding and 12% transactivation. Three severe ARS mutations (L16Q, T30P, R53P) did not bind DNA at all and transactivated at 5–10% (Ref. 80). These findings were confirmed by Espinosa and co-workers studying the same T30P and R46W *PITX2* mutations (Ref. 81). (In the latter study the mutations were named T68P and R84W, respectively; the difference in names results from the fact that the nomenclature for the *PITX2* mutations was based on numbering amino acids starting at the beginning of the homeodomain in the first study, whereas the numbering started at the first methionine in the second study.) Espinosa and co-workers demonstrated that the R84W (R46W) mutant, with normal DNA-binding activity, could also transactivate a DLX2-coupled reporter gene in cell culture, albeit at 17% of the value for the wild-type PITX2 protein. As DLX2 is important for tooth development, and the majority of R84W patients have normal dentition, the authors concluded that the residual transactivating activity was sufficient for normal tooth development in these patients. By contrast, the T68P mutation, causing full-spectrum AR, did not bind DNA as well, and did not transactivate the DLX2 reporter above background levels (Ref. 81).

Same mutation, different phenotypes: the cellular context theory

A peculiar observation is that the same mutation can result in different manifestations in different members of the same ARS family. This is more difficult to explain than the differential biochemical properties discussed above. The phenomenon is also common for glaucoma patients with different aetiology. For example, in one family with full-spectrum ARS including systemic manifestations, the three affected individuals, all carrying a C insertion at position 1083 (a nonsense mutation), presented with different phenotypes (Ref. 82). Two had classical ARS abnormalities of the teeth, whereas one had normal teeth. The individual with normal teeth had RA in both eyes, and severe glaucoma with no light perception in only one eye. For the other two with tooth defects, one had IH and iridocorneal adhesions in both eyes; the other had PA in one eye, and IH and AA in the other eye. In another family described in the same study (Ref. 82), carrying a G-C 3' splice site mutation of intron 2, one of the two affected members had aniridia-like severe bilateral IH, and the other had bilateral RA with asymmetric refractive power

Accession information: DOI: 10.1017/S1462399405010082; Vol. 7; Issue 25; 8 November 2005
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https://doi.org/10.1017/S1462399405010082 Published online by Cambridge University Press

defects (anisometropia), joint hypermobility and an anteriorly placed anus. Both individuals had the classical ARS teeth and umbilical abnormalities.

The same type of variability, both in severity of the phenotype associated with different mutations, and variability of the same mutation between family members, is characteristic of *FOXC1*, *PAX6* and *PITX3* mutations (Refs 13, 61, 68, 83). There could be at least two possible explanations as to why these variations occur in a mendelian disorder: first, in some of the patients, there could be as-yet-undiscovered modifier mutations in *PITX2*, or in some other gene, that affect the phenotype. Second, particular alleles of downstream target genes or co-factor genes might demonstrate different sensitivity to a given *PITX2* mutation and this might contribute to the variability within the family. It has also been suggested that the clinical outcome of a given mutation in one gene is completely stochastically determined because there are so many intersecting genes and pathways involved (Ref. 84).

Animal and cell culture models

The PITX2 protein and mRNA are localised to the left side of several early developing vertebrate organ systems such as the heart, lungs and gut (Refs 85, 86, 87, 88, 89, 90). PITX2 participates downstream of Nodal and Lefty2 in the Nodal–Sonic-hedgehog (Shh) left–right determination pathway (Refs 85, 86, 88, 89, 90). Curiously, there have been no reports of asymmetry defects in ARS patients.

Homozygous mice with experimentally disrupted *Pitx2* die as mid-stage embryos from a combination of body-wall closure failure (omphalocele), abnormal cardiac positioning and pulmonary isomerism (Refs 73, 74, 91, 92). Some groups observed an increase in corneal thickness of $Pitx2^{-/-}$ mice (Refs 73, 74, 92). Most research groups (Refs 74, 91, 92) have reported heterozygous mice as normal, albeit with subtle gene expression changes, whereas others (Ref. 73) have reported that heterozygous mice display low-penetrance eye and tooth defects. The latter study also described a hypomorphic $Pitx2^{+/neo}$ mouse, resulting from an activated cryptic promoter in the neomycin resistance gene in the construct (Ref. 73). These hypomorphs displayed phenotypes intermediate to the $Pitx2^{-/-}$ and the $Pitx2^{+/-}$ mice, which suggests a need for sensitive regulation of *Pitx2* gene dosage for correct development. Thus, mouse models with a disrupted *Pitx2* gene do not primarily appear to exhibit severe haploinsufficiency traits, which is different to the situation in humans: most AR patients are heterozygous carriers of one defective and one normal *PITX2* allele, yet they are clinically affected. By contrast, mice heterozygous for *FoxC1* deletions display haploinsufficient phenotypes very reminiscent of the corresponding human manifestations in the eye (Ref. 93).

The effect of different *Pitx2* isoforms (*a*, *b* and *c*) on craniofacial and tooth development in mice has been studied by using combinations of knock-in constructs (Ref. 94). The results have indicated that, at least in the tooth, the isoforms are functionally redundant, and that regulation of target genes is dosage dependent rather that isoform dependent. The authors also uncovered evidence for the involvement of *Pitx2* in cell migration.

Many of the mutations detected in human *PITX2* are point mutations in the reading frame, and most of these are in the homeodomain (Ref. 2). Furthermore, most of these PITX2 mutants are defective in either DNA-binding assays in vitro or are defective in transactivation assays in cell culture (Ref. 2). One human ARS-causing PITX2 mutation, K88E, exhibits dominant-negative molecular behaviour in biochemical and cell culture transfection assays (Refs 95, 96). That is, the heterodimeric PITX2 protein dimer of one wild-type and one mutated form becomes incapable of synergising with co-factors (PIT1) needed for full transactivation of a given target gene (prolactin). Constructs carrying the ARS-causing mutation T68P, used as a control, were found not to disrupt transactivation of co-transfected wild-type PITX2 (Refs 95, 96). Curiously, this cell culture model thus also describes the T68P–wild-type heterodimer as functionally normal. This seems to contradict the clinical observations, since the T68P heterozygous patients have serious full-spectrum ARS, albeit the K88E mutation is clinically more severe than the T68P.

Another human ARS-causing PITX2 mutation, V45L, displays elevated transactivating properties in cell culture (Ref. 97). This finding is consistent with the recent report that mice overexpressing *PITX2A* in their corneas display hypertrophic corneas (similar to those of $Pitx2^{-/-}$ mice), iridocorneal adhesions, grey and tearing eyes, and severe apoptosis-associated retinal degeneration (Ref. 98). This is because an increase of expression

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level can be equivalent to an increase in functional activation. These findings can be interpreted as an indication that there are as-yet-undiscovered gain-of-function mutations involving *PITX2* that lead to defects in human ARS patients. These could be similar to the V45L, or they could be mutations that elevate the expression level. Other transcription factors are known to cause disorders by means of gain-of-function mechanisms. Gene duplications of the *FOXC1* gene cause glaucomas and IH (Ref. 99). Gain-of-function mutations in *MSX2* can cause Boston-type craniosynostosis (Refs 100, 101, 102). Both haploinsufficient and excess *PAX6* can result in the same small-eyes phenotype (Ref. 103). In addition, gain-of-function *Prop1* mice have been suggested to model some pituitary endocrinopathies in humans (Ref. 104). Prop1 is downregulated in Pitx2$^{-/-}$ pituitaries, and Prop1 is also a proposed target gene of Pitx2 (Refs 73, 91). Perhaps other transcriptional targets of Pitx2 that are transcription factors can elicit gain-of-function pathology, just like Pitx2 itself.

Expression and functional studies in mouse, rat and *Caenorhabditis elegans* have led to the conclusion that *Pitx2* is involved in development of the central nervous system (Refs 12, 87, 105, 106, 107, 108). However, only rarely do ARS patients present with mental defects (Refs 37, 38), and this is not considered part of the normal ARS clinical synopsis.

Zebrafish *Pitx2* and *Pitx3* genes have been identified and shown to have similar expression patterns, gene structure and sequence to other species (Refs 109, 110, 111). Both *Pitx2* and *Pitx3* are strongly expressed during zebrafish ocular development (*Pitx2* in the periocular mesenchyme and *Pitx3* in the developing lens). Morpholino-mediated knockdown of *Pitx3* function resulted in a phenotype highly similar to aphakia in mice. This underlines conservation of the pathway in eye development in vertebrates and opens new frontiers for studies of anterior segment dysgenesis/glaucoma phenotypes. The zebrafish model allows a combination of forward and reverse genetic approaches to be utilised to facilitate identification of critical genetic interactions required for the development and function of the eye.

In summary, studies in animal systems have indicated that Pitx2 is important for several developmental processes that are not primarily affected in ARS patients: brain development (Ref. 108), hindleg development (Ref. 67), left–right polarity determination (see above section), and stem cell development (Refs 72, 112). It is open for speculation, and further research, as to why this is so.

Pathways of gene regulation up- and downstream of PITX2

The PITX2 homeobox transcription factor is part of a large network of gene regulation, which is only partially characterised at present. In addition, there are probably several alternative networks for different tissues, developmental stages, and model animals. However, one of the more carefully elucidated pathways upstream of Pitx2 is the Nodal–Shh pathway (Fig. 4a). It is a major, although probably not the only, pathway for determining left–right polarity of mesoderm-derived organs such as heart, gut and lungs (Refs 86, 113, 114, 115, 116, 117, 118). At its core are merged two major signalling pathways involving members of the transforming growth factor β superfamily: Shh, signalling through its receptor Patched; and Nodal, signalling thought activin receptors linked to Smad internal signalling. The end output known so far is the upregulation/maintenance of *Pitx2* gene expression on the left side, but not on the right side, of some tissues. Only some Pitx2 isoforms are left-specific; furthermore, which isoform is left-specific varies in different model animals. Also, the direct upstream interactors of Pitx2 in this particular pathway are not yet known. Another, nonasymmetric pathway has been suggested for the regulation of Pitx2 in the developing heart and pituitary (Refs 119, 120) (Fig. 4b). Here, Pitx2 is downstream of the Wnt–Frizzled–β-catenin pathway. It is not known if or how the Wnt and Shh pathways interact in Pitx2 regulation. Several downstream target genes of Pitx2 have been proposed, based on a variety of biochemical and genetic experiments (Table 2). For example, in the pituitary the two transcription factors Pit1 and Pitx2 co-operatively upregulate transcription of the prolactin gene. Transcription of the important heart protein gene atrial natriuretic factor (ANF/NPPA) is upregulated by Pitx2 and its co-factor Nkx2.5. A related factor, Nkx2, is also involved in the left–right asymmetric expression of *Pitx2* via the Nkx2.5-binding site(s) in the *Pitx2* gene (Ref. 114). Much work remains to link up- and downstream signals that have Pitx2 in common. We deem it very likely that many additional upstream pathways exist to regulate

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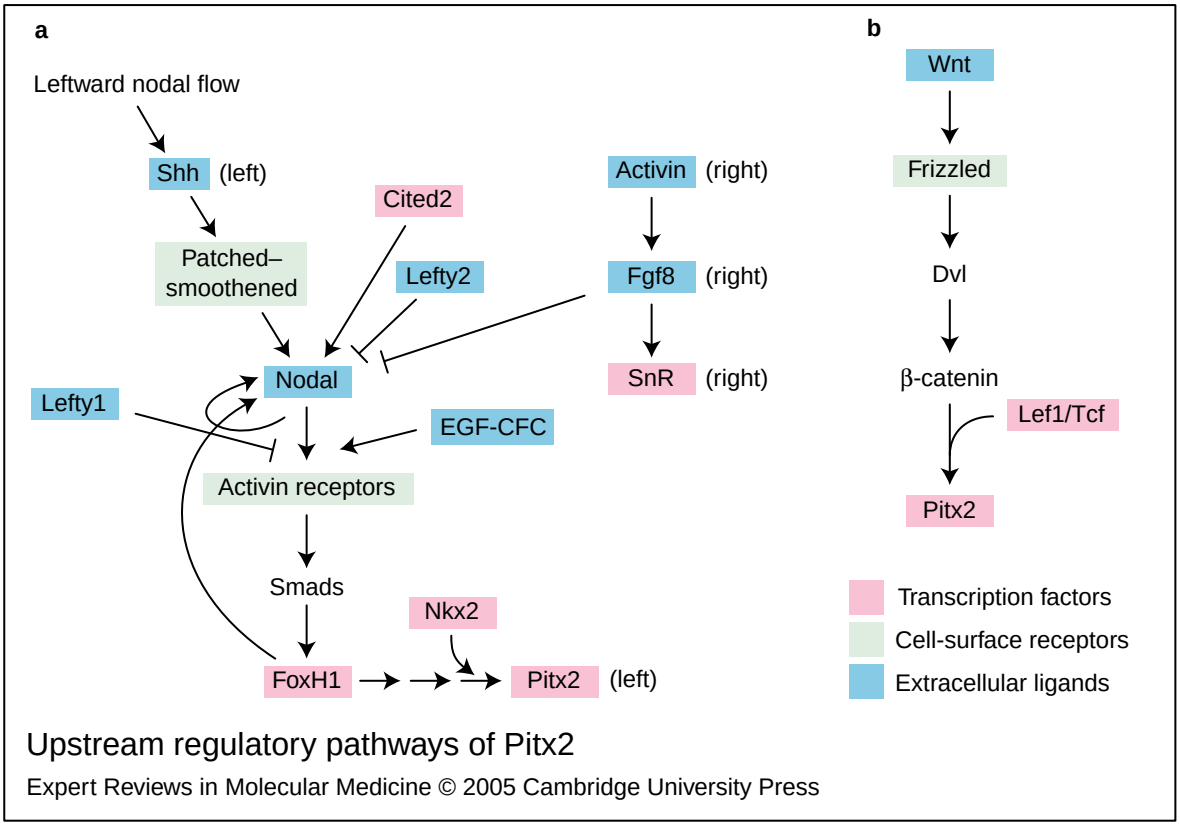


Figure 4. Upstream regulatory pathways of Pitx2. (a) In early mammalian and chicken development (but not in *Xenopus*), the initial left-specific signals come from a leftward flow in Hensen's node: the 'leftward nodal flow'. This results in the left-specific expression of Shh, a signalling molecule binding to the Patched–Smoothened cell-surface receptor complex, eliciting transactivation of the Nodal gene. Nodal, a BMP-family molecule that binds to activin receptors, can auto-upregulate itself. Nodal is also repressed by Lefty2, and on the right side by Fgf8. The EGF-CFC proteins stimulate Nodal binding to the activin receptors. Lefty1 inhibits Nodal–activin-receptor binding. Activin receptor dimerisation induced by Nodal binding triggers intracellular signalling via several Smad proteins to transactivate the transcription factor FoxH1. FoxH1 helps upregulate Nodal, and also indirectly or directly transactivates Pitx2. Nkx2 is a co-factor for Pitx2 regulation. (b) In cardiac and pituitary development, the Wnt signalling molecule can elicit transactivation of Pitx2 through the Frizzled–Dvl receptor complex, and the transactivators β-catenin and Lef1/Tcf. In both pathways, only the key molecules are depicted, and several intra- and extracellular steps have been omitted for the sake of clarity. Note also that Shh participates in several other important developmental pathways such as limb and digit formation. See text for references. Abbreviations: BMP, bone morphogenetic protein; Dvl, dishevelled 1; EGF-CFC, epidermal growth factor – cripto, Frl1, cryptic; Fgf, fibroblast growth factor; FoxH1, forkhead box H1; Lef1/Tcf, lymphoid enhancer-binding protein/T-cell-specific transcription factor; Nkx2, NK2 transcription fator related; Pitx2, pituitary homeobox 2; Shh, Sonic hedgehog; SnR, snail-related zinc finger protein.

Pitx2 in different cellular contexts. Similarly, there are probably several more downstream targets of Pitx2 in different tissues.

The two PITX2 downstream target genes that seem to have most relevance to ARS are probably the transcription factor DLX2 and the pro-collagen lysyl hydroxylase PLOD2. Dysregulation of DLX2 could explain the tooth phenotypes of ARS patients, and dysregulation of PLOD could explain some of the ocular ARS phenotypes. Indeed, data from human mutations in *PITX2* and their effect on patient's teeth match the corresponding response of *DLX2* reporter genes in cell culture (Ref. 81). Corneal overexpression of *PITX2A* is accompanied by a mild downregulation of PLOD2 and corneal clouding, collagen superstructure defects, and severe degeneration of the optic nerve (glaucoma) accompanied by retinal ganglion cell and whole retina degeneration (Ref. 98). It will be

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increasingly important to study the gene-regulatory pathways for individual cell types at different stages of development in order to clarify the role of a given transcription factor. It was recently revealed that the tissues in the eye important for these disorders develop from both neural crest and mesoderm, and that Pitx2 is active in both (Ref. 124).

Concluding remarks

The clinical consequences of ARS can be serious, such as in the case of glaucoma and omphalocele. However, it is hard to justify general prenatal screens since ARS disorders are very rare. Furthermore, genomic diagnostic kits are not feasible given the large number of loci underlying ARS: there are about 30 known full-spectrum-ARS-causing point mutations for *PITX2*, and about as many for ocular-only ARS for *FOXC1*. There are also many microdeletions in both genes. For several patients, the only genetic difference compared with a normal sibling is a point mutation in a single gene. To achieve full diagnostic saturation, one would have to use many markers to cover presently known sites/breakpoints, which still leaves out at least 30–40% of the cases for which no loci or genes have yet been described. Only in families with identified *PITX2*, *FOXC1* or *PAX6* mutations/deletions is the prenatal genetic diagnosis for ARS a possibility. In such families, individuals should be screened for glaucoma regularly throughout life given that about 50% of ARS patients develop the disease, uni- or bilaterally.

ARS serves as a good model system for the study of transcription-factor-based genetic disorders of development. As mentioned earlier, there are at least two major regulatory pathways identified upstream of Pitx2: the Wnt–β-catenin–Pitx2–CyclinD2, and the Nodal–Shh–Lefty2–Pitx2 pathways. Multiple direct downstream target genes of Pitx2 have been identified from many different functional groups: transcription factors, cell-cycle control proteins, growth factors, morphogens and modifying enzymes of extracellular matrix proteins. It is currently not known if or how these pathways branch together, if there are additional regulatory pathways upstream of Pitx2, or to which of the two identified upstream pathways the target genes relate best to. It is almost certain that additional Pitx2 target genes will be discovered. It also seems very likely that the identity of additional downstream target genes will vary with tissue type and developmental stage, and there may be a requirement for tissue- and stage-specific co-factors of Pitx2.

Researchers studying human gene mutations mostly limit their searches to the coding regions. However, splice-site mutations are occasionally found in introns. For the *PITX2* gene, there is as yet no published comprehensive study of mutations in the promoter region, or of regulatory regions further upstream of the gene. It has been known for some time that the correct dosage of *PITX2* is crucial for its function in many organ systems. Recent advances, including isolation of an apparent gain-of-function human mutation, as well as a study of *PITX2* overexpression in mice, point to the possibility of finding more gain-of-function mutations in human patient DNA. These could take many shapes and forms, perhaps most likely as destroyed silencers or destroyed negative regulatory elements. Gain-of-function pathology could also be caused by genetic defects in upstream repressor pathways of *PITX2*. As more than half of all detected cases of ARS cannot be attributed to any of the known genes/gene regions, it is at least plausible that some of them would represent such novel gain-of-function or loss-of-function mutations in *PITX2* regulatory regions. Identification of such mutations is an emerging theme in many genetic disorders (Ref. 125).

Acknowledgements and funding

We thank Peter Ekblom for critical reading of the manuscript, and the four referees for their anonymous peer review. This work was funded by grants from The Swedish Science Council, Kungliga Fysiografiska Sällskapet, Crafoordska Stiftelsen and Åke Wibergs Stiftelse (T.H.) and grants EY13606 and EY015518 from the National Eye Institute (E.S.).

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Accession information: DOI: 10.1017/S1462399405010082; Vol. 7; Issue 25; 8 November 2005
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Further reading, resources and contacts

Online Mendelian Inheritance in Man summaries genetic disorders, and advances in molecular biology (e.g. OMIM #180500: Rieger Syndrome, type 1, RIEG1):

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM

The National Eye Institute (USA) provides health information, grant support, and news:

http://www.nei.nih.gov

Handbook of ocular disease management, aimed at physicians:

http://www.revoptom.com/handbook/SECT34a.HTM

Glaucoma information:

http://www.emedicine.com/oph/byname/glaucoma-secondary-congenital.htm
http://www.glaucom.com

Useful clinical and molecular reference book:

Traboulsi, E.I., ed. (1998) Genetic Diseases of the Eye, Oxford University Press

Features associated with this article

Figures

Figure 1. Some clinical manifestations of Axenfeld–Rieger syndrome.
Figure 2. Some ocular phenotypes of Axenfeld–Rieger syndrome.
Figure 3. Isoforms of PITX2.
Figure 4. Upstream regulatory pathways of Pitx2.

Tables

Table 1. Summary of Axenfeld–Rieger syndrome phenotypes associated with nondeletion mutations in specific genes.
Table 2. Some proposed target genes of PITX2.

Citation details for this article

Tord A. Hjalt and Elena V. Semina (2005) Current molecular understanding of Axenfeld–Rieger syndrome. Expert Rev. Mol. Med. Vol. 7, Issue 25, 8 November, DOI: 10.1017/S1462399405010082

Accession information: DOI: 10.1017/S1462399405010082; Vol. 7; Issue 25; 8 November 2005
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