Clinical, Molecular, and Bioinformatics Analysis of Axenfeld-Rieger Syndrome

by

Morteza Seifi

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Medical Sciences - Medical Genetics

University of Alberta

Abstract

Axenfeld-Rieger syndrome (ARS) is an autosomal-dominant inherited disorder that primarily affects the development of structures in the anterior segment of the eye. Approximately half of patients with ARS develop glaucoma, a progressively blinding condition associated with increased intraocular pressure, which is the most severe common consequence of ARS. In almost 40% of cases, variations in the forkhead box C1 (*FOXC1*) or pituitary homeobox 2 (*PITX2*) genes are associated with ARS. However, in the remaining cases, the genetic basis of the ARS is still unknown and identifying new pathogenetic variants is becoming increasingly important for ARS genetic testing. Thus, reliable computational approaches are necessary to accurately prioritize harmful variants for functional tests to substantiate the association of variant with disruption to function.

The aim of this thesis is to identify rapid and efficient bioinformatics tools for clinical diagnostic lab researchers to prioritize predicted deleterious variants for further experimental characterization. Towards this goal, functional experiments and bioinformatics programs were performed on *FOXC1* and *PITX2* and then the results were analysed.

To this end, *FOXC1* and *PITX2* variations were identified and characterized (Chapter 2). The results showed a novel deletion involving the coding region of *PITX2* in a father-son pair associated with ARS. The proband (son) additionally, possessed a novel 2-bp deletion in a non-coding exon of the remaining *PITX2* allele predicted to alter correct splicing. It is hypothesized that the removal of the entire *PITX2* allele, plus a novel 2-bp deletion (observed in the proband) within the remaining *PITX2* allele together underlie the atypical ARS phenotypes in this family.

Then, the impact of variants on *FOXC1* and *PITX2* structure and function and the performance of bioinformatics tools for all missense variants reported in these genes were investigated (Chapter 3

and 4). Functional analysis indicated that c.378A> G (p.H128R), c.402G> A (p.C135Y), and c.481A> G(p.M161V) impair FOXC1 function via different mechanisms. C.1103C>A (p.T368N) variant was indistinguishable from wild-type FOXC1 in all tests, consistent with being a rare benign variant. Comparison of variants studied here and all previously characterized *FOXC1* missense variants, with predictions from commonly used *in silico* bioinformatics programs indicated that SIFT, PolyPhen-2, and MutPred can reliably be used to predict missense variant pathogenicity for forkhead transcription factors.

Regarding *PITX2*, the predictive value of bioinformatics programs was assessed by comparing their predictions to functional data for *PITX2* variants. The results showed that MutPred, Provean, and PMUT are the most reliable tools for predicting the pathogenicity of *PITX2* missense variants. The results of molecular modeling, performed on all the *PITX2* missense variations located in the homeodomain (HD), were compared with the findings of different protein stability programs and the results showed that I-mutant3.0 (sequence based) is the most reliable tool in predicting the effect of missense variations on PITX2 stability.

In the last chapter (Chapter 5), *in silico* analysis were used to identify and characterize the regulatory regions of *FOXC1* gene. Using an integration of three different bioinformatics programs, seven conserved non-coding elements (CNEs) resided up- and downstream of *FOXC1* were identified. Transactivation experiments indicated that none of the identified conserved regions have functional roles in the cell lines tested, suggesting that there is no association of expression of the *FOXC1* gene with my detected conserved regions.

As a conclusion, the results showed that in the absence of functional data, PMUT, Provean, MutPred, I-mutant3.0 and molecular modeling are all reliable means of predicting the pathogenicity of missense variations in the *FOXC1* forkhead domain (FHD) and *PITX2* HD. In

addition, due to the sequence homology between the FHDs of FOX class and HD of PITX transcription factors, it is hypothesized that these bioinformatics programs can be applied to determine the potential pathogenicity of missense variants within other FOX and PITX proteins and to prioritize variants for functional characterization.

Preface

This thesis is an original work by Morteza Seifi. Chapter 2 and 3 of this thesis have been previously published in journal of Acta Ophthalmologica and journal of Human Mutation, respectively.

Acknowledgements

First and foremost, I would like to thank my supervisor and Chair of our department, Dr. Michael Walter, for accepting me for your laboratory, and providing an inspiring and enjoyable environment for my research. Your excellence in research was an example for me to emulate. I really appreciate your patience, guidance, encouragement and providing an exciting research project.

I would like to express my gratitude to my committee members, Dr. Sherryl Taylor and Dr. Oana Caluseriu. Thank you for your constructive advice and suggestions, both for the Ph.D and the next stage of my career, are greatly appreciated. I am also grateful to Dr. Dennis E. Bulman for accepting to serve as my external examiner even when this task appears difficult to fit into your busy schedule.

Very special thanks are in order to Tim Footz for always helping me and guiding me whenever I was lost in the mazes of science. I would also like to thank my lab mates (Dr. Lance Doucette, Fahed Elian, and Adrian Lahola-Chomiak) who proved to be real friends and whose companionship was both joyful and beneficial. I wish to thank May Yu for giving me friendship and also for invaluable help with tissue culture.

I would also like to gratefully acknowledge the excellent assistance of Dr. Fred Berry for supporting my successful AIHS application, giving me great comments on my lab experiments, and providing me letters of reference for my postdoctoral application, really appreciate it. I would like to thank Dr. Ordan Lehmann for providing ARS patients Figures. I would like to thank Dr. Sarah Hughes for being a great graduate co-ordinator and for your help and encouragement throughout my program.

I would like to thank Dr. Georgina MacIntyre for giving me the confidence and the excellent guidance for my future academic life. Thank you to Lindsey Brown and Shari Barham for your kindness and also for helping me with administrative duties.

I would like to thank the Canadian Institutes of Health Research (CIHR), Alberta Innovates - Health Solutions (AIHS), and Izaak Walton Killam Memorial Scholarship for funding this research that would impossible without their financial supports.

Finally, I am deeply grateful to my wife, Nasim, for your love, sacrifice, and support. Your role in accomplishing this work cannot be denied. I express my warmest gratitude to my Mom and Dad. Your support and prayer are the key of my success.

Table of Contents

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Appendix

List of Symbols and Abbreviations

AA	Axenfeld anomaly
AC	Anterior chamber
AD	Transactivation domain
AF	Atrial fibrillation
AH	Aqueous humor
ANOLEA	Atomic nonlocal environment assessment
ARA	Axenfeld Rieger anomaly
ARS	Axenfeld-Rieger syndrome
AS	Axenfeld Syndrome
ASD	Atrial septal defects
°C	Degrees Celsius
CAD	Coronary artery disease
CHD	Congenital heart disease
CEU	Congenital Ectropion Uveae
CNE	Conserved non-coding elements
CRE	Cis-regulatory element
CRM	Cis-regulatory modules
Cx43	Connexin 43
DA	Dental anomaly
DAPI	4′,6-diamidine-2-phenylindole
dbSNP	Single nucleotide polymorphism database

DMEM	Dulbecco's modified Eagle's medium
DPE	Downstream promoter element
ECR	Evolutionarily conserved region
ELP	Ectopia Lentis et Pupilae
ELP4	Elongation protein 4 homolog
EMSA	Electrophoretic mobility shift assay
FHD	Forkhead domain
FOX	Forkhead Box
FOXC1	Forkhead box protein C1
GL	Glaucoma
H	Helix
HD	Homeodomain
HGMD	Human Gene Mutation Database
HSV-TK	Herpes simplex virus thymidine kinase
ICE	Iridocorneal Endothelial
ID	Inhibitory domain
IOP	Intraocular pressure
LMBR1	Limb region 1 homolog
MAPK	Mitogen-activated protein kinase
MCC	Matthews correlation coefficient
MIGS	Minimally-invasive glaucoma surgery
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing

NLS	Nuclear localization signals
NPV	Negative predictive value
NRQ	Normalized relative quantity
OAR	Orthopedia, Aristaless, and Rax
ODDD	Oculodentodigital Dysplasia
ORX	Orexin
PAX6	Paired box gene 6
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Phosphorylation domain
PITX2	Pituitary homeobox 2
PolyPhen	Polymorphism phenotyping
PROVEAN	Protein variation effect analyzer
RA	Rieger anomaly
RGC	Retinal ganglion cells
qPCR	Quantitative polymerase chain reaction
RQ	Relative quantity
RS	Rieger Syndrome
RU	Redundant periumbilical skin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHH	Sonic hedgehog
SIFT	Sorting intolerant from tolerant
SP1	Specificity protein 1

TFBS	Transcription factor binding site
TM	Trabecular meshwork
TOF	Tetralogy of Fallot
TSS	Transcriptional start site
USE	Upstream regulatory element
UTR	Untranslated region
VSD	Ventricular septal defects
W	Wing
WT	Wild-type
AA	Axenfeld anomaly

Chapter 1. General Introduction

Historical background

Axenfeld–Rieger syndrome (ARS; MIM# 602482) is transmitted in an autosomal dominant manner and impairs the development of structures of the anterior segment of the eye derived from the periocular mesenchyme (1, 2). In 1920, a patient presented with a line (ringlinie) in the posterior segment of the cornea (1 mm from the limbus) to which iris tissue was adherent and it was called "embryotoxon cornea posterius" (3). Later, in 1934 (4), two patients with similar phenotypes plus increased iris stroma and congenital pupillary malformations were described and this condition was named "dysgenesis mesodermalis corneae et iridis". It was thought to have arisen from a hindrance of development that occurred during the second month of gestation. The similarities of these presentations, especially anterior segment angle defects, lead to the suggestion that these abnormalities were features of a common group of disorders (4).

Axenfeld-Rieger disorders consisted of four different groups including Axenfeld anomaly (AA), Axenfeld syndrome (AS), Rieger anomaly (RA), and Rieger syndrome (RS). Axenfeld anomaly was the diagnosis for patients with posterior embryotoxon and peripheral anterior adhesions, while Axenfeld syndrome was the diagnosis for patients with both Axenfeld anomaly and non-ocular defects. Similarly, Rieger anomaly was the diagnosis for patient with ocular phenotypes including peripheral anterior adhesions, iris hypoplasia, polycoria, corectopia, and posterior embryotoxon while Rieger syndrome was used for patients with both Rieger anomaly and systemic malformations. As the four groups of disorders have overlapping combination of ocular and non-ocular abnormalities, and can result from variations of the same genes, the Axenfeld-Rieger group of disorders is now called Axenfeld-Rieger syndrome (ARS) (5).

Prevalence

ARS is a rare disorder, with prevalence estimated at 1 in 50,000 to 100,000 newborns. ARS has been described in different ethnic groups, including individuals from European countries, Africa, North and South America, and the Middle East.

Clinical characteristics of patients with ARS

The clinical manifestations of ARS can be divided into ocular and systemic features (Figure 1).

Ocular changes

Patients with ARS present with ocular malformations particularly in the iris, cornea and the chamber angle (Figure 1a). The ocular involvement in ARS is usually bilateral, however, it may be asymmetric and rarely unilateral (6). Iris hypoplasia is a condition of underdeveloped iris tissue (7). Corectopia is displacement of the pupil from its normal, central position, and is likely to be associated with high myopia or ectopia lentis (8). Polycoria, is a rare condition characterized by holes formed in the iris (9). The iris changes in ARS patients may range from very subtle to profound. In some cases, based on the location of the pupil, corectopia and polycoria contribute to abnormal intolerance to visual perception of light (photophobia) and cosmetic problems (10).

Anterior displacement of Schwalbe's line causes posterior embryotoxon, a corneal abnormality usually inherited as a dominant trait (11). The majority of patients with ARS present with posterior embryotoxon and, thus, it is a key factor in ARS diagnosis (12). The occurrence of posterior embryotoxon in a patient with an anterior segment disorder with no other corneal abnormalities, such as corneal opacity, sclerocornea, and megalocornea allows one to distinguish ARS from other anterior segment disorders (13).

In glaucoma, blockage of the canal of Schlemm causes increased intraocular pressure, leading to death of the retinal ganglion cells (RGCs) and blindness if left untreated (14). Glaucoma is the most serious consequence of ARS and develops in approximately 50% patients in whom it can cause complete permanent blindness within a few years (14, 15).

Systemic features

ARS can also cause non-ocular (systemic) changes (Figure 1b). The most common features include dental defects, mild craniofacial dysmorphism secondary to underdeveloped maxillary sinuses, and redundant periumblical skin which is occasionally hyperplastic (13). The most serious dental abnormalities are microdontia, hypodontia, oligodontia, anodontia, and cone-shaped teeth (16, 17). Facial features are the second systemic abnormalities that can help with diagnosis of ARS, especially in family members with a mild ocular phenotype (13). Craniofacial abnormalities include hypertelorism, telecanthus, maxillary hypoplasia), prominent forehead, a flattened mid-face with a broad, flat nasal bridge, thin upper lip and protruding lower lip (13, 16). In the abdominal region, defects of involution of the skin lead to extra folds of skin (redundant periumbilical skin), a condition that sometimes results in the erroneous diagnosis of an umbilical hernia for which unnecessary surgery may be performed. In some rare cases, the umbilical stump may be abnormally protruding. In serious ARS cases, individuals are dead at birth due to omphalocele, or abdominal wall closure defect (13, 16, 17). Additionally, ARS patients may present with a small anal opening (anal stenosis), pituitary gland abnormalities, growth retardation, hypospadias, empty sella syndrome, arachnoid cysts, and heart defects (13, 18, 19).

Types of ARS

There are three types of ARS (20). Type 1 is caused by variations in the homeobox transcription factor gene, *PITX2* (RefSeq NM 000325.5, MIM# 601542). ARS type 1 patients can present with ocular and systemic phenotypes. Dental and facial abnormalities are among the most common systemic features. ARS type 3 is caused by variations in transcription factor *FOXC1* gene (RefSeq NM 001453.2, MIM# 601090). ARS type 3 patients can present with ocular and systemic phenotypes although they more typically have only ocular features, especially anterior segment dysgenesis including anterior displacement of Schwalbe's line, iris stromal hypoplasia, corectopia, and glaucoma. ARS type 3 patients rarely present with dental anomalies and facial dysmorphism, and instead, sensorineural hearing loss and cardiac abnormalities appear to be more common (5). The gene that causes ARS type 2 is unknown (20). Umbilical defects and maxillary hypoplasia are less common in type 2.

The genetic basis of ARS

ARS describes a group of genetically heterogeneous disorders that affects primarily the anterior segment of the eye (21), although other parts of the body including the face, teeth, skeletal system and abdominal region can also be affected, particularly in ARS type 1 (22). Family-based studies, traditional genetic methods using linkage analysis and current advances in molecular genetics have identified two major genes, *PITX2* and *FOXC1*, revealing a wide spectrum of variations, which assist in the molecular diagnosis of the ARS type 1 and 3, respectively (23–25). It is reported that deletion of 13q14, supported by linkage analyses, can cause ARS type 2. However, variations in a causative gene are yet to be identified (26). Deletion of the *PAX6* gene at 11p13 and deletion of the 16q23-q24 region have also been rarely associated with ARS (27, 28). ARS patients with *PITX2* or *FOXC1*

variations will present the ocular phenotype with full penetrance. However, based on the type of variation, affected individuals exhibit variable expressivity with some limited genotype-phenotype correlations (13, 29, 30). Despite this substantial body of work, the molecular pathogenesis of ARS is only beginning to be understood; the underlying genetic defect in approximately 60% of cases remains unknown.

Forkhead box protein C1 (*FOXC1*)

Forkhead box protein C1 (*FOXC1*/FKHL7) is a member of the forkhead/winged-helix family of transcription factors characterized by a conserved 110-amino-acid motif known as the forkhead domain (FHD) (31). *FOXC1* has nuclear localization signals (NLSs) at either end of the FHD that allows it to transfer to the nucleus and bind DNA, regulating the expression of downstream target genes (Figure 2) (32). The FHD contains three N-terminal α-helices including H1, H2, H3, three β-strands named S1, S2, S3, two transactivation domains (AD, both located outside of the FHD) and a phosphorylated inhibitory domain (ID) (33–37). *FOXC1* has also a smaller fourth helix (H4) that is positioned between H2 and H3 (34). The anti-parallel β-strands interact with each other to generate a β-sheet. The FHD has two loop-like wings (W1 and W2) located between S2 and S3 (W1) and between S3 and the C-terminal (W2), giving the FHD a characteristic "winged-helix" motif (33, 35). *FOXC1* is located on 6p25 and has single exon that encodes a 553-amino-acid protein (38). The FOXC1 protein is evolutionary conserved and has a broad role in the developmental process. *FOXC1* is expressed in both fetal and adult human tissues including the eyes, brain, heart, and kidneys (39). In these tissues, FOXC1 acts as a pivotal regulator of embryogenesis, proliferation, cell migration, and cell differentiation (40, 41). Due to the role of FOXC1 in cell proliferation, it has been suggested that FOXC1 has a crucial role in cancer progression/prognosis and aging (42). Many FOX proteins

are involved in signalling pathways including the transforming growth factor β (TGF-β) and mitogen-activated protein kinase (MAPK) pathways, suggesting that these proteins are molecular integrators of extracellular signals (43).

To date, 54 *FOXC1* variants have been identified in ARS patients including missense (n=31), nonsense (n=6), and deletions/insertions/duplications (n=17) (Figure 3). Of the 31 missense variants in *FOXC1*, 22 have been molecularly investigated (44). Twenty-nine of the 31 missense variants affect amino acid residues within the forkhead domain (44). These variants impair FOXC1 function through different mechanisms including altering FOXC1 structure, nuclear localization, DNA-binding capacity, transactivation activity, DNA-binding specificity, and FOXC1 protein stability (1, 36, 39, 41, 44). The occurrence of *FOXC1* deletions and interstitial duplications in individuals with anterior segment dysgenesis demonstrates the importance of a tight control of FOXC1 expression levels (in temporal and spatial patterns) and activities for the normal development of ocular tissues. Therefore, any variations leading to an increase or decrease in FOXC1 levels can lead to ocular phenotypes in patients, 50-75% of whom continue to develop to glaucoma (37). Associations of *FOXC1* variations with Peter's anomaly (45, 46), primary congenital glaucoma (47), aniridia (48), and iris hypoplasia/iridogoniodysgenesis syndrome (49–51) have also been reported in rare cases.

Pituitary homeobox 2 (*PITX2*)

Pituitary homebox 2 (*PITX2*) was reported for the first time as an ARS-causing transcription factor in 1996 (52). As well as ARS, *PITX2* variations have been rarely associated with Peter's anomaly (53), ring dermoid of the cornea (54), and iris hypoplasia/iridogoniodysgenesis syndrome (55, 56).

PITX2 is a bicoid-related homeodomain (HD) protein; its gene is located on chromosome 4q25 (57). The *PITX2* gene is a member of the family of homeobox genes, which act during early embryonic

development to regulate the formation of ocular and several non-ocular tissues including the teeth, abdominal organs, brain, heart, and kidneys (58, 59). PITX2 protein plays a crucial role helping cells respond to oxidative stress in the adult eye (60).

PITX2 consists of eight alternatively spliced exons, which produce four different isoforms: PITX2A, PITX2B, PITX2C, and PITX2D (Figure 4). Except PITX2D, the remaining isoforms have an identical 60-amino-acid homeodomain and carboxy termini, but are different in their N-termini (61, 62). The 60-amino-acid homeodomain is responsible for localization of PITX2 to the nucleus, DNA binding, transactivation activity and protein-protein interaction (1, 21, 63–68). PITX2A (32 kDa) consists of 271 amino acids and has a short N-terminus preceding the homeodomain. PITX2B and PITX2C contain 317 and 324 amino acids, respectively, while, PITX2D has a truncated, non-functional homeodomain (52, 69–73).

In addition to the homeodomain, a second conserved region of PITX2, the OAR domain, is located within the common C-terminal region. The 14 amino acid OAR domain (first identified as a region conserved between the three paired-like activators Orthopedia, Aristaless, and Rax (74, 75)) is thought to mediate protein–protein interactions and self-inhibitory interactions with the N terminus (76). PITX2A, the most studied PITX2 isoform, contains two activation domains and two inhibitory domains (77). With respect to the functions of the isoforms of PITX2, there are contradictory theories; some studies indicated that the PITX2A, B and C isoforms have similar activities while others report that the isoforms have different activities (78–80). However, PITX2 isoforms A–C have been found to change, to different degrees, the expression levels of target genes, indicating their divergence functions (81, 82). PITX2D, has been reported to inhibit the function of other PITX2 isoforms (82). PITX2A, PITX2B and PITX2C are expressed in craniofacial areas and other tissues such as the pituitary and heart (83, 84).

To date, 87 *PITX2* variants have been identified in ARS patients including missense (n=33), nonsense (n=10), splice-site variants (n=6), and deletions/insertions/duplications (n=38) (Figure 3). *PITX2* (unlike *FOXC1*) variants often cause systemic features, in addition to anterior segment issues. The most common *PITX2* variants leading to ARS are point variants, including coding region frameshift, missense, and nonsense variants, which disrupt the function of PITX2 via different mechanisms. In addition, *PITX2* gene variations including copy number changes and small intragenic deletions alter the amount of functional PITX2 protein produced in cells. Having either too little or too much of this protein impairs the regulation of other genes required for normal development (46, 54, 56, 85–93). *PITX2* variants (particularly missense variants) have also been associated with other, non-ARS disease specially coronary artery disease (94–96).

FOXC1 **and** ***PITX2*** **interaction**

In 2006, a study for the first time showed FOXC1 and PITX2 are associated and their cooperation contributes to the sensitivity to *FOXC1* gene dose in ARS [1]. This study indicated that FOXC1 and PITX2 physically interact and colocalized within a common nuclear subcompartment. In addition, FOXC1 and PITX2 directly interact to negatively regulate FOXC1 transactivity, revealing an explanation of why elevated *FOXC1* gene dosage causes a phenotype similar to that of *PITX2* deletions and variations [1].

Further analysis showed that in cells expressing both FOXC1 and PITX2 proteins, *PITX2* target genes are expressed, while *FOXC1* target genes are hindered by FOXC1-PITX2 complexes. When there are *PITX2* loss of function variations, *PITX2* target genes expression is decreased, whereas *FOXC1* target genes are improperly activated [1].

Non-coding regulatory regions of *FOXC1* and *PITX2*

The appropriate functioning of processes in the body, including proliferation, differentiation, apoptosis and aging, needs tight temporal and spatial regulation of gene expression. Any changes to this regulation can lead to incorrect interactions between promoters and other cis-regulatory elements (CREs) and impair transcriptional regulation (97). Although disease-causing genetic changes commonly affect gene coding regions, variations or chromosomal rearrangements have been identified in non-coding regions that affect regulatory elements and/or disrupt the chromatin structure of the gene (98–101) The discovery of *FOXC1* and *PITX2* regulatory sequences provides an opportunity to probe the contribution of these regions to the ARS. Identification of regulatory sequences of ARS remains a challenge for gene characterization and certainly for understanding the clinical implications of changes to those regions (102–104). Interspecies analysis has been successful in recognizing genomic regions containing regulatory sequences, most importantly promoters and enhancers (105). For instance, the presence of regulatory elements upstream of *PITX2* is consistent with the previous reports of patients with ARS who have translocation breakpoints within the distant upstream region without disrupting the coding region of *PITX2* (106–108). Volkmann et al in 2011 (109) identified thirteen conserved non-coding regions located up to 1.1 Mb upstream of the *PITX2* gene. They showed that these conserved regions are involved in enhancer activities consistent with *PITX2* expression in the developing brain, eye, and craniofacial region. Unlike *PITX2*, we have no good understanding of the regulatory regions of *FOXC1*, or their roles and effects.

Genotype–phenotype correlation

ARS is genetically considered to be fully penetrant with variable expressivity. Unlike the patients with *FOXC1* variations that show a wide variety of manifestations with extraocular phenotypes, the

patients with duplication of the *FOXC1* show mainly eye abnormalities, particularly increased intraocular pressure (IOP) and glaucoma (29).

The same *FOXC1* variation can lead to considerable variable expressivity in clinical presentation. For instance, the L86F *FOXC1* variant has been shown to result in only ocular manifestations in one patient, but ARS type ocular features plus myocardial infarction, short stature, dental abnormality and obesity in another patient (29, 39, 40). While the phenotypic effect of P79T in an ARS patient appeared to be more serious than the P79L, presenting with ocular AR malformations and glaucoma, as well as systemic changes including maxillary hypoplasia and cardiac anomalies (110), functional analyses of the P79T and P79L variant show that they both impaired FOXC1 function to equivalent extents (29). In contrast to the P79L and P79T variants, biochemical analyses of the *FOXC1* R127H demonstrated that although this variant remarkably perturbed the FOXC1 mutant protein function, a patient with the *FOXC1* R127H variant had less severe clinical manifestations than a patient with the biochemically-milder P79T variant (39, 111). Therefore, there is no strong correlation between FOXC1 variant function and the severity of the phenotype (39).

Regarding *PITX2*, the same variation can also lead to considerable variable expression even within a particular pedigree (112, 113). In addition, there is no association between the location of the variation in the *PITX2* gene and the severity of the phenotype. For instance, two consecutive missense variants were reported within the homeodomain of *PITX2*: V83L that leads to ARS, and R84W which resulted in only iris hypoplasia (65, 67). Further functional analysis displayed that in contrast to the V83L variant which was a gain-of-function variation, the R84W variant led to reduced DNA binding and transactivation capacity of *PITX2*.

Diagnostic approach

ARS diagnosis is based on ophthalmologic and clinical examination including biomicroscopy of the anterior segment, intraocular pressure measurement, gonioscopy, and ophthalmoscopy (13, 63). The most typical tests are checking the pressure in the eye, examining the drainage angle, and looking at the optic nerve (114). If glaucoma is suspected, automated perimetry can help in the initial diagnosis as well as the follow up of glaucoma patients (63, 114, 115). As ARS patients typically present with systemic abnormalities, patients should also be examined for non-ocular changes such as face, teeth, and skeletal system malformations. The clinical diagnosis of ARS is made with common and uncommon features (Please see Table 1). In the light of clinical diagnosis, genetic testing is required to confirm the ARS diagnosis. Approximately, 40% of patients have a variation in either *FOXC1* or *PITX2*. Since patients with *PITX2* variations are more likely to have both ocular and non-ocular phenotypes, finding non-ocular features in a patient would suggest one to begin with *PITX2* variation screening prior to *FOXC1* screening for this patient.

Differential diagnosis

ICE (Iridocorneal Endothelial) Syndrome

A wide variety of disorders characterized by different degrees of corneal edema, glaucoma, and iris abnormalities are encompassed by three variations (1) Chandler syndrome, (2) essential iris atrophy, (3) Iris nevus (Cogan-Reese) syndrome. In ICE, the changes are unilateral and present in early adulthood. ICE mainly occurs in females and appears to be an acquired disease in which endothelial cells gain characteristics of epithelial cells (116). In *Chandler syndrome*, the pathologic changes are limited to the inner corneal surface and the endothelial pumps are impaired, leading to corneal edema. In *essential iris atrophy*, the abnormal endothelium proliferates onto the iris surface, leads to

contractile membranes and consequently iris atrophy, polycoria, and correctopia. In *Iris nevus (Cogan-Reese) syndrome*, contraction of the endothelial membranes on the surface of the iris underlie multiple pigmented iris nodules. The unilateral nature, corneal endothelial changes, manifestation in middle age, female predominance, and lack of systemic associations differentiate ICE from ARS (117, 118).

Peter's Anomaly

Peter's anomaly is a rare inherited condition characterized by corneal opacity due to anterior segment dysgenesis during development. Peter's anomaly can cause severe amblyopia by devastating corneal opacity in an infant. Nearly half of the patients with Peter's anomaly present with bilateral phenotypes, congenital glaucoma, aniridia, and microcornea. The affected individuals are likely to present systemic changes including hearing loss, CNS defects, heart defects, gastrointestinal and genitourinary defects, and developmental retardation (119). Peter's anomaly is mostly autosomal-recessive, although autosomal-dominant and sporadic cases have been identified (120, 121). Genetic variations within *FOXC1*, *PAX6*, *PITX2*, and *CYP1B1* can all result in Peter's anomaly (46, 53, 120, 122). The significant corneal changes are a key in differentiation of Peter's anomaly from ARS (123, 124).

Aniridia

Aniridia is a bilateral condition characterized by a complete or partial loss of the iris plus foveal hypoplasia. People with aniridia can also present with glaucoma (in late childhood or early adolescence), decreased vision, photophobia, underdeveloped optic nerves, and cataracts, contributing to progressive vision loss in 50-75% affected individuals (125). Aniridia occurs in 1 in

50,000 to 100,000 newborns worldwide and its inheritance is typically autosomal dominant. Sporadic variations in *PAX6* can be identified in over 30% of aniridia patients. Aniridia occurs either as an isolated ocular malformation without obvious systemic features or as part of the WAGR (Wilms tumor-aniridia-genital anomalies-retardation) contiguous gene syndrome in which there is deletion in both *PAX6* and Wilms tumor (WT1) genes. Gillespie syndrome is an autosomal recessive form of aniridia and accounts for 2% of all cases associated with cerebellar ataxia and mental retardation. The presence of foveal hypoplasia, is considered a key factor to differentiate aniridia from ARS (126, 127).

Congenital Ectropion Uveae (CEU)

Congenital Ectropion Uveae (CEU) is a rare, non-progressive anomaly characterized by the appearance of iris pigment epithelium on the anterior segment of the iris. The features associated with CEU include ARS, ICE, neurofibromatosis (most often), facial hemihypertophy, and Prader-Willi syndrome. Acquired EU is often associated with neovascularization of the iris and neovascular glaucoma (128–130). Unlike ARS, CEU is a unilateral condition and present with features of endothelialisation of the iridocorneal angle.

Ectopia Lentis et Pupilae (ELP)

Ectopia Lentis et Pupillae (ELP) is a rare, bilateral condition characterized by displacement of the pupil and dislocation of the lens usually in opposite directions. The pupil is irregular, usually slit shaped, and displaced from the normal position. The dislocated lens may bisect the pupil or may be fully luxated from the pupillary space (131). Other associated abnormalities are persistent pupillary membrane (87%), iridohyaloid adhesions, increased corneal diameters, elevated corneal thickness,

and axial myopia. This condition is also associated with microsperophakia, miosis, and poor pupillary dilation. Glaucoma is also a typical feature of ELP (131–133). ELP is autosomal recessive condition presented with changes of corneal endothelium which are important factors in its differentiating from ARS.

Oculodentodigital Dysplasia (ODDD)

Oculodentodigital Dysplasia (ODDD) is a rare and autosomal dominant disorder that is caused by heterozygous variations in the GJA1 gene (6q22-q23), which encodes the gap junction protein connexin 43 (Cx43) (134). In rare cases, ODDD can be inherited in an autosomal recessive pattern (135). ODDD affects many parts of the body, particularly the eyes (pigmentary retinopathy, iris coloboma, congenital cataract, glaucoma, microcornea, microphthalmos), teeth (defective enamel), fingers (syndactyly between the fourth and fifth fingers), and nose (thin nose). ODDD, unlike ARS, is progressive and more prevalent in females (136, 137). For differential diagnosis please see Table 2.

Genetic counseling

Offspring of a person with ARS have 50% chance of inheriting the trait. In ∼50%–70% of affected individuals, the variation is *de novo*. If clinical/periodontal examination, family history and laboratory tests initially indicate a genetic background, it is necessary to examine other family members for the presence of ARS. If ARS is suspected, the patient should be directed to a medical geneticist for additional clinical examination and specialized diagnostic tests. When probands have detectable *FOXC1* or *PITX2* pathogenic variants, molecular genetic testing of the parents is advised. If the proband carries a deletion or duplication of 4q25 on fluorescence in situ hybridization (FISH)

testing, FISH testing of both parents is indicated. If one of the parents is affected, their risk for having an affected child in their future pregnancies is 50%. If the *FOXC1* or *PITX2* pathogenic variant or deletion present in the proband cannot be found in either parent, the recurrence risk to the offspring is low, but higher than that of the general population because of the possibility of germline mosaicism in a patient.

Management and treatment

Current treatment of patients with ARS mostly involves managing glaucoma. As the risk of glaucoma is high and it often is diagnosed in childhood, it is crucial to follow patients with ARS frequently to monitor IOP and the optic nerve appearance. Current glaucoma therapies are aimed to reduce the build up of the aqueous humor (AH). Beta-blockers act against adrenaline-like substances by decreasing the production of intraocular fluid. Prostaglandin analogs work in glaucoma by elevating the outflow of fluid from the eye (138). Alpha-agonists work in glaucoma by both reducing the AH production and increasing AH drainage (139). Carbonic anhydrase inhibitors work in glaucoma by decreasing the AH production (140). Parasympathomimetic agents, especially miotics, act by constricting the pupils to increase AH outflow (139). Osmotic agents are another group of medications that increase the osmotic pressure of plasma relative to the aqueous and vitreous and also, decrease formation of AH. Osmotic agents are used to treat severe forms of glaucoma when, despite other treatments, IOP remains intensively high (141). Currently, many new classes of glaucoma drops are under development such as nitrous oxide latanoprostene, Rhopressa, and trabodenoson (142). In addition, there are several forms of laser therapy for glaucoma including laser iridotomy, laser trabeculoplasty, laser cyclo-ablation (143–145). Surgery is another procedure that can be applied in the ARS patients with glaucoma such as trabeculectomy, aqueous shunt devices,

and minimally-invasive glaucoma surgery (MIGS) that in the past few years has been under development (146–149). When considering the medical management of glaucoma, clinicians should also recognize their effects on body systems that are affected in ARS. For example, alpha agonists can complicate cardiac outflow further in a poorly developed system (150). Interdisciplinary communication is important in patients with ARS with the goal of collaborative care. It is important that ARS patients undergo complete examinations to define and monitor any systemic associations.

Prognosis of ARS

The prognosis of ARS mainly depends on when and if glaucoma is diagnosed. If glaucoma is detected prior to the occurrence of major optic nerve damage, the patient is compliant with the therapy recommended by the ophthalmologist. Optic nerve damage is irreversible and previously damaged optic nerves are more susceptible to extra damage. Therefore, a delayed diagnosis, for instance after serious optic nerve damage and field loss, necessitates more vigorous treatment and requires a prognosis for future visual loss, which is secured over the long term (151–153).

In summary, the molecular, cell biology and genetics of ARS are only beginning to be understood. Although 40% of ARS patients have variations in *FOXC1* and *PITX2*, the genetic basis of ARS is still unknown in about 60% of the patients, indicating the involvement of other genes and/or environmental factors in ARS disease etiology. The diagnosis of ARS is based on the clinical findings and genetic testing. The application of next generation sequencing (NGS) based targeted sequencing and exome sequencing will provide a cost-saving genetic testing and molecular diagnosis of patients. Further studies on identifying novel genes involved in ARS and new treatment strategies are underway and may open a new door to help patients with ARS.

Hypothesis and Rationale

In recent years, bioinformatics programs are becoming broadly available for estimating the effect of missense variants on protein structure and function. Bioinformatics tools are not based on the defined structure of a domain; thus, they could assess the likely pathogenicity of a massive number of missense variants. Understanding the pathogenicity of variants via experimental analysis such as complex association studies is costly, laborious and time consuming, and often even not possible. This complicated problem necessitates *in silico* approaches that use a large number of information about structure, function, conservation of genes and proteins to assess the impact of variants on the stability of the native protein structure, catalytic residues and binding properties, and protein function. I hypothesize that *in silico* bioinformatics tools can specifically and sensitively identify as pathogenic only *FOXC1* and *PITX2* variations with significant functional defects. I also hypothesize that there are regulatory elements located up- and downstream of *FOXC1* that control the expression of this gene. Thus, the overall objective of this thesis was to use an integration of functional analyses and bioinformatics tools to identify *FOXC1PITX2*the most reliable programs for clinical diagnostic lab researchers to prioritize predicted deleterious variations for further experimental characterization. This objective would be addressed by carrying out four experimental aims:

Aim 1: Identification and characterization of novel *PITX2* variations in ARS patients

Aim 2: Comparison of bioinformatics prediction, molecular modeling, and functional analyses of *FOXC1* variations in ARS patients

Aim 3: Determination of the accuracy of *in silico* bioinformatics tools and molecular modeling on all previously reported *PITX2* missense variants

Aim 4: Investigation of the upstream and downstream regions of *FOXC1* to discover regulatory sequences that control the expression of *FOXC1* and the neighboring genes

In chapters 2, the molecular consequences of two PIX2 deletions in one family were examined. I hypothesized that the deletion of the entire *PITX2* allele plus a novel 2-bp deletion within the remaining *PITX2* allele together cause an atypical ARS presentation. I also hypothesized that the 2-bp deletion of the *PITX2* has potential functional consequences and alter processing of *PITX2* mRNA and protein expression. This is the first study reporting on bi-allelic changes of *PITX2* potentially contributing to a more severe ARS phenotype.

In chapters 3 and 4, the molecular impacts of *FOXC1* and *PITX2* missense variations were investigated. I hypothesized that these variations alter the normal molecular characteristics of the FOXC1 and PITX2 proteins. The molecular features of FOXC1 and PITX2 including DNA binding, nuclear localization, transactivation activity, and protein stability are hypothesized to be altered by these variations. I suggest that these variations impair the molecular abilities of the FOXC1 and PITX2 and, thus, cause the malfunctioning of FOXC1 and PITX2 proteins during development, resulting in the ARS phenotype. In addition, the predictive performance of different *in silico* bioinformatics tools including mutation prediction programs, molecular modeling and protein stability predictor algorithms on all previously reported *FOXC1* and *PITX2* missense variants were also examined, as it is important to be able to predict which of the *FOXC1* and *PITX2* variations are likely to be the cause of disease.

Studying the CREs associated with *FOXC1* gene aid us to better understand the expression and developmental regulation of this gene, and shed light on the regulatory complexities of ocular development. In chapter 5, I used a comparative genomics approach for identifying evolutionarily conserved noncoding elements (CNEs) associated with *FOXC1*, and systematically validate the

function of the identified elements using a β-galactosidase reporter construct. I hypothesized that there are regulatory sequences flanking the 5' and 3' regions of *FOXC1* gene required for regulating its gene expression and that they are likely to be involved in its transcriptional activation. I proposed that the phenotypes observed in ARS patient with no variations in protein coding region of the *FOXC1* gene may be attributed to the misregulation of *FOXC1* expression, resulting from changes in CNEs.

Figure 1. Clinical features of ARS. Ocular changes; Iris hypoplasia (a), corectopia (b), and posterior embryotoxon (c, thick arrow). Photographs are courtesy of Dr. Ordan Lehmann (University of Alberta).

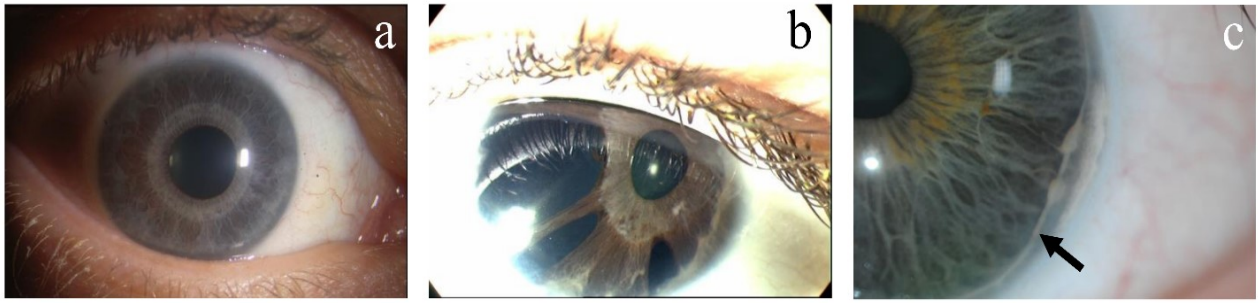

a
b
c

Figure 2. Localization of the structural domains of FOXC1 polypeptide chain. FOXC1 contains a conserved 110-amino-acid motif known as the forkhead domain (FHD). AD; transactivation domains, B; beta strands, FHD; forkhead domain, H; α-helice, ID/PD, inhibitory domain/phosphorylation domain, NLS; nuclear localization signal, W; loop-like wing. The orange boxes represent unknown structures.

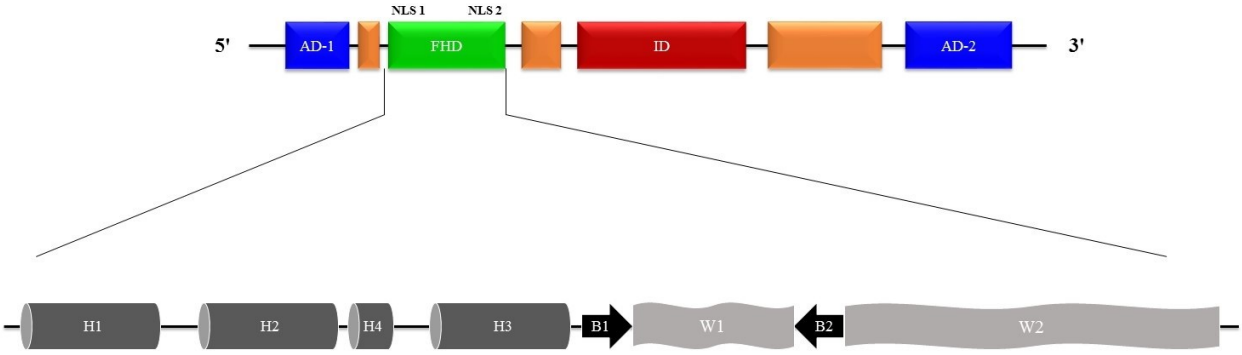

NLS 1
NLS 2
5'
AD-1
FHD
ID
AD-2
3'
H1
H2
H4
H3
B1
W1
B2
W2

Figure 3. Different types of *FOXC1* and *PITX2* genes mutations reported in the literature and their frequencies.

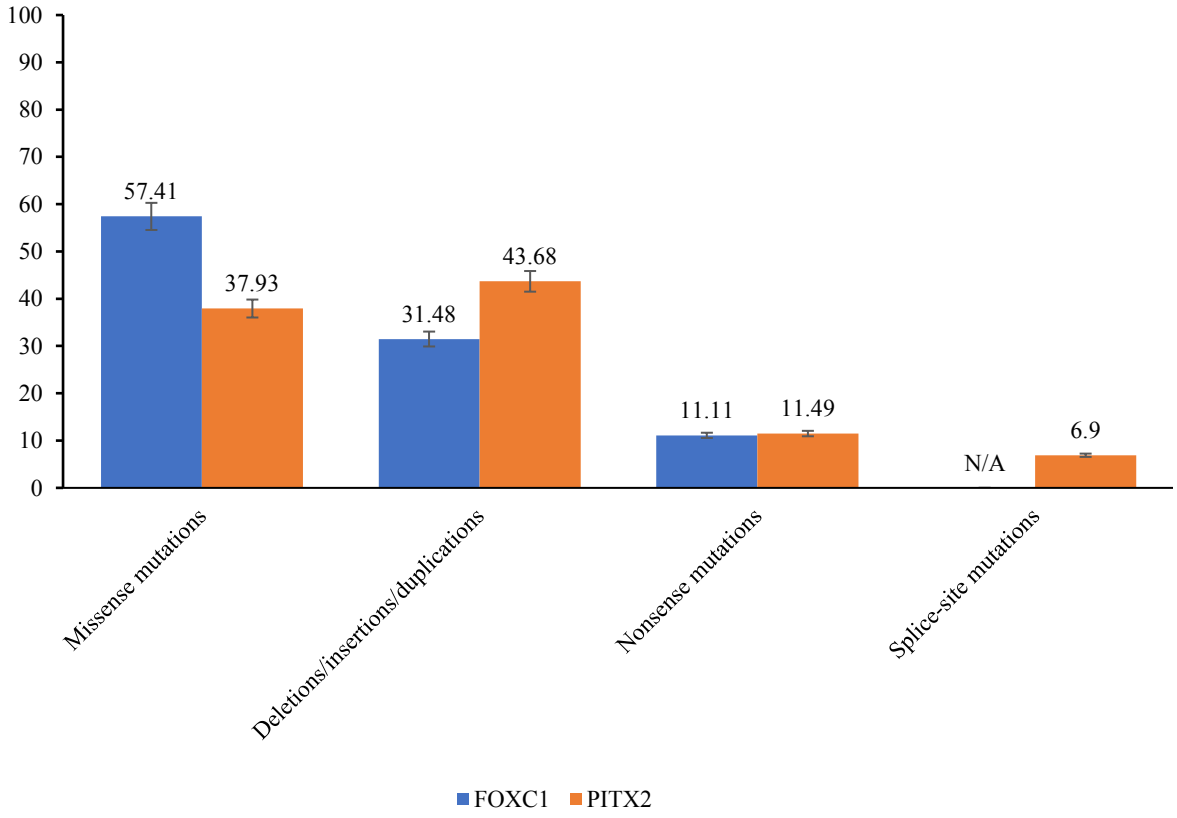

100
90
80
70
60
50
40
30
20
10
0
57.41
37.93
31.48
43.68
11.11
11.49
N/A
6.9
Missense mutations
Deletions/insertions/duplications
Nonsense mutations
Splice-site mutations
FOXC1
PITX2

Figure 4. Schematic drawing of the genomic isoforms of the human *PITX2*. Th isoforms have an identical 60-amino-acid homeodomain and carboxy termini, but are different in their N-termini. Isoform A and B have variant non-coding regions in their N-termini.

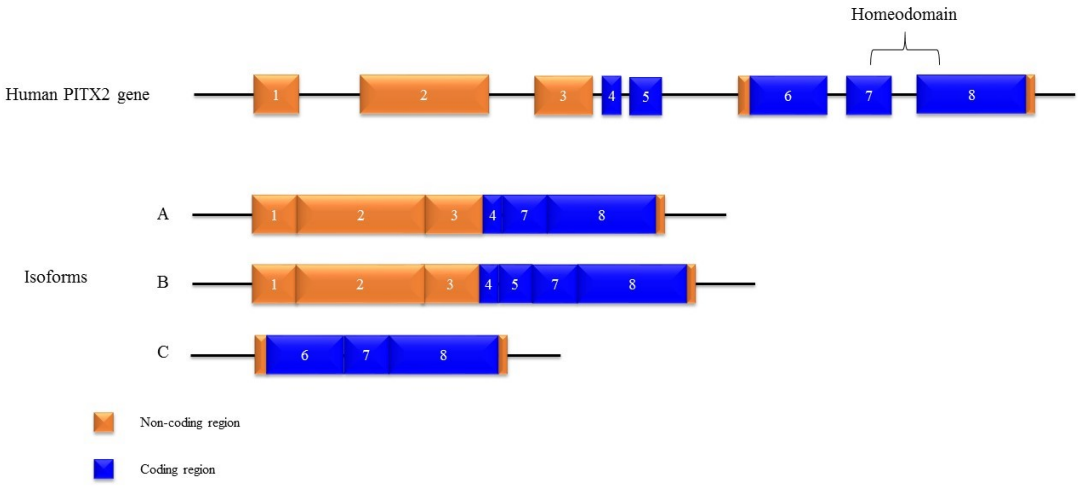

Homeodomain
Human PITX2 gene
1
2
3
4
5
6
7
8
Isoforms
A
1
2
3
4
7
8
B
1
2
3
4
5
7
8
C
6
7
8
Non-coding region
Coding region

Table 1. Common and uncommon features of ARS

	Common features	Uncommon features
Ocular	Iris hypoplasia, corectopia, polycoria, posterior embryotoxon, increased intraocular pressure, ectropion uveae	iridocorneal synechiae iridocorneal tissue adhesions, abnormal iris strands connecting the iridocorneal angle to the trabecular meshwork, adhesions, hypertelorism,
Systemic	dental defects, craniofacial dysmorphism, redundant periumblical skin	kidney abnormalities, heart defects,abnormal brain development, camptodactyly, hearing loss, ankyloglossia

Table 2. Differential diagnosis of ARS from other ocular-related disorders

	ARS	ICE syndrome	Peter's anomaly	Aniridia	CEU	ELP	ODDD
Congenital	×	-	×	×	×	×	×
Bilateral	×	-	×	×	-	×	×
Autosomal dominant	×	-	×	×	×	-	×
Corneal endothelial changes	-	×	×	×	×	×	-
Progressive	-	×	-	×	-	-	×
Sex related	-	×	-	-	-	-	×

ARS; Axenfeld-Rieger syndrome, CEU; congenital ectropion uveae, ELP; ectopia lentis et pupilae, ICE; Iridocorneal Endothelial, ODDD; oculodentodigital dysplasia

References

1. Berry,F.B., Lines,M.A., Oas,J.M., Footz,T., Underhill,D.A., Gage,P.J. and Walter,M.A. (2006) Functional interactions between FOXC1 and PITX2 underlie the sensitivity to FOXC1 gene dose in Axenfeld-Rieger syndrome and anterior segment dysgenesis. *Human molecular genetics*, **15**, 905–19.

2. Evans,A.L. and Gage,P.J. (2005) Expression of the homeobox gene Pitx2 in neural crest is required for optic stalk and ocular anterior segment development. *Human Molecular Genetics*, **14**, 3347–3359.

3. Axenfeld TH (1920) Embryotoxon corneaposterius. *Klin Monatsbl Augenheilkd*, **65**, 381–382.

4. Rieger H (1935) Dysgenesis mesodermalis corneae et iridis. *Z. Augenheilkd*, **86**, 333.

5. Alward,W.L.M. (2000) Axenfeld-Rieger syndrome in the age of molecular genetics. *American Journal of Ophthalmology*, **130**, 107–115.

6. Whitehead,M.T., Choudhri,A.F. and Salim,S. (2013) Magnetic resonance imaging findings in Axenfeld-Rieger syndrome. *Clinical ophthalmology (Auckland, N.Z.)*, **7**, 911–6.

7. Bergstrom,C.S., Saunders,R.A., Hutchinson,A.K. and Lambert,S.R. (2005) Iris hypoplasia and aorticopulmonary septal defect: A neurocristopathy. *Journal of AAPOS*, **9**, 264–267.

8. Kumar,V., Robinson,R. and Ainsworth,J.R. (2000) Idiopathic tractional corectopia. *The British journal of ophthalmology*, **84**, 1206.

9. Bhattacharjee,H., Bhattacharjee,K. and Tahiliani,P. (2013) Congenital polycoria, trichomegaly, and hereditary congenital cataract. *Journal of AAPOS*, **17**, 619–620.

10. Teus,M.A., Arranz-Marquez,E. and Lucea-Suescun,P. (2002) Incidence of iris colour change in

latanoprost treated eyes. *The British journal of ophthalmology*, **86**, 1085–1088.

11. Ito,Y.A. and Walter,M.A. (2014) Genomics and anterior segment dysgenesis: A review. *Clinical and Experimental Ophthalmology*, **42**, 13–24.

12. Saleh,M., Kamath,B.M. and Chitayat,D. (2016) Alagille syndrome: clinical perspectives. *The application of clinical genetics*, **9**, 75–82.

13. Tümer,Z. and Bach-Holm,D. (2009) Axenfeld-Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. *European journal of human genetics : EJHG*, **17**, 1527–39.

14. Doucette,L.P., Rasnitsyn,A., Seifi,M. and Walter,M.A. (2015) The interactions of genes, age, and environment in glaucoma pathogenesis. *Survey of Ophthalmology*, **60**, 310–326.

15. Anderson,D.R. (2011) Normal-tension glaucoma (Low-tension glaucoma). *Indian journal of ophthalmology*, **59 Suppl**, S97–S101.

16. O'Dwyer,E.M. and Jones,D.C. (2005) Dental anomalies in Axenfeld-Rieger syndrome. *International Journal of Paediatric Dentistry*, **15**, 459–463.

17. Jena,A.K. and Kharbanda,O.P. (2005) Axenfeld-Rieger syndrome: report on dental and craniofacial findings. *The Journal of clinical pediatric dentistry*, **30**, 83–8.

18. Klein,O.D., Oberoi,S., Huysseune,A., Hovorakova,M., Peterka,M. and Peterkova,R. (2013) Developmental disorders of the dentition: an update. *American journal of medical genetics. Part C, Seminars in medical genetics*, **163C**, 318–32.

19. Dressler,S., Meyer-Marcotty,P., Weisschuh,N., Jablonski-Momeni,A., Pieper,K., Gramer,G. and Gramer,E. (2010) Dental and Craniofacial Anomalies Associated with Axenfeld-Rieger Syndrome with PITX2 Mutation. *Case reports in medicine*, **2010**, 621984.

20. Kumari,A., Kumari,J., Singh,E. and Sharma,S. (2012) Genesis of axenfeld rieger syndrome: A review. *International Journal of Pharmaceutical Sciences Review and Research*, **14**, 64–68.

21. Seifi,M., Footz,T., Taylor,S.A.M., Elhady,G.M., Abdalla,E.M. and Walter,M.A. (2016) Novel PITX2 gene mutations in patients with Axenfeld-Rieger syndrome. *Acta Ophthalmologica*, **94**, e571–e579.

22. Chang,T.C., Summers,C.G., Schimmenti,L.A. and Grajewski,A.L. (2012) Axenfeld-Rieger syndrome: new perspectives. *The British journal of ophthalmology*, **96**, 318–22.

23. Waldron,J.M., McNamara,C., Hewson,A.R. and McNamara,C.M. (2010) ARTICLE: Axenfeld-Rieger Syndrome (ARS): A review and case report. *Special Care in Dentistry*, **30**, 218–222.

24. Acharya,M., Huang,L., Fleisch,V.C., Allison,W.T. and Walter,M.A. (2011) A complex regulatory network of transcription factors critical for ocular development and disease. *Human Molecular Genetics*, **20**, 1610–1624.

25. Berenstein-Aizman,G., Hazan-Molina,H., Drori,D. and Aizenbud,D. Axenfeld-Rieger syndrome: dentofacial manifestation and oral rehabilitation considerations. *Pediatric dentistry*, **33**, 440–4.

26. Phillips,J.C., del Bono,E.A., Haines,J.L., Pralea,A.M., Cohen,J.S., Greff,L.J. and Wiggs,J.L. (1996) A second locus for Rieger syndrome maps to chromosome 13q14. *American journal of human genetics*, **59**, 613–9.

27. Riise,R., Storhaug,K. and Brondum-Nielsen,K. (2001) Rieger syndrome is associated with PAX6 deletion. *Acta Ophthalmol Scand*, **79**, 201–3.

28. Prosser,J. and Van Heyningen,V. (1998) PAX6 mutations reviewed. *Human Mutation*, **11**, 93–

108.

29. Strungaru,M.H., Dinu,I. and Walter,M.A. (2007) Genotype-phenotype correlations in Axenfeld-Rieger malformation and glaucoma patients with FOXC1 and PITX2 mutations. *Investigative ophthalmology & visual science*, **48**, 228–37.

30. Hjalt,T.A. and Semina,E. V (2005) Current molecular understanding of Axenfeld-Rieger syndrome. *Expert reviews in molecular medicine*, **7**, 1–17.

31. Ito,Y.A., Footz,T.K., Berry,F.B., Mirzayans,F., Yu,M., Khan,A.O. and Walter,M.A. (2009) Severe molecular defects of a novel FOXC1 W152G mutation result in aniridia. *Investigative ophthalmology & visual science*, **50**, 3573–9.

32. Ito,Y.A., Footz,T.K., Murphy,T.C., Courtens,W. and Walter,M.A. (2007) Analyses of a novel L130F missense mutation in FOXC1. *Archives of ophthalmology (Chicago, Ill. : 1960)*, **125**, 128–35.

33. Weigelt,J., Climent,I., Dahlman-Wright,K. and Wikström,M. (2001) Solution Structure of the DNA Binding Domain of the Human Forkhead Transcription Factor AFX (FOXO4) †. *Biochemistry*, **40**, 5861–5869.

34. van Dongen,M.J., Cederberg,A., Carlsson,P., Enerbäck,S. and Wikström,M. (2000) Solution structure and dynamics of the DNA-binding domain of the adipocyte-transcription factor FREAC-11. *Journal of molecular biology*, **296**, 351–9.

35. Clark,K.L., Halay,E.D., Lai,E. and Burley,S.K. (1993) Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. *Nature*, **364**, 412–20.

36. Berry,F.B., Saleem,R.A. and Walter,M.A. (2002) FOXC1 transcriptional regulation is mediated

by N- and C-terminal activation domains and contains a phosphorylated transcriptional inhibitory domain. *The Journal of biological chemistry*, **277**, 10292–7.

37. Berry,F.B., Mirzayans,F. and Walter,M.A. (2006) Regulation of FOXC1 stability and transcriptional activity by an epidermal growth factor-activated mitogen-activated protein kinase signaling cascade. *The Journal of biological chemistry*, **281**, 10098–104.

38. Schachtschabel,D.O., Binninger,E.A. and Rohen,J.W. In vitro cultures of trabecular meshwork cells of the human eye as a model system for the study of cellular aging. *Archives of gerontology and geriatrics*, **9**, 251–62.

39. Saleem,R.A., Banerjee-Basu,S., Berry,F.B., Baxevanis,A.D. and Walter,M.A. (2003) Structural and functional analyses of disease-causing missense mutations in the forkhead domain of FOXC1. *Human Molecular Genetics*, **12**, 2993–3005.

40. Saleem,R.A., Murphy,T.C., Liebmann,J.M. and Walter,M.A. (2003) Identification and analysis of a novel mutation in the FOXC1 forkhead domain. *Investigative ophthalmology & visual science*, **44**, 4608–12.

41. Saleem,R.A., Banerjee-Basu,S., Berry,F.B., Baxevanis,A.D. and Walter,M.A. (2001) Analyses of the effects that disease-causing missense mutations have on the structure and function of the winged-helix protein FOXC1. *American journal of human genetics*, **68**, 627–41.

42. Han,B., Bhowmick,N., Qu,Y., Chung,S., Giuliano,A.E. and Cui,X. (2017) FOXC1: an emerging marker and therapeutic target for cancer. *Oncogene*, 10.1038/onc.2017.48.

43. Benayoun,B.A., Caburet,S. and Veitia,R.A. (2011) Forkhead transcription factors: key players in health and disease. *Trends in Genetics*, **27**, 224–232.

44. Seifi,M., Footz,T., Taylor,S.A.M. and Walter,M.A. (2016) Comparison of Bioinformatics Prediction, Molecular Modeling, and Functional Analyses of *FOXC1* Mutations in Patients with Axenfeld-Rieger Syndrome. *Human Mutation*, 10.1002/humu.23141.

45. Honkanen,R.A., Nishimura,D.Y., Swiderski,R.E., Bennett,S.R., Hong,S., Kwon,Y.H., Stone,E.M., Sheffield,V.C. and Alward,W.L.M. (2003) A family with Axenfeld-Rieger syndrome and Peter's Anomaly caused by a point mutation (Phe112Ser) in the FOXC1 gene. *American journal of ophthalmology*, **135**, 368–75.

46. Weisschuh,N., Wolf,C., Wissinger,B. and Gramer,E. (2008) A novel mutation in the FOXC1 gene in a family with Axenfeld-Rieger syndrome and Peter's' anomaly. *Clinical genetics*, **74**, 476–80.

47. Chakrabarti,S., Kaur,K., Rao,K.N., Mandal,A.K., Kaur,I., Parikh,R.S. and Thomas,R. (2009) The transcription factor gene FOXC1 exhibits a limited role in primary congenital glaucoma. *Investigative ophthalmology & visual science*, **50**, 75–83.

48. Khan,A.O., Aldahmesh,M.A. and Al-Amri,A. (2008) Heterozygous *FOXC1* Mutation (M161K) Associated with Congenital Glaucoma and Aniridia in an Infant and a Milder Phenotype in Her Mother. *Ophthalmic Genetics*, **29**, 67–71.

49. Mears,A.J., Jordan,T., Mirzayans,F., Dubois,S., Kume,T., Parlee,M., Ritch,R., Koop,B., Kuo,W.-L., Collins,C., *et al.* (1998) Mutations of the Forkhead/Winged-Helix Gene, FKHL7, in Patients with Axenfeld-Rieger Anomaly. *The American Journal of Human Genetics*, **63**, 1316–1328.

50. DeScipio,C., Schneider,L., Young,T.L., Wasserman,N., Yaeger,D., Lu,F., Wheeler,P.G., Williams,M.S., Bason,L., Jukofsky,L., *et al.* (2005) Subtelomeric deletions of chromosome

6p: Molecular and cytogenetic characterization of three new cases with phenotypic overlap with Ritscher?Schinzel (3C) syndrome. *American Journal of Medical Genetics Part A*, **134A**, 3–11.

51. Nishimura,D.Y., Searby,C.C., Alward,W.L., Walton,D., Craig,J.E., Mackey,D.A., Kawase,K., Kanis,A.B., Patil,S.R., Stone,E.M., *et al.* (2001) A Spectrum of FOXC1 Mutations Suggests Gene Dosage as a Mechanism for Developmental Defects of the Anterior Chamber of the Eye. *The American Journal of Human Genetics*, **68**, 364–372.

52. Semina,E. V., Reiter,R. and Leysens,N.J. (1996) Cloning and characterization of a novel bicoid-related homeobox trancription factor gene, RIEG, involved in Rieger syndrome. *Nat Genet.*, 10.1038/ng0496-417.

53. Doward,W., Perveen,R., Lloyd,I.C., Ridgway,A.E., Wilson,L. and Black,G.C. (1999) A mutation in the RIEG1 gene associated with Peter's' anomaly. *Journal of medical genetics*, **36**, 152–5.

54. Xia,K., Wu,L., Liu,X., Xi,X., Liang,D., Zheng,D., Cai,F., Pan,Q., Long,Z., Dai,H., *et al.* (2004) Mutation in PITX2 is associated with ring dermoid of the cornea. *Journal of medical genetics*, **41**, e129.

55. Alward,W.L., Semina,E. V, Kalenak,J.W., Héon,E., Sheth,B.P., Stone,E.M. and Murray,J.C. (1998) Autosomal dominant iris hypoplasia is caused by a mutation in the Rieger syndrome (RIEG/PITX2) gene. *American journal of ophthalmology*, **125**, 98–100.

56. Kulak,S.C., Kozlowski,K., Semina,E. V, Pearce,W.G. and Walter,M.A. (1998) Mutation in the RIEG1 gene in patients with iridogoniodysgenesis syndrome. *Human molecular genetics*, **7**, 1113–7.

57. Yoshioka,H., Meno,C., Koshiba,K., Sugihara,M., Itoh,H., Ishimaru,Y., Inoue,T., Ohuchi,H., Semina,E. V, Murray,J.C., *et al.* (1998) Pitx2, a bicoid-type homeobox gene, is involved in a lefty-signaling pathway in determination of left-right asymmetry. *Cell*, **94**, 299–305.

58. Semina,E. V, Reiter,R., Leysens,N.J., Alward,W.L., Small,K.W., Datson,N.A., Siegel-Bartelt,J., Bierke-Nelson,D., Bitoun,P., Zabel,B.U., *et al.* (1996) Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nature genetics*, **14**, 392–9.

59. Gage,P.J. and Camper,S.A. (1997) Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. *Human Molecular Genetics*, **6**, 457–464.

60. Strungaru,M.H., Footz,T., Liu,Y., Berry,F.B., Belleau,P., Semina,E. V, Raymond,V. and Walter,M.A. (2011) PITX2 is involved in stress response in cultured human trabecular meshwork cells through regulation of SLC13A3. *Investigative ophthalmology & visual science*, **52**, 7625–33.

61. Espinoza,H.M., Ganga,M., Vadlamudi,U., Martin,D.M., Brooks,B.P., Semina,E. V, Murray,J.C. and Amendt,B.A. (2005) Protein kinase C phosphorylation modulates N- and C-terminal regulatory activities of the PITX2 homeodomain protein. *Biochemistry*, **44**, 3942–54.

62. Amendt,B.A., Sutherland,L.B. and Russo,A.F. (1999) Multifunctional role of the Pitx2 homeodomain protein C-terminal tail. *Molecular and cellular biology*, **19**, 7001–10.

63. Chang,T.C., Summers,C.G., Schimmenti,L.A. and Grajewski,A.L. (2012) Axenfeld-Rieger syndrome: new perspectives: Figure 1. *British Journal of Ophthalmology*, **96**, 318–322.

64. Hjalt,T.A. and Semina,E. V. (2005) Current molecular understanding of Axenfeld–Rieger syndrome. *Expert Reviews in Molecular Medicine*, **7**, 1–17.

65. Kozlowski,K. and Walter,M.A. (2000) Variation in residual PITX2 activity underlies the phenotypic spectrum of anterior segment developmental disorders. *Human molecular genetics*, **9**, 2131–9.

66. Liu,Y. and Semina,E. V. (2012) pitx2 Deficiency Results in Abnormal Ocular and Craniofacial Development in Zebrafish. *PLoS ONE*, **7**, e30896.

67. Priston,M., Kozlowski,K., Gill,D., Letwin,K., Buys,Y., Levin,A. V, Walter,M.A. and Héon,E. (2001) Functional analyses of two newly identified PITX2 mutants reveal a novel molecular mechanism for Axenfeld-Rieger syndrome. *Human molecular genetics*, **10**, 1631–8.

68. Tümer,Z. and Bach-Holm,D. (2009) Axenfeld–Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. *European Journal of Human Genetics*, **17**, 1527–1539.

69. Cox,C.J., Espinoza,H.M., McWilliams,B., Chappell,K., Morton,L., Hjalt,T.A., Semina,E. V and Amendt,B.A. (2002) Differential Regulation of Gene Expression by PITX2 Isoforms. *Journal of Biological Chemistry*, **277**, 25001–25010.

70. Kitamura,K., Miura,H., Miyagawa-Tomita,S., Yanazawa,M., Katoh-Fukui,Y., Suzuki,R., Ohuchi,H., Suehiro,A., Motegi,Y., Nakahara,Y., *et al.* (1999) Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. *Development (Cambridge, England)*, **126**, 5749–58.

71. Ganga,M., Espinoza,H.M., Cox,C.J., Morton,L., Hjalt,T.A., Lee,Y. and Amendt,B.A. (2003) PITX2 isoform-specific regulation of atrial natriuretic factor expression: synergism and

repression with Nkx2.5. *The Journal of biological chemistry*, **278**, 22437–45.

72. Gage,P.J., Suh,H. and Camper,S.A. (1999) Dosage requirement of Pitx2 for development of multiple organs. *Development (Cambridge, England)*, **126**, 4643–51.

73. Charles,M.A., Suh,H., Hjalt,T.A., Drouin,J., Camper,S.A. and Gage,P.J. (2005) PITX genes are required for cell survival and Lhx3 activation. *Molecular endocrinology (Baltimore, Md.)*, **19**, 1893–903.

74. Furukawa,T., Kozak,C.A. and Cepko,C.L. (1997) rax, a novel paired-type homeobox gene, shows expression in the anterior neural fold and developing retina. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 3088–93.

75. Galliot,B., de Vargas,C. and Miller,D. (1999) Evolution of homeobox genes: Q50 Paired-like genes founded the Paired class. *Development genes and evolution*, **209**, 186–97.

76. Amendt,B.A., Semina,E. V and Alward,W.L. (2000) Rieger syndrome: a clinical, molecular, and biochemical analysis. *Cellular and molecular life sciences : CMLS*, **57**, 1652–66.

77. Footz,T., Idrees,F., Acharya,M., Kozlowski,K. and Walter,M.A. (2009) Analysis of mutations of the PITX2 transcription factor found in patients with Axenfeld-Rieger syndrome. *Investigative ophthalmology & visual science*, **50**, 2599–606.

78. Smidt,M.P., Cox,J.J., van Schaick,H.S., Coolen,M., Schepers,J., van der Kleij,A.M. and Burbach,J.P. (2000) Analysis of three Ptx2 splice variants on transcriptional activity and differential expression pattern in the brain. *Journal of neurochemistry*, **75**, 1818–25.

79. Schweickert,A., Campione1,M., Steinbeisser,H. and Blum,M. (2000) Pitx2 isoforms: involvement of Pitx2c but not Pitx2a or Pitx2b in vertebrate left-right asymmetry. *Mechanisms*

of development, **90**, 41–51.

80. Essner,J.J., Branford,W.W., Zhang,J. and Yost,H.J. (2000) Mesendoderm and left-right brain, heart and gut development are differentially regulated by pitx2 isoforms. *Development (Cambridge, England)*, **127**, 1081–93.

81. Ganga,M., Espinoza,H.M., Cox,C.J., Morton,L., Hjalt,T.A., Lee,Y. and Amendt,B.A. (2003) PITX2 isoform-specific regulation of atrial natriuretic factor expression: Synergism and repression with Nkx2.5. *Journal of Biological Chemistry*, **278**, 22437–22445.

82. Cox,C.J., Espinoza,H.M., McWilliams,B., Chappell,K., Morton,L., Hjalt,T. a, Semina,E. V and Amendt,B. a (2002) Differential regulation of gene expression by PITX2 isoforms. *The Journal of biological chemistry*, **277**, 25001–10.

83. Liu,C., Liu,W., Lu,M.F., Brown,N.A. and Martin,J.F. (2001) Regulation of left-right asymmetry by thresholds of Pitx2c activity. *Development (Cambridge, England)*, **128**, 2039–48.

84. Hjalt,T.A., Semina,E. V, Amendt,B.A. and Murray,J.C. (2000) The Pitx2 protein in mouse development. *Developmental dynamics : an official publication of the American Association of Anatomists*, **218**, 195–200.

85. Wang,Y., Zhao,H., Zhang,X. and Feng,H. (2003) Novel identification of a four-base-pair deletion mutation in PITX2 in a Rieger syndrome family. *Journal of dental research*, **82**, 1008–12.

86. Vieira,V., David,G., Roche,O., de la Houssaye,G., Boutboul,S., Arbogast,L., Kobetz,A., Orssaud,C., Camand,O., Schorderet,D.F., *et al.* (2006) Identification of four new PITX2 gene

mutations in patients with Axenfeld-Rieger syndrome. *Molecular vision*, **12**, 1448–60.

87. Saadi,I., Toro,R., Kuburas,A., Semina,E., Murray,J.C. and Russo,A.F. (2006) An unusual class of PITX2 mutations in Axenfeld-Rieger syndrome. *Birth defects research. Part A, Clinical and molecular teratology*, **76**, 175–81.

88. Maciolek,N.L., Alward,W.L.M., Murray,J.C., Semina,E. V and McNally,M.T. (2006) Analysis of RNA splicing defects in PITX2 mutants supports a gene dosage model of Axenfeld-Rieger syndrome. *BMC medical genetics*, **7**, 59.

89. Lines,M.A., Kozlowski,K., Kulak,S.C., Allingham,R.R., Héon,E., Ritch,R., Levin,A. V, Shields,M.B., Damji,K.F., Newlin,A., *et al.* (2004) Characterization and prevalence of PITX2 microdeletions and mutations in Axenfeld-Rieger malformations. *Investigative ophthalmology & visual science*, **45**, 828–33.

90. Kniestedt,C., Taralczak,M., Thiel,M.A., Stuermer,J., Baumer,A. and Gloor,B.P. (2006) A novel PITX2 mutation and a polymorphism in a 5-generation family with Axenfeld-Rieger anomaly and coexisting Fuchs' endothelial dystrophy. *Ophthalmology*, **113**, 1791.e1-8.

91. Idrees,F., Bloch-Zupan,A., Free,S.L., Vaideanu,D., Thompson,P.J., Ashley,P., Brice,G., Rutland,P., Bitner-Glindzicz,M., Khaw,P.T., *et al.* (2006) A novel homeobox mutation in the PITX2 gene in a family with Axenfeld-Rieger syndrome associated with brain, ocular, and dental phenotypes. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*, **141B**, 184–91.

92. D'haene,B., Meire,F., Claerhout,I., Kroes,H.Y., Plomp,A., Arens,Y.H., de Ravel,T., Casteels,I., De Jaegere,S., Hooghe,S., *et al.* (2011) Expanding the spectrum of FOXC1 and PITX2 mutations and copy number changes in patients with anterior segment malformations.

Investigative ophthalmology & visual science, **52**, 324–33.

93. Brooks,B.P., Moroi,S.E., Downs,C.A., Wiltse,S., Othman,M.I., Semina,E. V and Richards,J.E. (2004) A novel mutation in the PITX2 gene in a family with Axenfeld-Rieger syndrome. *Ophthalmic genetics*, **25**, 57–62.

94. Sun,Y.M., Wang,J., Qiu,X.B., Yuan,F., Xu,Y.J., Li,R.G., Qu,X.K., Huang,R.T., Xue,S. and Yang,Y.Q. (2016) PITX2 loss-of-function mutation contributes to tetralogy of Fallot. *Gene*, **577**, 258–264.

95. Mommersteeg,M.T.M., Brown,N.A., Prall,O.W.J., De Gier-De Vries,C., Harvey,R.P., Moorman,A.F.M. and Christoffels,V.M. (2007) Pitx2c and Nkx2-5 are required for the formation and identity of the pulmonary myocardium. *Circulation Research*, **101**, 902–909.

96. Bamforth,S.D., Bragança,J., Farthing,C.R., Schneider,J.E., Broadbent,C., Michell,A.C., Clarke,K., Neubauer,S., Norris,D., Brown,N. a, *et al.* (2004) Cited2 controls left-right patterning and heart development through a Nodal-Pitx2c pathway. *Nature genetics*, **36**, 1189–1196.

97. Weingarten-Gabbay,S. and Segal,E. (2014) The grammar of transcriptional regulation. *Human genetics*, **133**, 701–11.

98. Kleinjan,D. a and van Heyningen,V. (2005) Long-range control of gene expression: emerging mechanisms and disruption in disease. *American journal of human genetics*, **76**, 8–32.

99. Manco,L., Ribeiro,M.L., Máximo,V., Almeida,H., Costa,A., Freitas,O., Barbot,J., Abade,A. and Tamagnini,G. (2000) A new PKLR gene mutation in the R-type promoter region affects the gene transcription causing pyruvate kinase deficiency. *British journal of haematology*, **110**,

993–7.

100. Masotti,C., Armelin-Correa,L.M., Splendore,A., Lin,C.J., Barbosa,A., Sogayar,M.C. and Passos-Bueno,M.R. (2005) A functional SNP in the promoter region of TCOF1 is associated with reduced gene expression and YY1 DNA-protein interaction. *Gene*, **359**, 44–52.

101. Solis,C., Aizencang,G.I., Astrin,K.H., Bishop,D.F. and Desnick,R.J. (2001) Uroporphyrinogen III synthase erythroid promoter mutations in adjacent GATA1 and CP2 elements cause congenital erythropoietic porphyria. *The Journal of clinical investigation*, **107**, 753–62.

102. Niu,M., Tabari,E.S. and Su,Z. (2014) De novo prediction of cis-regulatory elements and modules through integrative analysis of a large number of ChIP datasets. *BMC Genomics*, **15**, 1047.

103. Narlikar,L. and Ovcharenko,I. (2009) Identifying regulatory elements in eukaryotic genomes. *Briefings in functional genomics & proteomics*, **8**, 215–30.

104. Barrera,L.O. and Ren,B. (2006) The transcriptional regulatory code of eukaryotic cells – insights from genome-wide analysis of chromatin organization and transcription factor binding. *Current Opinion in Cell Biology*, **18**, 291–298.

105. Boffelli,D., Nobrega,M.A. and Rubin,E.M. (2004) Comparative genomics at the vertebrate extremes. *Nature reviews. Genetics*, **5**, 456–65.

106. Semina,E. V, Datson,N.A., Leysens,N.J., Zabel,B.U., Carey,J.C., Bell,G.I., Bitoun,P., Lindgren,C., Stevenson,T., Frants,R.R., *et al.* (1996) Exclusion of epidermal growth factor and high-resolution physical mapping across the Rieger syndrome locus. *American journal of human genetics*, **59**, 1288–96.

107. Flomen,R.H., Vatcheva,R., Gorman,P.A., Baptista,P.R., Groet,J., Barišić,I., Ligutic,I. and Nižetić,D. (1998) Construction and Analysis of a Sequence-Ready Map in 4q25: Rieger Syndrome Can Be Caused by Haploinsufficiency ofRIEG, but Also by Chromosome Breaks ≈90 kb Upstream of This Gene. *Genomics*, **47**, 409–413.

108. Flomen,R.H., Gorman,P.A., Vatcheva,R., Groet,J., Barisić,I., Ligutić,I., Sheer,D. and Nizetić,D. (1997) Rieger syndrome locus: a new reciprocal translocation t(4;12)(q25;q15) and a deletion del(4)(q25q27) both break between markers D4S2945 and D4S193. *Journal of medical genetics*, **34**, 191–5.

109. Volkmann,B.A., Zinkevich,N.S., Mustonen,A., Schilter,K.F., Bosenko,D. V., Reis,L.M., Broeckel,U., Link,B.A. and Semina,E. V. (2011) Potential novel mechanism for Axenfeld-Rieger syndrome: Deletion of a distant region containing regulatory elements of PITX2. *Investigative Ophthalmology and Visual Science*, **52**, 1450–1459.

110. Suzuki,T., Takahashi,K., Kuwahara,S., Wada,Y., Abe,T. and Tamai,M. (2001) A novel (Pro79Thr) mutation in the FKHL7 gene in a Japanese family with Axenfeld-Rieger syndrome. *American journal of ophthalmology*, **132**, 572–5.

111. Kawase,C., Kawase,K., Taniguchi,T., Sugiyama,K., Yamamoto,T., Kitazawa,Y., Alward,W.L., Stone,E.M., Nishimura,D.Y. and Sheffield,V.C. (2001) Screening for mutations of Axenfeld-Rieger syndrome caused by FOXC1 gene in Japanese patients. *Journal of glaucoma*, **10**, 477–82.

112. Li,D., Zhu,Q., Lin,H., Zhou,N. and Qi,Y. (2008) A novel PITX2 mutation in a Chinese family with Axenfeld-Rieger syndrome. *Molecular vision*, **14**, 2205–10.

113. Yun,J.W., Cho,H.-K., Oh,S.-Y., Ki,C.-S. and Kee,C. (2013) Novel c.300_301delinsT mutation

in PITX2 in a Korean family with Axenfeld-Rieger syndrome. *Annals of laboratory medicine*, **33**, 360–3.

114. Borges,A.S., Susanna,R., Carani,J.C.E., Betinjane,A.J., Alward,W.L., Stone,E.M., Sheffield,V.C. and Nishimura,D.Y. (2002) Genetic analysis of PITX2 and FOXC1 in Rieger Syndrome patients from Brazil. *Journal of glaucoma*, **11**, 51–6.

115. Gripp,K.W., Hopkins,E., Jenny,K., Thacker,D. and Salvin,J. (2013) Cardiac anomalies in Axenfeld-Rieger syndrome due to a novel *FOXC1* mutation. *American Journal of Medical Genetics Part A*, **161**, 114–119.

116. Sacchetti,M., Mantelli,F., Marenco,M., Macchi,I., Ambrosio,O. and Rama,P. (2015) Diagnosis and Management of Iridocorneal Endothelial Syndrome. *BioMed Research International*, **2015**, 1–9.

117. Shields,M.B. (2001) Axenfeld-Rieger and iridocorneal endothelial syndromes: two spectra of disease with striking similarities and differences. *Journal of glaucoma*, **10**, S36-8.

118. Kamińska,A., Sokołowska-Oracz,A., Pawluczyk-Dyjecińska,M. and Szaflik,J.P. (2007) [Variability of clinical manifestations in the family with Axenfeld-Rieger syndrome]. *Klinika oczna*, **109**, 321–6.

119. Kenyon,K.R. (1975) Mesenchymal dysgenesis in Peter's anomaly, sclerocornea and congenital endothelial dystrophy. *Experimental eye research*, **21**, 125–42.

120. Greenberg,D.S., Sunada,Y., Campbell,K.P., Yaffe,D. and Nudel,U. (1994) © 199 4 Nature Publishing Group http://www.nature.com/naturegenetics. *Nature*, **8**, 340–344.

121. Cascella,R., Strafella,C., Germani,C., Novelli,G., Ricci,F., Zampatti,S. and Giardina,E. (2015)

The Genetics and the Genomics of Primary Congenital Glaucoma: EBSCOhost. **2015**.

122. Vincent,A., Billingsley,G., Priston,M., Williams-Lyn,D., Sutherland,J., Glaser,T., Oliver,E., Walter,M. a, Heathcote,G., Levin, a, *et al.* (2001) Phenotypic heterogeneity of CYP1B1: mutations in a patient with Peter's' anomaly. *Journal of medical genetics*, **38**, 324–6.

123. Harissi-Dagher,M. and Colby,K. (2008) Anterior Segment Dysgenesis: Peter's Anomaly and Sclerocornea. *International Ophthalmology Clinics*, **48**, 35–42.

124. Bhandari,R., Ferri,S., Whittaker,B., Liu,M. and Lazzaro,D.R. (2011) Peter's Anomaly: Review of the Literature. *Cornea*, **30**, 939–944.

125. Hingorani,M. and Moore,A. (1993) Aniridia.

126. Ihnatko,R., Eden,U., Fagerholm,P. and Lagali,N. (2016) Congenital Aniridia and the Ocular Surface. *The Ocular Surface*, **14**, 196–206.

127. Weissbart,S.B. and Ayres,B.D. (2016) Management of aniridia and iris defects. *Current Opinion in Ophthalmology*, **27**, 244–249.

128. Kumar,V., Kumar,K., Chanana,B., Rohatgi,J. and Gupta,V.P. (2011) Congenital ectropion uveae with iris coloboma and telecanthus. *Contact Lens and Anterior Eye*, **34**, 147–148.

129. Laaks,D. and Freeman,N. (2013) Congenital iris ectropion uveae presenting with glaucoma in infancy. *Journal of American Association for Pediatric Ophthalmology and Strabismus*, **17**, 214–216.

130. Ziakas,N.G., Chranioti,A., Malamas,A., Leliopoulou,O. and Mataftsi,A. (2014) Congenital ectropion uveae presenting as acute glaucoma in a 3-year-old child. *International Ophthalmology*, **34**, 97–98.

131. Bernardes,C.S., Leite,L.V. de O. and Castro,F.A.A. de [Ectopia lentis et pupillae: case report]. *Arquivos brasileiros de oftalmologia*, **68**, 841–4.

132. Omulecki,W., Wilczynski,M. and Gerkowicz,M. Management of bilateral ectopia lentis et pupillae syndrome. *Ophthalmic surgery, lasers & imaging : the official journal of the International Society for Imaging in the Eye*, **37**, 68–71.

133. Ekonomidis,P., Androudi,S., Brazitikos,P. and Alexandridis,A. (2006) Ectopia lentis et pupillae: report of a unilateral case and surgical management. *Graefe's Archive for Clinical and Experimental Ophthalmology*, **244**, 878–879.

134. Vasconcellos,J.P.C., Melo,M.B., Schimiti,R.B., Bressanim,N.C., Costa,F.F. and Costa,V.P. (2005) A novel mutation in the GJA1 gene in a family with oculodentodigital dysplasia. *Archives of ophthalmology (Chicago, Ill. : 1960)*, **123**, 1422–6.

135. Rishi P, Koundanya VV,S.C. (2015) Using risk factors for detection and prognostication of uveal melanoma. *Indian Journal of Ophthalmology*, **63**, 110–6.

136. Paznekas,W.A., Karczeski,B., Vermeer,S., Lowry,R.B., Delatycki,M., Laurence,F., Koivisto,P.A., Van Maldergem,L., Boyadjiev,S.A., Bodurtha,J.N., *et al.* (2009) *GJA1* mutations, variants, and connexin 43 dysfunction as it relates to the oculodentodigital dysplasia phenotype. *Human Mutation*, **30**, 724–733.

137. Avshalumova,L., Fabrikant,J. and Koriakos,A. (2014) Overview of skin diseases linked to connexin gene mutations. *International Journal of Dermatology*, **53**, 192–205.

138. Sambhara,D. and Aref,A.A. (2014) Glaucoma management: relative value and place in therapy of available drug treatments. *Therapeutic advances in chronic disease*, **5**, 30–43.

139. McKinnon,S.J., Goldberg,L.D., Peeples,P., Walt,J.G. and Bramley,T.J. (2008) Current management of glaucoma and the need for complete therapy. *The American journal of managed care*, **14**, S20-7.

140. Parikh,R.S., Parikh,S.R., Navin,S., Arun,E. and Thomas,R. (2008) Practical approach to medical management of glaucoma. *Indian journal of ophthalmology*, **56**, 223–30.

141. Kanski,J.J. (1968) Carbonic anhydrase inhibitors and osmotic agents in glaucoma. Carbonic anhydrase inhibitors. *The British journal of ophthalmology*, **52**, 642–3.

142. Kawase,K., Vittitow,J.L., Weinreb,R.N., Araie,M. and JUPITER Study Group,F. the J.S. (2016) Long-term Safety and Efficacy of Latanoprostene Bunod 0.024% in Japanese Subjects with Open-Angle Glaucoma or Ocular Hypertension: The JUPITER Study. *Advances in therapy*, **33**, 1612–27.

143. Sihota,R. (2011) Lasers in primary open angle glaucoma. *Indian journal of ophthalmology*, **59 Suppl**, S114-7.

144. Meyer,J.J. and Lawrence,S.D. (2012) What's new in laser treatment for glaucoma? *Current Opinion in Ophthalmology*, **23**, 111–117.

145. Mahdy,M.A.-M.S. (2008) Efficacy and safety of selective laser trabeculoplasty as a primary procedure for controlling intraocular pressure in primary open angle glaucoma and ocular hypertensive patients. *Sultan Qaboos University medical journal*, **8**, 53–8.

146. Shaarawy,T. (2015) Glaucoma surgery: taking the sub-conjunctival route. *Middle East African journal of ophthalmology*, **22**, 53–8.

147. Richter,G.M. and Coleman,A.L. (2016) Minimally invasive glaucoma surgery: current status

and future prospects. *Clinical ophthalmology (Auckland, N.Z.)*, **10**, 189–206.

148. Kammer,J.A. and Mundy,K.M. (2015) Suprachoroidal devices in glaucoma surgery. *Middle East African journal of ophthalmology*, **22**, 45–52.

149. Brandão,L.M. and Grieshaber,M.C. (2013) Update on Minimally Invasive Glaucoma Surgery (MIGS) and New Implants. *Journal of ophthalmology*, **2013**, 705915.

150. Aantaa,R. and Jalonen,J. (2006) Perioperative use of α2-adrenoceptor agonists and the cardiac patient. *European Journal of Anaesthesiology*, **23**, 361–372.

151. Chen,L. and Gage,P.J. (2016) Heterozygous Pitx2 Null Mice Accurately Recapitulate the Ocular Features of Axenfeld-Rieger Syndrome and Congenital Glaucoma. *Investigative ophthalmology & visual science*, **57**, 5023–5030.

152. Liu,D., Cruz,F.M. and Subramanian,P.S. (2012) Bilateral vision loss associated with radiofrequency exposure. *Clinical ophthalmology (Auckland, N.Z.)*, **6**, 2069–73.

153. Shah,B.M., Dada,T., Panda,A., Tanwar,M., Bhartiya,S. and Dada,R. (2014) Novel occurrence of axenfeld: Rieger syndrome in a patient with blepharophimosis ptosis epicanthus inversus syndrome. *Indian journal of ophthalmology*, **62**, 358–60.

Chapter 2. Novel *PITX2* gene mutations in patients with Axenfeld-Rieger syndrome

This chapter was published in:

Seifi M, Footz T, Taylor SA, Elhady GM, Abdalla EM, Walter MA. Novel *PITX2* gene mutations in patients with Axenfeld-Rieger syndrome. Acta Ophthalmol. 94, e571–e579 (2016).

Note: All experiments were carried out by Morteza Seifi except for the *in silico* analysis presented in Figure 4, which was carried out by Dr. Sherry A. M. Taylor. The clinical examination of the patient, presented in Figure 1A-1F, was carried out by Dr. Ebtesam M. Abdalla.

Introduction

Axenfeld-Rieger Syndrome (ARS) is transmitted in an autosomal dominant manner with complete penetrance but variable expressivity. ARS describes a group of genetically heterogeneous disorders that affect multiple organ systems, especially including the anterior segment of the eye (1). Affected individuals manifest a spectrum of anterior segment phenotypes including iridocorneal synechiae, corectopia, iris hypoplasia, polycoria, posterior embryotoxon, abnormal iris strands connecting the iridocorneal angle to the trabecular meshwork, adhesions and a prominent Schwalbe's line (2–4). Patients may also present with systemic extraocular features including dental, jaw, and umbilical anomalies and/or redundant periumbilical skin. Approximately half of patients with ARS develop glaucoma, a progressively blinding condition associated with elevated intraocular pressure, which is the most severe common consequence of ARS. Disease-causing variations have been detected in two known genes, *PITX2* and *FOXC1*. These genes, which play pivotal roles in embryonic development and are responsible for ARS, map to chromosomes 4q25 (5) and 6p25 (6), respectively. *PITX2* is a member of the bicoid-class of homeodomain proteins involved in regulating the development of various tissues of anterior segment and several extraocular tissues such as heart and branchial arches (1, 7). Affected individuals with *PITX2* gene variations are more likely than those with *FOXC1* gene variations to have systemic abnormalities in addition to eye issues (8, 9). In rare cases, *PITX2* variations have also been associated with Peter's syndrome, iris hypoplasia/iridogoniodysgenesis anomaly and ring dermoid of the cornea (10–13).

A wide variety of genetic defects in *PITX2* including splice-site variants, coding region frameshift, nonsense and missense variants have been identified to underlie ARS. These three variants are thought to alter the expression of functional PITX2, leading to abnormal regulation of *PITX2* target genes (3, 11, 13–23). In addition, copy number losses of *PITX2*, because of large chromosome and

genomic rearrangements, small intragenic deletions and a rare duplication have also been reported (1, 23–26). Previous findings demonstrated that dominant negative effects and haploinsufficiency of *PITX2* are also disease-causing ARS mechanisms (1, 25, 27–29). In addition, identification and characterization of a hypermorphic allele of *PITX2* in an ARS patient (30) suggests that there are strict upper and lower thresholds of *PITX2* activity necessary for normal ocular development and function.

In this report, two ARS familial patients (father and son) have been screened for *FOXC1* and *PITX2* variations. A novel deletion involving the coding region of *PITX2* was identified in both patients. The novel deletion spans all known *PITX2* exons as well as one upstream regulatory element. The proband additionally possesses a novel 2-bp deletion in a non-coding exon of the remaining *PITX2* allele, possibly underlying the more severe phenotype in this patient.

Materials and Methods

Clinical report

Patient samples and information were collected with written informed consent. This research adhered to the tenets of the Declaration of Helsinki. The University of Alberta Health Research Ethics Board and the Ethics Committee of Medical Research Institute, University of Alexandria, Egypt approved the use of human subjects in this study. We analysed one family of Egyptian descent in which two patients were affected by ARS (Figure 1a). The father (III-3) is 37 years old and presented with bilateral cataracts in his mid-childhood, complicated later by glaucoma which progressed until he completely lost his vision in his mid 20's. The father also had non-ocular anomalies including dental defects (Figure 1b).

The proband (IV-8, a 4-year-old male patient) was born to consanguineous parents. The pregnancy and delivery history were otherwise uneventful. The proband was born at full term with an

omphalocele that necessitated an immediate surgical intervention. The proband had an average physical growth and normal intellectual and motor developmental milestones. At the age of 2 years, the mother noticed squint and sought ophthalmic assessment, which detected an "Axenfeld Rieger anomaly" (ARA). The proband had abnormal eyes with hypertelorism and microcornea, and a flat midface with a depressed nose. In addition, he had camptodactyly of the right fifth finger, clinodactyly of the left one and bilateral Simian creases (Figure 1b). After a plastic surgery for ankyloglossia, the proband was still unable to protrude his tongue. The proband's abdomen was distended with a surgical scar at the site of the umbilicus. External genitalia were normal and anthropometric measurements were within the normal range. On karyotyping, a normal male karyotype was found. Echocardiography revealed that the proband had atrial and ventricular septal defects (ASD & VSD), while audiogram reported normal hearing. Abdominal ultrasound was unremarkable except for slightly enlarged kidneys. The proband's younger brother (IV-6) who was born with an abnormal umbilicus, suffered from renal troubles and eventually died at the age of 10 months of a nephrotic syndrome. Subsequent urine analysis and renal function tests of the proband detected marked proteinuria for which he also received a regular follow up. Four additional pregnancies were lost in this family due to miscarriages (Figure 1a).

Sequence analysis

Genomic DNA was isolated from peripheral blood leucocytes using the EZ-10 Spin Column Genomic DNA Minipreps Kit (Bio Basic Inc., Markham, ON). *FOXC1* and *PITX2* genes were PCR amplified (Table S1) using the following conditions: denaturation at 95.0˚C for 3:00 followed by 5 cycles of 95.0˚C (0.30 min), 64.0-56.0˚C for 0:30 (2˚C decrease per cycle touchdown), 72.0˚C for 0:30 and then 30 cycles of 95.0˚C (0.30 min), 54.0˚C (0.30 min) and 0:30 min final extension at

72.0˚C. FailSafe buffer J (Epicentre Biotechnologies, Madison, WI) was used in conjunction with Taq polymerase (New England Biolabs, Whitby, ON). PCR products were purified on separation columns (Qiagen Inc. Toronto, ON), and sequenced on a 3130XL Genetic Analyzer at The Applied Genomics Core of the University of Alberta.

Real-Time quantitative PCR

SYBR Green I quantitative Realtime PCR (7900HT Fast Real-Time PCR System, Life Technologies, Burlington, ON) was used to quantify the copy number levels of *PITX2* and its neighbouring genes (Table S2). Primers set efficiencies (E) were determined by standard curve analysis using dilutions of a common reference normal control sample. Relative quantity (RQ) values of each amplicon were determined by $E^{\Delta Ct}$ where ΔCt was calculated as the difference in threshold cycle (Ct) values for the reference sample minus the patient sample. These RQ values were normalized (NRQ) to amplification of the GJA5 gene and the results of three replicates per experiment were averaged. Individual replicates were discarded if the Ct values were >0.5 units away from the median, but amplifications were repeated if two replicates both deviated >0.5 units from the median. Experiments were performed two to three times, depending on the availability of the limited-quantity DNA samples and the consistency of the results. Confidence intervals for normal dosage ranges of the amplicons were established by using DNA from 11-12 non-ARS individuals, and were not further normalized by the normal sample average. Patient results were interpreted as "2 copies" or "non-deleted" if the NRQ value fell within the normal 95% confidence interval (as calculated by Microsoft Excel), but were interpreted as "1 copy" or "deleted" if the NRQ value was lower than the normal interval's minimum.

Splice enhancer prediction

Alamut version 2.3.3 (Interactive Biosoftware, Rouen, France) was used for splice site and exon splice enhancer prediction (http://www.interactive-biosoftware.com/doc/alamut-visual/2.3/splicing.html).

Results

An ARS family was tested for *FOXC1* and *PITX2* variations to provide a possible explanation of the ARS phenotype in this family for genetic counselling purposes. The entire coding region of the single exon *FOXC1* gene was screened, but DNA sequencing detected no sequence variations in the *FOXC1* gene in either of the patients. However, investigation of coding and non-coding regions of the *PITX2* gene by PCR-based sequencing showed that the proband possesses a novel 2-bp deletion in a non-coding exon (exon 2) of *PITX2* (Figure 2, NM_001204397.1 c.-1447_1448delAT). Simultaneously, quantitative genomic PCR was used to assay deletions involving the *PITX2* gene. A novel deletion involving the coding region of *PITX2* was identified in both patients. The novel deletion involved one previously described upstream regulatory element (CE4) (31), *PITX2* and a minimum of 13 known human genes (Figure 3). The genes tested by qPCR are shown in Table 1. The qPCR amplicons for CE4 and upstream regulatory element 2 (USE2) are 106,587bp and 208,856bp away from *PITX2* exon 1, respectively. Since CE4 (but not USE2) is deleted in the patients, one deletion breakpoint lies between CE4 and USE2. qPCR primers for COL25A1 and DKK2 are located 1,315,327 and 3,581,127bp away from *PITX2*, respectively. As COL25A1 (but not DKK2) is deleted in the ARS patients, the other breakpoint of the deletion is located between these two amplicons. Therefore, the maximum size of the deletion is estimated to be 3,789,983bp. The minimum size of the deletion is 1,421,914bp in which reside the following 14 genes: *PITX2*, *ENPEP, ELOVL6, EGF, LRIT3, RRH, GAR1, CFI, PLA2G12A, CASP6, CCDC109B, SEC24B, SEC24B-AS1, COL25A1*

(Figure 3). Both ARS patients thus have only one copy of the *PITX2* gene in which the proband also has a novel 2-bp deletion. The 2-bp deletion lies within the non-coding *PITX2* exon 2. *In silico* analysis, however, indicate this variant is predicted to create additional splice enhancer sites and thus may alter splicing of the *PITX2* mRNA (Figure 4). In addition to these novel variations of the *PITX2* gene a previously recorded common SNP was also discovered, present hemizygously in the *PITX2* exon 2 of both patients (rs2739200).

Discussion

Variations in a number of transcription factors that are mandatory for organogenesis exhibit semi-dominant inheritance in humans and mice due to haploinsufficiency (32–35). Heterozygotes display variable, but less severe, phenotypes than null homozygotes because the presence of one functional allele cannot fully compensate for the loss of function of the other allele. This indicates that the function of these transcription factors is sensitive to gene dosage. The correct expression and dosage of *FOXC1* and *PITX2* are fundamental for the development of different tissues. The observation of disparate variations such as interstitial duplications and deletions of the *FOXC1* and *PITX2* genes in patients with ARS indicates the importance of stringent control of these genes' expression levels and activities for embryogenesis and, in particular, for the normal development of the skeletal, cardiovascular, urogenital, and ocular tissues (23, 36–38). Accordingly, any variation resulting in an increase or decrease in *FOXC1* or PITX2 expression levels would likely lead to ARS.

FOXC1 alterations are detected mainly in patients with isolated ARS or ARS with heart and hearing defects whereas *PITX2* variations often appear in patients with defects of nonocular tissues, such as teeth and umbilical anomalies (8, 39). The occurrences of systemic changes in the patients in this study who were subsequently found to have *PITX2* variants are consistent with these observations.

As mentioned in chapter 1, FOXC1 and PITX2 are functionally and physically interconnected (40). PITX2 is able to negatively regulate the transcriptional activation potential of FOXC1, and such regulation is thought to underlie sensitivity to FOXC1 dosage in the eye (40). As a result, variants leading to deletion in *PITX2* or duplication in *FOXC1* display similar phenotypes. In our study, real-time qPCR analysis identified that the entire *PITX2* gene is deleted in both patients. Therefore, this deletion of *PITX2* is expected to not only lead to reduction of *PITX2* target gene expression, but also to disrupt the regulation of *FOXC1* and its target genes. This complex regulatory disruption may contribute to the severity of the ocular phenotype in our subjects.

The clinical manifestations of our proband, however, are unusual and atypical of ARS. In particular, in addition to ocular abnormalities often observed in ARS patients including the proband's father, the proband presented with clinodactyly, ankyloglossia and marked proteinuria not typically observed in patients with *PITX2* deletions. The family also experienced the unexpected loss of four pregnancies, and the postnatal death of the proband's sibling, raising the possibility that the reported consanguinity of the parents has resulted in comorbid disease in the proband and his siblings distinct from ARS and the *PITX2* genotype. Quantitative genomic PCR showed that the entirety of the *PITX2* gene is deleted in both the proband and his father. Genomic analysis, however, identified an additional 2-bp deletion of unknown clinical significance within the remaining *PITX2* allele in the proband, as well as one previously reported SNP involving the *PITX2* non-coding region in both patients. Therefore, the proband is a compound heterozygote for two novel *PITX2* gene variants.

The second variant, present in the severely affected proband but not his father who presents with typical ARS, is a 2-bp deletion (NM_001204397.1 (*PITX2*), c.-1448_1447delAT). This variant is located in *PITX2* non-coding exon 2, which encodes a portion of the 5' untranslated region of the gene. This novel variant has not been previously described in the literature or variation databases.

While the possibility that it is a rare neutral variant cannot be ruled out, *in silico* analysis suggests potential functional consequences. The location of the 2-bp deletion borders a phylogenetically conserved portion of the *PITX2* 5'UTR (PhastCon Score maximum 1.0) and is close to one end of the exon which increases the likelihood of a functional impact. While this variant does not appear to alter consensus splice sites it does create two additional binding sites for the splice enhancers SF2/ASF (SRSF1) and SRp40 (Figure 4). Enhanced binding of these proteins could alter processing of *PITX2* mRNA and protein expression. The ability to respond to glucocorticoids in a cellular model of glaucoma showed that common SNPs in the glucocorticoid receptor gene correlated with differences in the binding of SRp40 (41). The result was alternative splicing of the glucocorticoid receptor, which affected its ability to bind glucocorticoids and transactivate responsive genes, and which was postulated to underlie variation in response to glucocorticoid treatment. SRSF1 is a protooncogene that regulates both pre-mRNA splicing and translation. SRSF1 has been implicated in cancer and alternative splicing in other human conditions (42). The novel 2-bp deletion is predicted to alter regulation of splicing of all *PITX2* mRNA isoforms, thus affecting the development of the wide range of tissues normally regulated by *PITX2*. This 2-bp deletion, nevertheless, must be classified as a novel variant of unknown significance until additional experiments or family studies can be performed to further evaluate the potential functional consequences.

It is also plausible that the presence of atypical clinical findings of ARS in the proband is due to deletion of genes neighbouring *PITX2*. As mentioned previously, the minimum and maximum sizes of the deletion are estimated to be 1,421,914bp and 3,789,983bp, respectively. The minimum size of the deletion, in addition to the removal of one upstream *PITX2* regulatory element (CE4) and all *PITX2* exons, also includes the deletion of 13 neighbouring genes. While human phenotypes have not been attributed to the most of these additional deleted genes, one of the neighbouring genes

(ENPEP) has been associated with human disease. Analyses of the frequency of variations in the aminopeptidase A encoding gene (ENPEP) in patients with proteinuric disease focal and segmental glomerulosclerosis (FSGS) revealed no obvious connection between variants in the ENPEP and FSGS, but some variants of ENPEP disrupt the aminopeptidase A (APA) activity, suggesting that ENPEP genetic variations may contribute to the development of renal disorders and increased susceptibility to glomerular damage (43). In addition, a large number of studies revealed that the APA protein elevates the risk of experimental acute proteinuria (44–48). Therefore, it is possible that the severe renal phenotype observed in some members of this family could be in part due to haploinsufficiency of ENPEP, with variable expressivity being the reason underlying the absence of atypical ARS features in the father (who also carries this deletion). However, there is no evidence connecting any of the deleted genes with either the omphalocele or digital findings. It is possible that the deletion results in abnormal proximity of *PITX2* regulatory regions to genes downstream of *PITX2*. Volkmann et al in 2011 identified thirteen conserved non-coding regions located within 1.1 Mb upstream of the *PITX2* gene. Further analysis showed that these conserved regions have enhancer activities consistent with zebrafish *pitx2* expression and therefore play important roles in the development of different organs (31). Ectopic expression resulting from position effects is a well-studied genetic phenomenon and is among an emerging group of disease-causing mechanisms (49–52). For instance, in patients with holoprosencephaly spectrum disorder and severe upper limb syndactyly with lower limb synpolydactyly, a large-scale intrachromosomal rearrangement places the sonic hedgehog (*SHH*) transcription unit near a limb bud enhancer and, as a result, leads to driving ectopic *SHH* expression (53). Similarly, in patients effected with adult-onset demyelinating leukodystrophy, a large heterozygous deletion has been shown to destroy a domain boundary which

allows heterologous forebrain-specific enhancers to inappropriately interact with the lamin-B1 (LMNB1) promoter (54).

It also remains a possibility that the more severe phenotype in the proband, plus the miscarriages and the death of a brother, might be caused by variations in other recessive genes in the consanguineous family. Future genomic screening, by whole exomic sequencing or homozygosity mapping, of the proband's DNA might allow detection of additional loci that have a role in the proband's phenotype. Unfortunately, access to the family to obtain additional samples for further study are complicated by geopolitical issues.

In summary, the atypical and severe phenotypes observed in our proband and his family have not been previously reported in ARS patients with *PITX2* variations, and have not been previously associated with deletions of the neighbouring genes also found deleted in our study (Table 2). Thus, it is possible that the 2-bp deletion in the proband, when compounded with the deletion of entire *PITX2* coding region on the second allele, caused the atypical and severe clinical manifestation not observed in his father who carries only the large deletion of *PITX2*. This atypical ARS phenotype in the proband has striking similarities with *Pitx2* null mice (55). This is the first study reporting on a human patient with compound heterozygous changes of *PITX2* potentially contributing to a more severe ARS phenotype.

Figure 1. Family with Axenfeld-Rieger Syndrome (ARS) and additional clinical features. A. Pedigree of a consanguineous family with Axenfeld-Rieger syndrome and additional clinical features. The asterisks show subjects who underwent clinical and molecular analysis. The arrow indicates the proband. B. Photographs of father (37 years, upper Figures) and son (4 years, lower Figures) with ARS, demonstrating the characteristic facial features (a, b, d) and dental defects (c, e). In addition, the son (proband) presents with camptodactyly of the right fifth finger (f).

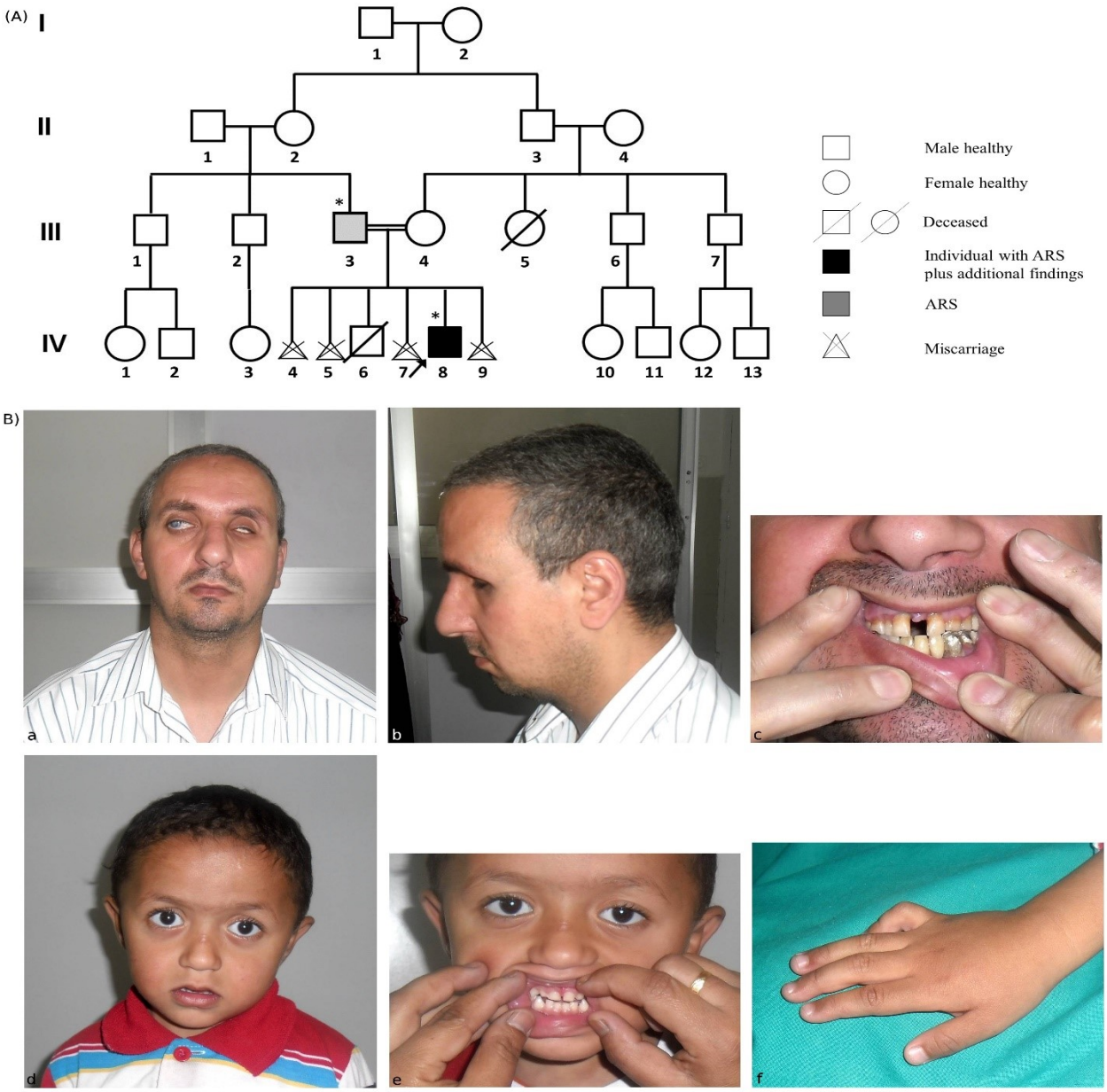

(A)
I
II
III
IV
Male healthy
Female healthy
Deceased
Individual with ARS plus additional findings
ARS
Miscarriage
B)
a
b
c
d
e
f

Figure 2. Detection of a novel 2-bp deletion in the proband by direct sequencing analysis of the noncoding region of *PITX2*. The lower sequence shows the non-heterozygous (hemizygous) 2-bp deletion variation (NM_001204397.1 c.-1447_1448delAT) identified in the proband, and the upper sequence is the corresponding wild-type sequence.

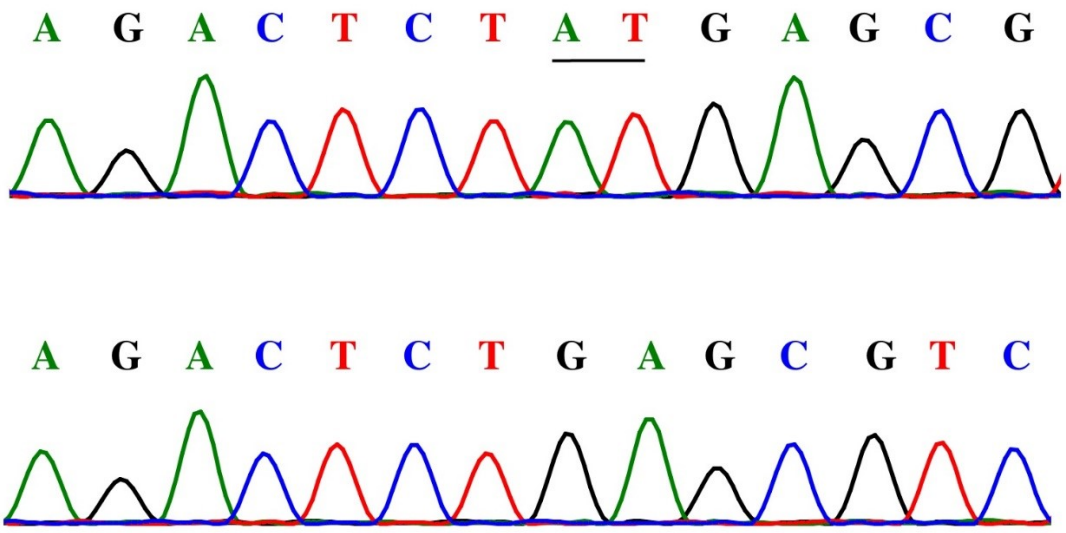

A G A C T C T A T G A G C G
A G A C T C T G A G C G T C

Figure 3. The size of the large chromosome 4q25 deletion detected in the ARS family, with its minimal and maximal boundaries. The orientation and names of the genes within this deletion are depicted (not to scale). A panel representing the exon structure of the *PITX2* gene is shown underneath with the position of 2-bp deletion. The red boxes show the coding regions and the purple boxes represent the non-coding regions.

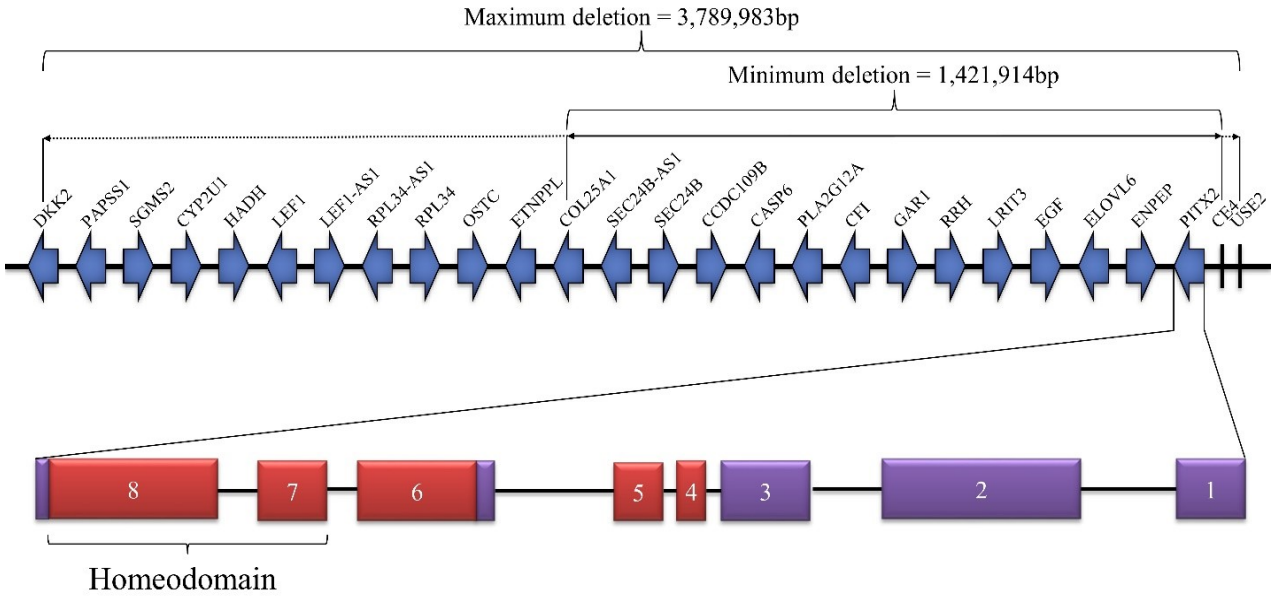

Maximum deletion = 3,789,983bp
Minimum deletion = 1,421,914bp
DKK2
PAPSS1
SGMS2
CYP2U1
HADH
LEF1
LEF1-AS1
RPL34-AS1
RPL34
OSTC
ETNPPL
COL25A1
SEC24B-AS1
SEC24B
CCDC109B
CASP6
PLA2G12A
CFI
GAR1
RRH
LRIT3
EGF
ELOVL6
ENPEP
PITX2
CE4
USE2
8
7
6
5
4
3
2
1
Homeodomain

Figure 4. Position of the 2-bp deletion of *PITX2* and *in silico* prediction of the consequences. Possible effects of 2-bp deletion were predicted using Alamut Visual 2.3.3 (Interactive Biosoftware, Rouen, France). The 2-bp deletion is predicted to produce two additional binding sites for the splice enhancers SF2/ASF (SRSF1) and SRp40. The red boxes show the coding regions and the purple boxes represent the non-coding regions.

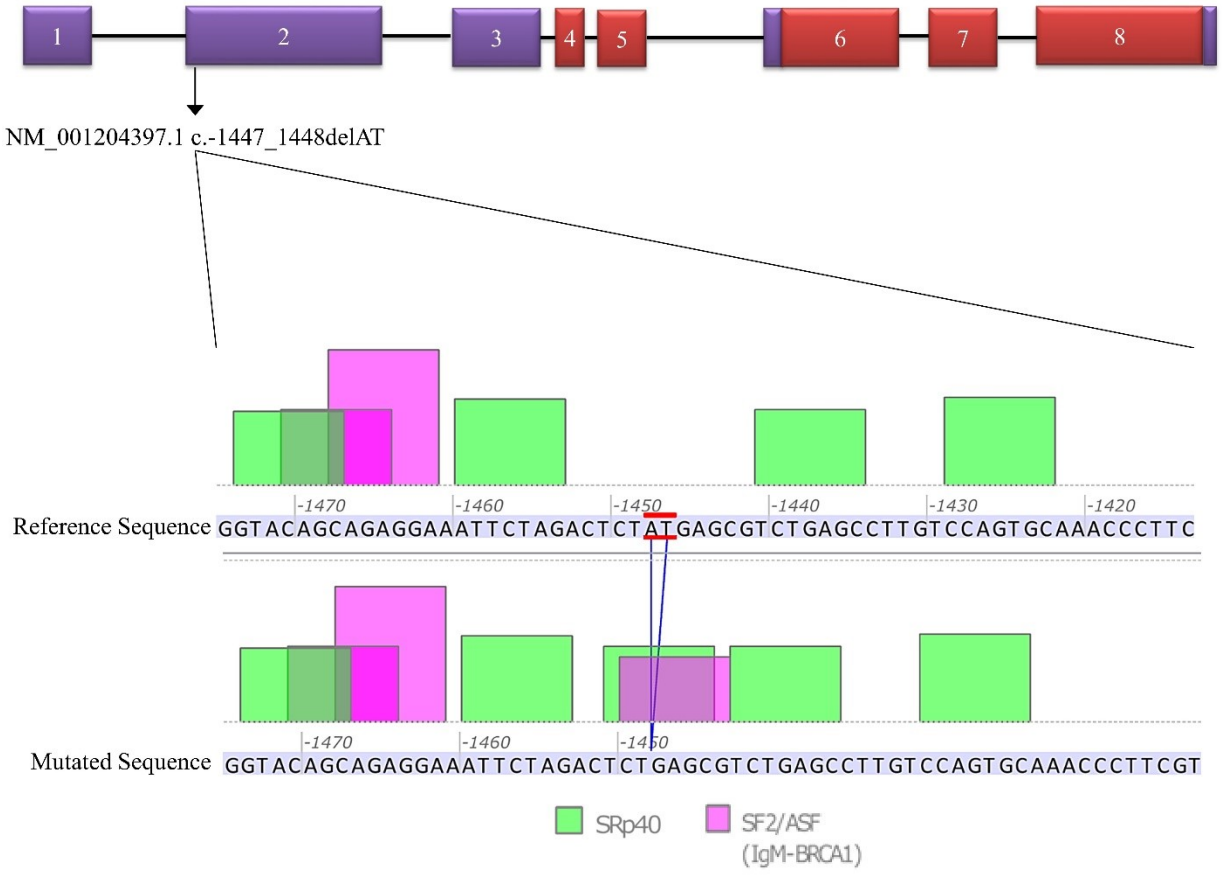

1
2
3
4
5
6
7
8
NM_001204397.1 c.-1447_1448delAT
-1470
-1460
-1450
-1440
-1430
-1420
Reference Sequence
GGTACAGCAGAGGAAATTCTAGACTCTATGAGCGTCTGAGCCTTGTCCAGTGCAAACCCTTC
-1470
-1460
-1450
Mutated Sequence
GGTACAGCAGAGGAAATTCTAGACTCTGAGCGTCTGAGCCTTGTCCAGTGCAAACCCTTCGT
SRp40
SF2/ASF
(IgM-BRCA1)

Table 1. qPCR analysis for *PITX2* and neighbouring genes.

		PITX2 USE2	*PITX2* CE4	*PITX2* ex2	*PITX2* ex8	ENPEP	ELOVL6	CASP6	SEC24B	COL25A1	DKK2
Controls	Mean NRQ	1.10	1.12	1.13	1.08	1.27	1.11	1.29	1.37	1.37	1.04
	95% Confidence	0.05	0.07	0.06	0.06	0.15	0.13	0.17	0.17	0.14	0.12
	2-copy Minimum NRQ	1.05	1.05	1.07	1.02	1.12	0.98	1.12	1.20	1.23	0.93
	2-copy Maximum NRQ	1.15	1.19	1.19	1.15	1.42	1.24	1.47	1.54	1.52	1.16
Patient	Patient average NRQ	1.10	0.49	0.54	0.48	0.55	0.56	0.36	0.25	0.71	0.99
	Within 2-copy interval?	Yes	No	No	No	No	No	No	No	No	Yes
	Interpretation	Normal	Deleted	Deleted	Deleted	Deleted	Deleted	Deleted	Deleted	Deleted	Normal

NRQ, normalized relative quantity

Table 2. Summary of chromosome rearrangements involving *PITX2*.

Reference	Rearrangement	Expected size or predicted effect	Main Clinical phenotypes
Reis et al, 2012 (9)	deletion: 4q25-q26 deletion: 4q25 deletion: 4q25-q28.2 deletion: (extent ND) deletion: (extent ND) deletion: distant upstream region (extent ND)	6.4Mb 1.1Mb 19.2Mb *PITX2* and other genes ND *PITX2* and other genes ND Regulatory region of *PITX2* and other genes	*Ocular features:* ARS, GL *Extraocular features:* DA, umbilicus, ventricular septal defect, hearing loss
Strehle et al, 2012 (56)	deletion: 111,310,828-130,503,896	19.2 Mb	*Ocular features:* ARS *Extraocular features:* craniofacial, digital, cardiovascular, gastrointestinal/urogenital, hearing impairment
Volkmann et al, 2011 (31)	deletion: 4q25-q26 region	7645 kb	*Ocular features:* ARS *Extraocular features:* thin upper lip, broad nasal bridge, DA, RU
Moreira et al, 2010 (57)	deletion: 4q25-q31	28MB	Ocular *features:* ARS, open anterior fontanelle, short and downslanting palpebral fissures, iris coloboma, microcornea, GL *Extraocular features:* flat face, hypertelorism, broad bulbous nose, short upper lip, high arched palate, mild micrognathia, low set posteriorly angulated ears, low posterior hairline, DA, bilateral transverse palmar creases, fifth ray clinodactyly and mild valgus deformity of the feet
Tanwar et al, 2009 (58)	deletion: 4q25-q27	ND	*Ocular features:* ARS *Extraocular features:* receding upper lip, DA, RU
D'haene et al, 2011 (23)	111760308–111761945 111648252–111934227 111161726–112223083 110200973–112725989 110322934–113076304	1.6 kb 286 kb 1.1 Mb 2.5 Mb 2.8 Mb	*Ocular features*: ARS, unilateral microcornea, atrophic iris, unilateral polycoria, dyscoria, Fuchs endothelial dystrophy
Engenheiro et al, 2007 (59)	translocation: t(4;17)(q25;q22) with a	1.9 Mb	*Ocular features:* ARS, deep anterior chambers *Extraocular features*: short philtrum

	deletion at the 4q breakpoint deletion: 4q25	550-950 kb	
Kamnasaran et al, 2003 (60)	deletion of exons 6 and 7 of *PITX2*	3.9 kb	*Extraocular features:* microcephaly, agenesis of the corpus callosum
Becker et al, 2003 (61)	deletion: 4q25-q27	ND	*Ocular features:* ARA *Extraocular features:* atrial septal defect, inclination of the 5th finger on the left hand, umbilical hernia, muscular hypotonia, dysmorphic facial features
Ogilvie et al, 1998 (62)	der(3,4,10,17)t(3; 10;4) (p22.2;q 11.22;q25)ins(17;3) (q25.3;p24.2p22.2)?t(10; 17) (q26.3;q25.3).	ND	*Ocular features:* cataract, coloboma *Extraocular features*: DA
Flomen et al, 1997 (63)	deletion: 4q23-27 t(4; 12)(q25;ql 5)	ND	*Ocular features:* ARS, microcornea, coloboma *Extraocular features:* DA, hypertelorism, dysplastic ears, hypoplasia
Schinzel et al, 1997 (64)	deletion: 4q25-q27	ND	*Ocular features:* ectopic pupils, alternate divergent squint, bilateral microcornea, microphthalmia, hypoplastic anterior chamber, greyish, hypoplastic irides *Extraocular features:* motor and mental retardation, DA
Kulharya et al, 1995 (65)	deletion: 4q21.1-q25 deletion: 4q25-q2	ND ND	*Extraocular features*: craniofacial and skeletal anomalies, hydrocephalus, congenital hypotonia and developmental delay
Vaux et al, 1992 (5)	deletion: 4q25-q27	ND	*Ocular features:* ARA *Extraocular features:* seizures, hearing loss
Raczenbek et al, 1991 (66)	deletion: 4q25-q27	ND	*Extraocular features*: marked hypotonia, cardiac abnormalities, cleft palate, micrognathia
Mitchell et al, 1981 (67)	deletion: 4q21.1-q25 deletion: 4q21.3 - q26 deletion: 4q27 - q31.3 terminal deletions 4q31-qter terminal deletion (4q33-qter)	ND ND ND ND ND	*Ocular features*: ARS, ARA

Abbreviations: ARS, Axenfeld-Rieger syndrome; ARA, Axenfeld-Rieger anomaly; DA, dental anomaly; GL, glaucoma, ND, not determined; RU, redundant periumbilical skin.

References

1. Semina,E. V., Reiter,R., Leysens,N.J., Alward,W.L.M., Small,K.W., Datson,N.A., Siegel-Bartelt,J., Bierke-Nelson,D., Bitoun,P., Zabel,B.U., *et al.* (1996) Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nature Genetics*, **14**, 392–399.

2. Lines,M.A., Kozlowski,K. and Walter,M.A. (2002) Molecular genetics of Axenfeld-Rieger malformations. *Human molecular genetics*, **11**, 1177–84.

3. Idrees,F., Bloch-Zupan,A., Free,S.L., Vaideanu,D., Thompson,P.J., Ashley,P., Brice,G., Rutland,P., Bitner-Glindzicz,M., Khaw,P.T., *et al.* (2006) A novel homeobox mutation in the PITX2 gene in a family with Axenfeld-Rieger syndrome associated with brain, ocular, and dental phenotypes. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, **141B**, 184–191.

4. Sowden,J.C. (2007) Molecular and developmental mechanisms of anterior segment dysgenesis. *Eye (London, England)*, **21**, 1310–8.

5. Vaux,C., Sheffield,L., Keith,C.G. and Voullaire,L. (1992) Evidence that Rieger syndrome maps to 4q25 or 4q27. *Journal of medical genetics*, **29**, 256–8.

6. Mirzayans,F., Gould,D.B., Héon,E., Billingsley,G.D., Cheung,J.C., Mears,A.J. and Walter,M.A. (2000) Axenfeld-Rieger syndrome resulting from mutation of the FKHL7 gene on chromosome 6p25. *European journal of human genetics : EJHG*, **8**, 71–4.

7. Gage,P.J. and Camper,S.A. (1997) Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. *Human*

Molecular Genetics, **6**, 457–464.

8. Hjalt,T.A., Semina,E. V, Amendt,B.A. and Murray,J.C. (2000) The Pitx2 protein in mouse development. *Developmental dynamics : an official publication of the American Association of Anatomists*, **218**, 195–200.

9. Reis,L.M., Tyler,R.C., Volkmann Kloss,B.A., Schilter,K.F., Levin,A. V, Lowry,R.B., Zwijnenburg,P.J.G., Stroh,E., Broeckel,U., Murray,J.C., *et al.* (2012) PITX2 and FOXC1 spectrum of mutations in ocular syndromes. *European journal of human genetics : EJHG*, **20**, 1224–33.

10. Alward,W.L., Semina,E. V, Kalenak,J.W., Héon,E., Sheth,B.P., Stone,E.M. and Murray,J.C. (1998) Autosomal dominant iris hypoplasia is caused by a mutation in the Rieger syndrome (RIEG/PITX2) gene. *American journal of ophthalmology*, **125**, 98–100.

11. Kulak,S.C., Kozlowski,K., Semina,E. V, Pearce,W.G. and Walter,M.A. (1998) Mutation in the RIEG1 gene in patients with iridogoniodysgenesis syndrome. *Human molecular genetics*, **7**, 1113–7.

12. Doward,W., Perveen,R., Lloyd,I.C., Ridgway,A.E., Wilson,L. and Black,G.C. (1999) A mutation in the RIEG1 gene associated with Peter's' anomaly. *Journal of medical genetics*, **36**, 152–5.

13. Xia,K., Wu,L., Liu,X., Xi,X., Liang,D., Zheng,D., Cai,F., Pan,Q., Long,Z., Dai,H., *et al.* (2004) Mutation in PITX2 is associated with ring dermoid of the cornea. *Journal of medical genetics*, **41**, e129.

14. Wang,Y., Zhao,H., Zhang,X. and Feng,H. (2003) Novel identification of a four-base-pair

deletion mutation in PITX2 in a Rieger syndrome family. *Journal of dental research*, **82**, 1008–12.

15. Brooks,B.P., Moroi,S.E., Downs,C.A., Wiltse,S., Othman,M.I., Semina,E. V and Richards,J.E. (2004) A novel mutation in the PITX2 gene in a family with Axenfeld-Rieger syndrome. *Ophthalmic genetics*, **25**, 57–62.

16. Lines,M.A., Kozlowski,K., Kulak,S.C., Allingham,R.R., He´on,E., Ritch,R., Levin,A. V., Shields,M.B., Damji,K.F., Newlin,A., *et al.* (2004) Characterization and Prevalence of *PITX2* Microdeletions and Mutations in Axenfeld-Rieger Malformations. *Investigative Opthalmology & Visual Science*, **45**, 828.

17. Idrees,F., Vaideanu,D., Fraser,S.G., Sowden,J.C. and Khaw,P.T. (2006) A review of anterior segment dysgeneses. *Survey of ophthalmology*, **51**, 213–31.

18. Kniestedt,C., Taralczak,M., Thiel,M.A., Stuermer,J., Baumer,A. and Gloor,B.P. (2006) A novel PITX2 mutation and a polymorphism in a 5-generation family with Axenfeld-Rieger anomaly and coexisting Fuchs' endothelial dystrophy. *Ophthalmology*, **113**, 1791.e1-8.

19. Maciolek,N.L., Alward,W.L.M., Murray,J.C., Semina,E. V and McNally,M.T. (2006) Analysis of RNA splicing defects in PITX2 mutants supports a gene dosage model of Axenfeld-Rieger syndrome. *BMC medical genetics*, **7**, 59.

20. Saadi,I., Toro,R., Kuburas,A., Semina,E., Murray,J.C. and Russo,A.F. (2006) An unusual class of PITX2 mutations in Axenfeld-Rieger syndrome. *Birth defects research. Part A, Clinical and molecular teratology*, **76**, 175–81.

21. Vieira,V., David,G., Roche,O., de la Houssaye,G., Boutboul,S., Arbogast,L., Kobetz,A.,

Orssaud,C., Camand,O., Schorderet,D.F., *et al.* (2006) Identification of four new PITX2 gene mutations in patients with Axenfeld-Rieger syndrome. *Molecular vision*, **12**, 1448–60.

22. Weisschuh,N., Dressler,P., Schuettauf,F., Wolf,C., Wissinger,B. and Gramer,E. (2006) Novel Mutations of FOXC1 and PITX2 in Patients with Axenfeld-Rieger Malformations. *Investigative Opthalmology & Visual Science*, **47**, 3846.

23. D'haene,B., Meire,F., Claerhout,I., Kroes,H.Y., Plomp,A., Arens,Y.H., de Ravel,T., Casteels,I., De Jaegere,S., Hooghe,S., *et al.* (2011) Expanding the spectrum of FOXC1 and PITX2 mutations and copy number changes in patients with anterior segment malformations. *Investigative ophthalmology & visual science*, **52**, 324–33.

24. Perveen,R., Lloyd,I.C., Clayton-Smith,J., Churchill,A., van Heyningen,V., Hanson,I., Taylor,D., McKeown,C., Super,M., Kerr,B., *et al.* (2000) Phenotypic variability and asymmetry of Rieger syndrome associated with PITX2 mutations. *Investigative ophthalmology & visual science*, **41**, 2456–60.

25. de la Houssaye,G., Bieche,I., Roche,O., Vieira,V., Laurendeau,I., Arbogast,L., Zeghidi,H., Rapp,P., Halimi,P., Vidaud,M., *et al.* (2006) Identification of the first intragenic deletion of the PITX2 gene causing an Axenfeld-Rieger Syndrome: case report. *BMC medical genetics*, **7**, 82.

26. Gripp,K.W., Hopkins,E., Jenny,K., Thacker,D. and Salvin,J. (2013) Cardiac anomalies in Axenfeld-Rieger syndrome due to a novel FOXC1 mutation. *American Journal of Medical Genetics Part A*, **161**, 114–119.

27. Saadi,I., Semina,E. V, Amendt,B.A., Harris,D.J., Murphy,K.P., Murray,J.C. and Russo,A.F.

(2001) Identification of a dominant negative homeodomain mutation in Rieger syndrome. *The Journal of biological chemistry*, **276**, 23034–41.

28. Saadi,I., Kuburas,A., Engle,J.J. and Russo,A.F. (2003) Dominant negative dimerization of a mutant homeodomain protein in Axenfeld-Rieger syndrome. *Molecular and cellular biology*, **23**, 1968–82.

29. Martin,D.M., Skidmore,J.M., Philips,S.T., Vieira,C., Gage,P.J., Condie,B.G., Raphael,Y., Martinez,S. and Camper,S.A. (2004) PITX2 is required for normal development of neurons in the mouse subthalamic nucleus and midbrain. *Developmental biology*, **267**, 93–108.

30. Priston,M., Kozlowski,K., Gill,D., Letwin,K., Buys,Y., Levin,A. V, Walter,M.A. and Héon,E. (2001) Functional analyses of two newly identified PITX2 mutants reveal a novel molecular mechanism for Axenfeld-Rieger syndrome. *Human molecular genetics*, **10**, 1631–8.

31. Volkmann,B.A., Zinkevich,N.S., Mustonen,A., Schilter,K.F., Bosenko,D. V, Reis,L.M., Broeckel,U., Link,B.A. and Semina,E. V (2011) Potential novel mechanism for Axenfeld-Rieger syndrome: deletion of a distant region containing regulatory elements of PITX2. *Investigative ophthalmology & visual science*, **52**, 1450–9.

32. Glaser,T., Jepeal,L., Edwards,J.G., Young,S.R., Favor,J. and Maas,R.L. (1994) PAX6 gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects. *Nature genetics*, **7**, 463–71.

33. Sheng,H.Z., Moriyama,K., Yamashita,T., Li,H., Potter,S.S., Mahon,K.A. and Westphal,H. (1997) Multistep control of pituitary organogenesis. *Science (New York, N.Y.)*, **278**, 1809–12.

34. Smith,R.S., Zabaleta,A., Kume,T., Savinova,O. V, Kidson,S.H., Martin,J.E., Nishimura,D.Y.,

Alward,W.L., Hogan,B.L. and John,S.W. (2000) Haploinsufficiency of the transcription factors FOXC1 and FOXC2 results in aberrant ocular development. *Human molecular genetics*, **9**, 1021–32.

35. Zou,C. and Levine,E.M. (2012) Vsx2 controls eye organogenesis and retinal progenitor identity via homeodomain and non-homeodomain residues required for high affinity DNA binding. *PLoS genetics*, **8**, e1002924.

36. Werner,W., Kraft,S., Callen,D.F., Bartsch,O. and Hinkel,G.K. (1997) A small deletion of 16q23.1-->16q24.2 [del(16)(q23.1q24.2).ish del(16)(q23.1q24.2)(D16S395+, D16S348-, P5432+)] in a boy with iris coloboma and minor anomalies. *American journal of medical genetics*, **70**, 371–6.

37. Lehmann,O.J., Ebenezer,N.D., Ekong,R., Ocaka,L., Mungall,A.J., Fraser,S., McGill,J.I., Hitchings,R.A., Khaw,P.T., Sowden,J.C., *et al.* (2002) Ocular developmental abnormalities and glaucoma associated with interstitial 6p25 duplications and deletions. *Investigative ophthalmology & visual science*, **43**, 1843–9.

38. Chanda,B., Asai-Coakwell,M., Ye,M., Mungall,A.J., Barrow,M., Dobyns,W.B., Behesti,H., Sowden,J.C., Carter,N.P., Walter,M.A., *et al.* (2008) A novel mechanistic spectrum underlies glaucoma-associated chromosome 6p25 copy number variation. *Human molecular genetics*, **17**, 3446–58.

39. Reis,L.M., Tyler,R.C., Volkmann Kloss,B.A., Schilter,K.F., Levin,A. V, Lowry,R.B., Zwijnenburg,P.J.G., Stroh,E., Broeckel,U., Murray,J.C., *et al.* (2012) PITX2 and FOXC1 spectrum of mutations in ocular syndromes. *European Journal of Human Genetics*, **20**, 1224–1233.

40. Berry,F.B., Lines,M.A., Oas,J.M., Footz,T., Underhill,D.A., Gage,P.J. and Walter,M.A. (2006) Functional interactions between FOXC1 and PITX2 underlie the sensitivity to FOXC1 gene dose in Axenfeld-Rieger syndrome and anterior segment dysgenesis. *Human molecular genetics*, **15**, 905–19.

41. Jain,A., Wordinger,R.J., Yorio,T. and Clark,A.F. (2012) Spliceosome protein (SRp) regulation of glucocorticoid receptor isoforms and glucocorticoid response in human trabecular meshwork cells. *Investigative ophthalmology & visual science*, **53**, 857–66.

42. Maslon,M.M., Heras,S.R., Bellora,N., Eyras,E. and Cáceres,J.F. (2014) The translational landscape of the splicing factor SRSF1 and its role in mitosis. *eLife*, **3**, e02028.

43. Tonna,S., Dandapani,S. V, Uscinski,A., Appel,G.B., Schlöndorff,J.S., Zhang,K., Denker,B.M. and Pollak,M.R. (2008) Functional genetic variation in aminopeptidase A (ENPEP): lack of clear association with focal and segmental glomerulosclerosis (FSGS). *Gene*, **410**, 44–52.

44. Mentzel,S., Assmann,K.J., Dijkman,H.B., de Jong,A.S., van Son,J.P., Wetzels,J.F. and Koene,R.A. (1996) Inhibition of aminopeptidase A activity causes an acute albuminuria in mice: an angiotensin II-mediated effect? *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*, **11**, 2163–9.

45. Mentzel,S., van Son,J.P., Dijkman,H.B., Wetzels,J.F. and Assmann,K.J. (1999) Induction of albuminuria in mice: synergistic effect of two monoclonal antibodies directed to different domains of aminopeptidase A. *Kidney international*, **55**, 1335–47.

46. Gerlofs-Nijland,M.E., Assmann,K.J., Dijkman,H.B., Dieker,J.W., van Son,J.P., Mentzel,S., van

Kats,J.P., Danser,A.H., Smithies,O., Groenen,P.J., *et al.* (2001) Albuminuria in mice after injection of antibodies against aminopeptidase A: role of angiotensin II. *Journal of the American Society of Nephrology : JASN*, **12**, 2711–20.

47. Gerlofs-Nijland,M.E., Assmann,K.J.M., van Son,J.P.H.F., Dijkman,H.B.P.M., te Loeke,N.A.J.M., van der Zee,R., Wetzels,J.F.M. and Groenen,P.J.T.A. (2003) Epitope mapping of monoclonal antibodies directed to aminopeptidase A and their relevance for albuminuria in mice. *Nephron. Experimental nephrology*, **94**, e25-34.

48. Dijkman,H.B.P.M., Gerlofs-Nijland,M.E., van der Laak,J.A.W.M., Wetzels,J.F.M., Groenen,P.J.T.A. and Assmann,K.J.M. (2003) Podocyte changes after induction of acute albuminuria in mice by anti-aminopeptidase A mAb. *Nephron. Experimental nephrology*, **94**, e85-93.

49. Wilson,C., Bellen,H.J. and Gehring,W.J. (1990) Position effects on eukaryotic gene expression. *Annual review of cell biology*, **6**, 679–714.

50. Spielmann,M., Brancati,F., Krawitz,P.M., Robinson,P.N., Ibrahim,D.M., Franke,M., Hecht,J., Lohan,S., Dathe,K., Nardone,A.M., *et al.* (2012) Homeotic arm-to-leg transformation associated with genomic rearrangements at the PITX1 locus. *American journal of human genetics*, **91**, 629–35.

51. Ibn-Salem,J., Köhler,S., Love,M.I., Chung,H.-R., Huang,N., Hurles,M.E., Haendel,M., Washington,N.L., Smedley,D., Mungall,C.J., *et al.* (2014) Deletions of chromosomal regulatory boundaries are associated with congenital disease. *Genome Biology*, **15**, 423.

52. Vernimmen,D. (2014) Uncovering enhancer functions using the α-globin locus. *PLoS genetics*,

10, e1004668.

53. Lettice,L.A., Daniels,S., Sweeney,E., Venkataraman,S., Devenney,P.S., Gautier,P., Morrison,H., Fantes,J., Hill,R.E. and FitzPatrick,D.R. (2011) Enhancer-adoption as a mechanism of human developmental disease. *Human mutation*, **32**, 1492–9.

54. Giorgio,E., Robyr,D., Spielmann,M., Ferrero,E., Di Gregorio,E., Imperiale,D., Vaula,G., Stamoulis,G., Santoni,F., Atzori,C., *et al.* (2015) A large genomic deletion leads to enhancer adoption by the lamin B1 gene: a second path to autosomal dominant adult-onset demyelinating leukodystrophy (ADLD). *Human molecular genetics*, **24**, 3143–54.

55. Tümer,Z. and Bach-Holm,D. (2009) Axenfeld–Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. *European Journal of Human Genetics*, **17**, 1527–1539.

56. Strehle,E.-M., Yu,L., Rosenfeld,J.A., Donkervoort,S., Zhou,Y., Chen,T.-J., Martinez,J.E., Fan,Y.-S., Barbouth,D., Zhu,H., *et al.* (2012) Genotype-phenotype analysis of 4q deletion syndrome: proposal of a critical region. *American journal of medical genetics. Part A*, **158A**, 2139–51.

57. Moreira,L., Schinzel,A., Baumer,A., Pinto,P., Góes,F., Falcão,M. de L., Barbosa,A.L. and Riegel,M. (2010) Longitudinal observation of a patient with Rieger syndrome and interstitial deletion 4 (q25-q31.1). *American journal of medical genetics. Part A*, **152A**, 977–81.

58. Tanwar,M., Kumar,R., Goyal,A., Kumar,M., Dada,T., Singh,G., Sihota,R. and Dada,R. (2009) Rieger syndrome with multiple chromosomal breaks and chromosome 4 deletion. *BMJ case reports*, **2009**, bcr0620080297-bcr0620080297.

59. Engenheiro,E., Saraiva,J., Carreira,I., Ramos,L., Ropers,H.H., Silva,E., Tommerup,N. and

Tümer,Z. (2007) Cytogenetically invisible microdeletions involving PITX2 in Rieger syndrome. *Clinical genetics*, **72**, 464–70.

60. Kamnasaran,D., O'Brien,P.C., Zackai,E.H., Muenke,M., Ferguson-Smith,M.A. and Cox,D.W. (2003) Rearrangement in the PITX2 and MIPOL1 genes in a patient with a t(4;14) chromosome. *European journal of human genetics : EJHG*, **11**, 315–24.

61. Becker,S.A., Popp,S., Rager,K. and Jauch,A. (2003) A new case of an interstitial deletion (4)(q25q27) characterised by molecular cytogenetic techniques and review of the literature. *European journal of pediatrics*, **162**, 267–70.

62. Ogilvie,C.M., Raymond,F.L., Harrison,R.H., Scriven,P.N. and Docherty,Z. (1998) A new approach to the elucidation of complex chromosome rearrangements illustrated by a case of Rieger syndrome. *Journal of medical genetics*, **35**, 234–7.

63. Flomen,R.H., Gorman,P.A., Vatcheva,R., Groet,J., Barisić,I., Ligutić,I., Sheer,D. and Nizetić,D. (1997) Rieger syndrome locus: a new reciprocal translocation t(4;12)(q25;q15) and a deletion del(4)(q25q27) both break between markers D4S2945 and D4S193. *Journal of medical genetics*, **34**, 191–5.

64. Schinzel,A., Brecevic,L., Dutly,F., Baumer,A., Binkert,F. and Largo,R.H. (1997) Multiple congenital anomalies including the Rieger eye malformation in a boy with interstitial deletion of (4) (q25-->q27) secondary to a balanced insertion in his normal father: evidence for haplotype insufficiency causing the Rieger malformation. *Journal of medical genetics*, **34**, 1012–4.

65. Kulharya,A.S., Maberry,M., Kukolich,M.K., Day,D.W., Schneider,N.R., Wilson,G.N. and

Tonk,V. (1995) Interstitial deletions 4q21.1q25 and 4q25q27: phenotypic variability and relation to Rieger anomaly. *American journal of medical genetics*, **55**, 165–70.

66. Raczenbek,C., Krassikoff,N. and Cosper,P. (1991) Second case report of del(4) (q25q27) and review of the literature. *Clinical genetics*, **39**, 463–6.

67. Mitchell,J.A., Packman,S., Loughman,W.D., Fineman,R.M., Zackai,E., Patil,S.R., Emanual,B., Bartley,J.A., Hanson,J.W. and Opitz,J.M. (1981) Deletions of different segments of the long arm of chromosome 4. *American journal of medical genetics*, **8**, 73–89.

Chapter 3. Comparison of bioinformatics prediction, molecular modeling, and functional analyses of *FOXC1* mutations in patients with Axenfeld-Rieger Syndrome

This chapter was published in:

Seifi M, Footz T, Taylor SA, Walter MA. Comparison of bioinformatics prediction, molecular modeling, and functional analyses of *FOXC1* mutations in patients with Axenfeld-Rieger Syndrome. Hum Mutat. 38(2), 169-179 (2017).

Note: All experiments were carried out by Morteza Seifi except for the molecular modeling analysis presented in Figure 2, which was carried out by Tim Footz and align-GVGD analysis presented in Table 1 and 2, which were carried out by Dr. Sherry A. M. Taylor.

Introduction

FOXC1 (6p25, RefSeq NM_001453.2, MIM# 601090), a single exon gene, is a member of the winged helix/forkhead family of transcription factors, which is characterized by a conserved 110 amino acid sequence, the forkhead domain (FHD) (1). Structurally, this DNA-binding domain is an evolutionarily highly conserved variant of the helix-turn-helix DNA binding motif and comprises 1 minor, and 3 major α-helixes and 2 β-sheets. Each β-sheet is followed by a wing-like loop (2). FOXC1 is able to interact with DNA using the FHD, which contains nuclear localization signals (NLS) at the N- and C- termini required for translocation of FOXC1 protein to cell nuclei (3). *FOXC1* is expressed in fetal human tissues and has a wide variety of roles in the developmental process. *FOXC1* is also extensively expressed in adult human tissues including the eyes, brain, heart, and kidneys. Increased expression of *FOXC1* is associated with the low chance of survival in cancers such as lung cancer (4) and hepatocellular carcinoma (5). *FOXC1* may be considered as a key diagnostic biomarker specific for basal-like breast cancer (5, 6). *FOXC1* acts as a pivotal regulator of embryogenesis, cell migration and cell differentiation (7). A large number of pathogenic variants, including insertion, deletions, nonsense and missense variations, in the FHD in the *FOXC1* gene have been documented. These variants impair the localization of FOXC1 to the nucleus, DNA-binding capacity, transactivation and DNA-binding specificity, leading to anterior segment disorders, particularly Axenfeld–Rieger syndrome (ARS; MIM# 602482) (2, 8–12).

ARS is a genetically heterogeneous group of abnormalities that map not only to 6p25, but also to 4q25 where variations in paired-like homeodomain transcription factor 2 (*PITX2*, RefSeq NM_000325.5, MIM# 601542) underlie ARS (13, 14). ARS is an autosomal dominant disorder and its primary feature is anterior segment dysgenesis of the eye. While ARS is genetically fully penetrant, affected individuals display variable expressivity that can involve both ocular and non-

ocular structures (15). The most critical consequence of ARS is glaucoma, a progressively blinding condition that develops in over 50% of ARS patients (10). Murine models of *Foxc1* null variations also show both ocular and extraocular malformations similar to those observed in humans, indicating critical developmental functions for FOXC1 (16). The observation of point variations, interstitial duplications and deletions of the *FOXC1* gene in patients with ARS implies the significance of a stringent control of FOXC1 expression levels and activity for embryogenesis and, particularly, for the normal development and function of skeletal, cardiovascular, urogenital, and ocular tissues. Taken together, these findings reveal that variants leading to an increase or decrease in FOXC1 levels contribute to ARS (17).

In the current chapter, I have analyzed four *FOXC1* missense variants including c.383A>G (p.H128R), c.404G>A (p.C135Y), c.481A>G (p.M161V), and c.1103C>A (p.T368N) to understand how dysfunction of *FOXC1* increases the risk of ocular abnormalities in patients. I investigated the impact of these variants on the structure and function of the FOXC1 protein by exploring their effect on molecular modeling, subcellular localization, DNA binding, transactivation activity, expression levels and protein stability. In addition, we also evaluated the predictive value of four common *in silico* bioinformatics tools including sorting intolerant from tolerant (SIFT), polymorphism phenotyping (PolyPhen-2), Align-GVGD and MutPred on these 4 novel and 27 previously reported *FOXC1* missense variants (for the variants and their location on FHD please see Figure 1). Understanding the consequences of *FOXC1* variations will allow better determination of how disruptions in *FOXC1* contribute to disease and aid in the development of new treatments for ARS patients.

Materials and Methods

Patient samples and variation analysis

The detailed clinical features of the patients included in our study have been reported elsewhere (12, 18, 19). Briefly, individuals with H128R and C135Y were affected with primary congenital glaucoma and elevated corneal diameter with increased intraocular pressure (IOP) and/or Haab's striae, corneal scarring, and optic disc changes. No symptoms of secondary glaucoma or other systemic features were observed in the patients. The ARS patient harboring *FOXC1* p.M161V had elevated IOP, posterior embryotoxon, iris hypoplasia, iridocorneal adhesions, corectopia, and systemic anomalies including umbilicus and middle-ear deafness. The patient with the p.T368N variant presented with unilateral microphthalmia and dense cataracts. Nucleotide numbering of the variations herein indicates cDNA numbering with +1 as the A of the ATG translation initiation codon in the NCBI reference sequence NM_001453.2, while the amino positions are based on the corresponding NCBI reference sequence NP_001444.2.

Molecular modeling

The homology model of *FOXC1* was generated by aligning residues 76-177 of *FOXC1* with residues 117-218 of the crystal structure of FOXA3 (RefSeq NM_004497.2, MIM# 602295) (20) in Swiss-PdbViewer (21), as described previously (22). The backbone atoms of *FOXC1* were "fitted" against the FOXA3, and the modeling project was analyzed by the SWISS-MODEL server (http://www.expasy.org/spdbv/; provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland). Finally, model structures of mutants were created in Swiss-

Pdb Viewer and investigated using the ANOLEA server (http://melolab.org/anolea), as described previously (22).

Plasmid construction and mutagenesis

Site-directed mutagenesis was conducted to introduce variations into the *FOXC1* complementary DNA using the QuikChange mutagenesis kit (Agilent Technologies Canada Inc., Mississauga, ON) and appropriate forward primers 5'-GAA CAG CAT CCG CCG CAA CCT CTC GCT CA-3', 5'-CTC TCG CTC AAC GAG TAC TTC GTC AAG GTG CCG C-3', 5'-CCG GAC TCC TAC AAC GTG TTC GAG AAC GGC A-3', and 5'-CCC GCG CTG GAG TTC TGG CTG CAG G-3' according to the manufacturer's protocol. Potential mutant constructs were sequenced by The Applied Genomics Core at the University of Alberta. Confirmed mutants were subcloned into the pcDNA4/HisMax© plasmid using Gateway technology (Life Technologies Inc., Burlington, ON) and the final clones were re-sequenced. For the electrophoretic mobility shift assay (EMSA) experiment, confirmed mutants were subcloned into the pcDNA4/HisMax© non-Gateway vector.

Cell culture

HeLa cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1X antibiotic-antimycotic solution (Gibco BRL). Cells were transfected with the plasmid constructs using Lipofectamine™ 2000 (Life Technologies Inc.), according to the manufacturer's protocol.

Immunoblot analysis

Forty-eight hours after transfection, media was aspirated and cells were rinsed two times with 5 mL of cold PBS. After adding 75 µL of lysis buffer (IGEPAL ® CA-680, 0.05 M Tris pH 8.0, 0.15 M NaCl, 1 mM PMSF, 0.05% Protease Inhibitor Cocktail) drop-wise over the whole plate, cells were harvested by gently scraping and then incubated on ice for 15 minutes. Next, cells were lysed by gentle sonication on ice and centrifuged at 14,000 g for 5 minutes at 4oC. The supernatants were transferred to a new cold microfuge tube, quantified, denatured at 95oC for 5 minutes and size-separated on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Xpress™ epitope-tagged FOXC1 proteins were detected by immunoblotting using a commercial anti-Xpress antibody (1:5000 dilutions; Life Technologies Inc.) and visualized by chemiluminescence (SuperSignal® West Femto Maximum Sensitivity Substrate, Thermo Scientific, Rockford, IL).

Immunofluorescence microscopy

HeLa cells were grown and transfected on glass coverslips. After twenty-four hours, cells were fixed with 2% paraformaldehyde for 20 minutes, followed by permeabilization and blocking (1% BSA, 0.5% Triton X-100 in PBS) for 15 minutes. The localization of FOXC1 protein was detected with anti-Xpress antibody and a secondary anti-mouse antibody conjugated with Cy3 (Jackson ImmunoResearch, West Grove, PA), both at a dilution of 1:500. Nuclei were visualized by staining with 4', 6-diamidine-2-phenylindole (DAPI) dye (10µg/ml). Cells transfected with wild-type and mutant FOXC1 proteins were scored for nuclear and cytoplasmic staining with a fluorescence microscope (Leica Microsystems Inc., Concord, ON).

Electrophoretic mobility shift assay (EMSA)

HeLa cell lysates containing mutant proteins were standardized to wild-type FOXC1 levels by western analysis. The whole-cell protein extracts (40 μg) were incubated for 20 minutes in the dark at room temperature with annealed fluorescently-labeled oligomer probe (0.5 μM) (forward, 5'-Cy3-GAT CCA AAG TAA ATA AAC AAC AGA-3'; and reverse, 5'-Cy3-GAT CTC TGT TGT TTA TTT ACT TTG-3') in buffer (1.25 mM dithiothreitol, 1 μg Poly (deoxyinosinic-deoxycytidylic) acid sodium salt, 5% glycerol). After pre-running the 6% polyacrylamide Tris/glycine/EDTA gel at 150V (10V/cm) for 15 minutes, the binding reactions were subjected to electrophoresis in the dark for 50 minutes and then visualized on the Image Station 4000MM (Eastman Kodak, Rochester, NY).

Reporter transactivation assay

HeLa cells plated in 15-mm well culture plates were transfected with 500 ng of the *FOXC1* pcDNA4His/Max construct, 60 ng of the pGL3-TK construct with 6×*FOXC1* binding sites (23) and 30 ng of pCMVβ transfection-control plasmid. Cells were harvested with 100 μl buffer (Reporter Lysis Buffer; Promega, Madison WI) 48 hours after transfection. Firefly luciferase activity was measured by luminometry (Turner Designs, Sunnyvale, CA) from 10 μl protein lysate mixed with 100 μl Luciferase Assay Reagent (Promega) and was standardized to the β-galactosidase (internal control) activity quantitated by the β-Galactosidase Enzyme Assay System (Promega) from 50 μl of protein lysate. Reactions were repeated three times in triplicate.

Protein stability

FOXC1–transfected HeLa cells were treated after 24 hours with cycloheximide (50 μg/ml) for different time periods (0 to 6 hours) to inhibit protein synthesis. Cell lysates were harvested, as described for immunoblot analysis, at different time points. Samples were subjected to immunoblotting with anti-Xpress and anti-α-tubulin loading control (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies. The bands were quantified and normalized against the loading control. Band intensities were measured on the Image Station 4000MM with Molecular Imaging Software version 4.0.5 (Eastman Kodak). The unpaired Student's t-test was employed to determine statistical significance using slopes over time-course of cycloheximide treatment. Three independent experiments were carried out to determine the rates of decay of FOXC1 proteins.

Bioinformatics mutation prediction tools

The predictive value of four *in silico* protein function predictor programs (SIFT (24) (http://sift.jcvi.org/), PolyPhen-2 (25) (http://genetics.bwh.harvard.edu/pph2/index.shtml), Align-GVGD (26) (http://agvgd.hci.utah.edu/) and MutPred (27) (http://mutpred.mutdb.org/)) was determined for all 22 functionally characterized FOXC1 amino acid substitutions (18 previously characterized *FOXC1* variants plus the four variants studied here; Figure 1, Table 1). SIFT, PolyPhen-2 and MutPred predictions were also obtained for nine additional, not functionally characterized, *FOXC1* missense variants (Figure 1, Table 2). Variants were defined as "deleterious" (a value of less than 0.05) or "neutral" by the SIFT program. PolyPhen-2, analyses report variants as 'benign', 'possibly damaging', 'probably damaging', or 'unknown'. Variants scored as benign were recorded as non-deleterious/neutral in our comparisons. Variants predicted by PolyPhen-2 as "possibly

damaging" and "probably damaging" were considered in our analyses as having PolyPhen-2 predicted-deleterious effects. The Align-GVGD web-based server integrates the biophysical features of amino acids and protein multiple sequence alignments to predict whether missense variants in genes of interest lead to deleterious or neutral effects. This program is an extension of the original Grantham difference to multiple sequence alignments and true simultaneous multiple comparisons (28). For Align-GVGD program, a value of C>0 was considered deleterious; otherwise a variant was considered to be neutral. MutPred was developed to categorize an amino acid substitution as a disease-causing change or benign and used the SIFT algorithm and gain/loss of 14 other structural and functional features (29). Predictions for the *FOXC1* missense variants used the default settings for all tools.

Results

Molecular modeling of forkhead domain of *FOXC1*

Molecular models for the *FOXC1* FHD were designed using threading algorithms to analyze *FOXC1* and examine how missense variants could disrupt FOXC1 structure (30). FOXA3 was used as a template for homology modeling of FOXC1, because the structure contains co-purified protein bound to DNA. The p.T368N variant was excluded from these molecular modeling analyses since p.T368 is not located in the FHD, but instead lies within a downstream region of unknown structure (Figure 1). H128, C135 and M161 were changed to arginine, tyrosine and valine respectively, to examine putative structural effects of the p.H128R, p.C135Y and p.M161V variants through ANOLEA mean force potential calculations. When H128 and M161 were changed to arginine and valine residues respectively, no substantially altered pairwise residue interactions were detected. Thus, this indicates

that these missense variants are predicted to have no effects on FOXC1's global structure (Figure 2). In contrast, when C135 was altered to a tyrosine residue, unfavorable effects were predicted, affecting multiple non-local amino acid sidechain contacts, suggesting that this variant changes the structure of the FOXC1 FHD (Figure 2).

Expression of *FOXC1*

Whole-cell extracts of HeLa cells transfected separately with each of the four variants tested here (p.H128R, p.C135Y, p.M161V, and p.T368N) were resolved by SDS-PAGE and subjected to immunoblot analysis. *FOXC1* variants p.H128R, p.M161V, and p.T368N expressed proteins at the same level and size (~65 kD) as the wild-type FOXC1 protein (Figure 2). In contrast, immunoblotting revealed that protein levels of the *FOXC1* p.C135Y variant were 5X lower than that of wild-type FOXC1 (Figure 3). Similar to the wild-type *FOXC1*, all four variants appeared to produce multiple migrating FOXC1 protein bands suggesting that these proteins contained analogous post-translational modification as wild-type FOXC1 (3, 31, 32).

Localization of *FOXC1* and mutant *FOXC1* proteins

Immunofluorescent microscopy was conducted against the vector-encoded Xpress epitope of the recombinant *FOXC1* molecules to determine if the mutant proteins localize to the nucleus, as demonstrated by the co-localization of recombinant FOXC1 proteins with DAPI stained nuclei. It has been previously reported that > 90% of wild-type expressed FOXC1 localizes to the cell nucleus (3, 24). HeLa cells were transiently transfected with the pcDNA4 His/Max wild-type *FOXC1* or missense-variant expressing vectors. *FOXC1* p.H128R and p.C135Y displayed altered localization

distributions (Figure 4). Only 42% and 38% of cells expressing p.H128R or p.C135Y protein respectively display exclusive nuclear localization of FOXC1, compared with 93% for wild-type protein. Similar to the wild-type *FOXC1*, *FOXC1* p.M161V and p.T368N variant proteins were exclusively in the nucleus in 85 and 90 percent of cells, respectively (Figure 4).

Electrophoretic mobility shift assay (EMSA)

The DNA-binding capability of the *FOXC1* missense variants, expressed in the HeLa cells, was tested by EMSA experiments using fluorescently-labeled oligonucleotides containing a canonical *FOXC1* binding site. The presence of a shifted band, indicative of a *FOXC1*-DNA complex, increased with increasing protein concentrations of wild-type *FOXC1* (Figure 5). In contrast, the p.H128R and p.C135Y variants resulted in FOXC1 proteins with impaired DNA binding capacities. The p.H128R and p.C135Y variants were unable to form detectable protein-DNA complexes even with 5X more protein, demonstrating that these variants disrupt the normal DNA-binding ability of the FOXC1 protein. In contrast the *FOXC1* p.M161V and p.T368N missense variants were able to bind the *FOXC1* site at levels similar to wild-type *FOXC1* (Figure 5).

Transactivation assays

It is known that some *FOXC1* missense variants hinder the ability of *FOXC1* to stimulate transcription (3, 24). Therefore, a transactivation assay was performed to investigate the ability of the four variant-containing proteins to regulate expression of a luciferase reporter gene containing 6 consensus *FOXC1* binding sites upstream of the herpes simplex–virus thymidine kinase (HSV-TK) promoter. HeLa cells were co-transfected with the TK-luciferase reporter construct and either wild-

type or variant-expressing *FOXC1* pcDNA4 His/Max. Wild-type FOXC1 was found to activate expression of the luciferase reporter, a 14X increase compared with an empty vector control (the TK promoter alone, Figure 6). In contrast, p.H128R only increased the transactivation of the reporter to 2.2X, when compared to the empty vector control. The transactivation capacity of p.C135Y was similar to the transactivation capacity of the empty vector control (10.9% vs. 7.1%, respectively), indicating that the *FOXC1* p.C135Y variant severely disrupts the transactivation activity of *FOXC1*. The p.M161V missense variant increased luciferase activity to 3.3X over empty vector control, indicating a transcription activation defect, whereas the *FOXC1* p.T368N variant had normal transactivation capacity (89.1% of wild-type levels, Figure 6).

Protein stability

To study protein turnover, the cells transfected with *FOXC1* tagged with Xpress epitope were exposed to cycloheximide treatment to inhibit protein synthesis. FOXC1 protein levels at different time points were determined using immunoblot analysis (Figure 7). The half-life of FOXC1 p.H128R and p.C135Y were detected to be 118.5 min and 41.7 min, respectively, compared to the wild-type FOXC1 half-life of 89.6 min. The p.C135Y protein was undetectable after two hours of cycloheximide treatment, consistent with shortened protein intracellular half-life for this variant (P=0.02). Interestingly, FOXC1 p.H128R appears to have a significantly longer half-life than wild-type FOXC1 (P=0.01). In contrast, the p.T368N and p.M161V FOXC1 proteins decayed at rates similar to wild-type FOXC1 (not statistically different, P=0.30 and P=0.40, respectively), indicating that these mutant proteins appear to be as stable as wild-type FOXC1.

Bioinformatics functional predictions

The four *FOXC1* variants studied here, plus 18 previously characterized *FOXC1* missense variants (Figure 1), were used to test the specificity and sensitivity of the SIFT, PolyPhen-2, Align-GVGD and MutPred bioinformatics prediction tools for *FOXC1* (Table 1). For *FOXC1*, MutPred was the most reliable of the tested bioinformatics tools in predicting the pathogenicity effects of all 22 characterized missense variants in *FOXC1*, with both sensitivity and specificity of 100% (Figure 8). SIFT and PolyPhen-2 both correctly predicted the pathogenicity effects of 21/22 (95%) missense variants, indicating high concordance between the two programs. Analysis of the sensitivity and specificity of SIFT and PolyPhen-2 showed that both had remarkable sensitivity (both 95%) and specificity (both 100%). In contrast, Align-GDGV analysis showed low sensitivity (86%), but the same specificity as compared with the other three programs, (100%) (Figure 8, Table 1).

Discussion

In this study, we used a variety of techniques, including molecular modeling, functional analyses and *in silico* mutation prediction software to determine the pathogenicity of missense variants of *FOXC1* found in ARS patients. We first used molecular modeling to predict how three different missense variants (p.H128R, p.C135Y and p.M161V) disrupt the structure and function of *FOXC1*. Molecular modeling of the effect of the p.H128R variant on the structure of the FOXC1 FHD predicts no change to the structure of the FHD (Figure 2). H128, however, is located in α-helix 3 of the FHD and is one of the main participants in the formation of the hydrophobic core. Helix 3 of FOX proteins interacts with the major groove of DNA and is responsible for DNA-binding specificity (2, 33). We predicted therefore that alteration of the normal H128 position to an arginine impairs the capacity of this helix

to interact with the major groove of the DNA, likely disrupting the DNA binding and transactivation abilities of FOXC1. Variation of the adjacent amino acid (c.380G>A (p.R127H)) results in a similar decrease in the ability of the mutant protein to interact with DNA (34). Molecular modeling of the *FOXC1* FHD predicts that p.C135Y perturbs helix 1, β-sheets 1, 2, and wing-2 of the *FOXC1* FHD, likely causing a global destabilization of the structure of FOXC1. Molecular modeling of p.M161V indicated that this variant does not grossly disrupt the structure of FOXC1, however, p.M161V alters slightly the required energy to maintain the proper folding of helix 1, β-sheet 1, and wing-2. Due to the role of wing-2 in DNA binding ability (22), p.M161V could disrupt this function. To test the predictions based upon these molecular modeling experiments, we characterized the molecular effects of these three missense variants plus p.T368N on FOXC1 structure and function.

The *FOXC1* p.H128R missense variant had severe effects on *FOXC1*, impairing nuclear localization, interaction with DNA which precluded formation of DNA-protein complexes, and hence transactivation capacity (Figure 4-6). The disruptions to the DNA binding affinity and transactivation ability of FOXC1 p.H128R are consistent with the pivotal role of helix 3 in FOXC1 function and are consistent with our predictions from molecular modeling of this variant.

The results for p.C135Y variant showed that substitution of cysteine in wild-type FOXC1 with tyrosine decreases nuclear localization of FOXC1 to nearly 40% of wild-type levels. While potential phosphorylation of the substituted tyrosine at this position might be predicted to disrupt cellular localization, western blot analyses showed no abnormal migration patterns of p.C135Y protein (Figure 3), consistent with normal post-translational modifications of this variant protein. It is possible that this mislocalization of p.C135Y results from altered interactions with other amino acids located within the FOXC1 FHD that impair interaction with the nuclear translocation machinery. Like p.H128R, FOXC1 p.C135Y is unable to bind to the FOXC1 binding site, even after increasing

by five times the amount of the mutant FOXC1 (Figure 5), consistent with an altered FHD. Similar to p.H128R, p.C135Y also severely impairs the transactivation potential of FOXC1 protein. This is consistent with the EMSA results, since the p.C135Y protein is not able to bind the canonical *FOXC1* DNA binding site that is a precondition for transactivation. Again, the functional investigations of the consequence of p.C135Y were consistent with our predictions from molecular modeling of this variant.

In contrast to these variants, p.M161V localizes to the nucleus efficiently, similar to the nuclear localization of wild-type FOXC1 (85% vs 93%, respectively, Figure 4). Analyses have previously found that other missense variants within the wing 2 region of the *FOXC1* FHD (c.482T>A (p.M161K), c.493G>A (p.G165R), and c.506G>C (p.R169P)), also result in proteins able to localize to the cell nucleus at similar levels as wild-type (22). These results suggest that variations in wing 2 do not impair nuclear localization. P.M161V reduced the DNA binding ability of mutant FOXC1 protein to approximately 70% of wild-type FOXC1 levels, demonstrating that formation of a normal wing 2 nevertheless is required for correct DNA-FOXC1 proteins complexes. The p.M161V missense variant led to the severely decreased transactivation potential of the FOXC1 mutant protein, suggesting a functional role of this position in transactivation activity. Consistent with this result, previous functional analysis of three missense variants in the *FOXC1* FHD (c.335T>C (p.F112S), c.378C>G (p.I126M) and c.493G>A (p.G165R)) revealed that these variants impaired transactivation, without perturbing DNA binding (3, 22). These data indicate that DNA binding and transactivation capacity are separable functions of the *FOXC1* FHD. It is possible that p.M161V disrupts protein–protein interactions, particularly intramolecular interactions required for transcription activation. Due to involvement of the N terminal portion of α-helix 1 in transactivation and DNA binding (23) and the role of wing 2 of FOXA3 in establishing a proper environment for

interaction between regions of α-helix 1 and wing 2 (20), we hypothesize that p.M161V hinders intramolecular interactions between wing 2 and α-helix 1 leading to disruption of the transactivation activity without affecting DNA-binding ability. These data are consistent with our predictions from molecular modeling of this variant.

One of the most interesting variants characterized in the present study was the substitution of threonine with asparagine at position 368. Position 368 is highly variable across species and is located outside of any known functional domains of *FOXC1* (19). It was not possible to perform molecular modelling of p.T368N since the region containing T368 has no known structure. This points to one of the fundamental drawbacks of molecular modeling; such analyses can only be conducted for domains with a previously determined three-dimensional structure, such as the FHD. Functional characterization of the p.T368N variant showed that the expression, localization, DNA binding ability, transactivation activity, protein stability of p.T368N *FOXC1* were all indistinguishable from wild-type *FOXC1* (Figure 3-7). This suggests that the p.T368N missense substitution is a rare non-pathogenic *FOXC1* variant, the first demonstration of a missense change of *FOXC1* that does not alter FOXC1 function. Our results indicate that molecular modeling prediction strongly correlated with the results of functional characterization for missense variants within *FOXC1* domains for which the dimensional structure could be predicted based upon homology to the known structures of other FOX proteins (20, 22). Bioinformatics mutation prediction tools typically do not rely upon the known structure of a domain, and thus can be applied to predict the likely pathogenicity of a wider range of missense variants. While direct sequencing of potential candidate genes has been one of the pivotal means of studying and diagnosing monogenic disease, determining if a variant is disease-causing in the absence of functional characterization remains difficult. Bioinformatics mutation prediction tools most commonly use measures of evolutionary conservation or the physical and chemical properties

of amino acid residues to assess the potential impact of missense variants on protein structure. Tools are being developed to predict the consequences of variants on other parameters including protein aggregation, solubility, stability, localization, post translation modification, and electrostatics (35). Alone or collectively these tools are used as one part of algorithms that assess whether a missense variant may be responsible for a clinically significant phenotype (36). There is enough similarity amongst these prediction tools that they are not used as independent predictors in those algorithms and therefore in the diagnostic setting their usefulness in assessing the pathogenicity of novel variants is limited. Of greater value clinically is the availability of reports of functional studies that assess whether a missense variant observed in an affected individual has had an impact on protein structure or function. Functional studies alone or in combination with the results of mutation prediction tools can result in a variant of unknown clinical significance being reclassified as likely pathogenic or pathogenic. The results reported here on the functional and bioinformatics assessment of *FOXC1* missense variants support the view put forward by a recently published paper (37) that these two approaches can be complementary and mutually supporting. We have evaluated four commonly used bioinformatics mutation prediction tools, for the first time, to test their ability in predicting the functional significance of *FOXC1* missense variants.

The application of SIFT, PolyPhen-2, Align-GVGD and MutPred bioinformatics tools to 22 functionally characterized *FOXC1* missense variants suggests that three of these tools were more reliable in their ability to predict pathogenicity (Table 1). The sensitivity and specificity of MutPred in recognizing deleterious variants in *FOXC1* were 100% while those of SIFT and PolyPhen-2 were over 95%. In contrast, Align-GVGD was found to be less reliable, having a sensitivity of only 86%. Our results show that these three programs, especially MutPred, can be used reliably to predict whether or not a *FOXC1* missense variant is likely to be deleterious. It is worth noting, however, that

in this study we could only apply these four *in silico* bioinformatics programs to assess 21 pathogenic and one benign variation. Analyses of more variants might be required to assess the specificity (true negative and false positive rates) of these programs. We then applied all of the programs (SIFT, PolyPhen-2, Align-GVGD and MutPred) to predict the likely pathogenicity of nine *FOXC1* missense variants found in ARS patients for which functional testing has not been done (Table 2). All four of the programs predict that these nine *FOXC1* variants are not-tolerated, and are thus consistent with these missense variants being ARS-causing missense variants.

My study indicates that, in the absence of functional data, SIFT, PolyPhen-2, and MutPred, and to a lesser extent Align-GVGD, are all reliable means of predicting the pathogenicity of missense substitutions with the *FOXC1* FHD. Given the sequence homology between the FHDs of FOX class transcription factors, I predict that these bioinformatics tools can be used to assess the potential pathogenicity of missense variants within other FOX proteins and to prioritize variants for functional studies.

Nevertheless, the results of these predictions should still be interpreted with caution, as previous studies demonstrated the low accuracy of these tools for gain-of-function variants (38, 39). Therefore, as per clinical guidelines for the interpretation of missense variants, the predictions made using bioinformatics prediction software should be interpreted together with the results of functional studies, data on population frequency and segregation in affected families.

Figure 1. Summary of all 31 known *FOXC1* missense variants. Uncharacterized variants are presented in italic type. The four missense variants molecularly characterized in this study are shown in bold type. AD, activation domain; FHD, forkhead domain; ID/PD, inhibitory domain/phosphorylation domain.

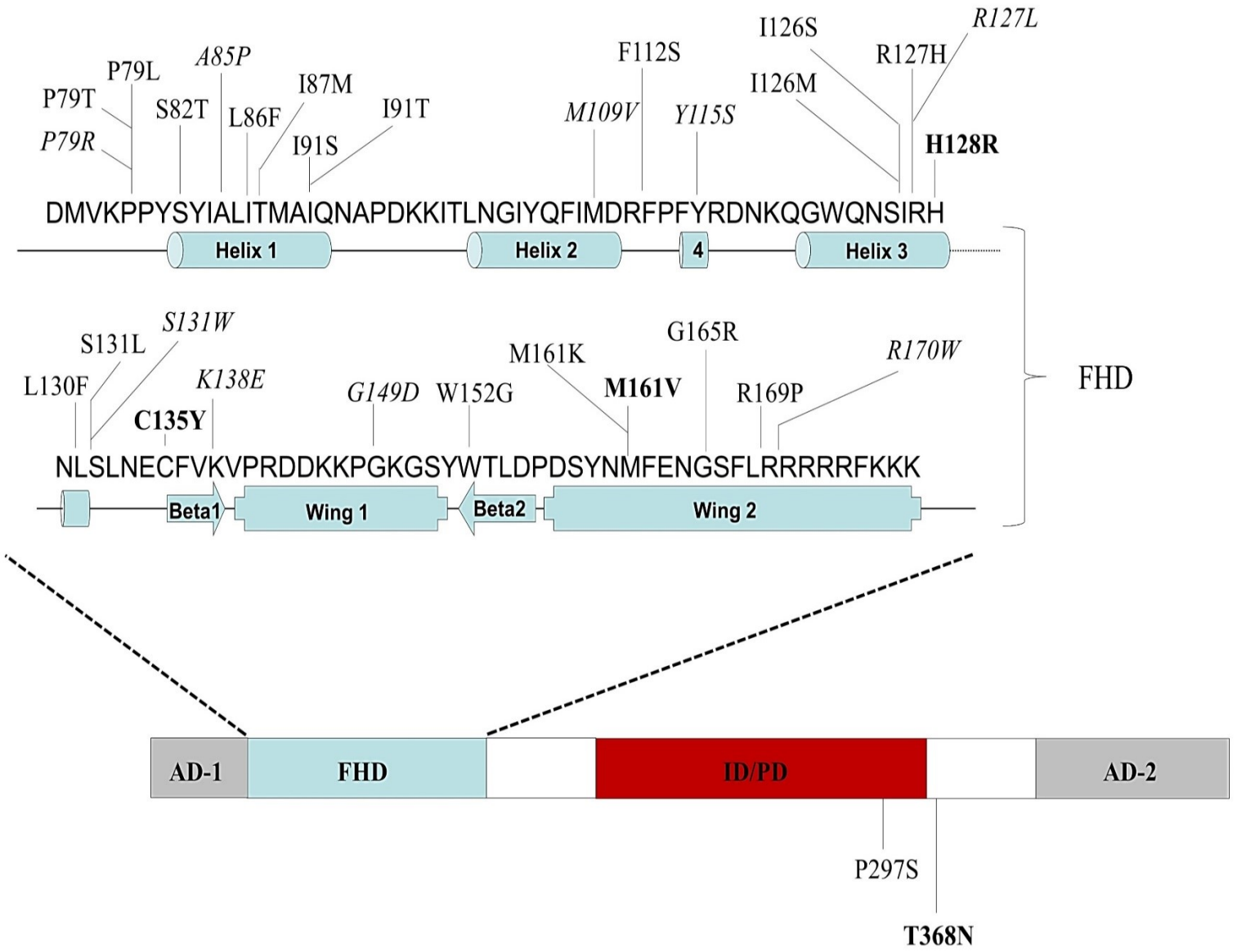

P79T
P79R
P79L
S82T
A85P
L86F
I87M
I91S
I91T
F112S
M109V
Y115S
I126S
I126M
R127H
R127L
H128R
DMVKPPYSYIALITMAIQNAPDKKITLNGIYQFIMDRFPFYRDNKQGWQNSIRH
Helix 1
Helix 2
4
Helix 3
S131W
S131L
L130F
K138E
C135Y
G149D
W152G
M161K
M161V
G165R
R169P
R170W
FHD
NLSLNECFVKVPRDDKKPGKGSYWTLDPDSYNMFENGSFLRRRRRFKKK
Beta1
Wing 1
Beta2
Wing 2
AD-1
FHD
ID/PD
AD-2
P297S
T368N

Figure 2. Homology models (left) and scatterplots (right) of *in silico* analyses of the c.383A>G (p.H128R), c.404G>A (p.C135Y) and c.481A>G (p.M161V) variants in the *FOXC1* gene. The FOXA3-derived homology model of *FOXC1* is presented with the protein backbone depicted in black ribbon, the co-crystallized DNA binding target in space-filled yellow model and positions 128, 135, and 161 in blue, green, and red, respectively. The wild-type and mutant-equivalent models were analyzed by the atomic nonlocal environment assessment (ANOLEA) server. Peaks on the scatterplots show the positions of amino acids that changed their pseudoenergy state, as a consequence of the mentioned variants. Energy differences are in E/KT units, where E represents energy; K, the Boltzmann constant; and T, absolute temperature. The c.1103C>A (p.T368N) variant was excluded from these molecular modeling analyses since p.T368 does not reside in the FHD.

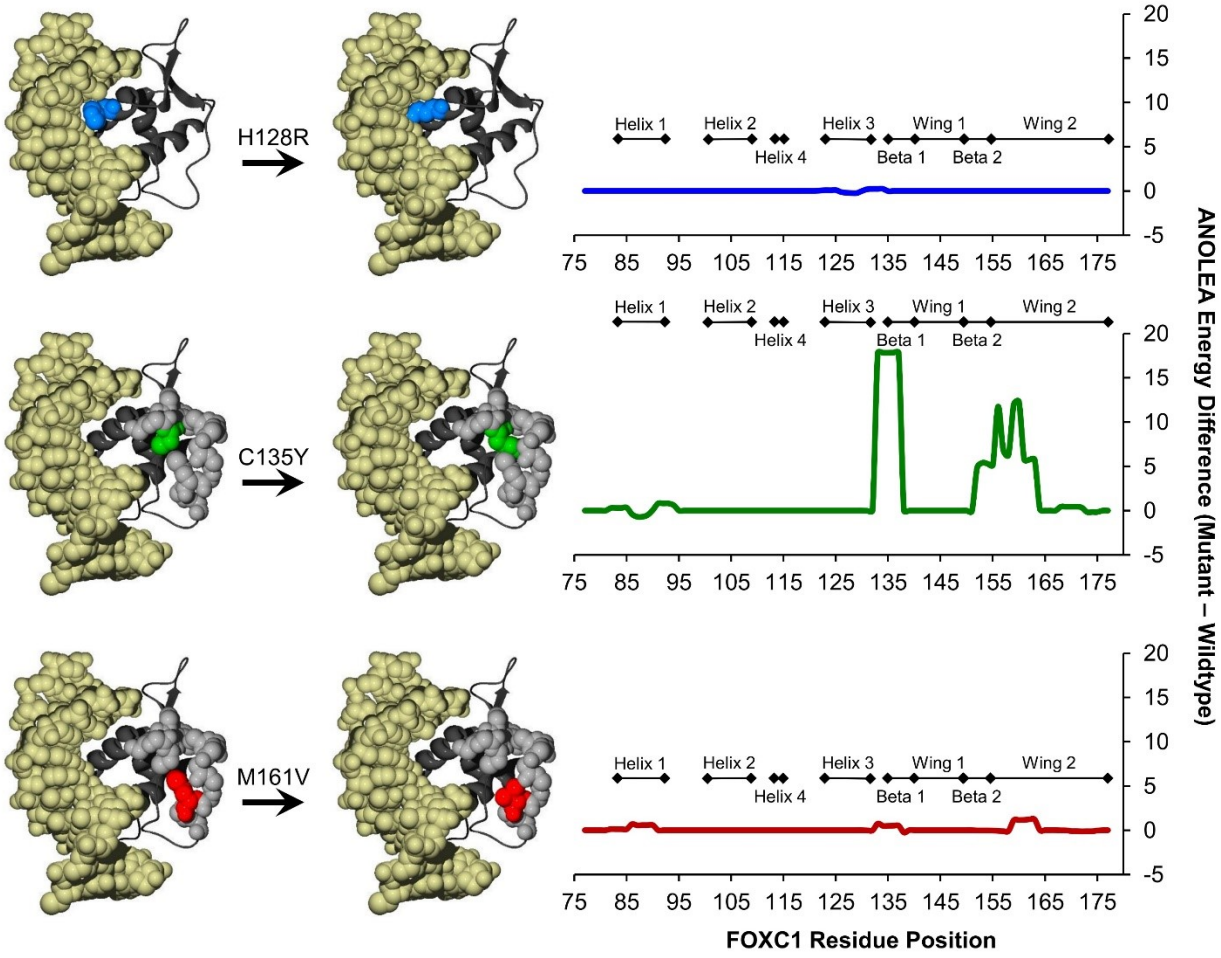

H128R
C135Y
M161V
Helix 1
Helix 2
Helix 3
Wing 1
Wing 2
Helix 4
Beta 1
Beta 2
20
15
10
5
0
-5
75 85 95 105 115 125 135 145 155 165 175
FOXC1 Residue Position
ANOLEA Energy Difference (Mutant – Wildtype)

Figure 3. The *FOXC1* wild-type (WT), p.T368N, p.H128R, p.C135Y, and p.M161V detected by immunoblotting. In this experiment, five times more protein lysate was used for C135Y, compared with the wild-type to obtain equivalent protein density. (Empty, pcDNA4 without FOXC1). All the proteins occurred as a doublet at approximately 65 kDa. The protein size marker is shown to the left. The experiment was repeated three times.

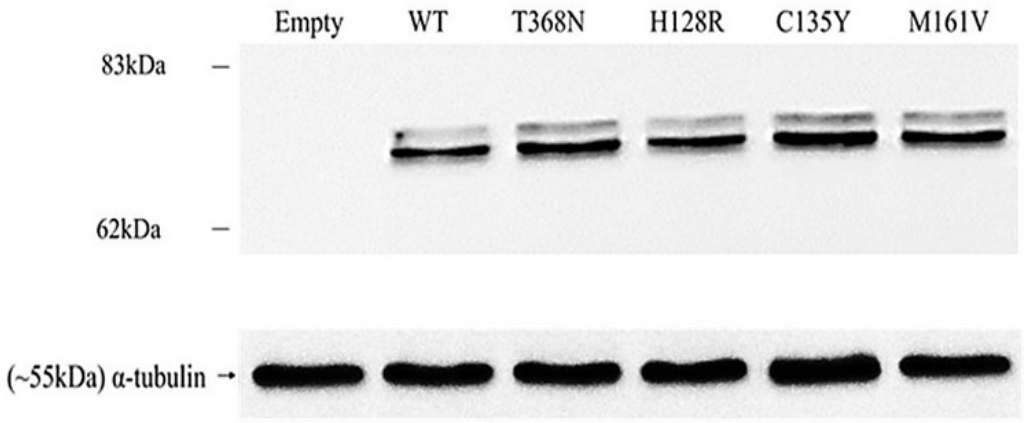

Empty
WT
T368N
H128R
C135Y
M161V
83kDa
62kDa
(~55kDa) α-tubulin

Figure 4. Localization patterns of FOXC1 wild-type, p.T368N, p.H128R, p.C135Y, and p.M161V proteins in HeLa cells. FOXC1 proteins were visualized by Cy3 fluorescence (red), whereas the cell nucleus was visualized by 4,6-diamidine-2-phenylindole staining (blue). The localization of FOXC1 proteins in cells were scored as N, nuclear; C, cytoplasm; or N+C, localized in both cell compartments. The p.M161V, p.T368N, and wild-type FOXC1 proteins all localized to cell nuclei. In contrast, the p.H128R and p.C135Y proteins, showed reduced localization, compared with FOXC1 wild-type. A total of 450 cells and 570 cells were counted for FOXC1 wild-type and variants, respectively. The 100× objective was used in this experiment. Scale bars = 50 μm.

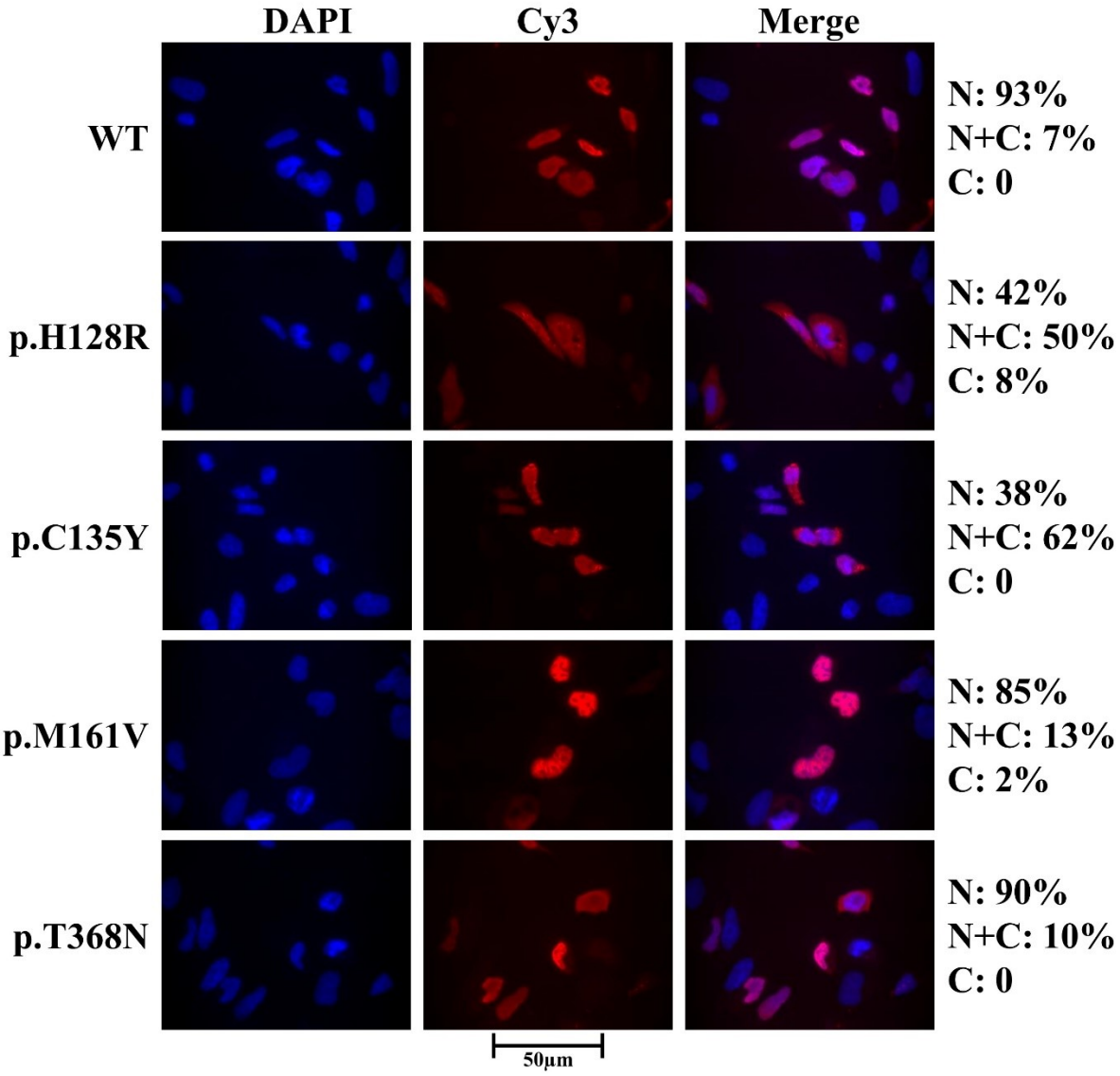

DAPI
Cy3
Merge
WT
N: 93%
N+C: 7%
C: 0
p.H128R
N: 42%
N+C: 50%
C: 8%
p.C135Y
N: 38%
N+C: 62%
C: 0
p.M161V
N: 85%
N+C: 13%
C: 2%
p.T368N
N: 90%
N+C: 10%
C: 0
50μm

Figure 5. Analysis of DNA binding capacities of *FOXC1* wild-type, p.T368N, p.H128R, p.C135Y, and p.M161V proteins. The FOXC1 wild-type, p.T368N, p.H128R, p.C135Y, and p.M161V proteins were incubated with Cy3-labeled double-stranded DNA containing a *FOXC1*-binding site. Unlike the p.T368N and p.M161V proteins, the p.H128R and p.Cys135Y proteins were unable to bind to the DNA probe even when the amounts of the FOXC1 p.H128R and p.C135Y proteins were increased 5×. (Empty, pcDNA4 without FOXC1).

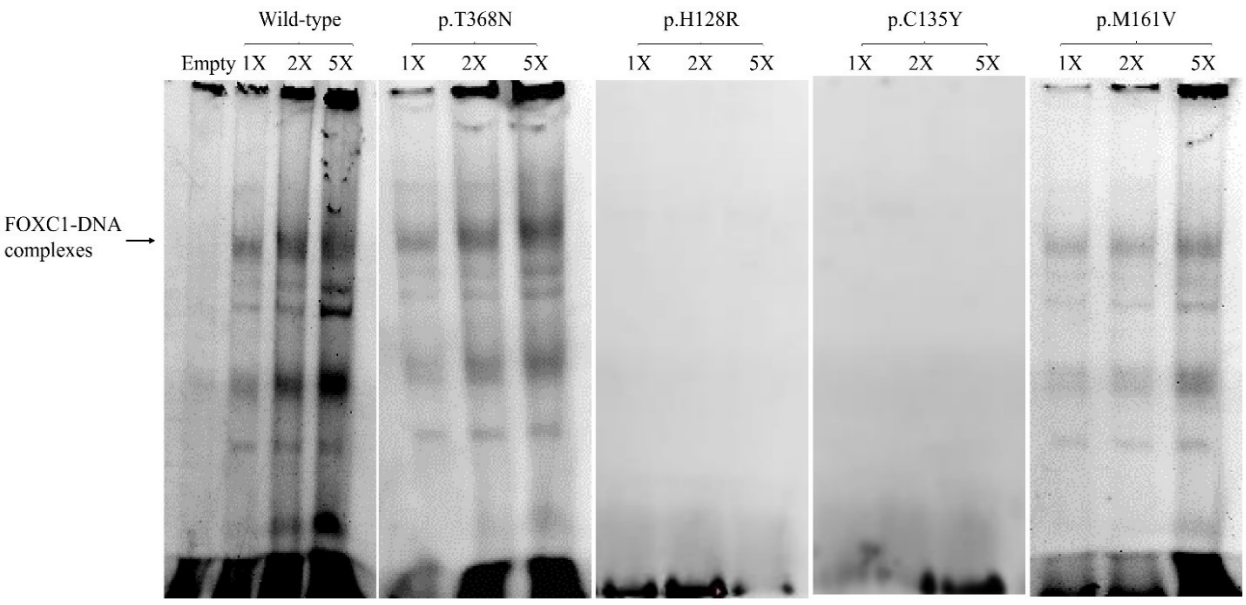

Wild-type
p.T368N
p.H128R
p.C135Y
p.M161V
Empty 1X 2X 5X
1X 2X 5X
1X 2X 5X
1X 2X 5X
1X 2X 5X
FOXC1-DNA complexes

Figure 6. Transactivation capacity of the FOXC1 p.T368N, p.H128R, p.C135Y, and p.M161V proteins. Luciferase activation was normalized to the β-galactosidase control. The p.H128R, p.C135Y, and p.M161V variations in *FOXC1* significantly disrupt transcriptional activation. The p.T368N was not different than wild-type FOXC1 in ability to transactivate the luciferase reporter. Asterisks: samples with a significant difference ($P < 0.05$) calculated from comparison with wild-type FOXC1. Error bars represent the SEM. WT indicates wild-type.

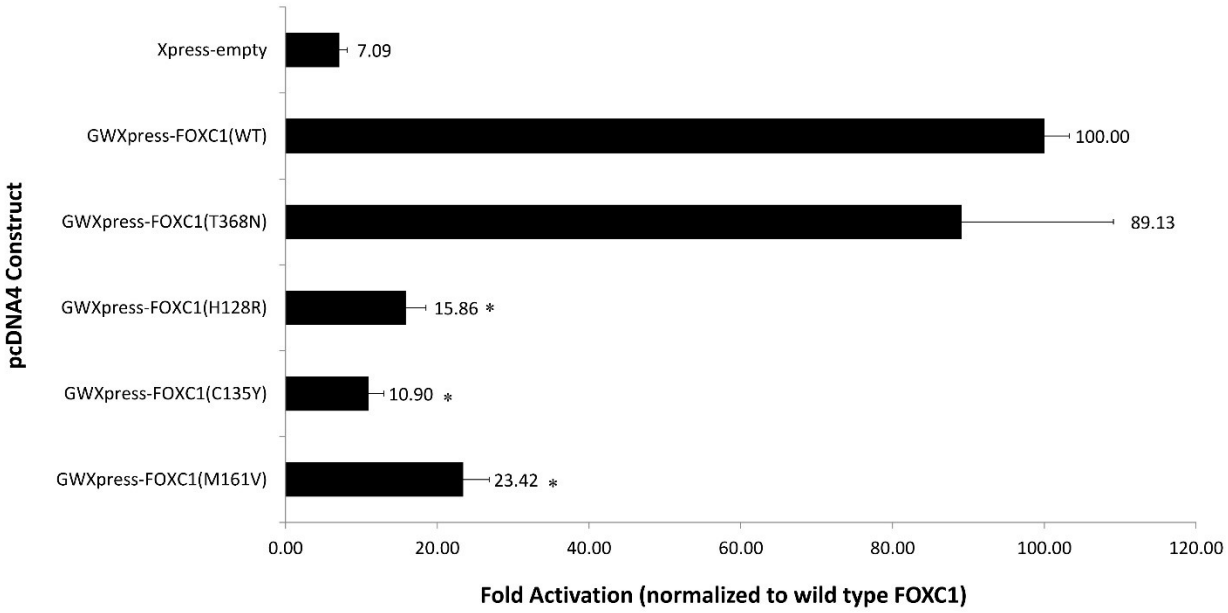
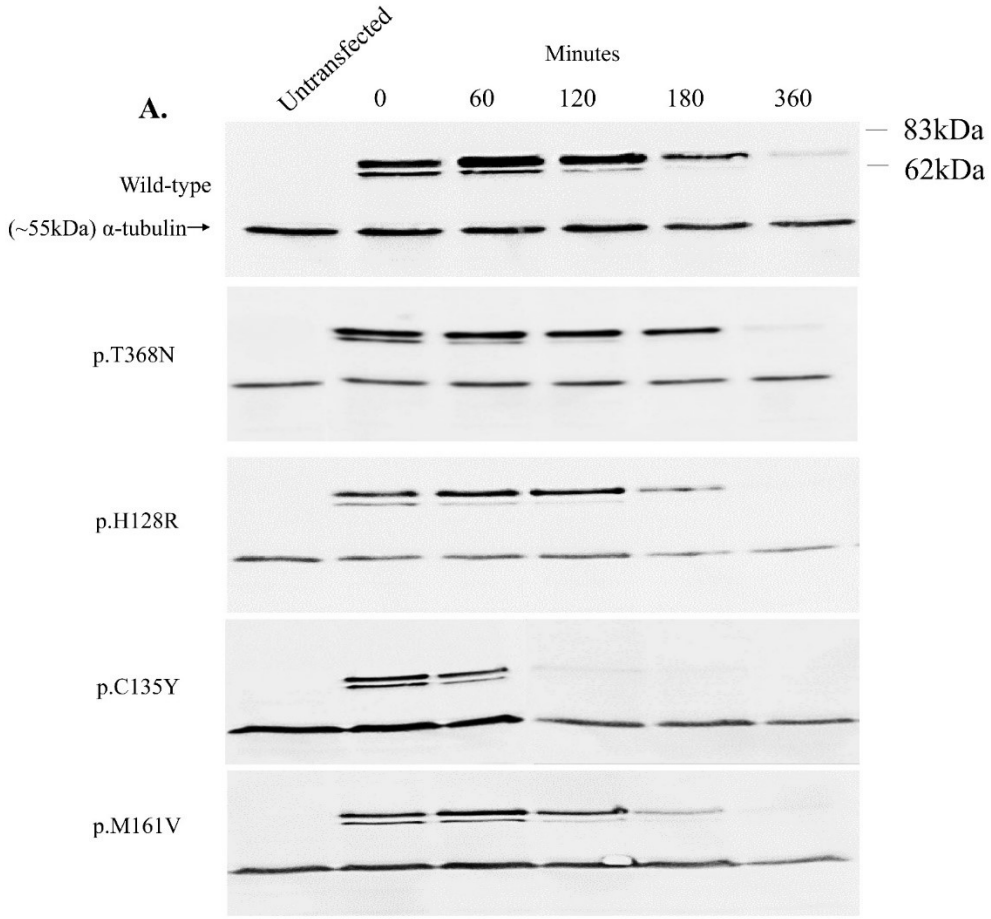

pcDNA4 Construct
Xpress-empty
GWXpress-FOXC1(WT)
GWXpress-FOXC1(T368N)
GWXpress-FOXC1(H128R)
GWXpress-FOXC1(C135Y)
GWXpress-FOXC1(M161V)
7.09
100.00
89.13
15.86 *
10.90 *
23.42 *
0.00
20.00
40.00
60.00
80.00
100.00
120.00
Fold Activation (normalized to wild type FOXC1)

Figure 7. Stability of FOXC1 wild-type, p.T368N, p.H128R, p.C135Y, and p.M161V. A: Transfected HeLa cells were treated with cycloheximide for the indicated times to inhibit protein synthesis. Western blots of cell extracts were probed with anti-Xpress antibodies and then with an antitubulin antibody. Immunoblots were scanned, and net pixel intensities of the bands were determined with the Image Station 4000MM (Eastman Kodak, Rochester, NY). FOXC1 values were normalized to α-tubulin. B: The slope of p.H128R and p.C135Y decay were significantly different from that of wild-type ($P < 0.05$). All the proteins occurred as a doublet at approximately 65 kDa. The protein size marker is shown to the right. The experiment was repeated three times.

A.

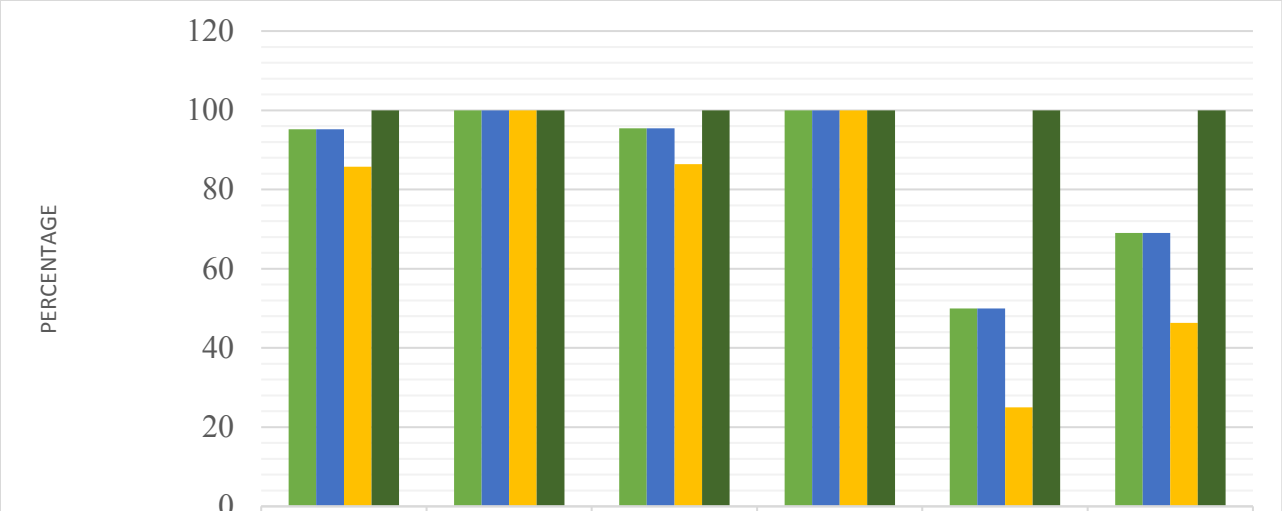


B.

Protein	Slope*	R2*	T ½ (min)*	T-test p-value
Wild-type (WT)	-0.0078	0.8237	89.6	NA
T368N	-0.0067	0.8063	107.0	0.30
H128R	-0.0059	0.8863	118.5	0.01
C135Y	-0.0167	0.9166	41.7	0.02
M161V	-0.0087	0.8979	81.5	0.40

*The data are based on average of three separate experiments. NA: not applicable.

Figure 8. Representation of the sensitivity, specificity, accuracy, precision, negative predictive value (NPV), and Matthews correlation coefficient (MCC) of SIFT, PolyPhen-2, Align-GVGD, and MutPred on all functionally characterized missense variants in *FOXC1*. The statistics used were calculated as follows: Sensitivity = TP/(TP + FN); Specificity = TN/(TN + FP); Accuracy = (TP + TN)/(TP + TN + FP + FN); Precision = TP/(TP + FP); Negative predictive value (NPV) = TN/(TN + FN); Matthews correlation coefficient (MCC) = (TP × TN − FP × FN)/([TP + FP] × [TP + FN] × [TN + FP] × [TN + FN]R). True positives (TP) are missense variants correctly predicted to disrupt *FOXC1* protein function, and false negatives (FN) are those incorrectly predicted to be benign or tolerated. True negatives (TN) are neutral variants correctly predicted as benign or tolerated and false positives (FP) are neutral variants incorrectly predicted to disrupt *FOXC1* protein function. The number of TP, TN, FP, and FN variants for both SIFT and PolyPhen-2 was the same including 20, 1, 0, 1, respectively. These numbers for Align-GVGD were 18, 1, 0, 3, and for MutPred were 21, 1, 0, 0, respectively. The total of variants for all methods was 22. Values were converted to percentage.



	Sensitivity	Specificity	Accuracy	Precision	NPV	MCC
SIFT	95.23	100	95.45	100	50	69.01
PolyPhen	95.23	100	95.45	100	50	69.01
Align-GVGD	85.71	100	86.36	100	25	46.29
MutPred	100	100	100	100	100	100

Table 1. Comparison of SIFT, PolyPhen, Align-CVGD and MutPred program predictions of degrees of tolerance for 22 functionally-characterized *FOXC1* missense variants

			Functional characterization vs. bioinformatics programs			
			SIFT	**PolyPhen**	**Align-GVGD**	**MutPred**
No	**Missense variants**	**References**	**Probability Score [a]**	**Probability Score [b]**	**Probability Score [c]**	**Probability Score [d]**
1	c.236C>T p.P79L	Saleem et al, (2003) (34,40)	0 (√)	1.000 (√)	C65 (√)	0.940 (√)
2	c.235C>A p.P79T	Saleem et al, (2003) (34,40)	0 (√)	1.000 (√)	C35 (√)	0.949(√)
3	c.245G>C p.S82T	Saleem et al, (2001) (23)	0 (√)	0.999 (√)	C55 (√)	0.944(√)
4	c.256C>T p.L86F	Saleem et al, (2003) (34,40)	0 (√)	1.000 (√)	C15 (√)	0.934(√)
5	c.261C>G p.I87M	Saleem et al, (2001) (23)	0 (√)	1.000 (√)	C0 (×)	0.946(√)
6	c.272T>G p.I91S	Saleem et al, (2003) (34,40)	0 (√)	1.000 (√)	C65 (√)	0.969(√)
7	c.272T>C p.I91T	Saleem et al, (2003) (34,40)	0 (√)	1.000 (√)	C65 (√)	0.968(√)
8	c.335T>C p.F112S	Saleem et al, (2001) (23)	0 (√)	1.000 (√)	C65 (√)	0.960(√)
9	c.377T>G p.I126S	Medina-Trillo et al, (2015) (41)	0 (√)	1.000 (√)	C65 (√)	0.910(√)
10	c.378C>G p.I126M	Saleem et al, (2001) (23)	0 (√)	1.000 (√)	C0 (×)	0.934(√)
11	c.380G>A p.R127H	Saleem et al, (2003) (34,40)	0 (√)	1.000 (√)	C25 (√)	0.975(√)
12	c.383A>G p.H128R	This study	0 (√)	1.000 (√)	C25 (√)	0.980(√)
13	c.388C>T p.L130F	Ito et al, (2007) (32)	0 (√)	1.000 (√)	C15 (√)	0.956(√)

14	c.392C>T p.S131L	Saleem et al, (2001) (23)	0 (√)	1.000 (√)	C65 (√)	0.963(√)
15	c.404G>A p.C135Y	This study	0 (√)	1.000 (√)	C65 (√)	0.958(√)
16	c.454T>G p.W152G	Ito et al, (2009) (42)	0 (√)	1.000 (√)	C65 (√)	0.954(√)
17	c.482T>A p.M161K	Murphy et al, (2004) (22)	0 (√)	0.996 (√)	C65 (√)	0.850(√)
18	c.481A>G p.M161V	This study	0 (√)	0.932(√)	C15 (√)	0.870(√)
19	c.493G>A p.G165R	Murphy et al, (2004) (22)	0 (√)	1.000 (√)	C65 (√)	0.921(√)
20	c.506G>C p.R169P	Murphy et al, (2004) (22)	0 (√)	1.000 (√)	C65 (√)	0.897(√)
21	c.889C>T p.P297S	Fetterman et al, (2009) (43)	0.74 (×)	0.261 (×)	C0 (×)	0.743(√)
22	c.1103C>A p.T368N	This study	0.28 (√)	0.000 (√)	C0 (√)	0.162(√)

[a]Probability of being pathogenic; 0=highest; 1=lowest.

[b]Probability of being pathogenic; 0=lowest; 1=highest.

[c]AlignGVGD, where >0=probably not tolerated; 0=probably tolerated.

[d]Probability of being pathogenic; 0=lowest; 1=highest

√; correspond to functional characterization, ×; do not correspond to functional characterization

Table 2. Bioinformatics prediction of the degrees of tolerance for nine uncharacterized *FOXC1* missense variants

			Bioinformatics predictions			
			SIFT	PolyPhen	Align-GVGD	MutPred
No.	**Missense variants**	**References**	**Probability Score [a]**	**Probability Score [b]**	**Probability Score [c]**	**Probability Score [d]**
1	C.236C>G p.P79R	Weisschuh et al, (2006) (18)	0	1.000	C65	0.933
2	c.253G>C p.A85P	Fuse et al, (2007) (44)	0	1.000	C25	0.925
3	c.325A>G p.M109V	D'haene et al, (2011) (45)	0	0.990	C15	0.638
4	c.344A>C p.Y115S	Weisschuh et al, (2006) (18)	0	1.000	C65	0.982
5	c.380G>T p.R127L	Du et al, (2015) (46)	0	1.000	C65	0.967
6	c.392C>A p.S131W	D'haene et al, (2011) (45)	0	1.000	C65	0.921
7	c.412A>G p.K138E	D'haene et al, (2011) (45)	0	0.999	C55	0.688
8	c.446G>A p.G149D	Weisschuh et al, (2006) (18)	0	1.000	C65	0.967
9	c.508C>T p.R170W	Gripp et al, (2013) (47)	0	1.000	C65	0.674

[a] Probability of being pathogenic; 0=highest; 1=lowest.

[b] Probability of being pathogenic; 0=lowest; 1=highest.

[c] The prediction ranges from C0 to C65 (C0, C15, C25, C35, C45, C55, C65) where C65 is most likely to disrupt function and C0 is least likely to be disruptive (30).

[d] Probability of being pathogenic; 0=lowest; 1=highest

References

1. Kaufmann,E. and Knöchel,W. (1996) Five years on the wings of fork head. *Mechanisms of development*, **57**, 3–20.

2. Saleem,R.A., Banerjee-Basu,S., Murphy,T.C., Baxevanis,A. and Walter,M.A. (2004) Essential structural and functional determinants within the forkhead domain of FOXC1. *Nucleic acids research*, **32**, 4182–93.

3. Berry,F.B., Saleem,R.A. and Walter,M.A. (2002) FOXC1 transcriptional regulation is mediated by N- and C-terminal activation domains and contains a phosphorylated transcriptional inhibitory domain. *The Journal of biological chemistry*, **277**, 10292–7.

4. Wei,L.-X., Zhou,R.-S., Xu,H.-F., Wang,J.-Y. and Yuan,M.-H. (2013) High expression of FOXC1 is associated with poor clinical outcome in non-small cell lung cancer patients. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, **34**, 941–6.

5. Xia,L., Huang,W., Tian,D., Zhu,H., Qi,X., Chen,Z., Zhang,Y., Hu,H., Fan,D., Nie,Y., *et al.* (2013) Overexpression of forkhead box C1 promotes tumor metastasis and indicates poor prognosis in hepatocellular carcinoma. *Hepatology (Baltimore, Md.)*, **57**, 610–24.

6. Jensen,T.W., Ray,T., Wang,J., Li,X., Naritoku,W.Y., Han,B., Bellafiore,F., Bagaria,S.P., Qu,A., Cui,X., *et al.* (2015) Diagnosis of Basal-Like Breast Cancer Using a FOXC1-Based Assay. *Journal of the National Cancer Institute*, **107**, djv148-djv148.

7. Aldinger,K.A., Lehmann,O.J., Hudgins,L., Chizhikov,V. V, Bassuk,A.G., Ades,L.C., Krantz,I.D., Dobyns,W.B. and Millen,K.J. (2009) FOXC1 is required for normal cerebellar

development and is a major contributor to chromosome 6p25.3 Dandy-Walker malformation. *Nature genetics*, **41**, 1037–42.

8. Mirzayans,F., Gould,D.B., Héon,E., Billingsley,G.D., Cheung,J.C., Mears,A.J. and Walter,M.A. (2000) Axenfeld-Rieger syndrome resulting from mutation of the FKHL7 gene on chromosome 6p25. *European journal of human genetics : EJHG*, **8**, 71–4.

9. Nishimura,D.Y., Searby,C.C., Alward,W.L., Walton,D., Craig,J.E., Mackey,D.A., Kawase,K., Kanis,A.B., Patil,S.R., Stone,E.M., *et al.* (2001) A spectrum of FOXC1 mutations suggests gene dosage as a mechanism for developmental defects of the anterior chamber of the eye. *American journal of human genetics*, **68**, 364–72.

10. Strungaru,M.H., Dinu,I. and Walter,M.A. (2007) Genotype-phenotype correlations in Axenfeld-Rieger malformation and glaucoma patients with FOXC1 and PITX2 mutations. *Investigative ophthalmology & visual science*, **48**, 228–37.

11. Weisschuh,N., Wolf,C., Wissinger,B. and Gramer,E. (2008) A novel mutation in the FOXC1 gene in a family with Axenfeld-Rieger syndrome and Peter's' anomaly. *Clinical genetics*, **74**, 476–80.

12. Chakrabarti,S., Kaur,K., Rao,K.N., Mandal,A.K., Kaur,I., Parikh,R.S. and Thomas,R. (2009) The transcription factor gene FOXC1 exhibits a limited role in primary congenital glaucoma. *Investigative ophthalmology & visual science*, **50**, 75–83.

13. Semina,E. V, Reiter,R., Leysens,N.J., Alward,W.L., Small,K.W., Datson,N.A., Siegel-Bartelt,J., Bierke-Nelson,D., Bitoun,P., Zabel,B.U., *et al.* (1996) Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nature genetics*, **14**, 392–9.

14. Nishimura,D.Y., Swiderski,R.E., Alward,W.L., Searby,C.C., Patil,S.R., Bennet,S.R., Kanis,A.B., Gastier,J.M., Stone,E.M. and Sheffield,V.C. (1998) The forkhead transcription factor gene FKHL7 is responsible for glaucoma phenotypes which map to 6p25. *Nature genetics*, **19**, 140–7.

15. Kidson,S.H., Kume,T., Deng,K., Winfrey,V. and Hogan,B.L. (1999) The forkhead/winged-helix gene, Mf1, is necessary for the normal development of the cornea and formation of the anterior chamber in the mouse eye. *Developmental biology*, **211**, 306–22.

16. Smith,R.S., Zabaleta,A., Kume,T., Savinova,O. V, Kidson,S.H., Martin,J.E., Nishimura,D.Y., Alward,W.L., Hogan,B.L. and John,S.W. (2000) Haploinsufficiency of the transcription factors FOXC1 and FOXC2 results in aberrant ocular development. *Human molecular genetics*, **9**, 1021–32.

17. Lehmann,O.J., Ebenezer,N.D., Ekong,R., Ocaka,L., Mungall,A.J., Fraser,S., McGill,J.I., Hitchings,R.A., Khaw,P.T., Sowden,J.C., *et al.* (2002) Ocular developmental abnormalities and glaucoma associated with interstitial 6p25 duplications and deletions. *Investigative ophthalmology & visual science*, **43**, 1843–9.

18. Weisschuh,N., Dressler,P., Schuettauf,F., Wolf,C., Wissinger,B. and Gramer,E. (2006) Novel Mutations of *FOXC1* and *PITX2* in Patients with Axenfeld-Rieger Malformations. *Investigative Opthalmology & Visual Science*, **47**, 3846.

19. Kaur,K., Ragge,N.K. and Ragoussis,J. (2009) Molecular analysis of FOXC1 in subjects presenting with severe developmental eye anomalies. *Molecular vision*, **15**, 1366–73.

20. Clark,K.L., Halay,E.D., Lai,E. and Burley,S.K. (1993) Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. *Nature*, **364**, 412–420.

21. Guex,N. and Peitsch,M.C. (1997) SWISS-MODEL and the Swiss-Pdb Viewer: An environment for comparative protein modeling. *Electrophoresis*, **18**, 2714–2723.

22. Murphy,T.C., Saleem,R.A., Footz,T., Ritch,R., McGillivray,B. and Walter,M.A. (2004) The wing 2 region of the FOXC1 forkhead domain is necessary for normal DNA-binding and transactivation functions. *Investigative ophthalmology & visual science*, **45**, 2531–8.

23. Saleem,R.A., Banerjee-Basu,S., Berry,F.B., Baxevanis,A.D. and Walter,M.A. (2001) Analyses of the effects that disease-causing missense mutations have on the structure and function of the winged-helix protein FOXC1. *American journal of human genetics*, **68**, 627–41.

24. Ng,P.C. and Henikoff,S. (2006) Predicting the effects of amino acid substitutions on protein function. *Annual review of genomics and human genetics*, **7**, 61–80.

25. Adzhubei,I.A., Schmidt,S., Peshkin,L., Ramensky,V.E., Gerasimova,A., Bork,P., Kondrashov,A.S. and Sunyaev,S.R. (2010) A method and server for predicting damaging missense mutations. *Nature methods*, **7**, 248–9.

26. Tavtigian,S. V, Deffenbaugh,A.M., Yin,L., Judkins,T., Scholl,T., Samollow,P.B., de Silva,D., Zharkikh,A. and Thomas,A. (2006) Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. *Journal of medical genetics*, **43**, 295–305.

27. Li,B., Krishnan,V.G., Mort,M.E., Xin,F., Kamati,K.K., Cooper,D.N., Mooney,S.D. and Radivojac,P. (2009) Automated inference of molecular mechanisms of disease from amino acid substitutions. *Bioinformatics (Oxford, England)*, **25**, 2744–50.

28. Majhi,R.K., Saha,S., Kumar,A., Ghosh,A., Swain,N., Goswami,L., Mohapatra,P., Maity,A.,

Kumar Sahoo,V., Kumar,A., *et al.* (2015) Expression of temperature-sensitive ion channel TRPM8 in sperm cells correlates with vertebrate evolution. *PeerJ*, **3**, e1310.

29. Ng,P.C. and Henikoff,S. (2003) SIFT: Predicting amino acid changes that affect protein function. *Nucleic acids research*, **31**, 3812–4.

30. Bryant,S.H. and Lawrence,C.E. (1993) An empirical energy function for threading protein sequence through the folding motif. *Proteins*, **16**, 92–112.

31. Berry,F.B., Mirzayans,F. and Walter,M.A. (2006) Regulation of FOXC1 stability and transcriptional activity by an epidermal growth factor-activated mitogen-activated protein kinase signaling cascade. *The Journal of biological chemistry*, **281**, 10098–104.

32. Ito,Y.A., Footz,T.K., Murphy,T.C., Courtens,W. and Walter,M.A. (2007) Analyses of a novel L130F missense mutation in FOXC1. *Archives of ophthalmology (Chicago, Ill. : 1960)*, **125**, 128–35.

33. Chun,S. and Fay,J.C. (2009) Identification of deleterious mutations within three human genomes. *Genome Research*, **19**, 1553–1561.

34. Saleem,R.A., Banerjee-Basu,S., Berry,F.B., Baxevanis,A.D. and Walter,M.A. (2003) Structural and functional analyses of disease-causing missense mutations in the forkhead domain of FOXC1. *Human molecular genetics*, **12**, 2993–3005.

35. Niroula,A. and Vihinen,M. (2016) Variation Interpretation Predictors: Principles, Types, Performance, and Choice. *Human mutation*, **37**, 579–97.

36. Richards,S., Aziz,N., Bale,S., Bick,D., Das,S., Gastier-Foster,J., Grody,W.W., Hegde,M., Lyon,E., Spector,E., *et al.* (2015) Standards and guidelines for the interpretation of sequence

variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine*, **17**, 405–423.

37. Masica,D.L. and Karchin,R. (2016) Towards Increasing the Clinical Relevance of In Silico Methods to Predict Pathogenic Missense Variants. *PLoS computational biology*, **12**, e1004725.

38. Valdmanis,P.N., Verlaan,D.J. and Rouleau,G.A. (2009) The proportion of mutations predicted to have a deleterious effect differs between gain and loss of function genes in neurodegenerative disease. *Human mutation*, **30**, E481-9.

39. Flanagan,S.E., Patch,A.-M. and Ellard,S. (2010) Using SIFT and PolyPhen to predict loss-of-function and gain-of-function mutations. *Genetic testing and molecular biomarkers*, **14**, 533–7.

40. Saleem,R.A., Murphy,T.C., Liebmann,J.M. and Walter,M.A. (2003) Identification and analysis of a novel mutation in the FOXC1 forkhead domain. *Investigative ophthalmology & visual science*, **44**, 4608–12.

41. Medina-Trillo,C., Sánchez-Sánchez,F., Aroca-Aguilar,J.-D., Ferre-Fernández,J.-J., Morales,L., Méndez-Hernández,C.-D., Blanco-Kelly,F., Ayuso,C., García-Feijoo,J. and Escribano,J. (2015) Hypo- and hypermorphic FOXC1 mutations in dominant glaucoma: transactivation and phenotypic variability. *PloS one*, **10**, e0119272.

42. Ito,Y.A., Footz,T.K., Berry,F.B., Mirzayans,F., Yu,M., Khan,A.O. and Walter,M.A. (2009) Severe molecular defects of a novel FOXC1 W152G mutation result in aniridia. *Investigative ophthalmology & visual science*, **50**, 3573–9.

43. Fetterman,C.D., Mirzayans,F. and Walter,M.A. (2009) Characterization of a novel FOXC1

mutation, P297S, identified in two individuals with anterior segment dysgenesis. *Clinical genetics*, **76**, 296–9.

44. Fuse,N., Takahashi,K., Yokokura,S. and Nishida,K. (2007) Novel mutations in the FOXC1 gene in Japanese patients with Axenfeld-Rieger syndrome. *Molecular vision*, **13**, 1005–9.

45. D'haene,B., Meire,F., Claerhout,I., Kroes,H.Y., Plomp,A., Arens,Y.H., de Ravel,T., Casteels,I., De Jaegere,S., Hooghe,S., *et al.* (2011) Expanding the spectrum of FOXC1 and PITX2 mutations and copy number changes in patients with anterior segment malformations. *Investigative ophthalmology & visual science*, **52**, 324–33.

46. Du,R.-F., Huang,H., Fan,L.-L., Li,X.-P., Xia,K. and Xiang,R. (2016) A Novel Mutation of FOXC1 (R127L) in an Axenfeld-Rieger Syndrome Family with Glaucoma and Multiple Congenital Heart Diseases. *Ophthalmic genetics*, **37**, 111–5.

47. Gripp,K.W., Hopkins,E., Jenny,K., Thacker,D. and Salvin,J. (2013) Cardiac anomalies in Axenfeld-Rieger syndrome due to a novel *FOXC1* mutation. *American Journal of Medical Genetics Part A*, **161**, 114–119.

Chapter 4. Accurate prediction of functional, structural, and stability changes in *PITX2* mutations using *in silico* bioinformatics algorithms

M. Seifi and M. A. Walter, in preparation. All experiments were carried out by Morteza Seifi.

Introduction

Paired-like homeodomain transcription factor 2 (*PITX2*, RefSeq NM 000325.5, MIM# 601542) is located at 4q25 and is expressed in the developing eye, brain, pituitary, lungs, heart, and gut (1). Variations in human *PITX2* or the forkhead box transcription factor C1 (*FOXC1*; 6p25, RefSeq NM 001453.2, MIM# 601090) underlie the autosomal dominant disorder called Axenfeld-Rieger syndrome (ARS; MIM# 602482) (2–5). ARS is a full penetrant, but clinically and genetically heterogeneous disorder characterized by developmental anomalies involving both ocular and non-ocular structures (6). To date, 87 variants within the *PITX2* gene have been identified including deletions, insertions, splice-site variants, and coding region frameshift, nonsense and missense variants (7–13).

Identifying new disease-associated variants is becoming increasingly important for genetic testing and it is leading to a significant change in the scale and sensitivity of molecular genetic analysis (14). One of the most frequent approaches for detecting novel variants in target genes is using direct gene sequencing. However, due to increasing number of newly identified missense variants, it is often difficult to interpret the pathogenicity of these variants as not all the variants alter protein function, and the ones that do may also have different functional impacts in disease (15, 16). Thus, prior to detailed analyses, novel variants cannot be easily classified as either deleterious or neutral, because of their unknown functional and phenotypic consequences. Therefore, further research should be conducted to validate the genetic diagnosis when a novel missense variant is discovered. Preferably, *in vitro* characterization of novel variants should be undertaken; however, due to facility limitation, it is often not practicable to experimentally verify the impact of large number of variants on protein function (17). Another robust approach to substantiate the pathogenicity is using animal models by generating the homologous variant that recapitulates the human phenotype; but, similar to *in vitro*

studies, these are time-consuming, labor-intensive, difficult and expensive, making this approach unfeasible to experimentally determine the pathogenicity effects of all novel identified variants (18). To circumvent the above mentioned limitations and to provide fast and efficient methods for predicting the functional effect of nonsynonymous variants on protein stability, structure, and function, several computational tools have been developed (19–21).

Protein stability and structure are key factors affecting function, activity, and regulation of proteins. Conformational changes are necessary for many proteins' function and disease-causing variants can impair protein folding and stability. Missense variants are also capable of impairing protein structure, likely by affecting protein folding, protein-protein interaction, solubility or stability of protein molecules. The structural effect of mutational changes can be examined *in silico* on the basis of three-dimensional structure, multiple alignments of homologous sequences, and molecular dynamics (22–24). Therefore, analysing sequence data *in silico* first and detecting a small number of predicted deleterious variants for further experimental characterization is a key factor in today's genetic and genomic studies.

In general, bioinformatics prediction methods obtain information on amino acid conservation through alignment with homologous and distantly related sequences. The most common criteria considered in many bioinformatics programs for predicting the functional effect of an amino acid substitution are amino acid sequence conservation across multiple species, physicochemical properties of the amino acids involved, database annotations, and potential protein structural changes (23, 25, 26). As mentioned above, resources for *in vitro* and *in vivo* functional analysis of novel variants are constrained in most clinical laboratories. Therefore, identifying and reporting novel variants that are likely to be pathogenic often requires accurate prediction using computational tools.

In chapter 3, I examined the effect of *FOXC1* variants on protein structure and function by combining laboratory experiments and *in silico* techniques. My results showed that integration of different algorithms with *in vitro* functional characterization serves as a reliable means of prioritizing, and then functional analyzing, candidate *FOXC1* variants (27). Unlike most previous studies that focused on using only PolyPhen and SIFT to predict the pathogenicity of missense variants, here, I investigated the predictive value of SIFT, PolyPhen and five other prediction tools by comparing their predictions to *in vitro* functional data for *PITX2* variants. The bioinformatics programs found to be most reliable were then used to predict the likely consequences of 13 functionally-uncharacterized *PITX2* variants. I also performed molecular modeling on all the *PITX2* missense variants located in the homeodomain and compared the results with the findings of protein stability algorithms to identify the most reliable tools in predicting the effect of missense variants on PITX2 stability. To the best of my knowledge, this is the first study that incorporates the results of functional studies in conjunction with bioinformatics approaches for predicting the pathogenicity of variations in *PITX2* gene.

Material and Methods

Source of missense variants

Lists of *PITX2* missense variants were assembled from the previous literature and a search using the ClinVar (28), Human Gene Mutation Database (HGMD) (29) and the single nucleotide polymorphism database (dbSNP). This study found 33 *PITX2* missense variants (Figure 1); 31 of which were described in the literature as being associated with ARS or coronary artery disease (CAD). Of these, 18 of the 31 variants were classified as pathogenic based on functional studies utilizing site-directed mutagenesis, expression studies, and other functional analysis (Table 1). Thirteen of 31 variants were described as associated with ARS and CAD in the absence of functional

analyses on PITX2 structure or function. Two exonic non-synonymous SNPs, with population allele frequencies > 1.0% were also identified from the ClinVar. Based upon the allele frequency these have been labelled benign polymorphisms.

Predicting functional impact of missense variant

PITX2 amino acid and DNA sequences were obtained from National Center for Biotechnology Information (NCBI) in FASTA format. The functional context of missense variants was predicted using the default settings of seven different *in silico* prediction algorithms, SIFT (sorting intolerant from tolerant) (30), PolyPhen-2 (polymorphism phenotyping-2) (31), PANTHER-PSEP (PANTHER position-specific evolutionary preservation) (32), MutPred (33), MutationTaster (34), Provean (protein variation effect analyzer) (35), and PMUT (36). These programs were used to analyse 18 functionally characterized *PITX2* missense variants plus 13 additional, functionally uncharacterized *PITX2* missense variants.

SIFT program provides functional predictions for coding variants, based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences, collected by PSI-BLAST (position-specific iterative basic local alignment search tool) algorithm (37). The PolyPhen-2 server predicts possible effect of an amino acid change on the structure and function of a protein using several sources of information such as straightforward physical and comparative considerations (38). PANTHER-PSEP is a new application that analyses the length of time a given amino acid has been conserved in the lineage leading to the protein of interest. There is a direct association between the conservation time and the likelihood of functional impact (39). MutPred is a free web-based application that utilizes a random forest algorithm with data based upon the probabilities of loss or gain of properties relating to many protein structures and dynamics,

predicted functional properties, and amino acid sequence and evolutionary information (33). MutationTaster is a tool that combines information derived from various biomedical databases and uses established analysis programs. Unlike SIFT or PolyPhen-2 which work on DNA level, MutationTaster processes substitutions of single amino acids and allows insertions and deletions (34). Protein variation effect analyzer (PROVEAN) is a web server which uses an alignment-based score approach to generate predictions not only for single amino acid substitutions, but also for multiple amino acid substitutions, and in-frame insertions and deletions (35). PMUT focuses on the annotation and prediction of pathological variants. PMUT is trained with a massive database of human disease-causing and neutral variants. PMUT calculates mutational hot spots, which are provided by three different approaches, alanine scanning, genetically accessible variations, and a very large database of variation (36). Please see Table 2 for more information on the prediction tools used in this study.

Molecular modeling of the mutant protein structure

The NMR structure of the homeodomain of PITX2 complexed with a TAATCC DNA binding site (PDB: 2LKX) were analyzed by the SWISS-MODEL server (http://www.expasy.org/spdbv/; provided in the public domain by the Swiss Institute of Bioinformatics, Geneva, Switzerland). Model structures of wild-type and mutants were created in Swiss-Pdb Viewer and investigated using the ANOLEA server (http://melolab.org/anolea). For structure predictions of *PITX2*, sequence in FASTA format was obtained from NCBI database (NP_001191327.1).

Calculating changes in protein stability

Four different protein stability programs (DUET, I-Mutant3.0, MUpro, and iPTREE-STAB) were used to predict the effects of missense variants on the stability of PITX2 protein. DUET is a web

server that uses integrated computational approach to predict effect of missense variants on protein stability (40). DUET calculation is based on complementary data regarding the variation including secondary structure (41) and a pharmacophore vector (42). I-Mutant3.0 is a neural-network-based web server that predicts automatically protein stability changes upon single point protein variations based on either protein sequence or protein structure. I-Mutant3.0 can predict the severity effect of a variation on the stability of the folded protein (43). MUpro is a set of machine learning programs that accurately calculates protein stability alterations based on primary sequence information particularly where the tertiary structure is unrevealed, overcoming a major restriction of previous methods which are based on the tertiary structure (44). iPTREE-STAB is a web service and mainly provides two function modules of services including discriminating the stability of a protein upon single amino acid substitutions and predicting their numerical stability values (45). Please see Table 3 for more information on the stability predictors used in this study.

Variants classification

Previous analyses of missense variations in different human diseases predicted that the stability margin without any immediate effect on protein fitness is 1–3 kcal mol^{-1} (46–48). Variations that reduce the protein stability by >2 kcal mol^{-1} contribute to severe disease phenotypes (49, 50). Therefore, in this study, all variations were classified as predicted to be neutral ($-1.5 < \Delta\Delta G < 1.5$), stabilizing ($\Delta\Delta G > 1.5$) or destabilizing ($\Delta\Delta G < -1.5$).

Results

Bioinformatics functional predictions

The protein sequence and/or protein structure with mutational position and amino acid residue of 18 previously functionally characterized pathogenic *PITX2* missense variants, plus two SNPs with a population frequency of higher than 1.0% (thus considered benign polymorphisms), were used to test the predictive value of seven common bioinformatics prediction programs; SIFT, PolyPhen-2, PANTHER-PSEP, MutPred, MutationTaster, Provean, and PMUT (Table 4). To evaluate the performances of the programs, six measures (sensitivity, specificity, accuracy, precision, negative predictive value (NPV), and Matthews correlation coefficient (MCC)) were calculated by comparing the results of all programs with previously generated functional data.

For *PITX2*, MutPred, Provean, and PMUT were the most reliable of the bioinformatics tools in predicting the pathogenicity effects of all 18 functionally characterized missense variants in *PITX2*, with sensitivity and specificity of > 94% (Figure 2). Analysis of the sensitivity and specificity SIFT showed that this program had good sensitivity (72.22%) and specificity (100%). Although PolyPhen-2, MutationTaster and PANTHER-PSEP exhibited over 83% sensitivity, they were unable to identify the benign polymorphisms, showing specificity of 50% (PolyPhen-2) and 0 (MutationTaster and PANTHER-PSEP).

The most reliable programs found in this study's analyses (MutPred, Provean, and PMUT) were then used to predict the likely pathogenicity of 13 *PITX2* missense variants for which functional testing has not been performed (Table 5). Interestingly, the A30V variant unanimously was predicted as benign by all three programs. The remaining 12 *PITX2* variants were predicted to be disease-associated variants by all programs.

Molecular modeling of *PITX2*

Molecular models for the homeodomain of wild-type and variant-containing PITX2 proteins were designed using threading algorithms to assess impairment of PITX2 structure by missense variants. Three functionally characterized variants (N100D, L105V, and N108T) were excluded from these molecular modeling analyses since they are not located in the homeodomain, which is the only portion of PITX2 with a known structure. Wild-type amino acids were changed to variant residues to determine putative structural effects of the remaining 15 functionally analysed *PITX2* variants through ANOLEA mean force potential calculations. The molecular modeling identified three variants as high-risk (L54Q, V83L, and R91P) to change the structure of PITX2, particularly in the H1, H2, and H3 subdomains (Figure 3). The R91P variant was predicted to grossly disrupt the non-local amino acid side chain contacts. Similar, although less profound, effects were predicted when L54 and V83 were altered to glutamine and leucine, respectively. In contrast, the remaining twelve amino acid variants showed no predicted substantially altered pairwise interactions, indicating that these missense variants are predicted to have minor or no effects on PITX2's structure (Supplementary Figure 1).

Molecular modeling was also performed on the nine functionally uncharacterized *PITX2* missense variants located in the homeodomain. Four variants (F58L, V83F, W86C, and W86S) were predicted to change the structure of PITX2 (Figure 4), while, the remaining five variants (R62H, P64L, P64R, R69C, and R90P) were predicted to have minor or no impact on PITX2's structure (Supplementary Figure 2).

Evaluation of the different algorithms in predicting stability changes

To assess the performance of four different stability predictor programs (DUET, I-Mutant3.0, MUpro, and iPTREE-STAB) in predicting the effect of missense variants on PITX2 protein stability, the change in protein stability (ΔΔG) were computed for all 24 *PITX2* homeodomain variants (15 functionally characterized and 9 functionally uncharacterized variants) (Table 6). DUET 3.0 predicted that eleven variants [eight experimentally verified (R43W, L54Q, P64S, R69H, R62H, K88E, R90C, and R91P) and three uncharacterized variants (R69C, W86S, and R90P)] affect the stability of the PITX2 protein. The remaining variants showed neutral effects on protein structure. iPTREE-STAB, and MUpro identified 7 (H45Q, L54Q, R62H, P64L, R84W, R90P, and R91P) and 6 variants (L54Q, P64S, M66T, W86S, R91Q, and R91P) that decrease the stability of the PITX2 protein structure. Q49L was predicted by MUpro to increase stability of the structure. I-Mutant3.0 sequence based predicted five destabilizing variants (L54Q, P64S, R69H, W86S, and W86C) and I-Mutant3.0 structure based detected three variants (L54Q, P64S, and W86S) to decrease the stability of PITX2 protein. Of these four programs, I-Mutant3.0 sequence based data were consistent with the results of my molecular modeling, by identifying 3 of 7 destabilizing variants that were also predicted to be destabilizing by molecular modeling (L54Q, W86S, and W86C).

Discussion

Although *in silico* programs are not a substitute for wet-lab experiments, they can provide a supportive role in the experimental validation of disease-associated alleles and can help further diagnostic strategies by prioritizing the most likely pathogenic novel variants.

While many tools are available for assessing the functional significance of variants, determining the reliability of prediction results is challenging. In this context, the current study investigated the

combination of experimental findings, molecular modeling, *in silico* mutation prediction programs, and stability prediction software to assess the pathogenicity of *PITX2* missense variants. *In silico* methods that correctly identify deleterious variants do not inevitably work well for benign variants. The methods determined by this study to be preferred for analyses of *PITX2* variants were those best able to distinguish both pathogenic and benign variants thus yielding the highest accuracy.

My results showed that MutPred, Provean, and PMUT were the most accurate in predicting pathogenicity of *PITX2* missense variants (Figure 2). The sensitivity and specificity of these three tools in recognizing *PITX2* disease-causing variants were over 94%, indicating the strong performance of these programs in identifying as pathogenic only *PITX2* variants with significant functional defects. SIFT was predicted to be less reliable, having a sensitivity of only 72.22%. PolyPhen-2, MutationTaster, and PANTHER-PSEP showed weak or no ability in identifying benign variants, having specificity of 50% (PolyPhen-2) and 0 (MutationTaster and PANTHER-PSEP). My results showed, therefore, that MutPred, Provean, and PMUT can be utilized with high confidence to test whether or not a *PITX2* missense variant is likely to be deleterious. Interestingly, MutPred was the only *in silico* program that ranked in the top three programs in identifying both pathogenic and benign *PITX2* and *FOXC1* variants (27). A likely explanation for MutPred's high ranking is that it evaluates the most factors in making assessments. However, since the number of variants available for testing in this study were small, a larger dataset would confirm that my results are reproducible and generally applicable.

The three programs that were found to be the most reliable (MutPred, Provean, and PMUT) were then used to assess the likely pathogenicity of thirteen *PITX2* missense variants for which functional analyses have not been performed, but which have been associated with ARS or CAD (Table 5). My results showed that MutPred, Provean, and PMUT predicted as pathogenetic 12/13 of the variants.

The p.A30V variant was scored as non-pathogenetic/benign by all three programs. While it is possible that p.A30V is an example of a false negative for all three programs, it is also likely that this variant is benign. Functional testing of the p.A30V variant is needed to determine which of these possibilities is accurate.

Various intramolecular interactions are involved in stabilizing and folded state of protein, including hydrophobic, electrostatic, and hydrogen-bonding (51–54). The stability state of a protein is key factor in its proper functionality. In fact, up to 80% of Mendelian disease-causing variations in protein coding regions are predicted to be caused by altering protein stability (55). In recent years, due to the availability of high-throughput array-based genotyping methods (56) and next generation sequencing platforms (57, 58), a large number of SNPs has been reported. However, the association of missense variants with protein stability has often been difficult to predict. Fortunately, recent advances in computational prediction of protein stability offers potential insight into this question. I used two parallel prediction methods to investigate the possible effects on PITX2 protein structure and stability of missense variants.

Knowledge of a protein's 3D structure can be used to predict the functionality of protein and the possible impact of variants on protein conformation and structure. I thus first used molecular modelling analyses to assess and compared the total energy difference between native and mutated modeled structure of PITX2 proteins. The results predicted that while most *PITX2* variants did not dramatically affect the protein tertiary structure, seven variants (L54Q, F58L, V83F, V83L, W86C, W86S, and R91P) altered the total energy level in comparison with the native structure, suggesting that these amino acid substitutions changed the structure of the PITX2 protein. Molecular modeling of the *PITX2* homeodomain predicted that these variants impair the required energy to maintain the proper folding of helix 1-3 and cause global destabilization of the structure of PITX2. These seven

amino acids are either invariant (e.g., W86) or highly conserved in the approximately 300 homeobox proteins analyzed, consistent with a pivotal role of these residues in the homeodomain (59–61). These seven amino acids are tightly packed hydrophobic amino acids responsible for holding helices of the *PITX2* homeodomain together, supporting my molecular modeling predicting that variations of these amino acids disrupt PITX2 structure. For F58L, V83F, and V83L, the native wild-type residues and the introduced mutant residues differ in size, probably causing loss of hydrophobic interactions in the core of the protein, particularly involving helix 1-3. For L54Q, W86C, W86S and R91P, the wild-type residues and the mutant residues are different in both size and charge, likely disturb the local structure of protein thereby altering protein structure and function.

Residues V83, W86, and R91 are located within the third helix which is specifically responsible for binding with the major groove of the DNA (62). Thus, the prediction that these variants impair the capacity of this helix to interact with DNA is consistent with this knowledge and with previous functional characterizations that showed reduced DNA-binding capacities of theV83L and R91P mutant PITX2 proteins (5, 63). Consistent with bioinformatics predictions of deleterious affects of variation of W86, variations of the neighboring amino acids (c.250C>T (p.R84W) and c.262A>G (p.K88E)) have been shown to decrease the ability of the mutant proteins to interact with DNA (64, 65).

Residues L54 and F58 are located in helix 1 of the homeodomain, responsible for contacting with the minor groove of the DNA. Molecular modeling of L54Q is consistent with the suggestion that variations in these highly-conserved residues in helix 1 of the homeodomain might disturb the DNA-protein binding affinity. My prediction is supported by the fact that changing the leucine to a glutamine (L54Q) disrupts DNA–protein complex, indicating the necessity of leucine at position 54 for *PITX2* binding ability (66). Thus, consistent with my recent studies on FOXC1 protein presented

in chapter 3 (67), the results of molecular modeling of *PITX2* are strongly consistent with the functional characterization of *PITX2* missense variants.

The results from my molecular modeling analysis were also compared to the predictions of four stability predictor methods (DUET, I-mutant3.0, MUpro, and iSTABLE-TREE). Based on my analyses, it appears that I-mutant3.0 performs the best of the three methods (evaluated here) in predicting the effect of missense variants on PITX2 protein stability, with DUET, MUpro, and iTREE-STAB performing somewhat weaker, consistent with the results of previous studies (68, 69). My results indicate that further studies are required to improve ΔΔG predictions, especially for buried amino acids.

In this study, for the first time, I evaluated the impact of missense variants on PITX2 stability, structure and function by integrating stability prediction algorithms, bioinformatics mutation prediction tools, and molecular modeling. My results showed that MutPred, Provean, PMUT, molecular modeling, and I-mutant3.0 are reliable methods to assess PITX family missense variants in the absence of laboratory experiments. However, for my analyses, it must be noted that I used two SNPs as non-pathogenetic control variants to investigate the performance of prediction programs. Although SNPs with a population frequency of >1.0% are normally considered as benign and were defined as such in my study, I cannot formally exclude that these SNPs might have un-documented pathogenic effects on *PITX2*. In addition, while the prediction methods used in this study are not gene-specific, generalization of the performance of these programs to other human genes may be inappropriate without additional study. In particular, as per clinical guidelines for the interpretation of single substitution variants, the output of computational tools should be interpreted in the light of functional studies results, population frequency data and segregation in affected families.

Figure 1. Summary of all 33 known *PITX2* missense variants. Characterized variants are shown in bold type. Two SNPs as benign polymorphism are presented in italic type.

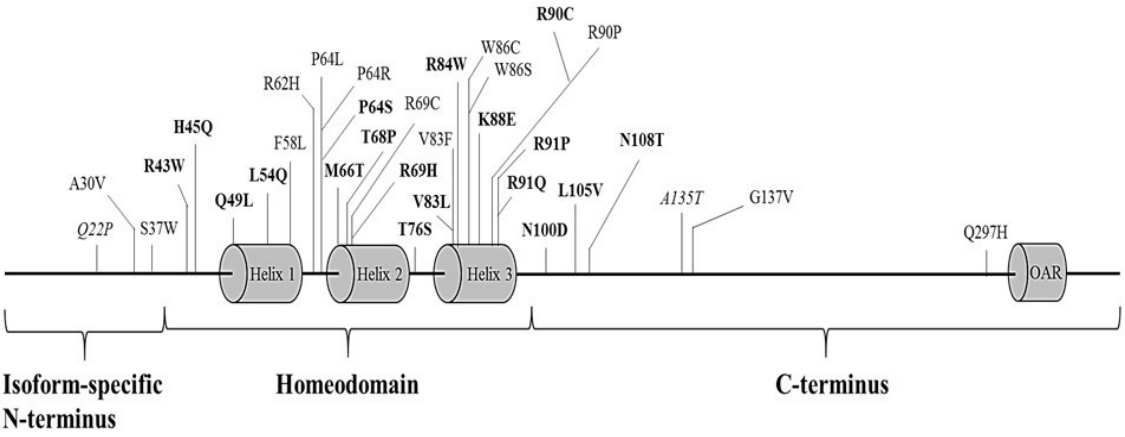
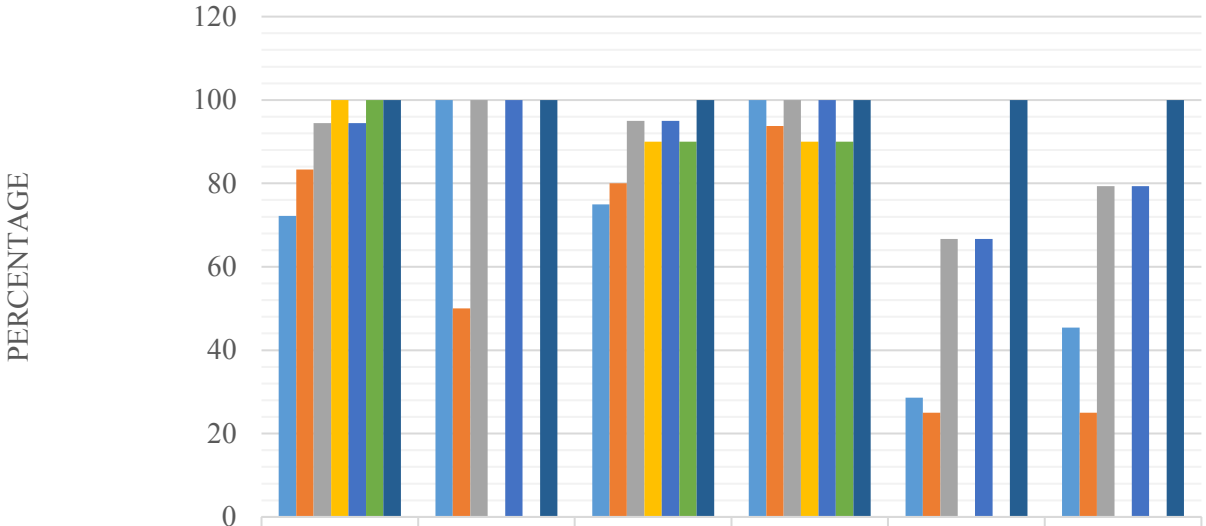

R90C
R90P
W86C
W86S
P64L
R84W
P64R
R62H
P64S
R69C
K88E
H45Q
T68P
V83F
R91P
N108T
F58L
R43W
M66T
R69H
R91Q
L105V
A30V
L54Q
Q49L
V83L
A135T
G137V
Q22P
S37W
T76S
N100D
Q297H
Helix 1
Helix 2
Helix 3
OAR
Isoform-specific N-terminus
Homeodomain
C-terminus

Figure 2. Reliability of seven programs used to analyze all 18 functionally characterized missense variants in *PITX2*. True positives (TP) are missense variants correctly predicted to disrupt *PITX2* protein function, and false negatives (FN) are those incorrectly predicted to be benign or tolerated. True negatives (TN) are neutral variants correctly predicted as benign or tolerated and false positives (FP) are neutral variants incorrectly predicted to disrupt *PITX2* protein function. The total of variants for all methods was 18. Values were converted to percentage. Values were converted to percentage. The statistics used were calculated as follows:

Sensitivity = TP/(TP + FN); Specificity = TN/(TN + FP); Accuracy = (TP + TN)/(TP + TN + FP + FN); Precision = TP/(TP + FP); Negative predictive value (NPV) = TN/(TN + FN); Matthews correlation coefficient (MCC) = (TP × TN − FP × FN)/-([TP + FP] × [TP + FN] × [TN + FP] × [TN + FN]R).



	Sensitivity	Specificity	Accuracy	Precision	NPV	MCC
SIFT	72.22	100	75	100	28.57	45.43
PolyPhen	83.33	50	80	93.75	25	25
MutPred	94.44	100	95	100	66.66	79.36
MutationTaster	100	0	90	90	0	0
Provean	94.44	100	95	100	66.66	79.36
PANTHER-PSEP	100	0	90	90	0	0
PMUT	100	100	100	100	100	100

Figure 3. Homology models (left) and scatterplots (right) of *in silico* analyses of the c.161T>A (p.L54Q), c.247G>C (p.V83L), and c.269G>C (p.R91P) variants in the *PITX2* gene. The 3D model of *PITX2* is presented with the protein backbone depicted in black ribbon, the co-crystallized DNA binding target in space-filled green model and the mutants positions in red. The wild-type and mutant-equivalent models were analyzed by the atomic nonlocal environment assessment (ANOLEA) server. Peaks on the scatterplots show the positions of amino acids that changed their pseudoenergy state, as a consequence of the mentioned variants.

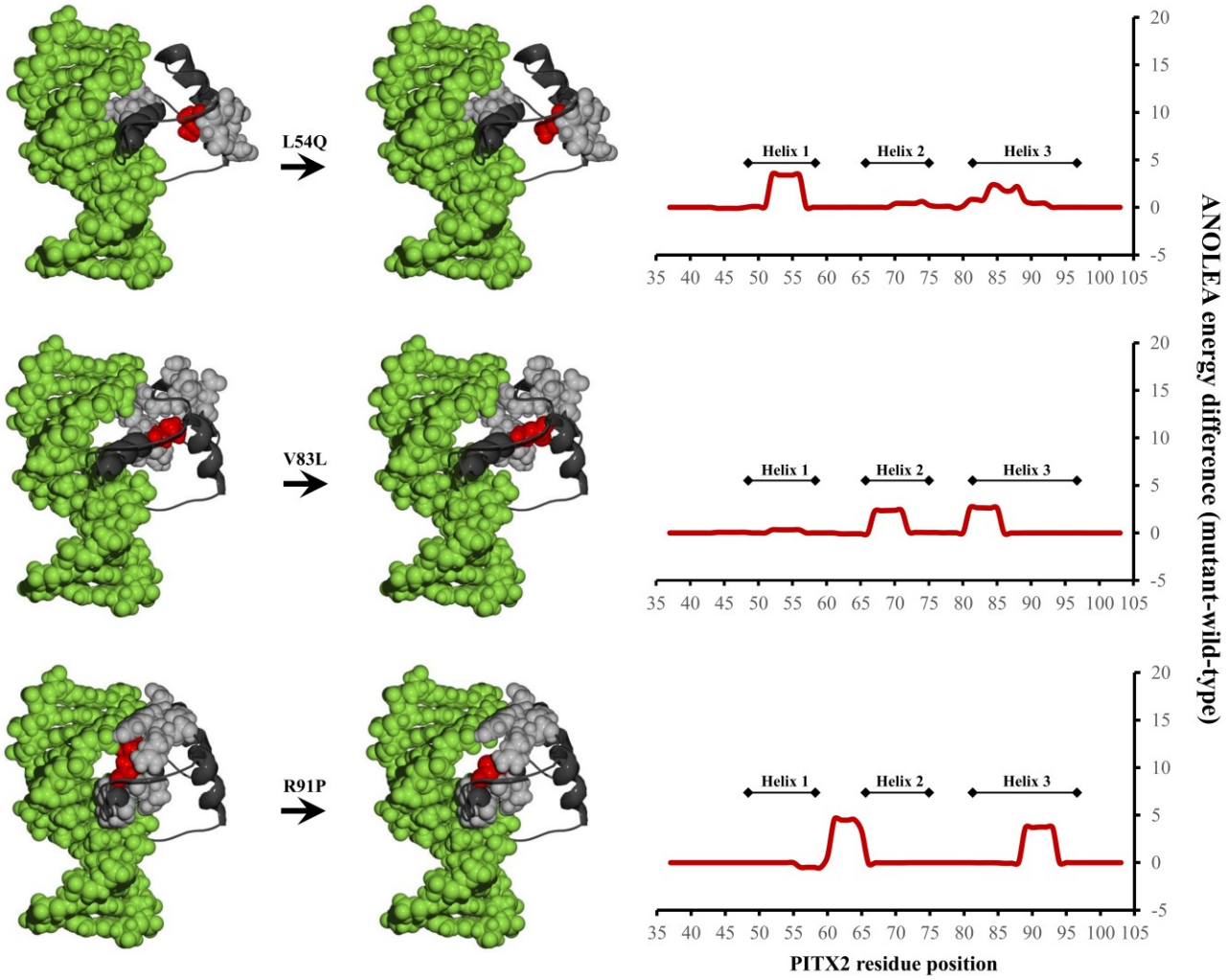

L54Q
V83L
R91P
Helix 1
Helix 2
Helix 3
20
15
10
5
0
-5
35 40 45 50 55 60 65 70 75 80 85 90 95 100 105
ANOLEA energy difference (mutant-wild-type)
PITX2 residue position

Figure 4. Homology models (left) and scatterplots (right) of *in silico* analyses of the c.172T>C (p.F58L), c.247G>T (p.V83F), c.247G>T (p.W86C), and c.257G>C (p.W86S) variants in the *PITX2* gene. The 3D model of *PITX2* is presented with the protein backbone depicted in black ribbon, the co-crystallized DNA binding target in space-filled green model and the mutants positions in red. The wild-type and mutant-equivalent models were analyzed by the atomic nonlocal environment assessment (ANOLEA) server. Peaks on the scatterplots show the positions of amino acids that changed their pseudoenergy state, as a consequence of the mentioned variants.

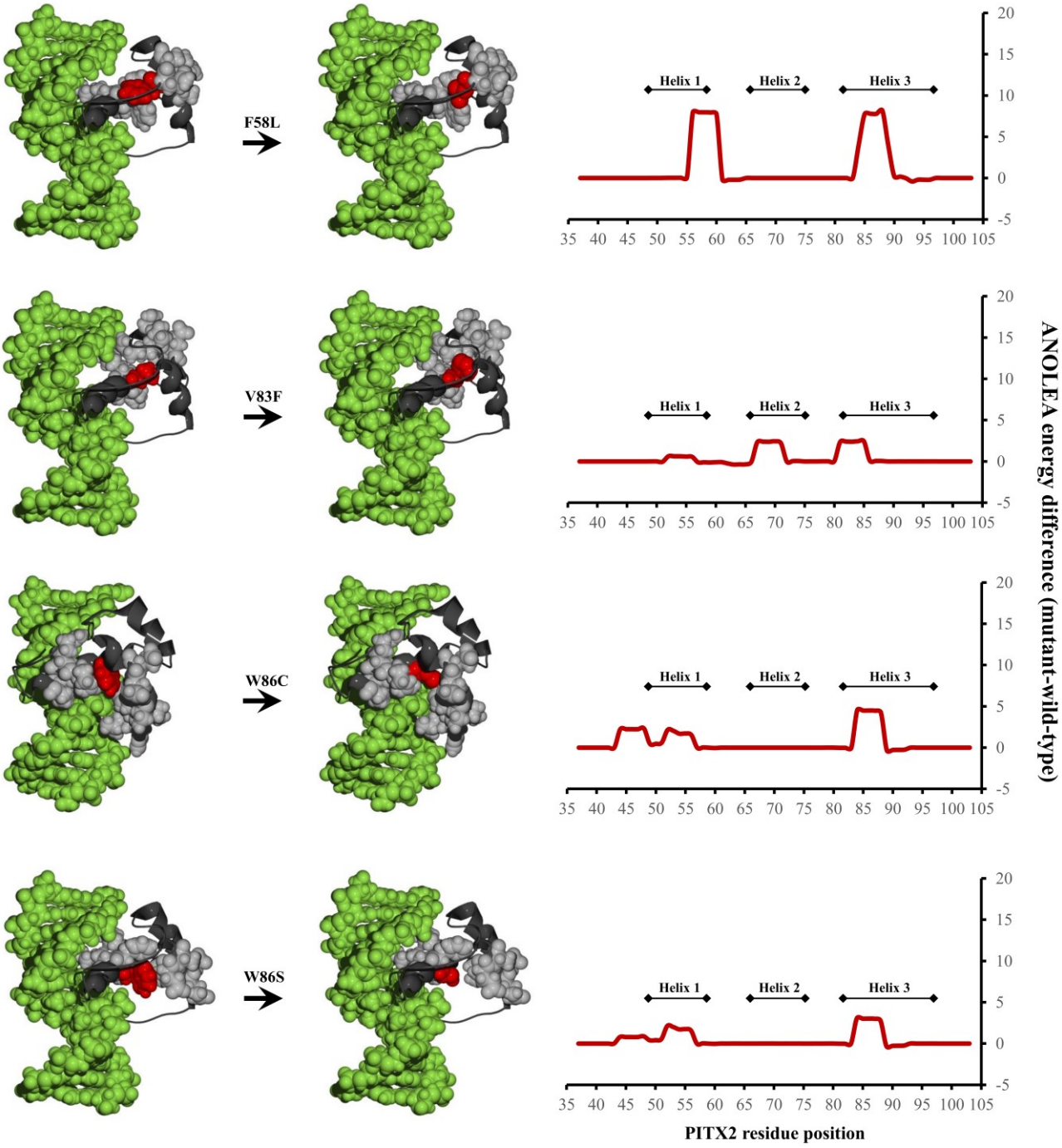

F58L
V83F
W86C
W86S
Helix 1
Helix 2
Helix 3
20
15
10
5
0
-5
35 40 45 50 55 60 65 70 75 80 85 90 95 100 105
ANOLEA energy difference (mutant-wild-type)
PITX2 residue position

Table 1. Position, effects on protein function and associated phenotype of *PITX2* missense variants.

Missense variants	Exon	Domain	Phenotype	Effect on protein function	Reference
c.127C>T p.R43W	5	HD	ARS	severely reduced DNA-binding and transcriptional activation	Idress et al, 2006 (70) Footz et al, 2009 (71)
c.294C>A p.H45Q	5	HD	CHD	significantly reduced transcriptional activity	Yuan et al, 2013 (72)
c.305A>T p.Q49L	5	HD	TOF	significantly reduced transcriptional activity, the variant markedly decreased the synergistic activation between *PITX2* and NKX2-5	Sun et al, 2016 (73)
c.161T>A p.L54Q	5	HD	ARS	Unable to bind DNA and deficient transactivation	Semina et al, 1996 (74) Amendt et al, 1998 (75)
c.185G>A p.R62H*	5	HD	ARS		Amendt et al, 2000 (76) Xia et al, 2004 (77)
c.349C>T p.P64S	5	HD	AF	significantly decreased transcriptional activity	Wang et al, 2014 (78)
c.356 T>C p.M66T	5	HD	CHD	significantly reduced transcriptional activity	Yuan et al, 2013 (72)
c.202A>C p.T68P	5	HD	ARS	Unable to bind DNA and deficient transactivation. *Pitx2* cannot transactivate Dlx2 promoter leading to abnormal tooth development.	Semina et al, 1996 (74) Amendt et al, 1998 (75) Amendt et al, 2000 (76) Kozlowski and Walter, 2000 (79) Espinoza et al, 2002 (80) Saadi et al, 2001 (81)
c.206G>A p.R69H	5	HD	ARS	Reduced DNA-binding activity.	Kulak et al, 1998 (82) Amendt et al, 2000 (76)

					Strungaru et al, 2007 (83) Kozlowski and Walter, 2000 (79)
c.385A>T T76S	5	HD	CHD	significantly diminished transcriptional activity	Wei et al, 2014 (84)
c.247G>C p.V83L	5	HD	ARS	Gain-of-function variant: decreased DNA binding, but increased transactivation.	Priston et al, 2001 (85)
c.250C>T p.R84W	5	HD	ARS	Reduced DNA binding and transactivation. Dlx2 promoter can be transactivated.	Alward et al, 1998 (86) Amendt et al, 2000 (76) Kozlowski and Walter, 2000 (79) Espinoza et al, 2002 (80)
c.262A>G p.K88E	6	HD	ARS	Defective DNA binding and transactivation, but has a dominant negative effect on wild-type protein.	Amendt et al, 2000 (76) Perveen et al, 2000 (87) Saadi et al, 2001 (81)
c.268C>T p.R90C	6	HD	ARS	severely reduced DNA-binding and transcriptional activation	Perveen et al, 2000 (87) Tootz et al, 2009 (71)
c.269G>C p.R91P	6	HD	ARS	Unable to bind DNA and deficient transactivation	Semina et al, 1996 (74) Amendt et al, 1998 (75) Amendt et al, 2000 (76) Priston et al, 2001 (85) Kozlowski and Walter, 2000 (79)
c.272G>A p.R91Q	6	HD	CHD	significantly diminished transactivational activity	Wei et al, 2014 (84)
c.457A>G	6	Downstream of HD	CHD	significantly reduced transactivational activity	Wang et al, 2013 (88)

p.N100D					
c.313C>G p.L105V	6	Downstream of HD	ARS	an altered mobility shift pattern of protein-DNA complexes	Phillips, 2002 (89) Footz et al, 2009 (71)
c.323A>C p.N108T	6	Downstream of HD	ARS	an altered mobility shift pattern and with slightly increased reporter transactivation and shortened protein half-life.	Phillips, 2002 (89) Footz et al, 2009 (71)

AF; atrial fibrillation (AF), ARS; Axenfeld-Rieger syndrome (ARS), CHD; congenital heart disease, HD; homeodomain, TOF; tetralogy of Fallot

Table 2. Amino acid substitution (AAS) prediction methods used in this study

Program	Input	Algorithm	Output	URL	Reference
SIFT	PS and AAS, protein sequence alignment and AAS, dbSNP id, or protein id	Uses sequence homology, scores assessment is based on position-specific scoring matrices with Dirichlet priors	Score ranges from 0 to 1, where <=0.05 is damaging and >0.05 is tolerated	http://sift.jcvi.org/www/SIFT_enst_submit.html	Ng and Henikoff, 2001 (90)
PolyPhen-2	PS and AAS, dbSNP id, HGVbASE id, or protein id	Uses sequence conservation and structure to model location of amino acid substitution, Swiss-Prot and TrEMBL annotation	Score ranges from 0 to 1, where <=0.05 is benign, and >0.05 is damaging	http://genetics.bwh.harvard.edu/pph2/	Ramensky et al, 2002 (91)
PANTHER-PSEP	PS and AAS	Uses sequence homology; scores are based on PANTHER Hidden Markov Model families	Probably damaging: time > 450my possibly damaging: 450my > time > 200my probably benign: time < 200my)	http://www.pantherdb.org/tools/csnpScoreForm.jsp	Tang and Thomas, 2016 (92)
MutPred	Protein id, PS, or multiple sequence alignment	Prediction is based on one of two neural networks which uses internal databases, secondary structure prediction, and sequence conservation	Score ranges from 0 to 1, where 0 is polymorphism and high scores are predicted to be deleterious/disease-associated	http://mutpred.mutdb.org/	Li et al, 2009 (93)
MutatioTaster	DNA sequence	Predictions are calculated by a naive Bayes classifier, which predicts the disease potential	Prediction is based one of four possible types: a) disease causing: probably deleterious b) disease causing automatic: known to be deleterious c) polymorphism: probably harmless d) polymorphism automatic: known to be harmless	http://www.mutationtaster.org/	Schwarz et al, 2014 (94)
Provean	PS and AAS	Uses an alignment-based score approach to generate predictions not only for single amino acid substitutions, but also for multiple amino acid substitutions, and in-frame insertions and deletions	the default score threshold is currently set at -2.5, in which >-2.5 is neutral, and <-2.5 is deleterious	http://provean.jcvi.org/index.php	Choi and Chan, 2015 (95)

PMUT	PS and AAS, dbSNP, Uniprot or PDB ID of protein	Based on the application of neural networks which uses internal databases, secondary structure prediction, and sequence conservation	Score ranges from 0 to 1, where <0.50 is neutral and >0.50 is disease associated	http://mmb.pcb.ub.es/pmut2017/analyses/new/	Ferrer-Costa et al, 2002 (96)

AAS; amino acid substitution, PS; protein sequence, PDB, protein data bank

Table 3. Protein stability predictor methods used in this study

Program	Input	Algorithm	Output	URL	Reference
DUET	protein structure	Prediction is made by combining the predictions from two methods (mCSM and SDM) in a non-linear way, using SVM regression with a Radial Basis Function kernel, and RSA	Score ranges from negative to positive numbers, where negative number denote destabilizing, and positive number denote stabilizing	http://bleoberis.bioc.cam.ac.uk/duet/	Pires et al, 2014 (97)
I-Mutant3.0	protein sequence alone or protein structure	Using SVM regression with a Radial Basis Function kernel, and RSA	Score ranges from negative to positive numbers, where negative number denote destabilizing, and positive number denote stabilizing	http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi	Capriotti et al, 2006 (98)
MUpro	protein sequence	Uses feed-forward neural networks and SVMs	A score near 0 means unchanged stability. Score near -1 means high confidence in decreased stability. Score near +1 means high confidence in increased stability	http://www.ics.uci.edu/~baldig/mutation.html	Cheng et al, 2006 (99)
iPTREE-STAB	protein sequence	Based on the neighboring residues of short window length	Score ranges from negative to positive numbers, where negative number denote destabilizing, and positive number denote stabilizing	http://210.60.98.19/IPTREEr/iptree.htm	Huang et al, 2007 (100)

Table 4. Comparison of SIFT, PolyPhen-2, MutPred, MutationTaster, Provean, PANTHE-PSEP, and PMUT program predictions of degrees of tolerance for 18 functionally-characterized *PITX2* missense variants

	Functional characterization vs. bioinformatics programs						
	SIFT	**PolyPhen-2**	**MutPred**	**MutationTaster**	**Provean**	**PANTHER-PSEP**	**PMUT**
Missense variants	**Probability Score**	**Probability Score**	**Probability Score**	**Probability Score**	**Probability Score**	**Probability Score**	**Probability Score**
c.127C>T p.R43W	0 (√)	0.003 (×)	0.952 (√)	101 (√)	-7.125 (√)	PD (√)	0.91 (√)
c.294C>A p.H45Q	0.21 (×)	1.000 (√)	0.372 (√)	24 (√)	-7.176 (√)	PD (√)	0.87 (√)
c.305A>T p.Q49L	0.25 (×)	0.995 (√)	0.498 (√)	113 (√)	-6.498 (√)	PD (√)	0.76 (√)
c.161T>A p.L54Q	0 (√)	0.997 (√)	0.959 (√)	113 (√)	-5.598 (√)	PD (√)	0.91 (√)
c.349C>T p.P64S	0 (√)	0.999 (√)	0.867 (√)	74 (√)	-7.547 (√)	PD (√)	0.85 (√)
c.356 T>C p.M66T	0 (√)	0.995 (√)	0.566 (√)	81 (√)	-5.555 (√)	PD (√)	0.88 (√)
c.202A>C p.T68P	0 (√)	0.946 (√)	0.854 (√)	38 (√)	-5.094 (√)	PD (√)	0.87 (√)
c.206G>A p.R69H	0 (√)	0.007 (×)	0.985 (√)	29 (√)	-4.733 (√)	PD (√)	0.90 (√)
c.385A>T T76S	0 (√)	0.995 (√)	0.655 (√)	58 (√)	-3.652 (√)	PD (√)	0.89 (√)
c.247G>C p.V83L	0.01 (√)	0.902 (√)	0.944 (√)	32 (√)	-2.758 (√)	PD (√)	0.89 (√)
c.250C>T p.R84W	0 (√)	0.994 (√)	0.841 (√)	101 (√)	-7.350 (√)	PD (√)	0.88 (√)
c.262A>G p.K88E	0 (√)	0.008 (×)	0.828 (√)	56 (√)	-3.800 (√)	PD (√)	0.88 (√)

c.268C>T p.R90C	0 (√)	0.957 (√)	0.975 (√)	180 (√)	-7.599 (√)	PD (√)	0.91 (√)
c.269G>C p.R91P	0 (√)	0.998 (√)	0.959 (√)	103 (√)	-6.649 (√)	PD (√)	0.91 (√)
c.272G>A p.R91Q	0 (√)	0.997 (√)	0.918 (√)	43 (√)	-3.800 (√)	PD (√)	0.91 (√)
c.457A>G p.N100D	0.2 (×)	0.863 (√)	0.365 (×)	23 (√)	-4.013 (√)	PD (√)	0.81 (√)
c.313C>G p.L105V	0.06 (×)	0.974 (√)	0.788 (√)	32 (√)	-1.894 (×)	PD (√)	0.80 (√)
c.323A>C p.N108T	0.24 (×)	0.990 (√)	0.789 (√)	65 (√)	-3.332 (√)	PD (√)	0.68 (√)

√; correspond to functional characterization, ×; do not correspond to functional characterization.

Table 5. Bioinformatics prediction of the degree of tolerance for 13 uncharacterized *PITX2* missense variants

				Functional characterization vs. bioinformatics programs		
				MutPred	**Provean**	**PMUT**
No	**Missense variants**	**References**	**Phenotype**	**Probability Score**	**Probability Score**	**Probability Score**
1	c.80C>T p.A30V	Zaidi et al, 2013 (101)	CHD	0.152	-0.948	0.10
2	c.101C>G p.S37W	Yang et al, 2013 (102)	AF	0.403	-1.074	0.81
3	c.172T>C p.F58L	Vieira et al, 2006 (103) D'haene et al, 2011 (104)	ARS	0.947	-5.560	0.90
4	c.185G>A p.R62H	Amendt et al, 2000 (76) Xia et al, 2004 (77)	ARS	0.856	-4.686	0.70
5	c.191C>T p.P64L	Phillips JC, 2002 (89) Weisschuh et al, 2006 (105) Meyer-Marcotty et al, 2008 (106) Dressler et al, 2010 (107)	ARS	0.973	-9.421	0.81
6	c.191C>G p.P64R	Weisschuh et al, 2006 (105)	ARS	0.944	-8.496	0.84
7	c.205C>T p.R69C	Kimura et al, 2014 (108)	ARS	0.960	-7.575	0.91
8	c.247G>T p.V83F	Reis et al, 2012 (109)	ARS	0.912	-4.643	0.91
9	c.257G>C p.W86S	Dandan et al, 2008 (110)	ARS	0.950	-12.282	0.91
10	c.258G>T p.W86C	Reis et al, 2012 (109)	ARS	0.868	-13.298	0.91
11	c.269G>C	Phillips JC, 2002 (89)	ARS	0.960	-6.649	0.91

	p.R90P					
12	c.410G>T p.G137V	Kniestedt et al, 2006 (111)	ARS	0.816	-1.902 Neutral	0.61
13	c. 891C>A p.Q297H	Huang et al, 2015 (112)	ARS	0.682	-3.966 deleterious	0.91

AF; Atrial fibrillation (AF), ARS; Axenfeld-Rieger syndrome (ARS), CHD; Congenital heart disease

Table 6. Evaluation of stability changes of 15 functionally characterized and 9 functionally uncharacterized *PITX2* homeodomain missense variants using DUET, I-Mutant3.0, MUpro, and iPTREE-STAB.

No.	Variations	DUET	I-Mutant3.0 SEQ	I-Mutant3.0 Structure	MUpro	iPTREE-STAB
	Characterized variants					
1	R43W	-1.773	0.00	-0.13	-0.162	0.0337
2	H45Q	0.158	0.07	0.17, I	-0.112	-2.9050
3	Q49L	0.471	0.38	0.68, I	1	0.9422
4	L54Q	-2.892	-1.65	-1.50	-1	-1.8488
5	P64S	-2.069	-1.59	-1.57	-1	-1.0233
6	M66T	0.444	-1.20	-0.32	-1	1.0943
7	T68P	-0.359	-0.90	-0.68	0.155	-1.0594
8	R69H	-2.369	-1.56	-1.29	-0.633	-1.3667
9	T76S	-1.35	-0.69	-0.26	-0.014	0.9377
10	V83L	-0.305	-0.91	-0.72	0.224	-1.3883
11	R84W	-1.056	-0.52	-0.41	-0.966	-2.9167
12	K88E	-1.759	-0.32	-0.24	-0.024	-0.9691
13	R90C	-2.014	-0.86	-0.89	-0.567	-0.6385
14	R91P	-2.225	-0.82	-0.93	-1	-2.7464
15	R91Q	-1.308	-0.95	-1.04	-1	0.3362
	Uncharacterized variants					
1	F58L	-0.868	-0.69	-0.71	0.446	-1.3492
2	R62H	-1.839	-1.24	-1.17	-0.634	-2.1794
3	P64L	-0.55	-0.07	-0.64	-0.260	-4.1000
4	P64R	-0.979	-0.83	-1.09	-0.892	-0.8385
5	R69C	-2.183	-1.12	-1.07	-0.183	0.2429
6	V83F	-1.437	-1.16	-1.12	-0.496	-1.3883
7	W86S	-2.327	-1.64	-1.55	-1	-0.6167
8	W86C	-0.931	-1.52	-1.40	-0.971	0.6923
9	R90P	-1.623	-0.71	-0.74	-0.346	-2.8825

Variations were considered neutral ($-1.5 < \Delta\Delta G < 1.5$), stabilizing ($\Delta\Delta G < 1.5$) or destabilizing ($\Delta\Delta G > 1.5$).

References

1. Vaux,C., Sheffield,L., Keith,C.G. and Voullaire,L. (1992) Evidence that Rieger syndrome maps to 4q25 or 4q27. *J Med Genet*, **29**, 256–8.

2. Tümer,Z. and Bach-Holm,D. (2009) Axenfeld-Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. *European journal of human genetics : EJHG*, **17**, 1527–39.

3. Strungaru,M.H., Dinu,I. and Walter,M.A. (2007) Genotype-Phenotype Correlations in Axenfeld-Rieger Malformation and Glaucoma Patients with *FOXC1* and *PITX2* Mutations. *Investigative Opthalmology & Visual Science*, **48**, 228.

4. Lines,M.A., Kozlowski,K. and Walter,M.A. (2002) Molecular genetics of Axenfeld-Rieger malformations. *Human molecular genetics*, **11**, 1177–84.

5. Kozlowski,K. and Walter,M.A. (2000) Variation in residual PITX2 activity underlies the phenotypic spectrum of anterior segment developmental disorders. *Human molecular genetics*, **9**, 2131–9.

6. Shields,M.B. (1983) Axenfeld-Rieger syndrome: a theory of mechanism and distinctions from the iridocorneal endothelial syndrome. *Transactions of the American Ophthalmological Society*, **81**, 736–84.

7. Xia,K., Wu,L., Liu,X., Xi,X., Liang,D., Zheng,D., Cai,F., Pan,Q., Long,Z., Dai,H., *et al.* (2004) Mutation in PITX2 is associated with ring dermoid of the cornea. *Journal of medical genetics*, **41**, e129.

8. Seifi,M., Footz,T., Taylor,S.A.M., Elhady,G.M., Abdalla,E.M. and Walter,M.A. (2016) Novel PITX2 gene mutations in patients with Axenfeld-Rieger syndrome. *Acta Ophthalmologica*, **94**, e571–e579.

9. Saadi,I., Toro,R., Kuburas,A., Semina,E., Murray,J.C. and Russo,A.F. (2006) An unusual class

of PITX2 mutations in Axenfeld-Rieger syndrome. *Birth defects research. Part A, Clinical and molecular teratology*, **76**, 175–81.

10. D'haene,B., Meire,F., Claerhout,I., Kroes,H.Y., Plomp,A., Arens,Y.H., de Ravel,T., Casteels,I., De Jaegere,S., Hooghe,S., *et al.* (2011) Expanding the spectrum of FOXC1 and PITX2 mutations and copy number changes in patients with anterior segment malformations. *Investigative ophthalmology & visual science*, **52**, 324–33.
11. Brooks,B.P., Moroi,S.E., Downs,C.A., Wiltse,S., Othman,M.I., Semina,E. V and Richards,J.E. (2004) A novel mutation in the PITX2 gene in a family with Axenfeld-Rieger syndrome. *Ophthalmic genetics*, **25**, 57–62.
12. Weisschuh,N., Dressler,P., Schuettauf,F., Wolf,C., Wissinger,B. and Gramer,E. (2006) Novel Mutations of FOXC1 and PITX2 in Patients with Axenfeld-Rieger Malformations. *Investigative Opthalmology & Visual Science*, **47**, 3846.
13. Perveen,R., Lloyd,I.C., Clayton-Smith,J., Churchill,A., van Heyningen,V., Hanson,I., Taylor,D., McKeown,C., Super,M., Kerr,B., *et al.* (2000) Phenotypic variability and asymmetry of Rieger syndrome associated with PITX2 mutations. *Investigative ophthalmology & visual science*, **41**, 2456–60.
14. Judkins,T., Hendrickson,B.C., Deffenbaugh,A.M. and Scholl,T. (2005) Single nucleotide polymorphisms in clinical genetic testing: the characterization of the clinical significance of genetic variants and their application in clinical research for BRCA1. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, **573**, 168–179.
15. Young,D.L. and Fields,S. (2015) The role of functional data in interpreting the effects of genetic variation. *Molecular biology of the cell*, **26**, 3904–8.
16. Campuzano,O., Allegue,C., Fernandez,A., Iglesias,A. and Brugada,R. (2015) Determining the

Pathogenicity of Genetic Variants Associated with Cardiac Channelopathies. *Scientific Reports*, **5**, 7953.

17. Jia,L., Yarlagadda,R., Reed,C.C., Haeberlein,M., Chen,H. and Ma,S. (2015) Structure Based Thermostability Prediction Models for Protein Single Point Mutations with Machine Learning Tools. *PLOS ONE*, **10**, e0138022.
18. Flanagan,S.E., Patch,A.-M. and Ellard,S. (2010) Using SIFT and PolyPhen to predict loss-of-function and gain-of-function mutations. *Genetic testing and molecular biomarkers*, **14**, 533–537.
19. Tokuriki,N. and Tawfik,D.S. (2009) Stability effects of mutations and protein evolvability. *Current Opinion in Structural Biology*, **19**, 596–604.
20. Gromiha,M.M. (2007) Prediction of protein stability upon point mutations. *Biochemical Society Transactions*, **35**, 1569–1573.
21. Choi,Y., Sims,G.E., Murphy,S., Miller,J.R. and Chan,A.P. (2012) Predicting the Functional Effect of Amino Acid Substitutions and Indels. *PLoS ONE*, **7**, e46688.
22. Stefl,S., Nishi,H., Petukh,M., Panchenko,A.R. and Alexov,E. (2013) Molecular mechanisms of disease-causing missense mutations. *Journal of molecular biology*, **425**, 3919–36.
23. Laskowski,R.A., Tyagi,N., Johnson,D., Joss,S., Kinning,E., McWilliam,C., Splitt,M., Thornton,J.M., Firth,H. V, DDD Study, the D., *et al.* (2016) Integrating population variation and protein structural analysis to improve clinical interpretation of missense variation: application to the WD40 domain. *Human molecular genetics*, **25**, 927–35.
24. Bao,L. and Cui,Y. (2005) Prediction of the phenotypic effects of non-synonymous single nucleotide polymorphisms using structural and evolutionary information. *Bioinformatics*, **21**, 2185–2190.

25. Wallqvist,A., Fukunishi,Y., Murphy,L.R., Fadel,A. and Levy,R.M. (2000) Iterative sequence/secondary structure search for protein homologs: comparison with amino acid sequence alignments and application to fold recognition in genome databases. *Bioinformatics (Oxford, England)*, **16**, 988–1002.

26. Geourjon,C., Combet,C., Blanchet,C. and Deléage,G. (2001) Identification of related proteins with weak sequence identity using secondary structure information. *Protein science : a publication of the Protein Society*, **10**, 788–97.

27. Seifi,M., Footz,T., Taylor,S.A.M. and Walter,M.A. (2017) Comparison of Bioinformatics Prediction, Molecular Modeling, and Functional Analyses of FOXC1 Mutations in Patients with Axenfeld-Rieger Syndrome. *Human Mutation*, **38**, 169–179.

28. Landrum,M.J., Lee,J.M., Riley,G.R., Jang,W., Rubinstein,W.S., Church,D.M. and Maglott,D.R. (2014) ClinVar: public archive of relationships among sequence variation and human phenotype. *Nucleic Acids Research*, **42**, D980–D985.

29. Stenson,P.D., Mort,M., Ball,E. V., Shaw,K., Phillips,A.D. and Cooper,D.N. (2014) The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. *Human Genetics*, **133**, 1–9.

30. Ng,P.C. and Henikoff,S. (2001) Predicting Deleterious Amino Acid Substitutions. *Genome Research*, **11**, 863–874.

31. Ramensky,V., Bork,P. and Sunyaev,S. (2002) Human non-synonymous SNPs: server and survey. *Nucleic acids research*, **30**, 3894–900.

32. Tang,H. and Thomas,P.D. (2016) PANTHER-PSEP: predicting disease-causing genetic variants using position-specific evolutionary preservation. *Bioinformatics*, **32**, 2230–2232.

33. Li,B., Krishnan,V.G., Mort,M.E., Xin,F., Kamati,K.K., Cooper,D.N., Mooney,S.D. and Radivojac,P. (2009) Automated inference of molecular mechanisms of disease from amino acid substitutions. *Bioinformatics (Oxford, England)*, **25**, 2744–50.

34. Schwarz,J.M., Cooper,D.N., Schuelke,M. and Seelow,D. (2014) MutationTaster2: mutation prediction for the deep-sequencing age. *Nature methods*, **11**, 361–2.

35. Choi,Y. and Chan,A.P. (2015) PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics (Oxford, England)*, **31**, 2745–7.

36. Ferrer-Costa,C., Orozco,M. and de la Cruz,X. (2002) Characterization of disease-associated single amino acid polymorphisms in terms of sequence and structure properties. *Journal of Molecular Biology*, **315**, 771–786.

37. Kumar,P., Henikoff,S. and Ng,P.C. (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nature Protocols*, **4**, 1073–1081.

38. Krogh,A., Larsson,B., von Heijne,G. and Sonnhammer,E.L.. (2001) Predicting transmembrane protein topology with a hidden markov model: application to complete genomes11Edited by F. Cohen. *Journal of Molecular Biology*, **305**, 567–580.

39. Mi,H. and Thomas,P. (2009) PANTHER Pathway: An Ontology-Based Pathway Database Coupled with Data Analysis Tools. In.pp. 123–140.

40. Pires,D.E. V, Ascher,D.B. and Blundell,T.L. (2014) DUET: a server for predicting effects of mutations on protein stability using an integrated computational approach. *Nucleic acids research*, **42**, W314-9.

41. Pires,D.E. V., Ascher,D.B. and Blundell,T.L. (2014) mCSM: predicting the effects of mutations in proteins using graph-based signatures. *Bioinformatics*, **30**, 335–342.

42. Worth,C.L., Preissner,R. and Blundell,T.L. (2011) SDM--a server for predicting effects of

mutations on protein stability and malfunction. *Nucleic Acids Research*, **39**, W215–W222.

43. Capriotti,E., Calabrese,R. and Casadio,R. (2006) Predicting the insurgence of human genetic diseases associated to single point protein mutations with support vector machines and evolutionary information. *Bioinformatics (Oxford, England)*, **22**, 2729–34.

44. Cheng,J., Randall,A. and Baldi,P. (2006) Prediction of protein stability changes for single-site mutations using support vector machines. *Proteins*, **62**, 1125–32.

45. Huang,L.-T., Gromiha,M.M. and Ho,S.-Y. (2007) iPTREE-STAB: interpretable decision tree based method for predicting protein stability changes upon mutations. *Bioinformatics (Oxford, England)*, **23**, 1292–3.

46. Tokuriki,N. and Tawfik,D.S. (2009) Stability effects of mutations and protein evolvability. *Current Opinion in Structural Biology*, **19**, 596–604.

47. Mayer,S., Rüdiger,S., Ang,H.C., Joerger,A.C. and Fersht,A.R. (2007) Correlation of levels of folded recombinant p53 in escherichia coli with thermodynamic stability in vitro. *Journal of molecular biology*, **372**, 268–76.

48. Calloni,G., Zoffoli,S., Stefani,M., Dobson,C.M. and Chiti,F. (2005) Investigating the Effects of Mutations on Protein Aggregation in the Cell. *Journal of Biological Chemistry*, **280**, 10607–10613.

49. Randles,L.G., Lappalainen,I., Fowler,S.B., Moore,B., Hamill,S.J. and Clarke,J. (2006) Using model proteins to quantify the effects of pathogenic mutations in Ig-like proteins. *The Journal of biological chemistry*, **281**, 24216–26.

50. Lindberg,M.J., Byström,R., Boknäs,N., Andersen,P.M. and Oliveberg,M. (2005) Systematically perturbed folding patterns of amyotrophic lateral sclerosis (ALS)-associated SOD1 mutants. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 9754–

9.

51. Aydin,H., Al-Khooly,D. and Lee,J.E. (2014) Influence of hydrophobic and electrostatic residues on SARS-coronavirus S2 protein stability: Insights into mechanisms of general viral fusion and inhibitor design. *Protein Science*, **23**, 603–617.

52. Aydin,H., Al-Khooly,D. and Lee,J.E. (2014) Influence of hydrophobic and electrostatic residues on SARS-coronavirus S2 protein stability: insights into mechanisms of general viral fusion and inhibitor design. *Protein science : a publication of the Protein Society*, **23**, 603–17.

53. Pace,C.N., Fu,H., Lee Fryar,K., Landua,J., Trevino,S.R., Schell,D., Thurlkill,R.L., Imura,S., Scholtz,J.M., Gajiwala,K., *et al.* (2014) Contribution of hydrogen bonds to protein stability. *Protein science : a publication of the Protein Society*, **23**, 652–61.

54. Vagenende,V., Yap,M.G.S. and Trout,B.L. (2009) Mechanisms of Protein Stabilization and Prevention of Protein Aggregation by Glycerol. *Biochemistry*, **48**, 11084–11096.

55. Wang,Z. and Moult,J. (2001) SNPs, protein structure, and disease. *Human Mutation*, **17**, 263–270.

56. Gunderson,K.L., Steemers,F.J., Ren,H., Ng,P., Zhou,L., Tsan,C., Chang,W., Bullis,D., Musmacker,J., King,C., *et al.* (2006) Whole-Genome Genotyping. In *Methods in enzymology*.Vol. 410, pp. 359–376.

57. Wheeler,D.A., Srinivasan,M., Egholm,M., Shen,Y., Chen,L., McGuire,A., He,W., Chen,Y.-J., Makhijani,V., Roth,G.T., *et al.* (2008) The complete genome of an individual by massively parallel DNA sequencing. *Nature*, **452**, 872–876.

58. Metzker,M.L. (2010) Sequencing technologies — the next generation. *Nature Reviews Genetics*, **11**, 31–46.

59. Qian,Y.Q., Billeter,M., Otting,G., Müller,M., Gehring,W.J. and Wüthrich,K. (1989) The

structure of the Antennapedia homeodomain determined by NMR spectroscopy in solution: comparison with prokaryotic repressors. *Cell*, **59**, 573–80.

60. Kornberg,T.B. (1993) Understanding the homeodomain. *The Journal of biological chemistry*, **268**, 26813–6.

61. Gehring,W.J., Affolter,M. and Burglin,T. (1994) Homeodomain Proteins. *Annual Review of Biochemistry*, **63**, 487–526.

62. Chaney,B.A., Clark-Baldwin,K., Dave,V., Ma,J. and Rance,M. (2005) Solution structure of the K50 class homeodomain PITX2 bound to DNA and implications for mutations that cause Rieger syndrome. *Biochemistry*, **44**, 7497–511.

63. Priston,M., Kozlowski,K., Gill,D., Letwin,K., Buys,Y., Levin,A. V, Walter,M.A. and Héon,E. (2001) Functional analyses of two newly identified PITX2 mutants reveal a novel molecular mechanism for Axenfeld-Rieger syndrome. *Human molecular genetics*, **10**, 1631–8.

64. Saadi,I., Semina,E. V, Amendt,B.A., Harris,D.J., Murphy,K.P., Murray,J.C. and Russo,A.F. (2001) Identification of a dominant negative homeodomain mutation in Rieger syndrome. *The Journal of biological chemistry*, **276**, 23034–41.

65. Espinoza,H.M., Cox,C.J., Semina,E. V and Amendt,B.A. (2002) A molecular basis for differential developmental anomalies in Axenfeld-Rieger syndrome. *Human molecular genetics*, **11**, 743–53.

66. Amendt,B.A., Sutherland,L.B., Semina,E. V and Russo,A.F. (1998) The molecular basis of Rieger syndrome. Analysis of Pitx2 homeodomain protein activities. *The Journal of biological chemistry*, **273**, 20066–72.

67. Seifi,M., Footz,T., Taylor,S.A.M. and Walter,M.A. (2016) Comparison of Bioinformatics Prediction, Molecular Modeling, and Functional Analyses of *FOXC1* Mutations in Patients

with Axenfeld-Rieger Syndrome. *Human Mutation*, 10.1002/humu.23141.

68. Doss,C.G.P., Rajith,B., Garwasis,N., Mathew,P.R., Raju,A.S., Apoorva,K., William,D., Sadhana,N.R., Himani,T. and Dike,I. (2012) Screening of mutations affecting protein stability and dynamics of FGFR1—A simulation analysis. *Applied & Translational Genomics*, **1**, 37–43.

69. Khan,S. and Vihinen,M. (2010) Performance of protein stability predictors. *Human Mutation*, **31**, 675–684.

70. Idrees,F., Bloch-Zupan,A., Free,S.L., Vaideanu,D., Thompson,P.J., Ashley,P., Brice,G., Rutland,P., Bitner-Glindzicz,M., Khaw,P.T., *et al.* (2006) A novel homeobox mutation in the PITX2 gene in a family with Axenfeld-Rieger syndrome associated with brain, ocular, and dental phenotypes. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*, **141B**, 184–91.

71. Footz,T., Idrees,F., Acharya,M., Kozlowski,K. and Walter,M.A. (2009) Analysis of mutations of the PITX2 transcription factor found in patients with Axenfeld-Rieger syndrome. *Investigative ophthalmology & visual science*, **50**, 2599–606.

72. Yuan,F., Zhao,L., Wang,J., Zhang,W., Li,X., Qiu,X.-B., Li,R.-G., Xu,Y.-J., Xu,L., Qu,X.-K., *et al.* (2013) PITX2c loss-of-function mutations responsible for congenital atrial septal defects. *International journal of medical sciences*, **10**, 1422–9.

73. Sun,Y.-M., Wang,J., Qiu,X.-B., Yuan,F., Xu,Y.-J., Li,R.-G., Qu,X.-K., Huang,R.-T., Xue,S. and Yang,Y.-Q. (2016) PITX2 loss-of-function mutation contributes to tetralogy of Fallot. *Gene*, **577**, 258–64.

74. Semina,E. V, Reiter,R., Leysens,N.J., Alward,W.L., Small,K.W., Datson,N.A., Siegel-Bartelt,J., Bierke-Nelson,D., Bitoun,P., Zabel,B.U., *et al.* (1996) Cloning and characterization

of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nature genetics*, **14**, 392–9.

75. Amendt,B.A., Sutherland,L.B., Semina,E. V and Russo,A.F. (1998) The molecular basis of Rieger syndrome. Analysis of Pitx2 homeodomain protein activities. *The Journal of biological chemistry*, **273**, 20066–72.

76. Amendt,B.A., Semina,E. V and Alward,W.L. (2000) Rieger syndrome: a clinical, molecular, and biochemical analysis. *Cellular and molecular life sciences : CMLS*, **57**, 1652–66.

77. Xia,K., Wu,L., Liu,X., Xi,X., Liang,D., Zheng,D., Cai,F., Pan,Q., Long,Z., Dai,H., *et al.* (2004) Mutation in PITX2 is associated with ring dermoid of the cornea. *Journal of medical genetics*, **41**, e129.

78. Wang,J., Zhang,D.-F., Sun,Y.-M. and Yang,Y.-Q. (2014) A novel PITX2c loss-of-function mutation associated with familial atrial fibrillation. *European journal of medical genetics*, **57**, 25–31.

79. Kozlowski,K. and Walter,M.A. (2000) Variation in residual PITX2 activity underlies the phenotypic spectrum of anterior segment developmental disorders. *Human molecular genetics*, **9**, 2131–9.

80. Espinoza,H.M., Cox,C.J., Semina,E. V and Amendt,B.A. (2002) A molecular basis for differential developmental anomalies in Axenfeld-Rieger syndrome. *Human molecular genetics*, **11**, 743–53.

81. Saadi,I., Semina,E. V, Amendt,B.A., Harris,D.J., Murphy,K.P., Murray,J.C. and Russo,A.F. (2001) Identification of a dominant negative homeodomain mutation in Rieger syndrome. *The Journal of biological chemistry*, **276**, 23034–41.

82. Kulak,S.C., Kozlowski,K., Semina,E. V, Pearce,W.G. and Walter,M.A. (1998) Mutation in the

RIEG1 gene in patients with iridogoniodysgenesis syndrome. *Human molecular genetics*, **7**, 1113–7.

83. Strungaru,M.H., Dinu,I. and Walter,M.A. (2007) Genotype-phenotype correlations in Axenfeld-Rieger malformation and glaucoma patients with FOXC1 and PITX2 mutations. *Investigative ophthalmology & visual science*, **48**, 228–37.

84. Wei,D., Gong,X.-H., Qiu,G., Wang,J. and Yang,Y.-Q. (2014) Novel PITX2c loss-of-function mutations associated with complex congenital heart disease. *International journal of molecular medicine*, **33**, 1201–8.

85. Priston,M., Kozlowski,K., Gill,D., Letwin,K., Buys,Y., Levin,A. V, Walter,M.A. and Héon,E. (2001) Functional analyses of two newly identified PITX2 mutants reveal a novel molecular mechanism for Axenfeld-Rieger syndrome. *Human molecular genetics*, **10**, 1631–8.

86. Alward,W.L., Semina,E. V, Kalenak,J.W., Héon,E., Sheth,B.P., Stone,E.M. and Murray,J.C. (1998) Autosomal dominant iris hypoplasia is caused by a mutation in the Rieger syndrome (RIEG/PITX2) gene. *American journal of ophthalmology*, **125**, 98–100.

87. Perveen,R., Lloyd,I.C., Clayton-Smith,J., Churchill,A., van Heyningen,V., Hanson,I., Taylor,D., McKeown,C., Super,M., Kerr,B., *et al.* (2000) Phenotypic variability and asymmetry of Rieger syndrome associated with PITX2 mutations. *Investigative ophthalmology & visual science*, **41**, 2456–60.

88. Wang,J., Xin,Y.-F., Xu,W.-J., Liu,Z.-M., Qiu,X.-B., Qu,X.-K., Xu,L., Li,X. and Yang,Y.-Q. (2013) Prevalence and spectrum of PITX2c mutations associated with congenital heart disease. *DNA and cell biology*, **32**, 708–16.

89. Phillips,J.C. Four novel mutations in the PITX2 gene in patients with Axenfeld-Rieger syndrome. *Ophthalmic research*, **34**, 324–6.

90. Ng,P.C. and Henikoff,S. (2001) Predicting deleterious amino acid substitutions. *Genome research*, **11**, 863–74.

91. Ramensky,V., Bork,P. and Sunyaev,S. (2002) Human non-synonymous SNPs: server and survey. *Nucleic acids research*, **30**, 3894–900.

92. Tang,H. and Thomas,P.D. (2016) PANTHER-PSEP: predicting disease-causing genetic variants using position-specific evolutionary preservation. *Bioinformatics (Oxford, England)*, **32**, 2230–2.

93. Li,B., Krishnan,V.G., Mort,M.E., Xin,F., Kamati,K.K., Cooper,D.N., Mooney,S.D. and Radivojac,P. (2009) Automated inference of molecular mechanisms of disease from amino acid substitutions. *Bioinformatics (Oxford, England)*, **25**, 2744–50.

94. Schwarz,J.M., Cooper,D.N., Schuelke,M. and Seelow,D. (2014) MutationTaster2: mutation prediction for the deep-sequencing age. *Nature methods*, **11**, 361–2.

95. Choi,Y. and Chan,A.P. (2015) PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. *Bioinformatics (Oxford, England)*, **31**, 2745–7.

96. Ferrer-Costa,C., Orozco,M. and de la Cruz,X. (2002) Characterization of disease-associated single amino acid polymorphisms in terms of sequence and structure properties. *Journal of molecular biology*, **315**, 771–86.

97. Pires,D.E. V, Ascher,D.B. and Blundell,T.L. (2014) DUET: a server for predicting effects of mutations on protein stability using an integrated computational approach. *Nucleic acids research*, **42**, W314-9.

98. Capriotti,E., Calabrese,R. and Casadio,R. (2006) Predicting the insurgence of human genetic diseases associated to single point protein mutations with support vector machines and evolutionary information. *Bioinformatics (Oxford, England)*, **22**, 2729–34.

99. Cheng,J., Randall,A. and Baldi,P. (2006) Prediction of protein stability changes for single-site mutations using support vector machines. *Proteins*, **62**, 1125–32.

100. Huang,L.-T., Gromiha,M.M. and Ho,S.-Y. (2007) iPTREE-STAB: interpretable decision tree based method for predicting protein stability changes upon mutations. *Bioinformatics (Oxford, England)*, **23**, 1292–3.

101. Zaidi,S., Choi,M., Wakimoto,H., Ma,L., Jiang,J., Overton,J.D., Romano-Adesman,A., Bjornson,R.D., Breitbart,R.E., Brown,K.K., *et al.* (2013) De novo mutations in histone-modifying genes in congenital heart disease. *Nature*, **498**, 220–3.

102. Yang,Y.-Q., Xu,Y.-J., Li,R.-G., Qu,X.-K., Fang,W.-Y. and Liu,X. (2013) Prevalence and spectrum of PITX2c mutations associated with familial atrial fibrillation. *International journal of cardiology*, **168**, 2873–6.

103. Vieira,V., David,G., Roche,O., de la Houssaye,G., Boutboul,S., Arbogast,L., Kobetz,A., Orssaud,C., Camand,O., Schorderet,D.F., *et al.* (2006) Identification of four new PITX2 gene mutations in patients with Axenfeld-Rieger syndrome. *Molecular vision*, **12**, 1448–60.

104. D'haene,B., Meire,F., Claerhout,I., Kroes,H.Y., Plomp,A., Arens,Y.H., de Ravel,T., Casteels,I., De Jaegere,S., Hooghe,S., *et al.* (2011) Expanding the spectrum of FOXC1 and PITX2 mutations and copy number changes in patients with anterior segment malformations. *Investigative ophthalmology & visual science*, **52**, 324–33.

105. Weisschuh,N., Dressler,P., Schuettauf,F., Wolf,C., Wissinger,B. and Gramer,E. (2006) Novel mutations of FOXC1 and PITX2 in patients with Axenfeld-Rieger malformations. *Investigative ophthalmology & visual science*, **47**, 3846–52.

106. Meyer-Marcotty,P., Weisschuh,N., Dressler,P., Hartmann,J. and Stellzig-Eisenhauer,A. (2008) Morphology of the sella turcica in Axenfeld-Rieger syndrome with PITX2 mutation. *Journal*

of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology, **37**, 504–10.

107. Dressler,S., Meyer-Marcotty,P., Weisschuh,N., Jablonski-Momeni,A., Pieper,K., Gramer,G. and Gramer,E. (2010) Dental and Craniofacial Anomalies Associated with Axenfeld-Rieger Syndrome with PITX2 Mutation. *Case reports in medicine*, **2010**, 621984.
108. Kimura,M., Tokita,Y., Machida,J., Shibata,A., Tatematsu,T., Tsurusaki,Y., Miyake,N., Saitsu,H., Miyachi,H., Shimozato,K., *et al.* (2014) A novel PITX2 mutation causing iris hypoplasia. *Human genome variation*, **1**, 14005.
109. Reis,L.M., Tyler,R.C., Volkmann Kloss,B.A., Schilter,K.F., Levin,A. V, Lowry,R.B., Zwijnenburg,P.J.G., Stroh,E., Broeckel,U., Murray,J.C., *et al.* (2012) PITX2 and FOXC1 spectrum of mutations in ocular syndromes. *European journal of human genetics : EJHG*, **20**, 1224–33.
110. Li,D., Zhu,Q., Lin,H., Zhou,N. and Qi,Y. (2008) A novel PITX2 mutation in a Chinese family with Axenfeld-Rieger syndrome. *Molecular vision*, **14**, 2205–10.
111. Kniestedt,C., Taralczak,M., Thiel,M.A., Stuermer,J., Baumer,A. and Gloor,B.P. (2006) A novel PITX2 mutation and a polymorphism in a 5-generation family with Axenfeld-Rieger anomaly and coexisting Fuchs' endothelial dystrophy. *Ophthalmology*, **113**, 1791.e1-8.
112. Huang,X., Xiao,X., Jia,X., Li,S., Li,M., Guo,X., Liu,X. and Zhang,Q. (2015) Mutation analysis of the genes associated with anterior segment dysgenesis, microcornea and microphthalmia in 257 patients with glaucoma. *International journal of molecular medicine*, **36**, 1111–7.

Chapter 5. Identification and functional characterization of conserved non-coding elements around *FOXC1*

No part of this thesis has been previously published. All experiments were carried out by Morteza Seifi.

Introduction

The regulation of gene expression, which takes place with the participation of many biological components, can be modulated at several stages including transcription (a major stage), posttranscriptional modification of mRNA, translation, and post-translational modification of a protein (1). The precise temporal and spatial expression of genes often requires the presence of cis-regulatory elements (CREs). CREs comprise clusters of transcription factor binding sites (TFBSs) called cis-regulatory modules (CRMs), which play important roles in developmental pattern formation and in producing the complex body plan of the adult organism. The CRMs are typically up to a few hundred nucleotides long, and can be comprised of specific binding sites for ~3-10 transcription factors (1, 2). Transcription factors or trans-acting regulatory proteins bind to CREs and control the expression level of target genes connected with the CREs in a tissue- and cell-specific manner (3).

The location of certain CREs can be different, either residing close to (a few kilobases from the exons or within the introns of the gene they control) or far away (usually several kilobases) from the basal promoter region and the target genes (4). In some cases, CREs are located in the introns of nearby genes. For example, the limb enhancer of the *SHH* gene resides in the 5th intron of the neighbouring limb region 1 homolog (*LMBR1*) gene; and the retina enhancer of the *PAX6* gene is embedded in the intron of the neighbouring elongation protein 4 homolog (*ELP4*) gene (4, 5). CREs have different roles, for example, some CREs may act as "silencers" that negatively regulate transcriptional activity. In contrast, some others appear to function as "enhancers", inducing the expression of target genes (4).

In the post-genome era, understanding the mechanisms involved in genome expression is one of the main questions for geneticists to answer. Genetic variations directly or indirectly underlie different

diseases, but since some of them occur in the non-coding part of the genome, we don't know the exact mechanisms by which they cause the disease. Variations in CREs including the core or the proximal promoter regions, single enhancer, silencer, insulator elements, and whole locus control regions can lead to deep effects on phenotype, since these variations may disrupt their interaction with the promoter or affect the chromatin structure of the locus, changing gene expression (6, 7). CRE variations can be attributed to different human disease phenotypes than those resulting from coding region variations, because such variations may affect only a subset of tissues in which a gene is expressed (7).

The presence of regulatory elements in the *PITX2* gene has been reported and studied (8). However, the roles of CREs in *FOXC1* have not been addressed. It is unclear how *FOXC1* is regulated and what factors are involved in the expression patterns of this gene. It is thought that the deletion of the *FOXC1* gene or its regulatory elements contribute to ocular abnormalities of the anterior segment, such as iris hypoplasia and posterior embryotoxon which are frequently observed in ARS (9). *FOXC1* is flanked by GMDS located downstream and the FOXF2, FOXQ1, and LINC01622 genes positioned upstream of *FOXC1* (Figure 1). Uncovering the mechanism of expression in our gene of interest and its regulatory elements will broaden our knowledge and pave the ground for better understanding the etiology of the ARS.

I hypothesized that these regions around *FOXC1* contain conserved sequences involved in transcriptional regulation of *FOXC1* and possibly of neighboring genes. Thus, I investigated the sequences surrounding the *FOXC1* gene to identify and characterize the regulatory regions crucial for the expression of *FOXC1*.

Material and Methods

FOXC1 non-coding conserved regions and primers

To identify putative regulatory sequences responsible for regulation of the expression of *FOXC1*, the sequences around *FOXC1* were scanned for noncoding genomic elements conserved between human, mice, chicken, rabbit, lizard, zebrafish, and frog genomes using molecular biology and bioinformatics techniques, including the multiple species alignment tool at UCSC Genome Browser (https://genome.ucsc.edu/), the evolutionarily conserved region (ECR) browser (https://ecrbrowser.dcode.org/) and BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) based on the criterion equal to or > 70% identity over 100 bp of sequence (10). The evolutionarily conserved region (ECR) browser is a dynamic graphical interface that allows us to visualize and assess CREs in genomes of different species. Briefly, this tool compares the level of identity between the base sequence and the sequence being compared. Different parts of compared genes including coding exons, introns, untranslated regions (UTRs), conserved intergenic regions, and repetitive elements are illustrated with different colors (11).

Primers corresponding to the conserved regions were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). Primers surrounding *FOXC1* conserved regions are presented in Table 1.

pGL3 and pGL3.TK plasmid constructs

I was unable to PCR amplify two of my identified conserved regions, even after redesigning and using new primers, and thus were not considered for further analysis. The functions of the remaining individual conserved non-coding elements (CNEs) were assayed by cloning them into the pGL3.TK expression plasmid. The primers, with Bgl II restriction sites added to facilitate cloning, were

amplified by PCR with genomic DNA. PCR amplicons were fractionated on TAE agarose gels, excised from gels, and purified by the Geneclean II kit (Qbiogene, USA). The purified product after digestion was cloned into the Bgl II site of pGL3.TK (Promega) using the pGMT-Easy cloning system (Promega Madison, Wisconsin, United States). The orientation and sequences of the conserved regions were verified by sequencing on a 3130XL Genetic Analyzer at The Applied Genomics Core of the University of Alberta.

Luciferase transactivation assay

CNEs were tested using luciferase reporter assays to determine whether these regions had functional activity. Three different cell lines, HeLa, TM, and ODM-2 (4×10^4 cells per 15-mm well), were seeded per well on 24 well plates and grown overnight in DMEM. Transfections were performed using Lipofectamine2000® reagent (Invitrogen Carlsbad California, United States), as per manufacturer instructions diluted in OptiMEM. Cells within wells were transfected in triplicate with 500 ng of the pGL3.TK constructs containing each of the CNEs, 30 ng of pCMVβ transfection-control plasmid (Promega), 60 ng of the pGL3.TK construct. Additionally, two wells were transfected with 100ng of pGL3.TK (negative control) and pGL3.TK-SV40 enhancer-containing construct as a positive control. Each condition was done in triplicate and each experiment was repeated at least three times.

Two days after transfection, luciferase transactivation assays were performed using the Promega dual-luciferase reporter assay system as per manufacturer's protocol. Briefly, following two washes of PBS, cells were lysed with 100 µl of 1x Reporter Lysis Buffer (Reporter Lysis Buffer; Promega, Madison,WI), frozen for 15 minutes at -80^{o}C, then thawed on a shaker for 15 minutes. Lysates were collected into 1.5ml tubes and spun at maximum speed for 3 minutes at 4^{o}C. 50 µl of lysate were

combined with 50µl of 2x Assay Buffer (Promega), mixed and incubated for 30 minutes at 37°C, the reaction was then stopped with addition of 900µl of 1M Sodium Carbonate and β galactosidase values were measured at O.D.420 on a spectrometer. While the β-galactosidase control was incubating at 37°C, luciferase activation was measured on the TD-20/20 luminometer (Turner Design Sunnyvale, CA, United States) by adding 10µl of protein lysate to 100ul luciferase assay reagent (LAR I, Promega), vortexing the lysate mix for 3 seconds and measuring luciferase activation. Firefly luciferase activity was standardized to the β-galactosidase (internal control) activity quantitated by the β-Galactosidase enzyme assay system (Promega) from 50 µl of protein lysate. Reactions were repeated three times in triplicate.

Results

In this study, I identified nine putative CNEs positioned up- or downstream of *FOXC1*. I was not able to PCR-amplified two putative CNEs even after using new primers and thus these two elements were not assayed further. The remaining seven conserved elements (Figure 2) were cloned independently into pGL3.TK to assess the functional activity of these conserved regions. The empty pGL3.TK reporter was used to control for basal activation level while pGL3.TK-SV40 was used as a positive control. While the pGL3.TK-SV40 positive control increased luciferase activity 20 times as compared to an empty pGL3.TK reporter, no significant changes in luciferase activation were identified when three different cells (HeLa, TM, and ODM-2 cells) were transfected with pGL3.TK containing each of the conserved regions (Figure 3-5). These results indicate that none of the identified regions has functional activity in these cells.

Discussion

Unlike protein coding regions, which contain firm positional, directional and compositional constraints, CREs have flexible features which make their detection much more difficult (12). Although our understanding about CREs is limited and in their infancy, the core promoters that are positioned nearly 50-100 base pairs upstream or downstream of the transcriptional start site (TSS) are the most studied regulatory sequences (13). Core promoters play important roles in the assembly of the many sequence-specific factors and coregulators, acting sites of binding for preinitiation complexes necessary for transcription to be initiated (13, 14). Prediction of core promoters has allowed experimental verification of TSSs of many genes, and the increase increased knowledge of various promoter types. For instance, it was previously believed that every gene contained a TATA-box (an A/T-rich region), however, further studies revealed that except for a small proportion of all Pol II genes in humans, the remaining genes contain different promoter structures such as downstream promoter element (*DPE*) and specificity protein 1 (*SP1*) (15–17).

Currently, however, there are no sequence traits available to give us a reasonable connection of genes to their CREs such as their promoter, enhancer or insulator function. However, a wide variety of experimental and computational techniques have been used to identify possible sequences with cis-regulatory activity. Interspecies analysis has been increasingly utilized to identify genomic regions accommodating regulatory elements, most importantly promoters and enhancers (18–20). Phylogenetic footprinting can be used to identify CREs in DNA of interest by comparing orthologous sequences in different species (21). For precise prediction, sufficient knowledge about such regulatory elements needs to be obtained.

As mentioned, initiation of transcription is the main key control point in gene expression, and is modulated by binding of transcription factors to their regulatory elements (1). The extent to which

alterations in *cis*- and *trans*-regulatory sequences lead to gene regulatory evolution are mostly unclear (22, 23). However, findings from previous studies on investigating functional activity of CREs demonstrated that alterations in CREs are critical and lead to different levels and patterns of gene expression. For instance, studies in yeast showed that changes in CREs are responsible for 25% of alterations in expression patterns between species (24, 25). Wittkopp et al. studied *Drosophila melanogaster* and *D. simulans* and showed that 90% of studied variations in gene expression between the two species could be due to changes in CREs (26). In a study that applied microarrays and genome-wide linkage, Morley et al. mapped gene expression phenotypes to investigate the level of expression in a large number of genes and observed that many of the significant associations (19%) were mapped to the regulatory regions (27). Taken together, these results signify that CREs possess functional activity and alterations in the expression profiles of genes due to variations in CREs are functionally critical.

For *FOXC1* and *PITX2*, previous literature has demonstrated that variations in the coding regions of these genes underlie ARS syndrome (28–33). To date, there has been only one study in which the regulatory elements of *PITX2* were identified (34). This information correlates with the reports of ARS patients with translocation breakpoints occurring within the distant upstream region of *PITX2* that did not impair the protein-coding region of *PITX2* (35–37). In the *PITX2* regulatory region study, thirteen conserved non-coding regions positioned about 1.1 Mb far upstream of the *PITX2* gene were discovered (34). Further analysis showed that these conserved regions are playing important role as enhancers, indicating the importance of these regions in regulating *PITX2* expression during brain, eye, and craniofacial development (34).

There has been no similar study investigating the regulatory regions of *FOXC1* before my study reported here. The FOX family of transcriptional factors is associated with a number of genetic

abnormalities and shows specific expression patterns during development that are conserved in vertebrates (38–40). The regions involved in the unique expression of FOX proteins are largely unknown. Therefore, determining the regulatory sequences of FOX proteins will help us understand the control of FOX gene expression and investigate the association of these regions and their interacting proteins with human disease.

In this study, I compared genomes of different species to identify CREs of *FOXC1* in the human genome. The longer the phylogenetic distance, the higher are the chances that the neutrally evolving regions would have diverged completely leaving behind footprints of highly conserved sequences representing functional elements (41). To identify regulatory elements of *FOXC1*, I included zebrafish as it diverged about 450 million years ago from the mammalian lineage and thus showing the most distantly related bony vertebrates (42). I used three different computational methods and identified 7 CNEs surrounding *FOXC1* that were longer than 100 bp and with greater than 70% identity. I then conducted BLAST against human, mice, chicken, rabbit, lizard, zebrafish, and frog genomes to determine the level of conservation of all the identified elements. To validate the functional activity of our novel CNEs, I performed transactivation activity experiments using three different cell lines. Interestingly, I found that none of the seven putative CNEs altered luciferase activity, indicating no association of expression of the *FOXC1* gene with our detected conserved regions.

There are several possible explanations for why my identified regions had no detectable functional activity. First, in this study I focused on limited number of cell lines (HeLa, TM and ODM-2 cells), and it is possible that the putative CNEs direct *FOXC1* expression in other cells. Second, in this study I examined the expression pattern of *FOXC1* in *in vitro* transactivation experiments, only, transgenic animal models with different combination of CNEs would allow investigation of the effects of these

CNEs in different tissues and at different developmental stages. However, as our CNEs were not conserved between at least three species, it is possible that the putative CNEs I found actually have no regulatory functional activity. It is known that high sequence conservation does not always imply potential regulatory activity of a sequence (43–45). In fact, there is no direct link between the degree of functional constraint and conservation of sequence. For instance, 32 CREs were detected following comparison of the sequences around the DACH gene in the human, mouse and *fugu*. Functional analysis using mouse transgenic assays of nine of these CREs showed that two of them did not possess any enhancer activity (44). Pennacchio et al. examined the functional activity of 84 CREs using transgenic mice and showed that 33 of them were not functionally conserved and did not have any regulatory functions in directing tissue-specific reporter gene expression at different developmental stages (45). A study in zebrafish showed that 20-30% of conserved regions near *nkx3.2, pax9, otx1b* and *foxa2* genes can act as enhancers (46). Further analysis showed that many of these enhancers act in a synergistic pattern and are not conserved at the sequence level. It was shown that Foxa1 binds to the otx1b non-conserved enhancer to direct its activity in forebrain and otic vesicle of zebrafish (46).

In contrast, it is likely that regions with weak sequence conservation have constrained functions (47–49). Blow et al. used ChIP-Seq analysis with the enhancer-associated protein p300, and identified some regulatory sequences that were critical in heart development. They further discovered that the detected sequences have little conservation between vertebrates (47). In another study, a multiple alignment algorithm called MLAGAN was used to compare 50 human forebrain genes with their orthologs in mouse and fugu (50). Functional characterization of the regulatory regions of orexin (ORX) gene have identified no conserved regulatory sequences based on the criteria used for defining CNEs, but functional analysis of the regulatory region of the *fugu* ORX revealed that the function of

detected enhancers were extensively conserved between fish and mammals, indicating that functional information is highly conserved in these vertebrate sequences at levels below the metric (70% identity over 100bp) used for identifying CREs (50). In another study, regions (both conserved and non-conserved sequences) around the zebrafish paired-like homeobox gene (phox2b) were examined for enhancer activity and the results showed that approximately 42-51% of regulatory regions were overlooked by using standard measures of evolutionary constraint (51). Further analysis showed that nonaligned sequences in the phox2b locus have conserved TFBSs that would distinguish them from non-functional regions. However, these nonaligned sequences are difficult to be identified by alignment alone as they are distributed at a low density (51). It is likely that orthologous cis-regulatory sequences regulate the expression of these genes, however, these sequences have diverged beyond detection using sequence searching algorithms via small alterations in TFBSs, rearrangement of these binding sites or multiple coevolved changes that give rise to compensatory variations within the CRE because of a stabilizing selection process (52, 53). Such weak constraint on functional sequence could result from sequence degeneracy of binding sites, redundancy of individual functional elements, or the need for secondary structure that is only indirectly associated with primary sequence (54). It is likely that our identified CNEs of *FOXC1* gene locus belongs to one of these categories of regulatory elements.

Taken together, it seems that if no CREs are identified in a gene of interest, it should not be considered as an indication of non-functional sequences or that different species genes are regulated using different pathways. In fact, functional annotation of 1% of the human genome by the ENCODE project has shown that although a huge number of experimentally validated functional CREs are under evolutionary constraint, many are variable across mammals (55, 56).

Figure 1. Schematic drawing of the genomic organization of human *FOXC1* and neighbouring genes. The numbers on top of each box show the length of the corresponding gene. The distance between genes is also shown below the boxes. Diagram is not to scale.

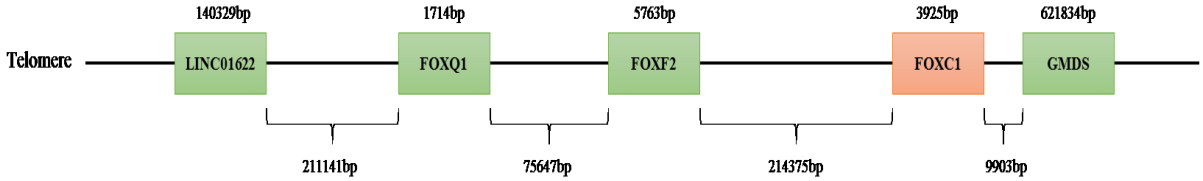

140329bp
1714bp
5763bp
3925bp
621834bp
Telomere
LINC01622
FOXQ1
FOXF2
FOXC1
GMDS
211141bp
75647bp
214375bp
9903bp

Figure 2. Schematic drawing of the genomic organization of human FOXC1 and the neighbouring genes along with seven identified conserved non-coding elements (CNEs). The numbers on top of each box show the length of the corresponding gene. The numbered black boxes are the identified CNEs (1-7). Diagram is not to scale.

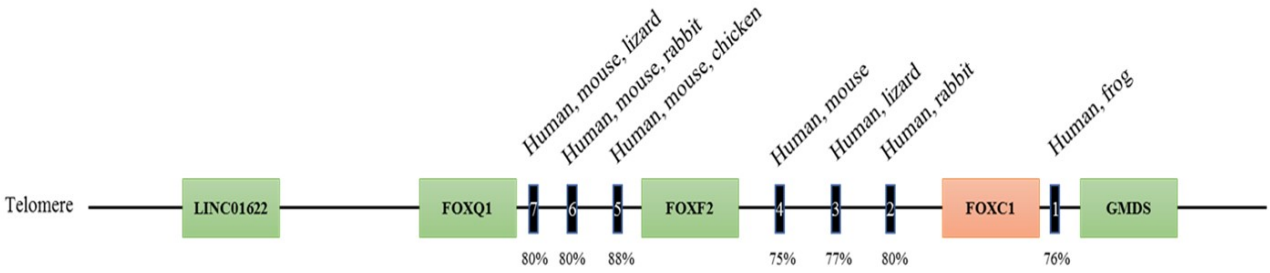
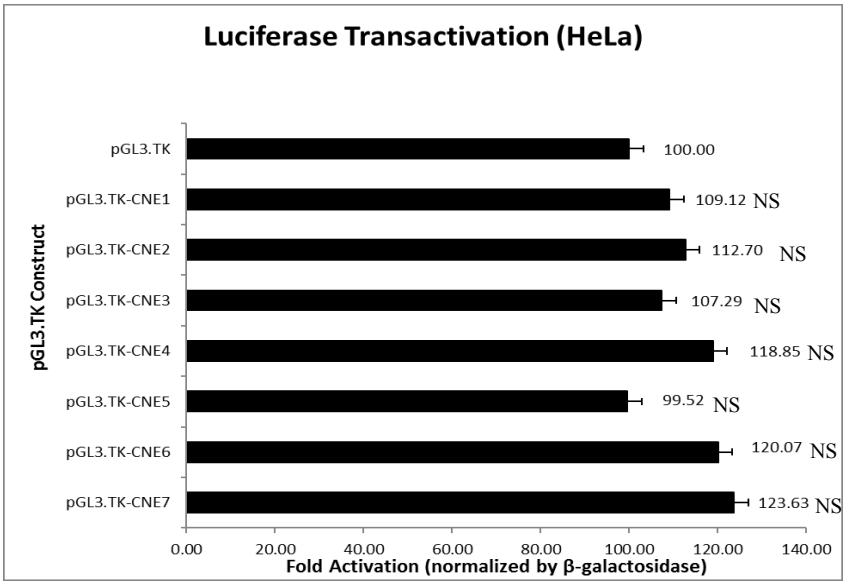

Human, mouse, lizard
Human, mouse, rabbit
Human, mouse, chicken
Human, mouse
Human, lizard
Human, rabbit
Human, frog
Telomere
LINC01622
FOXQ1
7
6
5
FOXF2
4
3
2
FOXC1
1
GMDS
80%
80%
88%
75%
77%
80%
76%

Figure 3. Transactivation capacity of a) the conserved non-coding elements (CNEs) 1-7 and b) SV40 enhancer in HeLa cells. Luciferase activation was normalized to the β-galactosidase control. NS; Not significant (P>0.05)), calculated from comparison with pGL3.TK (negative control).

a)


Luciferase Transactivation (HeLa)
pGL3.TK Construct
pGL3.TK
pGL3.TK-CNE1
pGL3.TK-CNE2
pGL3.TK-CNE3
pGL3.TK-CNE4
pGL3.TK-CNE5
pGL3.TK-CNE6
pGL3.TK-CNE7
100.00
109.12 NS
112.70 NS
107.29 NS
118.85 NS
99.52 NS
120.07 NS
123.63 NS
0.00
20.00
40.00
60.00
80.00
100.00
120.00
140.00
Fold Activation (normalized by β-galactosidase)

b)

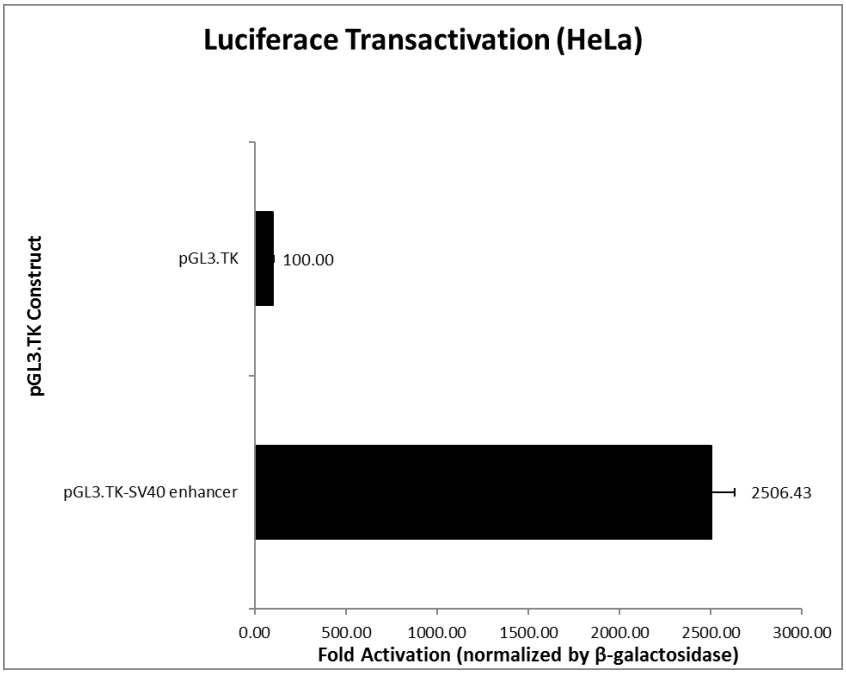
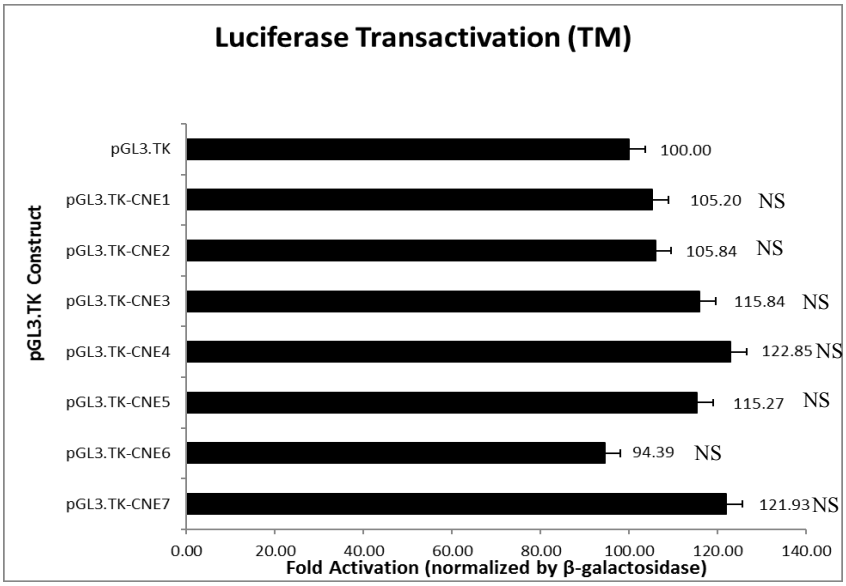

Luciferace Transactivation (HeLa)
pGL3.TK Construct
pGL3.TK
pGL3.TK-SV40 enhancer
100.00
2506.43
0.00
500.00
1000.00
1500.00
2000.00
2500.00
3000.00
Fold Activation (normalized by β-galactosidase)

Figure 4. Transactivation capacity of a) the conserved non-coding elements (CNEs) 1-7 and b) SV40 enhancer in TM cells. Luciferase activation was normalized to the β-galactosidase control. NS; Not significant (P>0.05)), calculated from comparison with pGL3.TK (negative control).

a)


Luciferase Transactivation (TM)
pGL3.TK Construct
pGL3.TK
pGL3.TK-CNE1
pGL3.TK-CNE2
pGL3.TK-CNE3
pGL3.TK-CNE4
pGL3.TK-CNE5
pGL3.TK-CNE6
pGL3.TK-CNE7
100.00
105.20 NS
105.84 NS
115.84 NS
122.85 NS
115.27 NS
94.39 NS
121.93 NS
0.00
20.00
40.00
60.00
80.00
100.00
120.00
140.00
Fold Activation (normalized by β-galactosidase)

b)

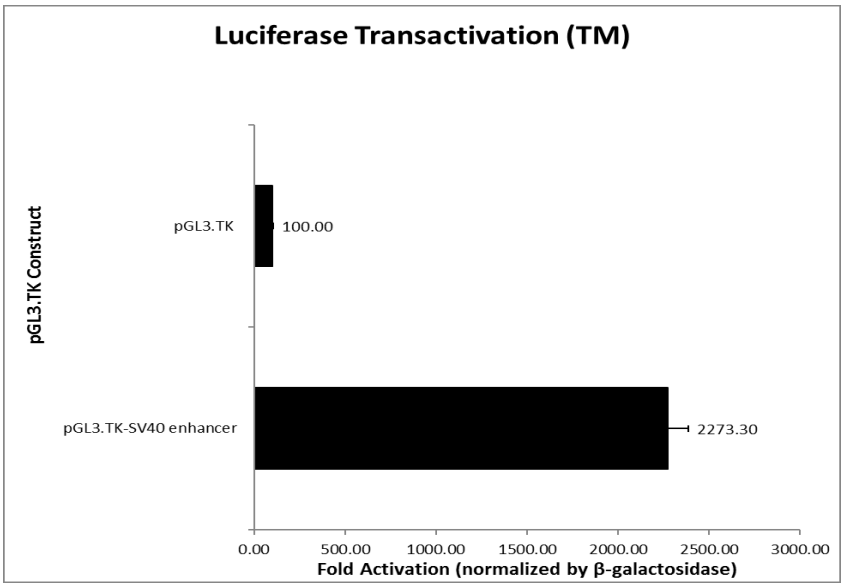
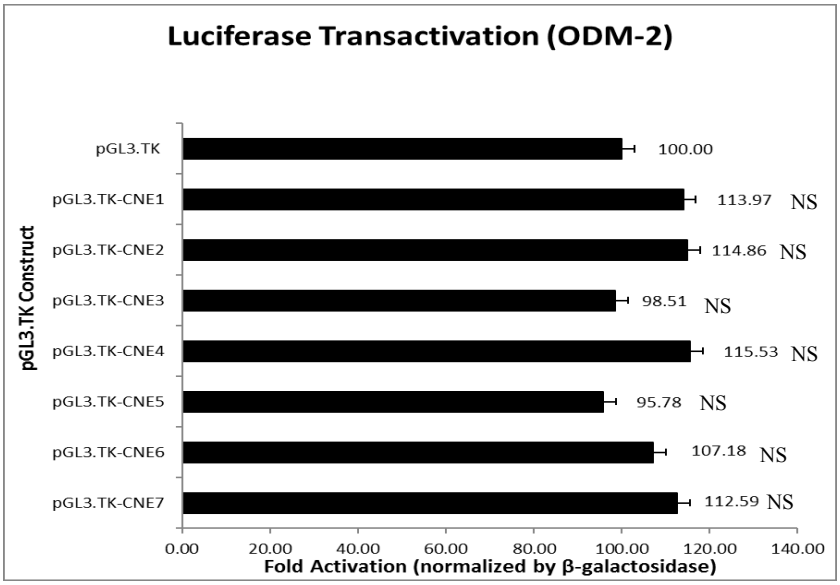

Luciferase Transactivation (TM)
pGL3.TK Construct
pGL3.TK
pGL3.TK-SV40 enhancer
100.00
2273.30
0.00
500.00
1000.00
1500.00
2000.00
2500.00
3000.00
Fold Activation (normalized by β-galactosidase)

Figure 5. Transactivation capacity of a) the conserved non-coding elements (CNEs) 1-7 and b) SV40 enhancer in ODM-2 cells. Luciferase activation was normalized to the β-galactosidase control. NS; Not significant (P>0.05)), calculated from comparison with pGL3.TK (negative control).

a)


Luciferase Transactivation (ODM-2)
pGL3.TK Construct
pGL3.TK
pGL3.TK-CNE1
pGL3.TK-CNE2
pGL3.TK-CNE3
pGL3.TK-CNE4
pGL3.TK-CNE5
pGL3.TK-CNE6
pGL3.TK-CNE7
100.00
113.97 NS
114.86 NS
98.51 NS
115.53 NS
95.78 NS
107.18 NS
112.59 NS
0.00
20.00
40.00
60.00
80.00
100.00
120.00
140.00
Fold Activation (normalized by β-galactosidase)

b)

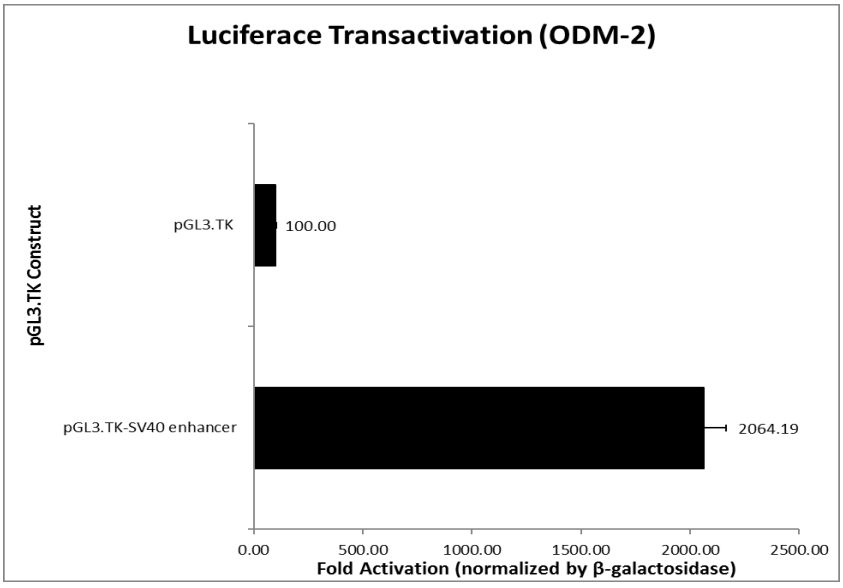

Luciferace Transactivation (ODM-2)
pGL3.TK Construct
pGL3.TK
pGL3.TK-SV40 enhancer
100.00
2064.19
0.00
500.00
1000.00
1500.00
2000.00
2500.00
Fold Activation (normalized by β-galactosidase)

Table 1. List of forward and reverse primers designed for seven identified conserved non-coding elements (CNEs).

Location	**Conserved non-coding elements (CNEs)**	**Sense**	**Antisense**
GMDS-*FOXC1* Chr6: 1,623,800-1,613,897	CNE1	AGATCTTCGAGCCAATGTGTGAGTATG	AGATCTCAGCAAGCAAGGATTCATGT
FOXC1-FOXF2 Chr6:1,609,972-1,395,597	CNE2	AGATCTTATGCAACAACCTGCCTTCA	AGATCTCCACAATGCCTCATCCTCA
	CNE3	AGATCTTCAGCCAAAGCAGGAAAGAC	AGATCTTCAGACCCTTTTCGAGGCTA
	CNE4	AGATCTGGATGGAAAAGCCATCACAT	AGATCTCTGGCCTCGTGGTTGTAAAT
FOXF2-FOXQ1 Chr6:1,389,834-1,314,187	CNE5	AGATCTCATTCGAGGCAGCCACAT	AGATCTTCTGGGAAGAAGCTGAGGT
	CNE6	AGATCTTGCACACCCAAACCTATTTGT	AGATCTCATTTCAATAACATTTTTGCATTGTA
	CNE7	AGATCTGGCCAGAGTCACGTGGAG	AGATCTCCACACCGAGGACTGTGTT
		Sense	**Antisense**
	SV40-Enhancer	AGATCTCGATGGAGCGGAGAATGG	AGATCTGCTGTGGAATGTGTGTCA

References

1. Chen,K. and Rajewsky,N. (2007) The evolution of gene regulation by transcription factors and microRNAs. *Nature Reviews Genetics*, **8**, 93–103.

2. Gotea,V., Visel,A., Westlund,J.M., Nobrega,M.A., Pennacchio,L.A. and Ovcharenko,I. (2010) Homotypic clusters of transcription factor binding sites are a key component of human promoters and enhancers. *Genome research*, **20**, 565–77.

3. Reinke,V., Krause,M. and Okkema,P. (2013) Transcriptional regulation of gene expression in C. elegans. *WormBook : the online review of C. elegans biology*, 10.1895/wormbook.1.45.2.

4. Lettice,L.A., Heaney,S.J.H., Purdie,L.A., Li,L., de Beer,P., Oostra,B.A., Goode,D., Elgar,G., Hill,R.E. and de Graaff,E. (2003) A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Human molecular genetics*, **12**, 1725–35.

5. Kleinjan,D.A., Seawright,A., Schedl,A., Quinlan,R.A., Danes,S. and van Heyningen,V. (2001) Aniridia-associated translocations, DNase hypersensitivity, sequence comparison and transgenic analysis redefine the functional domain of PAX6. *Human molecular genetics*, **10**, 2049–59.

6. Maston,G.A., Evans,S.K. and Green,M.R. (2006) Transcriptional Regulatory Elements in the Human Genome. *Annual Review of Genomics and Human Genetics*, **7**, 29–59.

7. Kleinjan,D.A. and van Heyningen,V. (2005) Long-range control of gene expression: emerging mechanisms and disruption in disease. *American journal of human genetics*, **76**, 8–32.

8. Volkmann,B.A., Zinkevich,N.S., Mustonen,A., Schilter,K.F., Bosenko,D. V, Reis,L.M., Broeckel,U., Link,B.A. and Semina,E. V (2011) Potential novel mechanism for Axenfeld-Rieger syndrome: deletion of a distant region containing regulatory elements of PITX2.

Investigative ophthalmology & visual science, **52**, 1450–9.

9. Tümer,Z. and Bach-Holm,D. (2009) Axenfeld-Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. *European journal of human genetics : EJHG*, **17**, 1527–39.

10. Loots,G.G., Locksley,R.M., Blankespoor,C.M., Wang,Z.E., Miller,W., Rubin,E.M. and Frazer,K.A. (2000) Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. *Science (New York, N.Y.)*, **288**, 136–40.

11. Ovcharenko,I., Nobrega,M.A., Loots,G.G. and Stubbs,L. ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes. 10.1093/nar/gkh355.

12. Rodríguez-Trelles,F., Tarrío,R. and Ayala,F.J. (2003) Evolution of cis-regulatory regions versus codifying regions. *The International journal of developmental biology*, **47**, 665–73.

13. Roy,A.L. and Singer,D.S. (2015) Core promoters in transcription: old problem, new insights. *Trends in biochemical sciences*, **40**, 165–71.

14. He,Y., Fang,J., Taatjes,D.J. and Nogales,E. (2013) Structural visualization of key steps in human transcription initiation. *Nature*, **495**, 481–6.

15. Zhou,T. and Chiang,C.-M. (2002) Sp1 and AP2 regulate but do not constitute TATA-less human TAF(II)55 core promoter activity. *Nucleic acids research*, **30**, 4145–57.

16. Yang,M.Q., Laflamme,K., Gotea,V., Joiner,C.H., Seidel,N.E., Wong,C., Petrykowska,H.M., Lichtenberg,J., Lee,S., Welch,L., *et al.* (2011) Genome-wide detection of a TFIID localization element from an initial human disease mutation. *Nucleic acids research*, **39**, 2175–87.

17. Lee,D.-H., Gershenzon,N., Gupta,M., Ioshikhes,I.P., Reinberg,D. and Lewis,B.A. (2005) Functional characterization of core promoter elements: the downstream core element is recognized by TAF1. *Molecular and cellular biology*, **25**, 9674–86.

18. Narlikar,L. and Ovcharenko,I. (2009) Identifying regulatory elements in eukaryotic genomes.

Briefings in Functional Genomics and Proteomics, **8**, 215–230.

19. Deyneko,I. V, Weiss,S. and Leschner,S. (2012) An integrative computational approach to effectively guide experimental identification of regulatory elements in promoters. *BMC bioinformatics*, **13**, 202.
20. Danko,C.G., McIlvain,V.A., Qin,M., Knox,B.E. and Pertsov,A.M. (2007) Bioinformatic identification of novel putative photoreceptor specific cis-elements. *BMC bioinformatics*, **8**, 407.
21. Blanchette,M. and Tompa,M. (2002) Discovery of Regulatory Elements by a Computational Method for Phylogenetic Footprinting. *Genome Research*, **12**, 739–748.
22. Wray,G.A. (2007) The evolutionary significance of cis-regulatory mutations. *Nature Reviews Genetics*, **8**, 206–216.
23. Wittkopp,P.J. and Kalay,G. (2011) Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. *Nature reviews. Genetics*, **13**, 59–69.
24. Emerson,J.J., Hsieh,L.-C., Sung,H.-M., Wang,T.-Y., Huang,C.-J., Lu,H.H.-S., Lu,M.-Y.J., Wu,S.-H. and Li,W.-H. (2010) Natural selection on cis and trans regulation in yeasts. *Genome Research*, **20**, 826–836.
25. Li,C.-M., Tzeng,J.-N. and Sung,H.-M. (2012) Effects of cis and trans regulatory variations on the expression divergence of heat shock response genes between yeast strains. *Gene*, **506**, 93–7.
26. Wittkopp,P.J., Haerum,B.K. and Clark,A.G. (2008) Regulatory changes underlying expression differences within and between Drosophila species. *Nature Genetics*, **40**, 346–350.
27. Morley,M., Molony,C.M., Weber,T.M., Devlin,J.L., Ewens,K.G., Spielman,R.S. and Cheung,V.G. (2004) Genetic analysis of genome-wide variation in human gene expression.

Nature, **430**, 743–747.

28. Nishimura,D.Y., Searby,C.C., Alward,W.L., Walton,D., Craig,J.E., Mackey,D.A., Kawase,K., Kanis,A.B., Patil,S.R., Stone,E.M., *et al.* (2001) A spectrum of FOXC1 mutations suggests gene dosage as a mechanism for developmental defects of the anterior chamber of the eye. *American journal of human genetics*, **68**, 364–72.

29. Mirzayans,F., Gould,D.B., Héon,E., Billingsley,G.D., Cheung,J.C., Mears,A.J. and Walter,M.A. (2000) Axenfeld-Rieger syndrome resulting from mutation of the FKHL7 gene on chromosome 6p25. *European journal of human genetics : EJHG*, **8**, 71–4.

30. Murphy,T.C., Saleem,R.A., Footz,T., Ritch,R., McGillivray,B. and Walter,M.A. (2004) The wing 2 region of the FOXC1 forkhead domain is necessary for normal DNA-binding and transactivation functions. *Investigative ophthalmology & visual science*, **45**, 2531–8.

31. Weisschuh,N., Dressler,P., Schuettauf,F., Wolf,C., Wissinger,B. and Gramer,E. (2006) Novel Mutations of *FOXC1* and *PITX2* in Patients with Axenfeld-Rieger Malformations. *Investigative Opthalmology & Visual Science*, **47**, 3846.

32. Wang,Y., Zhao,H., Zhang,X. and Feng,H. (2003) Novel identification of a four-base-pair deletion mutation in PITX2 in a Rieger syndrome family. *Journal of dental research*, **82**, 1008–12.

33. Maciolek,N.L., Alward,W.L.M., Murray,J.C., Semina,E. V and McNally,M.T. (2006) Analysis of RNA splicing defects in PITX2 mutants supports a gene dosage model of Axenfeld-Rieger syndrome. *BMC medical genetics*, **7**, 59.

34. Volkmann,B.A., Zinkevich,N.S., Mustonen,A., Schilter,K.F., Bosenko,D. V, Reis,L.M., Broeckel,U., Link,B.A. and Semina,E. V (2011) Potential novel mechanism for Axenfeld-Rieger syndrome: deletion of a distant region containing regulatory elements of PITX2.

Investigative ophthalmology & visual science, **52**, 1450–9.

35. Flomen,R.H., Gorman,P.A., Vatcheva,R., Groet,J., Barisić,I., Ligutić,I., Sheer,D. and Nizetić,D. (1997) Rieger syndrome locus: a new reciprocal translocation t(4;12)(q25;q15) and a deletion del(4)(q25q27) both break between markers D4S2945 and D4S193. *Journal of medical genetics*, **34**, 191–5.
36. Flomen,R.H., Vatcheva,R., Gorman,P.A., Baptista,P.R., Groet,J., Barisić,I., Ligutic,I. and Nizetić,D. (1998) Construction and analysis of a sequence-ready map in 4q25: Rieger syndrome can be caused by haploinsufficiency of RIEG, but also by chromosome breaks approximately 90 kb upstream of this gene. *Genomics*, **47**, 409–13.
37. Semina,E. V, Datson,N.A., Leysens,N.J., Zabel,B.U., Carey,J.C., Bell,G.I., Bitoun,P., Lindgren,C., Stevenson,T., Frants,R.R., *et al.* (1996) Exclusion of epidermal growth factor and high-resolution physical mapping across the Rieger syndrome locus. *American journal of human genetics*, **59**, 1288–96.
38. Benayoun,B.A., Caburet,S. and Veitia,R.A. (2011) Forkhead transcription factors: key players in health and disease. *Trends in Genetics*, **27**, 224–232.
39. Hannenhalli,S. and Kaestner,K.H. (2009) The evolution of Fox genes and their role in development and disease. *Nature reviews. Genetics*, **10**, 233–40.
40. Myatt,S.S. and Lam,E.W.-F. (2007) The emerging roles of forkhead box (Fox) proteins in cancer. *Nature Reviews Cancer*, **7**, 847–859.
41. Lenhard,B., Sandelin,A., Mendoza,L., Engström,P., Jareborg,N. and Wasserman,W.W. (2003) Identification of conserved regulatory elements by comparative genome analysis. *Journal of Biology*, **2**, 13.
42. Hedges,S.B. and Kumar,S. (1998) A molecular timescale for vertebrate evolution. *Nature*, **392**,

917–920.

43. Ahituv,N., Zhu,Y., Visel,A., Holt,A., Afzal,V., Pennacchio,L.A. and Rubin,E.M. (2007) Deletion of ultraconserved elements yields viable mice. *PLoS biology*, **5**, e234.

44. Nobrega,M.A., Ovcharenko,I., Afzal,V. and Rubin,E.M. (2003) Scanning Human Gene Deserts for Long-Range Enhancers. *Science*, **302**, 413–413.

45. Pennacchio,L.A., Ahituv,N., Moses,A.M., Prabhakar,S., Nobrega,M.A., Shoukry,M., Minovitsky,S., Dubchak,I., Holt,A., Lewis,K.D., *et al.* (2006) In vivo enhancer analysis of human conserved non-coding sequences. *Nature*, **444**, 499–502.

46. Chatterjee,S., Bourque,G. and Lufkin,T. (2011) Conserved and non-conserved enhancers direct tissue specific transcription in ancient germ layer specific developmental control genes. *BMC developmental biology*, **11**, 63.

47. Blow,M.J., McCulley,D.J., Li,Z., Zhang,T., Akiyama,J.A., Holt,A., Plajzer-Frick,I., Shoukry,M., Wright,C., Chen,F., *et al.* (2010) ChIP-Seq identification of weakly conserved heart enhancers. *Nature genetics*, **42**, 806–10.

48. Prabhakar,S., Poulin,F., Shoukry,M., Afzal,V., Rubin,E.M., Couronne,O. and Pennacchio,L.A. (2006) Close sequence comparisons are sufficient to identify human cis-regulatory elements. *Genome research*, **16**, 855–63.

49. Visel,A., Prabhakar,S., Akiyama,J.A., Shoukry,M., Lewis,K.D., Holt,A., Plajzer-Frick,I., Afzal,V., Rubin,E.M. and Pennacchio,L.A. (2008) Ultraconservation identifies a small subset of extremely constrained developmental enhancers. *Nature genetics*, **40**, 158–60.

50. MATHAVAN,K.J. (2008) Identification and characterization of conserved regulatory elements by comparative genomics.

51. McGaughey,D.M., Vinton,R.M., Huynh,J., Al-Saif,A., Beer,M.A. and McCallion,A.S. (2008)

Metrics of sequence constraint overlook regulatory sequences in an exhaustive analysis at phox2b. *Genome research*, **18**, 252–60.

52. Fisher,S., Grice,E.A., Vinton,R.M., Bessling,S.L. and McCallion,A.S. (2006) Conservation of RET regulatory function from human to zebrafish without sequence similarity. *Science (New York, N.Y.)*, **312**, 276–9.

53. Ludwig,M.Z., Bergman,C., Patel,N.H. and Kreitman,M. (2000) Evidence for stabilizing selection in a eukaryotic enhancer element. *Nature*, **403**, 564–7.

54. Cooper,G.M. and Brown,C.D. (2008) Qualifying the relationship between sequence conservation and molecular function. *Genome research*, **18**, 201–5.

55. ENCODE Project Consortium,E., Birney,E., Stamatoyannopoulos,J.A., Dutta,A., Guigó,R., Gingeras,T.R., Margulies,E.H., Weng,Z., Snyder,M., Dermitzakis,E.T., *et al.* (2007) Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. *Nature*, **447**, 799–816.

56. Margulies,E.H., Cooper,G.M., Asimenos,G., Thomas,D.J., Dewey,C.N., Siepel,A., Birney,E., Keefe,D., Schwartz,A.S., Hou,M., *et al.* (2007) Analyses of deep mammalian sequence alignments and constraint predictions for 1% of the human genome. *Genome research*, **17**, 760–74.

Chapter 6. General discussion and future directions

ARS is a clinically and genetically heterogeneous group of developmental disorders that primarily present with malformations in the anterior segment of the eye. As well, ARS patients show non-ocular abnormalities in different organs including brain, heart, and kidney (1–3). Approximately 40% of ARS patients have variations in *FOXC1* and *PITX2*. Thus, the discovery and analyses of variations in these two genes will provide information of how they are involved in the ARS disease, and more importantly gain an understanding of how they influence the expression level of these transcription factors, aiding us to expand our understanding of the ARS disease pathogenicity.

After summarizing the current understanding of ARS clinical presentation and its underlying genetic basis in chapter 1, in chapter 2, I reported the discovery and characterization of novel *PITX2* deletions in two familial patients (father and son) from a consanguineous family. Using PCR-based sequencing and copy number variation by SYBR Green quantitative polymerase chain reaction (PCR) analysis, I identified a novel deletion involving the coding region of *PITX2* in both patients.

As discussed in chapter two, the level of *PITX2* expression needs to be stringently regulated and any alteration in the expression and dosage of this gene impair development of different tissues (4–6). The observation of deletions of the *PITX2* gene in our ARS patients demonstrates the necessity of tight regulation of *PITX2* gene expression levels and activities for embryogenesis and, specially, for the normal development of ocular and non-ocular tissues. In the proband (son), I also identified a novel 2-bp deletion that lies in a non-coding exon of the remaining *PITX2* allele. *In silico* analysis predicted that this variant creates additional splice enhancer sites for the splice enhancers the shuttling Serine/Arginine rich (SR) protein SRSF1 and serine-arginine rich protein 40 (SRp40). SRSF1 is involved in many different cellular processes, including cell cycle progression, RNA splicing, and mRNA translation, suggesting its role in changing correct splicing of the *PITX2* mRNA and affecting the development of the wide range of tissues normally regulated by *PITX2* (7). It has been reported

that relative levels of SRp40 along with SRp20, SRp30, regulate glucocorticoid receptor (GR) splicing and the glucocorticoid (GC) response in the trabecular meshwork cells (8). Various levels and/or functionality of these SRps likely cause differential GC sensitivity in the normal and glaucoma individuals (8).

My findings implicate a novel deletion of the *PITX2* gene in the pathogenesis of ARS in the affected family. The phenotypic manifestations in the proband were more severe than that of the father. Since no evidence have been identified to indicate the association of any of the deleted genes with either the omphalocele or digital findings, I hypothesize that the deletion of the entire *PITX2* allele plus a novel 2-bp deletion (observed in the proband) within the remaining *PITX2* allele together contributed to the atypical ARS presentation in this family. Consistent with my findings, molecular analyses of the mutant PITX2 proteins have shown that ARS patients with *PITX2* gene in addition to eye abnormalities, are more likely than those with *FOXC1* gene variations to have severe disruptions to the normal abilities of the PITX2 protein and present with systemic abnormalities such as teeth and umbilical defects (9, 10). Regarding the novel 2-bp deletion, additional functional analysis will be required to explore further the effect of this *PITX2* two base pair deletion on *PITX2* RNA splicing. Advances in bioinformatics tools have enabled the interpretation of the functional consequences of variant alleles in molecular genetics (11–14). In chapter 3, I used a combination of functional analysis and bioinformatics programs to investigate the effect of four ARS missense variants including p.H128R, p.C135Y), p.M161V, and p.T368N on FOXC1 structure and function, and examined the predictive value of four *in silico* programs (SIFT, PolyPhen-2, align-GVGD, and MutPred) for all 31 *FOXC1* missense variants identified to date. Molecular modeling analysis assesses that p.C135Y changes FOXC1's structure. In contrast, p.H128R and p.M161V are not estimated to alter FOXC1's structure, suggesting that these missense variants are predicted to have no impacts on FOXC1's

structure. Functional experiments showed that p.H128R decreased DNA binding, transactivation, nuclear localization, and has a longer protein half-life than normal. p.C135Y markedly impairs *FOXC1*'s DNA binding, transactivation, and nuclear localization. p.M161V decreases transactivation activity and has no effect on other FOXC1 functions. p.T368N is similar to wild-type *FOXC1* in all features, indicating as a rare benign variant. Comparison of these four variants and 18 previously analysed *FOXC1* missense variants with the results of four *in silico* bioinformatics tools revealed that MutPred program is a reliable tool in determining the pathogenicity impacts of all 22 characterized missense variants in *FOXC1*. SIFT and PolyPhen-2 can also sensitively predict deleterious variants, indicating the reliability of MutPred, SIFT, and PolyPhen-2 in predicting the pathogenicity of *FOXC1* missense substitutions in the absence of functional analysis. It should be noted that in this study, however, I only used four bioinformatics tools to predict the effect of *FOXC1* missense variants. As well, the number of variants studied here was small. Therefore, using more bioinformatics prediction tools along with a large number of variants are required to examine the pathogenicity of these variants on *FOXC1* and to assess the predictive value of these programs. The results of these predictions should still be interpreted with caution. In a study, Flanagan et al. used 141 missense variants in three genes including ABCC8, GCK, and KCNJ11 to examine the predictive value of SIFT and PolyPhen (15). They found that while these two programs are likely aid in recognizing variants that may be lead to a loss of protein function, they have low accuracy for detecting gain of function variations (15). Thus, it is worth noting that, for the interpretation of missense variants, the results of bioinformatics prediction programs should be interpreted in the light of functional results, data on population frequency, segregation in affected families, and further evidence to support/disprove pathogenicity should be considered prior to reporting novel missense variants. As mentioned, direct sequencing of potential candidate genes could have conducted to identify novel variants and diagnose

the associated disease. However, sequencing studies result in a large number of variants, and, as result, analyzing the functional effect of all these variants using *in vitro* experiments such as genotyping would be expensive, laborious and time consuming. Therefore, computational methods are required to recognize deleterious variants. Thus, over the recent decade, bioinformatics programs have been designed to analyse and aid in the interpretation of novel sequence variations. Now, there are wide variety of prediction software that can assess the association of missense variants with protein structure and function. These programs can predict effects on different features such as protein aggregation, stability, localization, and post translation modification (16).

To date, there has been no study to investigate the efficiency of bioinformatics tools in predicting the association of *PITX2* variants with ARS. Thus, in chapter 4, I evaluated the performance of seven different bioinformatics tools (SIFT, PolyPhen-2, PANTHER-PSEP, MutPred, MutationTaster, Provean, and PMUT) in predicting the pathogenicity of *PITX2* sequence variations. In addition, I used molecular modeling and different protein stability prediction tools (DUET, I-Mutant3.0, MUpro, and iPTREE-STAB) to gain knowledge of molecules structure of PITX2 and to identify how PITX2 missense variants impair the structure of the protein and consequently destabilize it. The result showed that MutPred, Provean, and PMUT have the highest performance of the tested bioinformatics tools in identifying the pathogenicity effects of all 18 characterized missense variants in *PITX2*, all with the sensitivity and specificity of over 94%. After these three programs, PolyPhen-2 and SIFT both showed higher sensitivity and specificity.

MutationTaster and PANTHER-PSEP in spite of good sensitivity, were unable to predict the neutral variants. Applying the most reliable programs (MutPred, Provean, and PMUT) to assess the likely pathogenicity of 13 previously uncharacterized *PITX2* missense variants predicted 12/13 of these variants as deleterious, except A30V that was predicted to be a benign polymorphism.

The molecular modeling of all *PITX2* variations identified seven variants that grossly disrupt the structure of PITX2, including three characterized (L54Q, V83L, and R91P) and four uncharacterized (F58L, V83F, W86C, and W86S). These variants were predicted to change the structure of PITX2 homeodomain, particularly in H1, H2, and H3 subdomains. These seven amino acids create densely packed hydrophobic amino acids which are involved in keeping helices of the PITX2 homeodomain together, supporting our molecular modeling findings for these variants. Since each amino acid has unique size, charge, and hydrophobicity value (17), changes in these features of seven variants may disrupt the structure and function of protein. For instance, in F58L, V83L, and V83F, difference in size of the native wild type residues and the introduced mutant residues may impair hydrophobic interactions in the core of the protein, and consequently interfere with the core structure of helix 1 and 3. Alternatively, substitution of L54, W86, and W86 to uncharged polar residues could create empty spaces in the core of the protein. As the side chains of native wild-type residues are required for hydrophobic interactions with the residues within neighboring α-helices, these variants could increase structural perturbations via disruption in hydrophobic interactions. Evaluating the change in protein stability (ΔΔG) using four different stability prediction methods showed that I-mutant3.0 is the most reliable program in determining the stability of *PITX2* variants. DUET, MUpro, and iTREE-STAB were indicated to be weaker which is in line with the results of a previous study (18).

As mentioned previously, our analysis predicted the p.A30V to be a benign polymorphism, however, further functional characterization of the p.A30V variant is necessary to validate our prediction. In addition, further functional analyses of *PITX2* variations (particularly proteins stability) would be required to assess the accuracy of our suggested programs in larger dataset. It would be interesting to perform functional and bioinformatics analyses on other PITX family proteins to test the predictive

value of our suggested mutation prediction programs and to see that they are applicable to all these PITX transcription factor proteins.

Strict regulation of gene expression is very important and any increase or decrease in the level of expression could lead to abnormalities in mammalian organogenesis (19, 20). In some disorders, screening the coding region may reveal no variations, with further investigations revealing phenotype-associated variations or breakpoints located in non-coding sequences and found in different parts of the regulator elements such as in the promoter regions, single enhancer, silencer or insulator elements (21–24). The association of deletion in non-coding regulatory regions of the *PITX2* gene with ARS has been reported (21). The most apparent cases of transcriptional misregulation as the cause of genetic disease are associated with visible chromosomal rearrangements. For example, aniridia and related eye anomalies are caused mainly by haploinsufficiency of paired box 6 (*PAX6*) gene at human chromosome 11p13 (25). A number of aniridia human subjects have been described with no detectable variation in the transcription region (24, 26, 27). Instead, chromosomal rearrangements that impair the region downstream of the *PAX6* transcription region have been implicated. Detailed mapping of the breakpoints positioned them about 125 kb beyond the final exon. Analysis of the region beyond this breakpoint showed the presence of a downstream regulatory region resided about 200 kb away and within the intron of the adjacent ubiquitously expressed *ELP4* gene (24). Deletion of this downstream regulatory region showed that it is mandatory for expression of *PAX6* in the retina and iris, even in the presence of more proximal known retinal enhancers, and describes why the aniridia manifestation in 'position effect' patients is not distinct from aniridia in patients harboring variations in coding sequences of *PAX6* (28).

Variations in cis-regulatory elements (CREs) lead to a wide variety of phenotypes by different mechanisms. For instance, alterations at transcription factor binding sites (TFBSs) impair the interaction of specific proteins with their corresponding places which are substantial for RNA polymerase II (pol II)-dependent synthesis of mRNA transcripts (29, 30). Elevating or decreasing gene expression is another mechanism by which changes in CREs lead to human disease (31–34). There is a poor understanding of the regulatory regions of *FOXC1* gene and its connection with ARS. Therefore, in chapter 5, I investigated the sequences surrounding *FOXC1* gene in ARS patients to detect and characterize the regulatory regions. Using a combination of different bioinformatics algorithm, I identified seven conserved non-coding elements (CNEs) located up- and downstream of *FOXC1*. Further transactivation experiments showed that none of the conserved regions has functional activity, suggesting no association of spatiotemporal specific expression of the *FOXC1* gene with our detected conserved regions. There are some possibilities about why my identified regions are not capable of expressing luciferase reporter gene; first, I used limited cell lines (HeLa, TM, and ODM-2) in our analysis and, thus, it is possible that these conserved regions show their functional activity in specific cell lines. Second, I only performed *in vitro* transactivation experiments to validate the detected conserved regions. Using animal models such as zebrafish with different combination of CNEs may aid us to examine the expression pattern of these regions in different tissues and at different developmental stages. Treatment strategies for many human diseases and disorders have resulted from studies in animal models and now, transgenic zebrafish and mouse models are thought to be essential for testing gene functions in whole animals (35–37).

In addition, it would be interesting to investigate promoter-CREs interaction specificity in *FOXC1* and *PITX2*. The position of the CREs could be placed many kilobases upstream or downstream of the promoter of the controlled gene. They could reside even in a megabase range around the promoter,

encompassing other target genes (22). For appropriate gene modulation in complex loci, specific enhancer(s)–promoter interactions are necessary (38). Insulators or boundary elements can inhibit the communication of promoters with enhancers (39). A wide spectrum of studies has provided evidence that the core promoter sequence context is able to dramatically affect the responsiveness of a given gene to gene-specific DNA-binding activators and repressors (40–43). There are some factors that are fundamental in the competition of promoters for a distinct enhancer. One of the major elements is the distance of promoters from a particular enhancer. For instance, in the homeobox D (HOXD) cluster, the genes that are close to a specific enhancer have a higher chance of communication than distal ones (44). Distance is also expected to be relevant in terms of spacing between CREs. Structural studies show that the flexibility and conformation of the chromatin template will restrict the distance between two elements forming a loop (45). Promoter affinity is another crucial factor in gene competition that relies on interaction of transcription factors with the CREs (46). In another study, evidence of a promoter targeting sequence (PTS) was identified in Drosophila (46). PTS plays as an anti-insulator role by expediting the interaction of an enhancer with its promoter positioned far from each other (47). In addition, despite several promoters located in the same transgene, a PTS particularly activates just a specific promoter (48). In some cases, CREs affect the transcription of more than one gene. For example, regarding bidirectional promoters, CREs positioned between the two different promoters are capable of controlling the expression of paralogous loci that reside on opposite DNA strand (49). Thus, besides the impact of CREs on *FOXC1* and *PITX2* expression, it would be interesting to investigate their neighbouring genes including GMDS, FOXF2, FOXQ1, ENPEP, and ELOVL6.

As a conclusion, in this thesis, I performed a wide variety of molecular techniques to identify novel variants in *FOXC1* and *PITX2* and then investigated the mechanisms by which these variants disrupt

FOXC1 and *PITX2* expression, structure and function. Next, I applied a combination of bioinformatics tools to predict the pathogenicity of variants in FOXC and PITX2. Finally, to assess the performance of these bioinformatics programs in identifying deleterious variant, I compared the results of my functional analysis and previous experimental data with bioinformatics findings. The results showed that in the absence of functional data, PMUT, Provean, MutPred, I-mutant3.0 and molecular modeling are all reliable means of predicting the pathogenicity of missense substitutions with the *FOXC1* FHD and *PITX2* HD. In addition, due to the sequence homology between the FHDs of FOX class and HD of PITX transcription factors, I predict that these bioinformatics programs can be applied to determine the potential pathogenicity of missense variants within other FOX and PITX proteins and to prioritize variants for functional analysis. My work has the potentiality to narrow the gap between the generation of massively parallel sequencing output and the ability to process, analyze and interpret the resulting data. Although the bioinformatics programs used in this thesis are not gene-specific, generalization of the performance of these programs to other human genes require additional studies and also a larger dataset would confirm that our results are reproducible and generally applicable.

References

1. Semina,E. V, Reiter,R., Leysens,N.J., Alward,W.L., Small,K.W., Datson,N.A., Siegel-Bartelt,J., Bierke-Nelson,D., Bitoun,P., Zabel,B.U., *et al.* (1996) Cloning and characterization of a novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. *Nature genetics*, **14**, 392–9.

2. Nishimura,D.Y., Swiderski,R.E., Alward,W.L., Searby,C.C., Patil,S.R., Bennet,S.R., Kanis,A.B., Gastier,J.M., Stone,E.M. and Sheffield,V.C. (1998) The forkhead transcription factor gene FKHL7 is responsible for glaucoma phenotypes which map to 6p25. *Nature genetics*, **19**, 140–7.

3. Nishimura,D.Y., Searby,C.C., Alward,W.L., Walton,D., Craig,J.E., Mackey,D.A., Kawase,K., Kanis,A.B., Patil,S.R., Stone,E.M., *et al.* (2001) A spectrum of FOXC1 mutations suggests gene dosage as a mechanism for developmental defects of the anterior chamber of the eye. *American journal of human genetics*, **68**, 364–72.

4. Lehmann,O.J., Ebenezer,N.D., Ekong,R., Ocaka,L., Mungall,A.J., Fraser,S., McGill,J.I., Hitchings,R.A., Khaw,P.T., Sowden,J.C., *et al.* (2002) Ocular developmental abnormalities and glaucoma associated with interstitial 6p25 duplications and deletions. *Investigative ophthalmology & visual science*, **43**, 1843–9.

5. D'haene,B., Meire,F., Claerhout,I., Kroes,H.Y., Plomp,A., Arens,Y.H., de Ravel,T., Casteels,I., De Jaegere,S., Hooghe,S., *et al.* (2011) Expanding the spectrum of FOXC1 and PITX2 mutations and copy number changes in patients with anterior segment malformations. *Investigative ophthalmology & visual science*, **52**, 324–33.

6. Chanda,B., Asai-Coakwell,M., Ye,M., Mungall,A.J., Barrow,M., Dobyns,W.B., Behesti,H.,

Sowden,J.C., Carter,N.P., Walter,M.A., *et al.* (2008) A novel mechanistic spectrum underlies glaucoma-associated chromosome 6p25 copy number variation. *Human molecular genetics*, **17**, 3446–58.

7. Maslon,M.M., Heras,S.R., Bellora,N., Eyras,E. and Cáceres,J.F. (2014) The translational landscape of the splicing factor SRSF1 and its role in mitosis. *eLife*, **3**, e02028.

8. Jain,A., Wordinger,R.J., Yorio,T. and Clark,A.F. (2012) Spliceosome protein (SRp) regulation of glucocorticoid receptor isoforms and glucocorticoid response in human trabecular meshwork cells. *Investigative ophthalmology & visual science*, **53**, 857–66.

9. Reis,L.M., Tyler,R.C., Volkmann Kloss,B.A., Schilter,K.F., Levin,A. V, Lowry,R.B., Zwijnenburg,P.J.G., Stroh,E., Broeckel,U., Murray,J.C., *et al.* (2012) PITX2 and FOXC1 spectrum of mutations in ocular syndromes. *European Journal of Human Genetics*, **20**, 1224–1233.

10. Hjalt,T.A., Semina,E. V, Amendt,B.A. and Murray,J.C. (2000) The Pitx2 protein in mouse development. *Developmental dynamics : an official publication of the American Association of Anatomists*, **218**, 195–200.

11. Wang,Z., Rolish,M.E., Yeo,G., Tung,V., Mawson,M. and Burge,C.B. (2004) Systematic Identification and Analysis of Exonic Splicing Silencers. *Cell*, **119**, 831–845.

12. Smith,P.J., Zhang,C., Wang,J., Chew,S.L., Zhang,M.Q. and Krainer,A.R. (2006) An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. *Human Molecular Genetics*, **15**, 2490–2508.

13. Goren,A., Ram,O., Amit,M., Keren,H., Lev-Maor,G., Vig,I., Pupko,T. and Ast,G. (2006)

Comparative Analysis Identifies Exonic Splicing Regulatory Sequences—The Complex Definition of Enhancers and Silencers. *Molecular Cell*, **22**, 769–781.

14. Fairbrother,W.G., Yeh,R.-F., Sharp,P.A. and Burge,C.B. (2002) Predictive identification of exonic splicing enhancers in human genes. *Science (New York, N.Y.)*, **297**, 1007–13.

15. Flanagan,S.E., Patch,A.-M. and Ellard,S. (2010) Using SIFT and PolyPhen to predict loss-of-function and gain-of-function mutations. *Genetic testing and molecular biomarkers*, **14**, 533–7.

16. Niroula,A. and Vihinen,M. (2016) Variation Interpretation Predictors: Principles, Types, Performance, and Choice. *Human mutation*, **37**, 579–97.

17. Biro,J.C. (2006) Amino acid size, charge, hydropathy indices and matrices for protein structure analysis. *Theoretical biology & medical modelling*, **3**, 15.

18. Khan,S. and Vihinen,M. (2010) Performance of protein stability predictors. *Human mutation*, **31**, 675–84.

19. Yilmaz,A. and Grotewold,E. (2010) Components and Mechanisms of Regulation of Gene Expression. In *Methods in molecular biology (Clifton, N.J.)*.Vol. 674, pp. 23–32.

20. Kadonaga,J.T. (2004) Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. *Cell*, **116**, 247–57.

21. Volkmann,B.A., Zinkevich,N.S., Mustonen,A., Schilter,K.F., Bosenko,D. V, Reis,L.M., Broeckel,U., Link,B.A. and Semina,E. V (2011) Potential novel mechanism for Axenfeld-Rieger syndrome: deletion of a distant region containing regulatory elements of PITX2. *Investigative ophthalmology & visual science*, **52**, 1450–9.

22. Lettice,L.A., Heaney,S.J.H., Purdie,L.A., Li,L., de Beer,P., Oostra,B.A., Goode,D., Elgar,G., Hill,R.E. and de Graaff,E. (2003) A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Human molecular genetics*, **12**, 1725–35.

23. Kleinjan,D.A. and van Heyningen,V. (2005) Long-range control of gene expression: emerging mechanisms and disruption in disease. *American journal of human genetics*, **76**, 8–32.

24. Kleinjan,D.A., Seawright,A., Schedl,A., Quinlan,R.A., Danes,S. and van Heyningen,V. (2001) Aniridia-associated translocations, DNase hypersensitivity, sequence comparison and transgenic analysis redefine the functional domain of PAX6. *Human molecular genetics*, **10**, 2049–59.

25. van Heyningen,V. and Williamson,K.A. (2002) PAX6 in sensory development. *Human molecular genetics*, **11**, 1161–7.

26. Fantes,J., Redeker,B., Breen,M., Boyle,S., Brown,J., Fletcher,J., Jones,S., Bickmore,W., Fukushima,Y. and Mannens,M. (1995) Aniridia-associated cytogenetic rearrangements suggest that a position effect may cause the mutant phenotype. *Human molecular genetics*, **4**, 415–22.

27. Cheng,F., Song,W., Kang,Y., Yu,S. and Yuan,H. (2011) A 556 kb deletion in the downstream region of the PAX6 gene causes familial aniridia and other eye anomalies in a Chinese family. *Molecular vision*, **17**, 448–55.

28. Kleinjan,D.A., Seawright,A., Mella,S., Carr,C.B., Tyas,D.A., Simpson,T.I., Mason,J.O., Price,D.J. and van Heyningen,V. (2006) Long-range downstream enhancers are essential for Pax6 expression. *Developmental biology*, **299**, 563–81.

29. Wittwer,J., Marti-Jaun,J. and Hersberger,M. (2006) Functional polymorphism in ALOX15 results in increased allele-specific transcription in macrophages through binding of the transcription factor SPI1. *Human mutation*, **27**, 78–87.

30. Kamanu,F.K., Medvedeva,Y.A., Schaefer,U., Jankovic,B.R., Archer,J.A.C. and Bajic,V.B. (2012) Mutations and Binding Sites of Human Transcription Factors. *Frontiers in Genetics*, **3**, 100.

31. Nokes,E.B., Van Der Linden,A.M., Winslow,C., Mukhopadhyay,S., Ma,K. and Sengupta,P. (2009) Cis-regulatory mechanisms of gene expression in an olfactory neuron type in Caenorhabditis elegans. *Developmental dynamics : an official publication of the American Association of Anatomists*, **238**, 3080–92.

32. Narlikar,L. and Ovcharenko,I. (2009) Identifying regulatory elements in eukaryotic genomes. *Briefings in functional genomics & proteomics*, **8**, 215–30.

33. Mathelier,A., Lefebvre,C., Zhang,A.W., Arenillas,D.J., Ding,J., Wasserman,W.W. and Shah,S.P. (2015) Cis-regulatory somatic mutations and gene-expression alteration in B-cell lymphomas. *Genome biology*, **16**, 84.

34. Chabot,A., Shrit,R.A., Blekhman,R. and Gilad,Y. (2007) Using reporter gene assays to identify cis regulatory differences between humans and chimpanzees. *Genetics*, **176**, 2069–76.

35. Casal,M. and Haskins,M. (2006) Large animal models and gene therapy. *European journal of human genetics : EJHG*, **14**, 266–72.

36. Lozier,J. (2004) Gene therapy of the hemophilias. *Seminars in hematology*, **41**, 287–96.

37. Buch,P.K., Bainbridge,J.W. and Ali,R.R. (2008) AAV-mediated gene therapy for retinal

disorders: from mouse to man. *Gene Therapy*, **15**, 849–857.

38. Atkinson,T.J. and Halfon,M.S. (2014) Regulation of gene expression in the genomic context. *Computational and structural biotechnology journal*, **9**, e201401001.

39. Parnell,T.J. and Geyer,P.K. (2000) Differences in insulator properties revealed by enhancer blocking assays on episomes. *The EMBO journal*, **19**, 5864–74.

40. So,A.Y.-L., Chaivorapol,C., Bolton,E.C., Li,H. and Yamamoto,K.R. (2007) Determinants of cell- and gene-specific transcriptional regulation by the glucocorticoid receptor. *PLoS genetics*, **3**, e94.

41. Pauw,B., Hilliou,F.A.O., Martin,V.S., Chatel,G., de Wolf,C.J.F., Champion,A., Pré,M., van Duijn,B., Kijne,J.W., van der Fits,L., *et al.* (2004) Zinc finger proteins act as transcriptional repressors of alkaloid biosynthesis genes in Catharanthus roseus. *The Journal of biological chemistry*, **279**, 52940–8.

42. Deckert,J. and Struhl,K. (2001) Histone acetylation at promoters is differentially affected by specific activators and repressors. *Molecular and cellular biology*, **21**, 2726–35.

43. Fragiadakis,G.S., Tzamarias,D. and Alexandraki,D. (2004) Nhp6 facilitates Aft1 binding and Ssn6 recruitment, both essential for FRE2 transcriptional activation. *The EMBO journal*, **23**, 333–42.

44. Kmita,M., Fraudeau,N., Hérault,Y. and Duboule,D. (2002) Serial deletions and duplications suggest a mechanism for the collinearity of Hoxd genes in limbs. *Nature*, **420**, 145–50.

45. Rippe,K. (2001) Making contacts on a nucleic acid polymer. *Trends in biochemical sciences*, **26**, 733–40.

46. Lin,Q., Chen,Q., Lin,L., Smith,S. and Zhou,J. (2007) Promoter targeting sequence mediates enhancer interference in the Drosophila embryo. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 3237–42.

47. Chen,Q., Lin,L., Smith,S., Lin,Q. and Zhou,J. (2005) Multiple Promoter Targeting Sequences exist in Abdominal-B to regulate long-range gene activation. *Developmental biology*, **286**, 629–36.

48. Chen,Y.-H., Wang,Y.-H., Chang,M.-Y., Lin,C.-Y., Weng,C.-W., Westerfield,M. and Tsai,H.-J. (2007) Multiple upstream modules regulate zebrafish myf5 expression. *BMC developmental biology*, **7**, 1.

49. Trinklein,N.D., Aldred,S.F., Hartman,S.J., Schroeder,D.I., Otillar,R.P. and Myers,R.M. (2004) An abundance of bidirectional promoters in the human genome. *Genome research*, **14**, 62–6.

Bibliography

Aantaa R, Jalonen J. 2006. Perioperative use of α2-adrenoceptor agonists and the cardiac patient. European Journal of Anaesthesiology 23:361–372.

Acharya M, Huang L, Fleisch VC, Allison WT, Walter MA. 2011. A complex regulatory network of transcription factors critical for ocular development and disease. Human Molecular Genetics 20:1610–1624.

Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, Kondrashov AS, Sunyaev SR. 2010. A method and server for predicting damaging missense mutations. Nature methods 7:248–9.

Ahituv N, Zhu Y, Visel A, Holt A, Afzal V, Pennacchio LA, Rubin EM. 2007. Deletion of ultraconserved elements yields viable mice. PLoS biology 5:e234.

Aldinger KA, Lehmann OJ, Hudgins L, Chizhikov V V, Bassuk AG, Ades LC, Krantz ID, Dobyns WB, Millen KJ. 2009. FOXC1 is required for normal cerebellar development and is a major contributor to chromosome 6p25.3 Dandy-Walker malformation. Nature genetics 41:1037–42.

Alward WL, Semina E V, Kalenak JW, Héon E, Sheth BP, Stone EM, Murray JC. 1998. Autosomal dominant iris hypoplasia is caused by a mutation in the Rieger syndrome (RIEG/PITX2) gene. American journal of ophthalmology 125:98–100.

Amendt BA, Semina E V, Alward WL. 2000. Rieger syndrome: a clinical, molecular, and biochemical analysis. Cellular and molecular life sciences : CMLS 57:1652–66.

Amendt BA, Sutherland LB, Russo AF. 1999. Multifunctional role of the Pitx2 homeodomain protein C-terminal tail. Molecular and cellular biology 19:7001–10.

Amendt BA, Sutherland LB, Semina E V, Russo AF. 1998a. The molecular basis of Rieger syndrome. Analysis of Pitx2 homeodomain protein activities. The Journal of biological chemistry 273:20066–72.

Atkinson TJ, Halfon MS. 2014. Regulation of gene expression in the genomic context. Computational and structural biotechnology journal 9:e201401001.

Avshalumova L, Fabrikant J, Koriakos A. 2014. Overview of skin diseases linked to connexin gene mutations. International Journal of Dermatology 53:192–205.

Axenfeld TH. 1920. Embryotoxon corneaposterius. Klin Monatsbl Augenheilkd 65:381–382.

Aydin H, Al-Khooly D, Lee JE. 2014a. Influence of hydrophobic and electrostatic residues on SARS-coronavirus S2 protein stability: Insights into mechanisms of general viral fusion and inhibitor design. Protein Science 23:603–617.

Bamforth SD, Bragança J, Farthing CR, Schneider JE, Broadbent C, Michell AC, Clarke K, Neubauer S, Norris D, Brown N a, Anderson RH, Bhattacharya S. 2004. Cited2 controls left-right patterning and heart development through a Nodal-Pitx2c pathway. Nature genetics 36:1189–1196.

Bao L, Cui Y. 2005. Prediction of the phenotypic effects of non-synonymous single nucleotide polymorphisms using structural and evolutionary information. Bioinformatics 21:2185–2190.

Barrera LO, Ren B. 2006. The transcriptional regulatory code of eukaryotic cells – insights from genome-wide analysis of chromatin organization and transcription factor binding. Current Opinion in Cell Biology 18:291–298.

Becker SA, Popp S, Rager K, Jauch A. 2003. A new case of an interstitial deletion (4)(q25q27) characterised by molecular cytogenetic techniques and review of the literature. European journal of pediatrics 162:267–70.

Benayoun BA, Caburet S, Veitia RA. 2011. Forkhead transcription factors: key players in health and disease. Trends in Genetics 27:224–232.

Berenstein-Aizman G, Hazan-Molina H, Drori D, Aizenbud D. Axenfeld-Rieger syndrome: dentofacial manifestation and oral rehabilitation considerations. Pediatric dentistry 33:440–4.

Bernardes CS, Leite LV de O, Castro FAA de. [Ectopia lentis et pupillae: case report]. Arquivos brasileiros de oftalmologia 68:841–4.

Berry FB, Lines MA, Oas JM, Footz T, Underhill DA, Gage PJ, Walter MA. 2006a. Functional interactions between FOXC1 and PITX2 underlie the sensitivity to FOXC1 gene dose in Axenfeld-Rieger syndrome and anterior segment dysgenesis. Human molecular genetics 15:905–19.

Berry FB, Mirzayans F, Walter MA. 2006. Regulation of FOXC1 stability and transcriptional activity by an epidermal growth factor-activated mitogen-activated protein kinase signaling cascade. The Journal of biological chemistry 281:10098–104.

Berry FB, Saleem RA, Walter MA. 2002. FOXC1 transcriptional regulation is mediated by N- and C-terminal activation domains and contains a phosphorylated transcriptional inhibitory domain. The Journal of biological chemistry 277:10292–7.

Bhandari R, Ferri S, Whittaker B, Liu M, Lazzaro DR. 2011. Peters Anomaly: Review of the Literature. Cornea 30:939–944.

Bhattacharjee H, Bhattacharjee K, Tahiliani P. 2013. Congenital polycoria, trichomegaly, and hereditary congenital cataract. Journal of AAPOS 17:619–620.

Biro JC. 2006. Amino acid size, charge, hydropathy indices and matrices for protein structure analysis. Theoretical biology & medical modelling 3:15.

Blanchette M, Tompa M. 2002. Discovery of Regulatory Elements by a Computational Method for Phylogenetic Footprinting. Genome Research 12:739–748.

Blow MJ, McCulley DJ, Li Z, Zhang T, Akiyama JA, Holt A, Plajzer-Frick I, Shoukry M, Wright C, Chen F, Afzal V, Bristow J, et al. 2010. ChIP-Seq identification of weakly conserved heart enhancers. Nature genetics 42:806–10.

Boffelli D, Nobrega MA, Rubin EM. 2004. Comparative genomics at the vertebrate extremes. Nature reviews Genetics 5:456–65.

Borges AS, Susanna R, Carani JCE, Betinjane AJ, Alward WL, Stone EM, Sheffield VC, Nishimura DY. 2002. Genetic analysis of PITX2 and FOXC1 in Rieger Syndrome patients from Brazil. Journal of glaucoma 11:51–6.

Brandão LM, Grieshaber MC. 2013. Update on Minimally Invasive Glaucoma Surgery (MIGS) and New Implants. Journal of ophthalmology 2013:705915.

Brooks BP, Moroi SE, Downs CA, Wiltse S, Othman MI, Semina E V, Richards JE. 2004. A novel mutation in the PITX2 gene in a family with Axenfeld-Rieger syndrome. Ophthalmic genetics 25:57–62.

Bryant SH, Lawrence CE. 1993. An empirical energy function for threading protein sequence through the folding motif. Proteins 16:92–112.

Buch PK, Bainbridge JW, Ali RR. 2008. AAV-mediated gene therapy for retinal disorders: from mouse to man. Gene Therapy 15:849–857.

Calloni G, Zoffoli S, Stefani M, Dobson CM, Chiti F. 2005. Investigating the Effects of Mutations on Protein Aggregation in the Cell. Journal of Biological Chemistry 280:10607–10613.

Campuzano O, Allegue C, Fernandez A, Iglesias A, Brugada R. 2015. Determining the Pathogenicity of Genetic Variants Associated with Cardiac Channelopathies. Scientific Reports 5:7953.

Capriotti E, Calabrese R, Casadio R. 2006a. Predicting the insurgence of human genetic diseases associated to single point protein mutations with support vector machines and evolutionary information. Bioinformatics (Oxford, England) 22:2729–34.

Casal M, Haskins M. 2006. Large animal models and gene therapy. European journal of human genetics : EJHG 14:266–72.

Cascella R, Strafella C, Germani C, Novelli G, Ricci F, Zampatti S, Giardina E. 2015. The Genetics and the Genomics of Primary Congenital Glaucoma: EBSCOhost. 2015:.

Chabot A, Shrit RA, Blekhman R, Gilad Y. 2007. Using reporter gene assays to identify cis regulatory differences between humans and chimpanzees. Genetics 176:2069–76.

Chakrabarti S, Kaur K, Rao KN, Mandal AK, Kaur I, Parikh RS, Thomas R. 2009. The transcription factor gene FOXC1 exhibits a limited role in primary congenital glaucoma. Investigative ophthalmology & visual science 50:75–83.

Chanda B, Asai-Coakwell M, Ye M, Mungall AJ, Barrow M, Dobyns WB, Behesti H, Sowden JC, Carter NP, Walter MA, Lehmann OJ. 2008. A novel mechanistic spectrum underlies glaucoma-associated chromosome 6p25 copy number variation. Human molecular genetics 17:3446–58.

Chaney BA, Clark-Baldwin K, Dave V, Ma J, Rance M. 2005. Solution structure of the K50 class homeodomain PITX2 bound to DNA and implications for mutations that cause Rieger syndrome. Biochemistry 44:7497–511.

Chang TC, Summers CG, Schimmenti LA, Grajewski AL. 2012a. Axenfeld-Rieger syndrome: new perspectives. The British journal of ophthalmology 96:318–22.

Charles MA, Suh H, Hjalt TA, Drouin J, Camper SA, Gage PJ. 2005. PITX genes are required for cell survival and Lhx3 activation. Molecular endocrinology (Baltimore, Md) 19:1893–903.

Chatterjee S, Bourque G, Lufkin T. 2011. Conserved and non-conserved enhancers direct tissue specific transcription in ancient germ layer specific developmental control genes. BMC developmental biology 11:63.

Chen K, Rajewsky N. 2007. The evolution of gene regulation by transcription factors and microRNAs. Nature Reviews Genetics 8:93–103.

Chen L, Gage PJ. 2016. Heterozygous Pitx2 Null Mice Accurately Recapitulate the Ocular Features of Axenfeld-Rieger Syndrome and Congenital Glaucoma. Investigative ophthalmology & visual science 57:5023–5030.

Chen Q, Lin L, Smith S, Lin Q, Zhou J. 2005. Multiple Promoter Targeting Sequences exist in Abdominal-B to regulate long-range gene activation. Developmental biology 286:629–36.

Chen Y-H, Wang Y-H, Chang M-Y, Lin C-Y, Weng C-W, Westerfield M, Tsai H-J. 2007. Multiple upstream modules regulate zebrafish myf5 expression. BMC developmental biology 7:1.

Cheng F, Song W, Kang Y, Yu S, Yuan H. 2011. A 556 kb deletion in the downstream region of the PAX6 gene causes familial aniridia and other eye anomalies in a Chinese family. Molecular vision 17:448–55.

Cheng J, Randall A, Baldi P. 2006a. Prediction of protein stability changes for single-site mutations using support vector machines. Proteins 62:1125–32.

Choi Y, Chan AP. 2015a. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. Bioinformatics (Oxford, England) 31:2745–7.

Choi Y, Sims GE, Murphy S, Miller JR, Chan AP. 2012. Predicting the Functional Effect of Amino Acid Substitutions and Indels. PLoS ONE 7:e46688.

Chun S, Fay JC. 2009. Identification of deleterious mutations within three human genomes. Genome Research 19:1553–1561.

Clark KL, Halay ED, Lai E, Burley SK. 1993. Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. Nature 364:412–20.

Cooper GM, Brown CD. 2008. Qualifying the relationship between sequence conservation and molecular function. Genome research 18:201–5.

Cox CJ, Espinoza HM, McWilliams B, Chappell K, Morton L, Hjalt T a, Semina E V, Amendt B a. 2002a. Differential regulation of gene expression by PITX2 isoforms. The Journal of biological chemistry 277:25001–10.

D'haene B, Meire F, Claerhout I, Kroes HY, Plomp A, Arens YH, Ravel T de, Casteels I, Jaegere S De, Hooghe S, Wuyts W, Ende J van den, et al. 2011. Expanding the spectrum of FOXC1 and PITX2 mutations and copy number changes in patients with anterior segment malformations. Investigative ophthalmology & visual science 52:324–33.

Danko CG, McIlvain VA, Qin M, Knox BE, Pertsov AM. 2007. Bioinformatic identification of novel putative photoreceptor specific cis-elements. BMC bioinformatics 8:407.

Deckert J, Struhl K. 2001. Histone acetylation at promoters is differentially affected by specific activators and repressors. Molecular and cellular biology 21:2726–35.

DeScipio C, Schneider L, Young TL, Wasserman N, Yaeger D, Lu F, Wheeler PG, Williams MS, Bason L, Jukofsky L, Menon A, Geschwindt R, et al. 2005. Subtelomeric deletions of chromosome 6p: Molecular and cytogenetic characterization of three new cases with phenotypic overlap with Ritscher?Schinzel (3C) syndrome. American Journal of Medical Genetics Part A 134A:3–11.

Deyneko I V, Weiss S, Leschner S. 2012. An integrative computational approach to effectively guide experimental identification of regulatory elements in promoters. BMC bioinformatics 13:202.

Dijkman HBPM, Gerlofs-Nijland ME, Laak JAWM van der, Wetzels JFM, Groenen PJTA, Assmann KJM. 2003. Podocyte changes after induction of acute albuminuria in mice by anti-aminopeptidase A mAb. Nephron Experimental nephrology 94:e85-93.

Dongen MJ van, Cederberg A, Carlsson P, Enerbäck S, Wikström M. 2000. Solution structure and dynamics of the DNA-binding domain of the adipocyte-transcription factor FREAC-11. Journal of molecular biology 296:351–9.

Doss CGP, Rajith B, Garwasis N, Mathew PR, Raju AS, Apoorva K, William D, Sadhana NR, Himani T, Dike I. 2012. Screening of mutations affecting protein stability and dynamics of FGFR1—A simulation analysis. Applied & Translational Genomics 1:37–43.

Doucette LP, Rasnitsyn A, Seifi M, Walter MA. 2015. The interactions of genes, age, and environment in glaucoma pathogenesis. Survey of Ophthalmology 60:310–326.

Doward W, Perveen R, Lloyd IC, Ridgway AE, Wilson L, Black GC. 1999. A mutation in the RIEG1 gene associated with Peters' anomaly. Journal of medical genetics 36:152–5.

Dressler S, Meyer-Marcotty P, Weisschuh N, Jablonski-Momeni A, Pieper K, Gramer G, Gramer E. 2010. Dental and Craniofacial Anomalies Associated with Axenfeld-Rieger Syndrome with PITX2 Mutation. Case reports in medicine 2010:621984.

Du R-F, Huang H, Fan L-L, Li X-P, Xia K, Xiang R. 2016. A Novel Mutation of FOXC1 (R127L) in an Axenfeld-Rieger Syndrome Family with Glaucoma and Multiple Congenital Heart Diseases. Ophthalmic genetics 37:111–5.

Ekonomidis P, Androudi S, Brazitikos P, Alexandridis A. 2006. Ectopia lentis et pupillae: report of a unilateral case and surgical management. Graefe's Archive for Clinical and Experimental Ophthalmology 244:878–879.

Emerson JJ, Hsieh L-C, Sung H-M, Wang T-Y, Huang C-J, Lu HH-S, Lu M-YJ, Wu S-H, Li W-H. 2010. Natural selection on cis and trans regulation in yeasts. Genome Research 20:826–836.

ENCODE Project Consortium E, Birney E, Stamatoyannopoulos JA, Dutta A, Guigó R, Gingeras TR, Margulies EH, Weng Z, Snyder M, Dermitzakis ET, Thurman RE, Kuehn MS, et al. 2007. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature 447:799–816.

Engenheiro E, Saraiva J, Carreira I, Ramos L, Ropers HH, Silva E, Tommerup N, Tümer Z. 2007. Cytogenetically invisible microdeletions involving PITX2 in Rieger syndrome. Clinical genetics 72:464–70.

Espinoza HM, Cox CJ, Semina E V, Amendt BA. 2002a. A molecular basis for differential developmental anomalies in Axenfeld-Rieger syndrome. Human molecular genetics 11:743–53.

Espinoza HM, Ganga M, Vadlamudi U, Martin DM, Brooks BP, Semina E V, Murray JC, Amendt BA. 2005. Protein kinase C phosphorylation modulates N- and C-terminal regulatory activities of the PITX2 homeodomain protein. Biochemistry 44:3942–54.

Essner JJ, Branford WW, Zhang J, Yost HJ. 2000. Mesendoderm and left-right brain, heart and gut development are differentially regulated by pitx2 isoforms. Development (Cambridge, England) 127:1081–93.

Evans AL, Gage PJ. 2005. Expression of the homeobox gene Pitx2 in neural crest is required for optic stalk and ocular anterior segment development. Human Molecular Genetics 14:3347–3359.

Fairbrother WG, Yeh R-F, Sharp PA, Burge CB. 2002. Predictive identification of exonic splicing enhancers in human genes. Science (New York, NY) 297:1007–13.

Fantes J, Redeker B, Breen M, Boyle S, Brown J, Fletcher J, Jones S, Bickmore W, Fukushima Y, Mannens M. 1995. Aniridia-associated cytogenetic rearrangements suggest that a position effect may cause the mutant phenotype. Human molecular genetics 4:415–22.

Ferrer-Costa C, Orozco M, la Cruz X de. 2002a. Characterization of disease-associated single amino acid polymorphisms in terms of sequence and structure properties. Journal of Molecular Biology 315:771–786.

Fetterman CD, Mirzayans F, Walter MA. 2009. Characterization of a novel FOXC1 mutation, P297S, identified in two individuals with anterior segment dysgenesis. Clinical genetics 76:296–9.

Fisher S, Grice EA, Vinton RM, Bessling SL, McCallion AS. 2006. Conservation of RET regulatory function from human to zebrafish without sequence similarity. Science (New York, NY) 312:276–9.

Flanagan SE, Patch A-M, Ellard S. 2010. Using SIFT and PolyPhen to predict loss-of-function and gain-of-function mutations. Genetic testing and molecular biomarkers 14:533–7.

Flomen RH, Gorman PA, Vatcheva R, Groet J, Barisić I, Ligutić I, Sheer D, Nizetić D. 1997. Rieger syndrome locus: a new reciprocal translocation t(4;12)(q25;q15) and a deletion del(4)(q25q27) both break between markers D4S2945 and D4S193. Journal of medical genetics 34:191–5.

Flomen RH, Vatcheva R, Gorman PA, Baptista PR, Groet J, Barisić I, Ligutic I, Nizetić D. 1998. Construction and analysis of a sequence-ready map in 4q25: Rieger syndrome can be caused by haploinsufficiency of RIEG, but also by chromosome breaks approximately 90 kb upstream of this gene. Genomics 47:409–13.

Footz T, Idrees F, Acharya M, Kozlowski K, Walter MA. 2009. Analysis of mutations of the PITX2 transcription factor found in patients with Axenfeld-Rieger syndrome. Investigative ophthalmology & visual science 50:2599–606.

Fragiadakis GS, Tzamarias D, Alexandraki D. 2004. Nhp6 facilitates Aft1 binding and Ssn6 recruitment, both essential for FRE2 transcriptional activation. The EMBO journal 23:333–42.

Furukawa T, Kozak CA, Cepko CL. 1997. rax, a novel paired-type homeobox gene, shows expression in the anterior neural fold and developing retina. Proceedings of the National Academy of Sciences of the United States of America 94:3088–93.

Fuse N, Takahashi K, Yokokura S, Nishida K. 2007. Novel mutations in the FOXC1 gene in Japanese patients with Axenfeld-Rieger syndrome. Molecular vision 13:1005–9.

Gage PJ, Camper SA. 1997. Pituitary homeobox 2, a novel member of the bicoid-related family of homeobox genes, is a potential regulator of anterior structure formation. Human Molecular Genetics 6:457–464.

Gage PJ, Suh H, Camper SA. 1999. Dosage requirement of Pitx2 for development of multiple organs. Development (Cambridge, England) 126:4643–51.

Galliot B, Vargas C de, Miller D. 1999. Evolution of homeobox genes: Q50 Paired-like genes founded the Paired class. Development genes and evolution 209:186–97.

Ganga M, Espinoza HM, Cox CJ, Morton L, Hjalt TA, Lee Y, Amendt BA. 2003a. PITX2 isoform-specific regulation of atrial natriuretic factor expression: synergism and repression with Nkx2.5. The Journal of biological chemistry 278:22437–45.

Gehring WJ, Affolter M, Burglin T. 1994. Homeodomain Proteins. Annual Review of Biochemistry 63:487–526.

Geourjon C, Combet C, Blanchet C, Deléage G. 2001. Identification of related proteins with weak sequence identity using secondary structure information. Protein science : a publication of the Protein Society 10:788–97.

Gerlofs-Nijland ME, Assmann KJ, Dijkman HB, Dieker JW, Son JP van, Mentzel S, Kats JP van, Danser AH, Smithies O, Groenen PJ, Wetzels JF. 2001. Albuminuria in mice after injection of antibodies against aminopeptidase A: role of angiotensin II. Journal of the American Society of Nephrology : JASN 12:2711–20.

Gerlofs-Nijland ME, Assmann KJM, Son JPHF van, Dijkman HBPM, Loeke NAJM te, Zee R van der, Wetzels JFM, Groenen PJTA. 2003. Epitope mapping of monoclonal antibodies directed to aminopeptidase A and their relevance for albuminuria in mice. Nephron Experimental nephrology 94:e25-34.

Giorgio E, Robyr D, Spielmann M, Ferrero E, Gregorio E Di, Imperiale D, Vaula G, Stamoulis G, Santoni F, Atzori C, Gasparini L, Ferrera D, et al. 2015. A large genomic deletion leads to enhancer adoption by the lamin B1 gene: a second path to autosomal dominant adult-onset demyelinating leukodystrophy (ADLD). Human molecular genetics 24:3143–54.

Glaser T, Jepeal L, Edwards JG, Young SR, Favor J, Maas RL. 1994. PAX6 gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects. Nature genetics 7:463–71.

Goren A, Ram O, Amit M, Keren H, Lev-Maor G, Vig I, Pupko T, Ast G. 2006. Comparative Analysis Identifies Exonic Splicing Regulatory Sequences—The Complex Definition of Enhancers and Silencers. Molecular Cell 22:769–781.

Gotea V, Visel A, Westlund JM, Nobrega MA, Pennacchio LA, Ovcharenko I. 2010. Homotypic clusters of transcription factor binding sites are a key component of human promoters and enhancers. Genome research 20:565–77.

Greenberg DS, Sunada Y, Campbell KP, Yaffe D, Nudel U. 1994. © 199 4 Nature Publishing Group http://www.nature.com/naturegenetics. Nature 8:340–344.

Gripp KW, Hopkins E, Jenny K, Thacker D, Salvin J. 2013. Cardiac anomalies in Axenfeld-Rieger syndrome due to a novel FOXC1 mutation. American Journal of Medical Genetics Part A 161:114–119.

Gromiha MM. 2007. Prediction of protein stability upon point mutations. Biochemical Society Transactions 35:1569–1573.

Guex N, Peitsch MC. 1997. SWISS-MODEL and the Swiss-Pdb Viewer: An environment for comparative protein modeling. Electrophoresis 18:2714–2723.

Gunderson KL, Steemers FJ, Ren H, Ng P, Zhou L, Tsan C, Chang W, Bullis D, Musmacker J, King C, Lebruska LL, Barker D, et al. 2006. Whole-Genome Genotyping. Methods in Enzymology, p 359–376.

Han B, Bhowmick N, Qu Y, Chung S, Giuliano AE, Cui X. 2017. FOXC1: an emerging marker and therapeutic target for cancer. Oncogene 1–7.

Hannenhalli S, Kaestner KH. 2009. The evolution of Fox genes and their role in development and disease. Nature reviews Genetics 10:233–40.

Harissi-Dagher M, Colby K. 2008. Anterior Segment Dysgenesis: Peters Anomaly and Sclerocornea. International Ophthalmology Clinics 48:35–42.

He Y, Fang J, Taatjes DJ, Nogales E. 2013. Structural visualization of key steps in human transcription initiation. Nature 495:481–6.

Hedges SB, Kumar S. 1998. A molecular timescale for vertebrate evolution. Nature 392:917–920.

Heyningen V van, Williamson KA. 2002. PAX6 in sensory development. Human molecular genetics 11:1161–7.

Hjalt TA, Semina E V, Amendt BA, Murray JC. 2000. The Pitx2 protein in mouse development. Developmental dynamics : an official publication of the American Association of Anatomists 218:195–200.

Hjalt TA, Semina E V. 2005a. Current molecular understanding of Axenfeld-Rieger syndrome. Expert reviews in molecular medicine 7:1–17.

Honkanen RA, Nishimura DY, Swiderski RE, Bennett SR, Hong S, Kwon YH, Stone EM, Sheffield VC, Alward WLM. 2003. A family with Axenfeld-Rieger syndrome and Peters Anomaly caused by a point mutation (Phe112Ser) in the FOXC1 gene. American journal of ophthalmology 135:368–75.

Huang L-T, Gromiha MM, Ho S-Y. 2007a. iPTREE-STAB: interpretable decision tree based method for predicting protein stability changes upon mutations. Bioinformatics (Oxford, England) 23:1292–3.

Huang X, Xiao X, Jia X, Li S, Li M, Guo X, Liu X, Zhang Q. 2015. Mutation analysis of the genes associated with anterior segment dysgenesis, microcornea and microphthalmia in 257 patients with glaucoma. International journal of molecular medicine 36:1111–7.

Ibn-Salem J, Köhler S, Love MI, Chung H-R, Huang N, Hurles ME, Haendel M, Washington NL, Smedley D, Mungall CJ, Lewis SE, Ott C-E, et al. 2014. Deletions of chromosomal regulatory boundaries are associated with congenital disease. Genome Biology 15:423.

Idrees F, Bloch-Zupan A, Free SL, Vaideanu D, Thompson PJ, Ashley P, Brice G, Rutland P, Bitner-Glindzicz M, Khaw PT, Fraser S, Sisodiya SM, et al. 2006. A novel homeobox mutation in the PITX2 gene in a family with Axenfeld-Rieger syndrome associated with brain, ocular, and dental phenotypes. American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics 141B:184–91.

Idrees F, Vaideanu D, Fraser SG, Sowden JC, Khaw PT. 2006b. A review of anterior segment dysgeneses. Survey of ophthalmology 51:213–31.

Ihnatko R, Eden U, Fagerholm P, Lagali N. 2016. Congenital Aniridia and the Ocular Surface. The Ocular Surface 14:196–206.

Ito YA, Footz TK, Berry FB, Mirzayans F, Yu M, Khan AO, Walter MA. 2009. Severe molecular defects of a novel FOXC1 W152G mutation result in aniridia. Investigative ophthalmology & visual science 50:3573–9.

Ito YA, Footz TK, Murphy TC, Courtens W, Walter MA. 2007. Analyses of a novel L130F missense mutation in FOXC1. Archives of ophthalmology (Chicago, Ill : 1960) 125:128–35.

Jain A, Wordinger RJ, Yorio T, Clark AF. 2012. Spliceosome protein (SRp) regulation of glucocorticoid receptor isoforms and glucocorticoid response in human trabecular meshwork cells. Investigative ophthalmology & visual science 53:857–66.

Jena AK, Kharbanda OP. 2005. Axenfeld-Rieger syndrome: report on dental and craniofacial findings. The Journal of clinical pediatric dentistry 30:83–8.

Jensen TW, Ray T, Wang J, Li X, Naritoku WY, Han B, Bellafiore F, Bagaria SP, Qu A, Cui X, Taylor CR, Ray PS. 2015. Diagnosis of Basal-Like Breast Cancer Using a FOXC1-Based Assay. Journal of the National Cancer Institute 107:djv148-djv148.

Jia L, Yarlagadda R, Reed CC, Haeberlein M, Chen H, Ma S. 2015. Structure Based Thermostability Prediction Models for Protein Single Point Mutations with Machine Learning Tools. PLOS ONE 10:e0138022.

Judkins T, Hendrickson BC, Deffenbaugh AM, Scholl T. 2005. Single nucleotide polymorphisms in clinical genetic testing: the characterization of the clinical significance of genetic variants and their application in clinical research for BRCA1. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 573:168–179.

Kadonaga JT. 2004. Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. Cell 116:247–57.

Kamanu FK, Medvedeva YA, Schaefer U, Jankovic BR, Archer JAC, Bajic VB. 2012. Mutations and Binding Sites of Human Transcription Factors. Frontiers in Genetics 3:100.

Kamińska A, Sokołowska-Oracz A, Pawluczyk-Dyjecińska M, Szaflik JP. 2007. [Variability of clinical manifestations in the family with Axenfeld-Rieger syndrome]. Klinika oczna 109:321–6.

Kammer JA, Mundy KM. 2015. Suprachoroidal devices in glaucoma surgery. Middle East African journal of ophthalmology 22:45–52.

Kamnasaran D, O'Brien PC, Zackai EH, Muenke M, Ferguson-Smith MA, Cox DW. 2003. Rearrangement in the PITX2 and MIPOL1 genes in a patient with a t(4;14) chromosome. European journal of human genetics : EJHG 11:315–24.

Kanski JJ. 1968. Carbonic anhydrase inhibitors and osmotic agents in glaucoma. Carbonic anhydrase inhibitors. The British journal of ophthalmology 52:642–3.

Kaufmann E, Knöchel W. 1996. Five years on the wings of fork head. Mechanisms of development 57:3–20.

Kaur K, Ragge NK, Ragoussis J. 2009. Molecular analysis of FOXC1 in subjects presenting with severe developmental eye anomalies. Molecular vision 15:1366–73.

Kawase C, Kawase K, Taniguchi T, Sugiyama K, Yamamoto T, Kitazawa Y, Alward WL, Stone EM, Nishimura DY, Sheffield VC. 2001. Screening for mutations of Axenfeld-Rieger syndrome caused by FOXC1 gene in Japanese patients. Journal of glaucoma 10:477–82.

Kawase K, Vittitow JL, Weinreb RN, Araie M, JUPITER Study Group F the JS. 2016. Long-term Safety and Efficacy of Latanoprostene Bunod 0.024% in Japanese Subjects with Open-Angle Glaucoma or Ocular Hypertension: The JUPITER Study. Advances in therapy 33:1612–27.

Kenyon KR. 1975. Mesenchymal dysgenesis in Peter's anomaly, sclerocornea and congenital endothelial dystrophy. Experimental eye research 21:125–42.

Khan AO, Aldahmesh MA, Al-Amri A. 2008. Heterozygous FOXC1 Mutation (M161K) Associated with Congenital Glaucoma and Aniridia in an Infant and a Milder Phenotype in Her Mother. Ophthalmic Genetics 29:67–71.

Khan S, Vihinen M. 2010. Performance of protein stability predictors. Human Mutation 31:675–684.

Kidson SH, Kume T, Deng K, Winfrey V, Hogan BL. 1999. The forkhead/winged-helix gene, Mf1, is necessary for the normal development of the cornea and formation of the anterior chamber in the mouse eye. Developmental biology 211:306–22.

Kimura M, Tokita Y, Machida J, Shibata A, Tatematsu T, Tsurusaki Y, Miyake N, Saitsu H, Miyachi H, Shimozato K, Matsumoto N, Nakashima M. 2014. A novel PITX2 mutation causing iris hypoplasia. Human genome variation 1:14005.

Kitamura K, Miura H, Miyagawa-Tomita S, Yanazawa M, Katoh-Fukui Y, Suzuki R, Ohuchi H, Suehiro A, Motegi Y, Nakahara Y, Kondo S, Yokoyama M. 1999. Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. Development (Cambridge, England) 126:5749–58.

Klein OD, Oberoi S, Huysseune A, Hovorakova M, Peterka M, Peterkova R. 2013. Developmental disorders of the dentition: an update. American journal of medical genetics Part C, Seminars in medical genetics 163C:318–32.

Kleinjan D a, Heyningen V van. 2005. Long-range control of gene expression: emerging mechanisms and disruption in disease. American journal of human genetics 76:8–32.

Kleinjan DA, Heyningen V van. 2005. Long-range control of gene expression: emerging mechanisms and disruption in disease. American journal of human genetics 76:8–32.

Kleinjan DA, Seawright A, Mella S, Carr CB, Tyas DA, Simpson TI, Mason JO, Price DJ, Heyningen V van. 2006. Long-range downstream enhancers are essential for Pax6 expression. Developmental biology 299:563–81.

Kleinjan DA, Seawright A, Schedl A, Quinlan RA, Danes S, Heyningen V van. 2001. Aniridia-associated translocations, DNase hypersensitivity, sequence comparison and transgenic analysis redefine the functional domain of PAX6. Human molecular genetics 10:2049–59.

Kmita M, Fraudeau N, Hérault Y, Duboule D. 2002. Serial deletions and duplications suggest a mechanism for the collinearity of Hoxd genes in limbs. Nature 420:145–50.

Kniestedt C, Taralczak M, Thiel MA, Stuermer J, Baumer A, Gloor BP. 2006. A novel PITX2 mutation and a polymorphism in a 5-generation family with Axenfeld-Rieger anomaly and coexisting Fuchs' endothelial dystrophy. Ophthalmology 113:1791.e1-8.

Kornberg TB. 1993. Understanding the homeodomain. The Journal of biological chemistry 268:26813–6.

Kozlowski K, Walter MA. 2000. Variation in residual PITX2 activity underlies the phenotypic spectrum of anterior segment developmental disorders. Human molecular genetics 9:2131–9.

Krogh A, Larsson B, Heijne G von, Sonnhammer EL. 2001. Predicting transmembrane protein topology with a hidden markov model: application to complete genomes11Edited by F. Cohen. Journal of Molecular Biology 305:567–580.

Kulak SC, Kozlowski K, Semina E V, Pearce WG, Walter MA. 1998. Mutation in the RIEG1 gene in patients with iridogoniodysgenesis syndrome. Human molecular genetics 7:1113–7.

Kulharya AS, Maberry M, Kukolich MK, Day DW, Schneider NR, Wilson GN, Tonk V. 1995. Interstitial deletions 4q21.1q25 and 4q25q27: phenotypic variability and relation to Rieger anomaly. American journal of medical genetics 55:165–70.

Kumar P, Henikoff S, Ng PC. 2009. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nature Protocols 4:1073–1081.

Kumar V, Kumar K, Chanana B, Rohatgi J, Gupta VP. 2011. Congenital ectropion uveae with iris coloboma and telecanthus. Contact Lens and Anterior Eye 34:147–148.

Kumar V, Robinson R, Ainsworth JR. 2000. Idiopathic tractional corectopia. The British journal of ophthalmology 84:1206.

la Houssaye G de, Bieche I, Roche O, Vieira V, Laurendeau I, Arbogast L, Zeghidi H, Rapp P, Halimi P, Vidaud M, Dufier J-L, Menasche M, et al. 2006. Identification of the first intragenic deletion of the PITX2 gene causing an Axenfeld-Rieger Syndrome: case report. BMC medical genetics 7:82.

Laaks D, Freeman N. 2013. Congenital iris ectropion uveae presenting with glaucoma in infancy. Journal of American Association for Pediatric Ophthalmology and Strabismus 17:214–216.

Landrum MJ, Lee JM, Riley GR, Jang W, Rubinstein WS, Church DM, Maglott DR. 2014. ClinVar: public archive of relationships among sequence variation and human phenotype. Nucleic Acids Research 42:D980–D985.

Laskowski RA, Tyagi N, Johnson D, Joss S, Kinning E, McWilliam C, Splitt M, Thornton JM, Firth H V, DDD Study the D, Wright CF. 2016. Integrating population variation and protein structural analysis to improve clinical interpretation of missense variation: application to the WD40 domain. Human molecular genetics 25:927–35.

Lee D-H, Gershenzon N, Gupta M, Ioshikhes IP, Reinberg D, Lewis BA. 2005. Functional characterization of core promoter elements: the downstream core element is recognized by TAF1. Molecular and cellular biology 25:9674–86.

Lehmann OJ, Ebenezer ND, Ekong R, Ocaka L, Mungall AJ, Fraser S, McGill JI, Hitchings RA, Khaw PT, Sowden JC, Povey S, Walter MA, et al. 2002. Ocular developmental abnormalities and glaucoma associated with interstitial 6p25 duplications and deletions. Investigative ophthalmology & visual science 43:1843–9.

Lenhard B, Sandelin A, Mendoza L, Engström P, Jareborg N, Wasserman WW. 2003. Identification of conserved regulatory elements by comparative genome analysis. Journal of Biology 2:13.

Lettice LA, Daniels S, Sweeney E, Venkataraman S, Devenney PS, Gautier P, Morrison H, Fantes J, Hill RE, FitzPatrick DR. 2011. Enhancer-adoption as a mechanism of human developmental disease. Human mutation 32:1492–9.

Lettice LA, Heaney SJH, Purdie LA, Li L, Beer P de, Oostra BA, Goode D, Elgar G, Hill RE, Graaff E de. 2003. A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. Human molecular genetics 12:1725–35.

Li B, Krishnan VG, Mort ME, Xin F, Kamati KK, Cooper DN, Mooney SD, Radivojac P. 2009. Automated inference of molecular mechanisms of disease from amino acid substitutions. Bioinformatics (Oxford, England) 25:2744–50.

Li C-M, Tzeng J-N, Sung H-M. 2012. Effects of cis and trans regulatory variations on the expression divergence of heat shock response genes between yeast strains. Gene 506:93–7.

Li D, Zhu Q, Lin H, Zhou N, Qi Y. 2008. A novel PITX2 mutation in a Chinese family with Axenfeld-Rieger syndrome. Molecular vision 14:2205–10.

Lin Q, Chen Q, Lin L, Smith S, Zhou J. 2007. Promoter targeting sequence mediates enhancer interference in the Drosophila embryo. Proceedings of the National Academy of Sciences of the United States of America 104:3237–42.

Lindberg MJ, Byström R, Boknäs N, Andersen PM, Oliveberg M. 2005. Systematically perturbed folding patterns of amyotrophic lateral sclerosis (ALS)-associated SOD1 mutants. Proceedings of the National Academy of Sciences of the United States of America 102:9754–9.

Lines MA, Kozlowski K, Kulak SC, Allingham RR, Héon E, Ritch R, Levin A V, Shields MB, Damji KF, Newlin A, Walter MA. 2004. Characterization and prevalence of PITX2 microdeletions and mutations in Axenfeld-Rieger malformations. Investigative ophthalmology & visual science 45:828–33.

Lines MA, Kozlowski K, Walter MA. 2002. Molecular genetics of Axenfeld-Rieger malformations. Human molecular genetics 11:1177–84.

Liu C, Liu W, Lu MF, Brown NA, Martin JF. 2001. Regulation of left-right asymmetry by thresholds of Pitx2c activity. Development (Cambridge, England) 128:2039–48.

Liu D, Cruz FM, Subramanian PS. 2012. Bilateral vision loss associated with radiofrequency exposure. Clinical ophthalmology (Auckland, NZ) 6:2069–73.

Liu Y, Semina E V. 2012. pitx2 Deficiency Results in Abnormal Ocular and Craniofacial Development in Zebrafish. PLoS ONE 7:e30896.

Loots GG, Locksley RM, Blankespoor CM, Wang ZE, Miller W, Rubin EM, Frazer KA. 2000. Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. Science (New York, NY) 288:136–40.

Lozier J. 2004. Gene therapy of the hemophilias. Seminars in hematology 41:287–96.

Ludwig MZ, Bergman C, Patel NH, Kreitman M. 2000. Evidence for stabilizing selection in a eukaryotic enhancer element. Nature 403:564–7.

Maciolek NL, Alward WLM, Murray JC, Semina E V, McNally MT. 2006. Analysis of RNA splicing defects in PITX2 mutants supports a gene dosage model of Axenfeld-Rieger syndrome. BMC medical genetics 7:59.

Mahdy MA-MS. 2008. Efficacy and safety of selective laser trabeculoplasty as a primary procedure for controlling intraocular pressure in primary open angle glaucoma and ocular hypertensive patients. Sultan Qaboos University medical journal 8:53–8.

Majhi RK, Saha S, Kumar A, Ghosh A, Swain N, Goswami L, Mohapatra P, Maity A, Kumar Sahoo V, Kumar A, Goswami C. 2015. Expression of temperature-sensitive ion channel TRPM8 in sperm cells correlates with vertebrate evolution. PeerJ 3:e1310.

Manco L, Ribeiro ML, Máximo V, Almeida H, Costa A, Freitas O, Barbot J, Abade A, Tamagnini G. 2000. A new PKLR gene mutation in the R-type promoter region affects the gene transcription causing pyruvate kinase deficiency. British journal of haematology 110:993–7.

Margulies EH, Cooper GM, Asimenos G, Thomas DJ, Dewey CN, Siepel A, Birney E, Keefe D, Schwartz AS, Hou M, Taylor J, Nikolaev S, et al. 2007. Analyses of deep mammalian sequence alignments and constraint predictions for 1% of the human genome. Genome research 17:760–74.

Martin DM, Skidmore JM, Philips ST, Vieira C, Gage PJ, Condie BG, Raphael Y, Martinez S, Camper SA. 2004. PITX2 is required for normal development of neurons in the mouse subthalamic nucleus and midbrain. Developmental biology 267:93–108.

Masica DL, Karchin R. 2016. Towards Increasing the Clinical Relevance of In Silico Methods to Predict Pathogenic Missense Variants. PLoS computational biology 12:e1004725.

Maslon MM, Heras SR, Bellora N, Eyras E, Cáceres JF. 2014. The translational landscape of the splicing factor SRSF1 and its role in mitosis. eLife 3:e02028.

Masotti C, Armelin-Correa LM, Splendore A, Lin CJ, Barbosa A, Sogayar MC, Passos-Bueno MR. 2005. A functional SNP in the promoter region of TCOF1 is associated with reduced gene expression and YY1 DNA-protein interaction. Gene 359:44–52.

Maston GA, Evans SK, Green MR. 2006. Transcriptional Regulatory Elements in the Human Genome. Annual Review of Genomics and Human Genetics 7:29–59.

MATHAVAN KJ. 2008. Identification and characterization of conserved regulatory elements by comparative genomics.

Mathelier A, Lefebvre C, Zhang AW, Arenillas DJ, Ding J, Wasserman WW, Shah SP. 2015. Cis-regulatory somatic mutations and gene-expression alteration in B-cell lymphomas. Genome biology 16:84.

Mayer S, Rüdiger S, Ang HC, Joerger AC, Fersht AR. 2007. Correlation of levels of folded recombinant p53 in escherichia coli with thermodynamic stability in vitro. Journal of molecular biology 372:268–76.

McGaughey DM, Vinton RM, Huynh J, Al-Saif A, Beer MA, McCallion AS. 2008. Metrics of sequence constraint overlook regulatory sequences in an exhaustive analysis at phox2b. Genome research 18:252–60.

McKinnon SJ, Goldberg LD, Peeples P, Walt JG, Bramley TJ. 2008. Current management of glaucoma and the need for complete therapy. The American journal of managed care 14:S20-7.

Mears AJ, Jordan T, Mirzayans F, Dubois S, Kume T, Parlee M, Ritch R, Koop B, Kuo W-L, Collins C, Marshall J, Gould DB, et al. 1998. Mutations of the Forkhead/Winged-Helix Gene, FKHL7, in Patients with Axenfeld-Rieger Anomaly. The American Journal of Human Genetics 63:1316–1328.

Medina-Trillo C, Sánchez-Sánchez F, Aroca-Aguilar J-D, Ferre-Fernández J-J, Morales L, Méndez-Hernández C-D, Blanco-Kelly F, Ayuso C, García-Feijoo J, Escribano J. 2015. Hypo- and hypermorphic FOXC1 mutations in dominant glaucoma: transactivation and phenotypic variability. PloS one 10:e0119272.

Mentzel S, Assmann KJ, Dijkman HB, Jong AS de, Son JP van, Wetzels JF, Koene RA. 1996. Inhibition of aminopeptidase A activity causes an acute albuminuria in mice: an angiotensin II-mediated effect? Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association 11:2163–9.

Mentzel S, Son JP van, Dijkman HB, Wetzels JF, Assmann KJ. 1999. Induction of albuminuria in mice: synergistic effect of two monoclonal antibodies directed to different domains of aminopeptidase A. Kidney international 55:1335–47.

Metzker ML. 2010. Sequencing technologies — the next generation. Nature Reviews Genetics 11:31–46.

Meyer JJ, Lawrence SD. 2012. What's new in laser treatment for glaucoma? Current Opinion in Ophthalmology 23:111–117.

Meyer-Marcotty P, Weisschuh N, Dressler P, Hartmann J, Stellzig-Eisenhauer A. 2008. Morphology of the sella turcica in Axenfeld-Rieger syndrome with PITX2 mutation. Journal of oral pathology

& medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology 37:504–10.

Mi H, Thomas P. 2009. PANTHER Pathway: An Ontology-Based Pathway Database Coupled with Data Analysis Tools. p 123–140.

Mirzayans F, Gould DB, Héon E, Billingsley GD, Cheung JC, Mears AJ, Walter MA. 2000. Axenfeld-Rieger syndrome resulting from mutation of the FKHL7 gene on chromosome 6p25. European journal of human genetics : EJHG 8:71–4.

Mitchell JA, Packman S, Loughman WD, Fineman RM, Zackai E, Patil SR, Emanual B, Bartley JA, Hanson JW, Opitz JM. 1981. Deletions of different segments of the long arm of chromosome 4. American journal of medical genetics 8:73–89.

Mommersteeg MTM, Brown NA, Prall OWJ, Gier-De Vries C De, Harvey RP, Moorman AFM, Christoffels VM. 2007. Pitx2c and Nkx2-5 are required for the formation and identity of the pulmonary myocardium. Circulation Research 101:902–909.

Moreira L, Schinzel A, Baumer A, Pinto P, Góes F, Falcão M de L, Barbosa AL, Riegel M. 2010. Longitudinal observation of a patient with Rieger syndrome and interstitial deletion 4 (q25-q31.1). American journal of medical genetics Part A 152A:977–81.

Morley M, Molony CM, Weber TM, Devlin JL, Ewens KG, Spielman RS, Cheung VG. 2004. Genetic analysis of genome-wide variation in human gene expression. Nature 430:743–747.

Murphy TC, Saleem RA, Footz T, Ritch R, McGillivray B, Walter MA. 2004. The wing 2 region of the FOXC1 forkhead domain is necessary for normal DNA-binding and transactivation functions. Investigative ophthalmology & visual science 45:2531–8.

Myatt SS, Lam EW-F. 2007. The emerging roles of forkhead box (Fox) proteins in cancer. Nature Reviews Cancer 7:847–859.

Narlikar L, Ovcharenko I. 2009. Identifying regulatory elements in eukaryotic genomes. Briefings in functional genomics & proteomics 8:215–30.

Ng PC, Henikoff S. 2001a. Predicting Deleterious Amino Acid Substitutions. Genome Research 11:863–874.

Ng PC, Henikoff S. 2003. SIFT: Predicting amino acid changes that affect protein function. Nucleic acids research 31:3812–4.

Ng PC, Henikoff S. 2006. Predicting the effects of amino acid substitutions on protein function. Annual review of genomics and human genetics 7:61–80.

Niroula A, Vihinen M. 2016. Variation Interpretation Predictors: Principles, Types, Performance, and Choice. Human mutation 37:579–97.

Nishimura DY, Searby CC, Alward WL, Walton D, Craig JE, Mackey DA, Kawase K, Kanis AB, Patil SR, Stone EM, Sheffield VC. 2001. A Spectrum of FOXC1 Mutations Suggests Gene

Dosage as a Mechanism for Developmental Defects of the Anterior Chamber of the Eye. The American Journal of Human Genetics 68:364–372.

Nishimura DY, Swiderski RE, Alward WL, Searby CC, Patil SR, Bennet SR, Kanis AB, Gastier JM, Stone EM, Sheffield VC. 1998. The forkhead transcription factor gene FKHL7 is responsible for glaucoma phenotypes which map to 6p25. Nature genetics 19:140–7.

Niu M, Tabari ES, Su Z. 2014. De novo prediction of cis-regulatory elements and modules through integrative analysis of a large number of ChIP datasets. BMC Genomics 15:1047.

Nobrega MA, Ovcharenko I, Afzal V, Rubin EM. 2003. Scanning Human Gene Deserts for Long-Range Enhancers. Science 302:413–413.

Nokes EB, Linden AM Van Der, Winslow C, Mukhopadhyay S, Ma K, Sengupta P. 2009. Cis-regulatory mechanisms of gene expression in an olfactory neuron type in Caenorhabditis elegans. Developmental dynamics : an official publication of the American Association of Anatomists 238:3080–92.

O'Dwyer EM, Jones DC. 2005. Dental anomalies in Axenfeld-Rieger syndrome. International Journal of Paediatric Dentistry 15:459–463.

Ogilvie CM, Raymond FL, Harrison RH, Scriven PN, Docherty Z. 1998. A new approach to the elucidation of complex chromosome rearrangements illustrated by a case of Rieger syndrome. Journal of medical genetics 35:234–7.

Omulecki W, Wilczynski M, Gerkowicz M. Management of bilateral ectopia lentis et pupillae syndrome. Ophthalmic surgery, lasers & imaging : the official journal of the International Society for Imaging in the Eye 37:68–71.

Ovcharenko I, Nobrega MA, Loots GG, Stubbs L. ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes.

Pace CN, Fu H, Lee Fryar K, Landua J, Trevino SR, Schell D, Thurlkill RL, Imura S, Scholtz JM, Gajiwala K, Sevcik J, Urbanikova L, et al. 2014. Contribution of hydrogen bonds to protein stability. Protein science : a publication of the Protein Society 23:652–61.

Parikh RS, Parikh SR, Navin S, Arun E, Thomas R. 2008. Practical approach to medical management of glaucoma. Indian journal of ophthalmology 56:223–30.

Parnell TJ, Geyer PK. 2000. Differences in insulator properties revealed by enhancer blocking assays on episomes. The EMBO journal 19:5864–74.

Pauw B, Hilliou FAO, Martin VS, Chatel G, Wolf CJF de, Champion A, Pré M, Duijn B van, Kijne JW, Fits L van der, Memelink J. 2004. Zinc finger proteins act as transcriptional repressors of alkaloid biosynthesis genes in Catharanthus roseus. The Journal of biological chemistry 279:52940–8.

Paznekas WA, Karczeski B, Vermeer S, Lowry RB, Delatycki M, Laurence F, Koivisto PA, Maldergem L Van, Boyadjiev SA, Bodurtha JN, Wang Jabs E. 2009. GJA1 mutations, variants,

and connexin 43 dysfunction as it relates to the oculodentodigital dysplasia phenotype. Human Mutation 30:724–733.

Pennacchio LA, Ahituv N, Moses AM, Prabhakar S, Nobrega MA, Shoukry M, Minovitsky S, Dubchak I, Holt A, Lewis KD, Plajzer-Frick I, Akiyama J, et al. 2006. In vivo enhancer analysis of human conserved non-coding sequences. Nature 444:499–502.

Perveen R, Lloyd IC, Clayton-Smith J, Churchill A, Heyningen V van, Hanson I, Taylor D, McKeown C, Super M, Kerr B, Winter R, Black GC. 2000. Phenotypic variability and asymmetry of Rieger syndrome associated with PITX2 mutations. Investigative ophthalmology & visual science 41:2456–60.

Phillips JC, Bono EA del, Haines JL, Pralea AM, Cohen JS, Greff LJ, Wiggs JL. 1996. A second locus for Rieger syndrome maps to chromosome 13q14. American journal of human genetics 59:613–9.

Phillips JC. Four novel mutations in the PITX2 gene in patients with Axenfeld-Rieger syndrome. Ophthalmic research 34:324–6.

Pires DE V, Ascher DB, Blundell TL. 2014b. DUET: a server for predicting effects of mutations on protein stability using an integrated computational approach. Nucleic acids research 42:W314-9.

Pires DE V., Ascher DB, Blundell TL. 2014a. mCSM: predicting the effects of mutations in proteins using graph-based signatures. Bioinformatics 30:335–342.

Prabhakar S, Poulin F, Shoukry M, Afzal V, Rubin EM, Couronne O, Pennacchio LA. 2006. Close sequence comparisons are sufficient to identify human cis-regulatory elements. Genome research 16:855–63.

Priston M, Kozlowski K, Gill D, Letwin K, Buys Y, Levin A V, Walter MA, Héon E. 2001. Functional analyses of two newly identified PITX2 mutants reveal a novel molecular mechanism for Axenfeld-Rieger syndrome. Human molecular genetics 10:1631–8.

Qian YQ, Billeter M, Otting G, Müller M, Gehring WJ, Wüthrich K. 1989. The structure of the Antennapedia homeodomain determined by NMR spectroscopy in solution: comparison with prokaryotic repressors. Cell 59:573–80.

Raczenbek C, Krassikoff N, Cosper P. 1991. Second case report of del(4) (q25q27) and review of the literature. Clinical genetics 39:463–6.

Ramensky V, Bork P, Sunyaev S. 2002a. Human non-synonymous SNPs: server and survey. Nucleic acids research 30:3894–900.

Randles LG, Lappalainen I, Fowler SB, Moore B, Hamill SJ, Clarke J. 2006. Using model proteins to quantify the effects of pathogenic mutations in Ig-like proteins. The Journal of biological chemistry 281:24216–26.

Reinke V, Krause M, Okkema P. 2013. Transcriptional regulation of gene expression in C. elegans. WormBook : the online review of C elegans biology 1–34.

Reis LM, Tyler RC, Volkmann Kloss BA, Schilter KF, Levin A V, Lowry RB, Zwijnenburg PJG, Stroh E, Broeckel U, Murray JC, Semina E V. 2012a. PITX2 and FOXC1 spectrum of mutations in ocular syndromes. European journal of human genetics : EJHG 20:1224–33.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL, et al. 2015. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genetics in Medicine 17:405–423.

Richter GM, Coleman AL. 2016. Minimally invasive glaucoma surgery: current status and future prospects. Clinical ophthalmology (Auckland, NZ) 10:189–206.

Riise R, Storhaug K, Brondum-Nielsen K. 2001. Rieger syndrome is associated with PAX6 deletion. Acta Ophthalmol Scand 79:201–3.

Rippe K. 2001. Making contacts on a nucleic acid polymer. Trends in biochemical sciences 26:733–40.

Rishi P, Koundanya VV SC. 2015. Using risk factors for detection and prognostication of uveal melanoma. Indian Journal of Ophthalmology 63:110–6.

Rodríguez-Trelles F, Tarrío R, Ayala FJ. 2003. Evolution of cis-regulatory regions versus codifying regions. The International journal of developmental biology 47:665–73.

Roy AL, Singer DS. 2015. Core promoters in transcription: old problem, new insights. Trends in biochemical sciences 40:165–71.

Saadi I, Kuburas A, Engle JJ, Russo AF. 2003. Dominant negative dimerization of a mutant homeodomain protein in Axenfeld-Rieger syndrome. Molecular and cellular biology 23:1968–82.

Saadi I, Semina E V, Amendt BA, Harris DJ, Murphy KP, Murray JC, Russo AF. 2001. Identification of a dominant negative homeodomain mutation in Rieger syndrome. The Journal of biological chemistry 276:23034–41.

Saadi I, Toro R, Kuburas A, Semina E, Murray JC, Russo AF. 2006. An unusual class of PITX2 mutations in Axenfeld-Rieger syndrome. Birth defects research Part A, Clinical and molecular teratology 76:175–81.

Sacchetti M, Mantelli F, Marenco M, Macchi I, Ambrosio O, Rama P. 2015. Diagnosis and Management of Iridocorneal Endothelial Syndrome. BioMed Research International 2015:1–9.

Saleem RA, Banerjee-Basu S, Berry FB, Baxevanis AD, Walter MA. 2001. Analyses of the effects that disease-causing missense mutations have on the structure and function of the winged-helix protein FOXC1. American journal of human genetics 68:627–41.

Saleem RA, Banerjee-Basu S, Berry FB, Baxevanis AD, Walter MA. 2003a. Structural and functional analyses of disease-causing missense mutations in the forkhead domain of FOXC1. Human Molecular Genetics 12:2993–3005.

Saleem RA, Banerjee-Basu S, Murphy TC, Baxevanis A, Walter MA. 2004. Essential structural and functional determinants within the forkhead domain of FOXC1. Nucleic acids research 32:4182–93.

Saleem RA, Murphy TC, Liebmann JM, Walter MA. 2003b. Identification and analysis of a novel mutation in the FOXC1 forkhead domain. Investigative ophthalmology & visual science 44:4608–12.

Saleh M, Kamath BM, Chitayat D. 2016. Alagille syndrome: clinical perspectives. The application of clinical genetics 9:75–82.

Sambhara D, Aref AA. 2014. Glaucoma management: relative value and place in therapy of available drug treatments. Therapeutic advances in chronic disease 5:30–43.

Schachtschabel DO, Binninger EA, Rohen JW. In vitro cultures of trabecular meshwork cells of the human eye as a model system for the study of cellular aging. Archives of gerontology and geriatrics 9:251–62.

Schinzel A, Brecevic L, Dutly F, Baumer A, Binkert F, Largo RH. 1997. Multiple congenital anomalies including the Rieger eye malformation in a boy with interstitial deletion of (4) (q25-->q27) secondary to a balanced insertion in his normal father: evidence for haplotype insufficiency causing the Rieger malformation. Journal of medical genetics 34:1012–4.

Schwarz JM, Cooper DN, Schuelke M, Seelow D. 2014a. MutationTaster2: mutation prediction for the deep-sequencing age. Nature methods 11:361–2.

Schweickert A, Campione1 M, Steinbeisser H, Blum M. 2000. Pitx2 isoforms: involvement of Pitx2c but not Pitx2a or Pitx2b in vertebrate left-right asymmetry. Mechanisms of development 90:41–51.

Seifi M, Footz T, Taylor SAM, Elhady GM, Abdalla EM, Walter MA. 2016a. Novel PITX2 gene mutations in patients with Axenfeld-Rieger syndrome. Acta Ophthalmologica 94:e571–e579.

Seifi M, Footz T, Taylor SAM, Walter MA. 2017. Comparison of Bioinformatics Prediction, Molecular Modeling, and Functional Analyses of FOXC1 Mutations in Patients with Axenfeld-Rieger Syndrome. Human Mutation 38:169–179.

Semina E V, Datson NA, Leysens NJ, Zabel BU, Carey JC, Bell GI, Bitoun P, Lindgren C, Stevenson T, Frants RR, Ommen G van, Murray JC. 1996b. Exclusion of epidermal growth factor and high-resolution physical mapping across the Rieger syndrome locus. American journal of human genetics 59:1288–96.

Semina E V, Reiter R, Leysens NJ, Alward WL, Small KW, Datson NA, Siegel-Bartelt J, Bierke-Nelson D, Bitoun P, Zabel BU, Carey JC, Murray JC. 1996c. Cloning and characterization of a

novel bicoid-related homeobox transcription factor gene, RIEG, involved in Rieger syndrome. Nature genetics 14:392–9.

Semina E V., Reiter R, Leysens NJ. 1996a. Cloning and characterization of a novel bicoid-related homeobox trancription factor gene, RIEG, involved in Rieger syndrome. Nat Genet 392–399.

Shaarawy T. 2015. Glaucoma surgery: taking the sub-conjunctival route. Middle East African journal of ophthalmology 22:53–8.

Shah BM, Dada T, Panda A, Tanwar M, Bhartiya S, Dada R. 2014. Novel occurrence of axenfeld: Rieger syndrome in a patient with blepharophimosis ptosis epicanthus inversus syndrome. Indian journal of ophthalmology 62:358–60.

Sheng HZ, Moriyama K, Yamashita T, Li H, Potter SS, Mahon KA, Westphal H. 1997. Multistep control of pituitary organogenesis. Science (New York, NY) 278:1809–12.

Shields MB. 1983. Axenfeld-Rieger syndrome: a theory of mechanism and distinctions from the iridocorneal endothelial syndrome. Transactions of the American Ophthalmological Society 81:736–84.

Shields MB. 2001. Axenfeld-Rieger and iridocorneal endothelial syndromes: two spectra of disease with striking similarities and differences. Journal of glaucoma 10:S36-8.

Sihota R. 2011. Lasers in primary open angle glaucoma. Indian journal of ophthalmology 59 Suppl:S114-7.

Smidt MP, Cox JJ, Schaick HS van, Coolen M, Schepers J, Kleij AM van der, Burbach JP. 2000. Analysis of three Ptx2 splice variants on transcriptional activity and differential expression pattern in the brain. Journal of neurochemistry 75:1818–25.

Smith PJ, Zhang C, Wang J, Chew SL, Zhang MQ, Krainer AR. 2006. An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. Human Molecular Genetics 15:2490–2508.

Smith RS, Zabaleta A, Kume T, Savinova O V, Kidson SH, Martin JE, Nishimura DY, Alward WL, Hogan BL, John SW. 2000. Haploinsufficiency of the transcription factors FOXC1 and FOXC2 results in aberrant ocular development. Human molecular genetics 9:1021–32.

So AY-L, Chaivorapol C, Bolton EC, Li H, Yamamoto KR. 2007. Determinants of cell- and gene-specific transcriptional regulation by the glucocorticoid receptor. PLoS genetics 3:e94.

Solis C, Aizencang GI, Astrin KH, Bishop DF, Desnick RJ. 2001. Uroporphyrinogen III synthase erythroid promoter mutations in adjacent GATA1 and CP2 elements cause congenital erythropoietic porphyria. The Journal of clinical investigation 107:753–62.

Sowden JC. 2007. Molecular and developmental mechanisms of anterior segment dysgenesis. Eye (London, England) 21:1310–8.

Spielmann M, Brancati F, Krawitz PM, Robinson PN, Ibrahim DM, Franke M, Hecht J, Lohan S, Dathe K, Nardone AM, Ferrari P, Landi A, et al. 2012. Homeotic arm-to-leg transformation

associated with genomic rearrangements at the PITX1 locus. American journal of human genetics 91:629–35.

Stefl S, Nishi H, Petukh M, Panchenko AR, Alexov E. 2013. Molecular mechanisms of disease-causing missense mutations. Journal of molecular biology 425:3919–36.

Stenson PD, Mort M, Ball E V., Shaw K, Phillips AD, Cooper DN. 2014. The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine. Human Genetics 133:1–9.

Strehle E-M, Yu L, Rosenfeld JA, Donkervoort S, Zhou Y, Chen T-J, Martinez JE, Fan Y-S, Barbouth D, Zhu H, Vaglio A, Smith R, et al. 2012. Genotype-phenotype analysis of 4q deletion syndrome: proposal of a critical region. American journal of medical genetics Part A 158A:2139–51.

Strungaru MH, Dinu I, Walter MA. 2007. Genotype-phenotype correlations in Axenfeld-Rieger malformation and glaucoma patients with FOXC1 and PITX2 mutations. Investigative ophthalmology & visual science 48:228–37.

Strungaru MH, Dinu I, Walter MA. 2007a. Genotype-Phenotype Correlations in Axenfeld-Rieger Malformation and Glaucoma Patients with FOXC1 and PITX2 Mutations. Investigative Opthalmology & Visual Science 48:228.

Strungaru MH, Footz T, Liu Y, Berry FB, Belleau P, Semina E V, Raymond V, Walter MA. 2011. PITX2 is involved in stress response in cultured human trabecular meshwork cells through regulation of SLC13A3. Investigative ophthalmology & visual science 52:7625–33.

Sun Y-M, Wang J, Qiu X-B, Yuan F, Xu Y-J, Li R-G, Qu X-K, Huang R-T, Xue S, Yang Y-Q. 2016. PITX2 loss-of-function mutation contributes to tetralogy of Fallot. Gene 577:258–64.

Suzuki T, Takahashi K, Kuwahara S, Wada Y, Abe T, Tamai M. 2001. A novel (Pro79Thr) mutation in the FKHL7 gene in a Japanese family with Axenfeld-Rieger syndrome. American journal of ophthalmology 132:572–5.

Tang H, Thomas PD. 2016a. PANTHER-PSEP: predicting disease-causing genetic variants using position-specific evolutionary preservation. Bioinformatics 32:2230–2232.

Tanwar M, Kumar R, Goyal A, Kumar M, Dada T, Singh G, Sihota R, Dada R. 2009. Rieger syndrome with multiple chromosomal breaks and chromosome 4 deletion. BMJ case reports 2009:bcr0620080297-bcr0620080297.

Tavtigian S V, Deffenbaugh AM, Yin L, Judkins T, Scholl T, Samollow PB, Silva D de, Zharkikh A, Thomas A. 2006. Comprehensive statistical study of 452 BRCA1 missense substitutions with classification of eight recurrent substitutions as neutral. Journal of medical genetics 43:295–305.

Teus MA, Arranz-Marquez E, Lucea-Suescun P. 2002. Incidence of iris colour change in latanoprost treated eyes. The British journal of ophthalmology 86:1085–1088.

Tokuriki N, Tawfik DS. 2009a. Stability effects of mutations and protein evolvability. Current Opinion in Structural Biology 19:596–604.

Tonna S, Dandapani S V, Uscinski A, Appel GB, Schlöndorff JS, Zhang K, Denker BM, Pollak MR. 2008. Functional genetic variation in aminopeptidase A (ENPEP): lack of clear association with focal and segmental glomerulosclerosis (FSGS). Gene 410:44–52.

Trinklein ND, Aldred SF, Hartman SJ, Schroeder DI, Otillar RP, Myers RM. 2004. An abundance of bidirectional promoters in the human genome. Genome research 14:62–6.

Tümer Z, Bach-Holm D. 2009b. Axenfeld–Rieger syndrome and spectrum of PITX2 and FOXC1 mutations. European Journal of Human Genetics 17:1527–1539.

Vagenende V, Yap MGS, Trout BL. 2009. Mechanisms of Protein Stabilization and Prevention of Protein Aggregation by Glycerol. Biochemistry 48:11084–11096.

Valdmanis PN, Verlaan DJ, Rouleau GA. 2009. The proportion of mutations predicted to have a deleterious effect differs between gain and loss of function genes in neurodegenerative disease. Human mutation 30:E481-9.

Vasconcellos JPC, Melo MB, Schimiti RB, Bressanim NC, Costa FF, Costa VP. 2005. A novel mutation in the GJA1 gene in a family with oculodentodigital dysplasia. Archives of ophthalmology (Chicago, Ill : 1960) 123:1422–6.

Vaux C, Sheffield L, Keith CG, Voullaire L. 1992. Evidence that Rieger syndrome maps to 4q25 or 4q27. Journal of medical genetics 29:256–8.

Vernimmen D. 2014. Uncovering enhancer functions using the α-globin locus. PLoS genetics 10:e1004668.

Vieira V, David G, Roche O, la Houssaye G de, Boutboul S, Arbogast L, Kobetz A, Orssaud C, Camand O, Schorderet DF, Munier F, Rossi A, et al. 2006. Identification of four new PITX2 gene mutations in patients with Axenfeld-Rieger syndrome. Molecular vision 12:1448–60.

Vincent A, Billingsley G, Priston M, Williams-Lyn D, Sutherland J, Glaser T, Oliver E, Walter M a, Heathcote G, Levin a, Héon E. 2001. Phenotypic heterogeneity of CYP1B1: mutations in a patient with Peters' anomaly. Journal of medical genetics 38:324–6.

Visel A, Prabhakar S, Akiyama JA, Shoukry M, Lewis KD, Holt A, Plajzer-Frick I, Afzal V, Rubin EM, Pennacchio LA. 2008. Ultraconservation identifies a small subset of extremely constrained developmental enhancers. Nature genetics 40:158–60.

Volkmann BA, Zinkevich NS, Mustonen A, Schilter KF, Bosenko D V, Reis LM, Broeckel U, Link BA, Semina E V. 2011. Potential novel mechanism for Axenfeld-Rieger syndrome: deletion of a distant region containing regulatory elements of PITX2. Investigative ophthalmology & visual science 52:1450–9.

Waldron JM, McNamara C, Hewson AR, McNamara CM. 2010. ARTICLE: Axenfeld-Rieger Syndrome (ARS): A review and case report. Special Care in Dentistry 30:218–222.

Wallqvist A, Fukunishi Y, Murphy LR, Fadel A, Levy RM. 2000. Iterative sequence/secondary structure search for protein homologs: comparison with amino acid sequence alignments and

application to fold recognition in genome databases. Bioinformatics (Oxford, England) 16:988–1002.

Wang J, Xin Y-F, Xu W-J, Liu Z-M, Qiu X-B, Qu X-K, Xu L, Li X, Yang Y-Q. 2013. Prevalence and spectrum of PITX2c mutations associated with congenital heart disease. DNA and cell biology 32:708–16.

Wang J, Zhang D-F, Sun Y-M, Yang Y-Q. 2014. A novel PITX2c loss-of-function mutation associated with familial atrial fibrillation. European journal of medical genetics 57:25–31.

Wang Y, Zhao H, Zhang X, Feng H. 2003. Novel identification of a four-base-pair deletion mutation in PITX2 in a Rieger syndrome family. Journal of dental research 82:1008–12.

Wang Z, Moult J. 2001. SNPs, protein structure, and disease. Human Mutation 17:263–270.

Wang Z, Rolish ME, Yeo G, Tung V, Mawson M, Burge CB. 2004. Systematic Identification and Analysis of Exonic Splicing Silencers. Cell 119:831–845.

Wei D, Gong X-H, Qiu G, Wang J, Yang Y-Q. 2014. Novel PITX2c loss-of-function mutations associated with complex congenital heart disease. International journal of molecular medicine 33:1201–8.

Wei L-X, Zhou R-S, Xu H-F, Wang J-Y, Yuan M-H. 2013. High expression of FOXC1 is associated with poor clinical outcome in non-small cell lung cancer patients. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine 34:941–6.

Weigelt J, Climent I, Dahlman-Wright K, Wikström M. 2001. Solution Structure of the DNA Binding Domain of the Human Forkhead Transcription Factor AFX (FOXO4) †. Biochemistry 40:5861–5869.

Weingarten-Gabbay S, Segal E. 2014. The grammar of transcriptional regulation. Human genetics 133:701–11.

Weissbart SB, Ayres BD. 2016. Management of aniridia and iris defects. Current Opinion in Ophthalmology 27:244–249.

Weisschuh N, Dressler P, Schuettauf F, Wolf C, Wissinger B, Gramer E. 2006b. Novel mutations of FOXC1 and PITX2 in patients with Axenfeld-Rieger malformations. Investigative ophthalmology & visual science 47:3846–52.

Weisschuh N, Wolf C, Wissinger B, Gramer E. 2008. A novel mutation in the FOXC1 gene in a family with Axenfeld-Rieger syndrome and Peters' anomaly. Clinical genetics 74:476–80.

Werner W, Kraft S, Callen DF, Bartsch O, Hinkel GK. 1997. A small deletion of 16q23.1-->16q24.2 [del(16)(q23.1q24.2).ish del(16)(q23.1q24.2)(D16S395+, D16S348-, P5432+)] in a boy with iris coloboma and minor anomalies. American journal of medical genetics 70:371–6.

Wheeler DA, Srinivasan M, Egholm M, Shen Y, Chen L, McGuire A, He W, Chen Y-J, Makhijani V, Roth GT, Gomes X, Tartaro K, et al. 2008. The complete genome of an individual by massively parallel DNA sequencing. Nature 452:872–876.

Whitehead MT, Choudhri AF, Salim S. 2013. Magnetic resonance imaging findings in Axenfeld-Rieger syndrome. Clinical ophthalmology (Auckland, NZ) 7:911–6.

Wilson C, Bellen HJ, Gehring WJ. 1990. Position effects on eukaryotic gene expression. Annual review of cell biology 6:679–714.

Wittkopp PJ, Haerum BK, Clark AG. 2008. Regulatory changes underlying expression differences within and between Drosophila species. Nature Genetics 40:346–350.

Wittkopp PJ, Kalay G. 2011. Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. Nature reviews Genetics 13:59–69.

Wittwer J, Marti-Jaun J, Hersberger M. 2006. Functional polymorphism in ALOX15 results in increased allele-specific transcription in macrophages through binding of the transcription factor SPI1. Human mutation 27:78–87.

Worth CL, Preissner R, Blundell TL. 2011. SDM--a server for predicting effects of mutations on protein stability and malfunction. Nucleic Acids Research 39:W215–W222.

Wray GA. 2007. The evolutionary significance of cis-regulatory mutations. Nature Reviews Genetics 8:206–216.

Xia K, Wu L, Liu X, Xi X, Liang D, Zheng D, Cai F, Pan Q, Long Z, Dai H, Hu Z, Tang B, et al. 2004. Mutation in PITX2 is associated with ring dermoid of the cornea. Journal of medical genetics 41:e129.

Xia L, Huang W, Tian D, Zhu H, Qi X, Chen Z, Zhang Y, Hu H, Fan D, Nie Y, Wu K. 2013. Overexpression of forkhead box C1 promotes tumor metastasis and indicates poor prognosis in hepatocellular carcinoma. Hepatology (Baltimore, Md) 57:610–24.

Yang MQ, Laflamme K, Gotea V, Joiner CH, Seidel NE, Wong C, Petrykowska HM, Lichtenberg J, Lee S, Welch L, Gallagher PG, Bodine DM, et al. 2011. Genome-wide detection of a TFIID localization element from an initial human disease mutation. Nucleic acids research 39:2175–87.

Yang Y-Q, Xu Y-J, Li R-G, Qu X-K, Fang W-Y, Liu X. 2013. Prevalence and spectrum of PITX2c mutations associated with familial atrial fibrillation. International journal of cardiology 168:2873–6.

Yilmaz A, Grotewold E. 2010. Components and Mechanisms of Regulation of Gene Expression. Methods in Molecular Biology (Clifton, N.J.), p 23–32.

Yoshioka H, Meno C, Koshiba K, Sugihara M, Itoh H, Ishimaru Y, Inoue T, Ohuchi H, Semina E V, Murray JC, Hamada H, Noji S. 1998. Pitx2, a bicoid-type homeobox gene, is involved in a lefty-signaling pathway in determination of left-right asymmetry. Cell 94:299–305.

Young DL, Fields S. 2015. The role of functional data in interpreting the effects of genetic variation. Molecular biology of the cell 26:3904–8.

Yuan F, Zhao L, Wang J, Zhang W, Li X, Qiu X-B, Li R-G, Xu Y-J, Xu L, Qu X-K, Fang W-Y, Yang Y-Q. 2013. PITX2c loss-of-function mutations responsible for congenital atrial septal defects. International journal of medical sciences 10:1422–9.

Yun JW, Cho H-K, Oh S-Y, Ki C-S, Kee C. 2013. Novel c.300_301delinsT mutation in PITX2 in a Korean family with Axenfeld-Rieger syndrome. Annals of laboratory medicine 33:360–3.

Zaidi S, Choi M, Wakimoto H, Ma L, Jiang J, Overton JD, Romano-Adesman A, Bjornson RD, Breitbart RE, Brown KK, Carriero NJ, Cheung YH, et al. 2013. De novo mutations in histone-modifying genes in congenital heart disease. Nature 498:220–3.

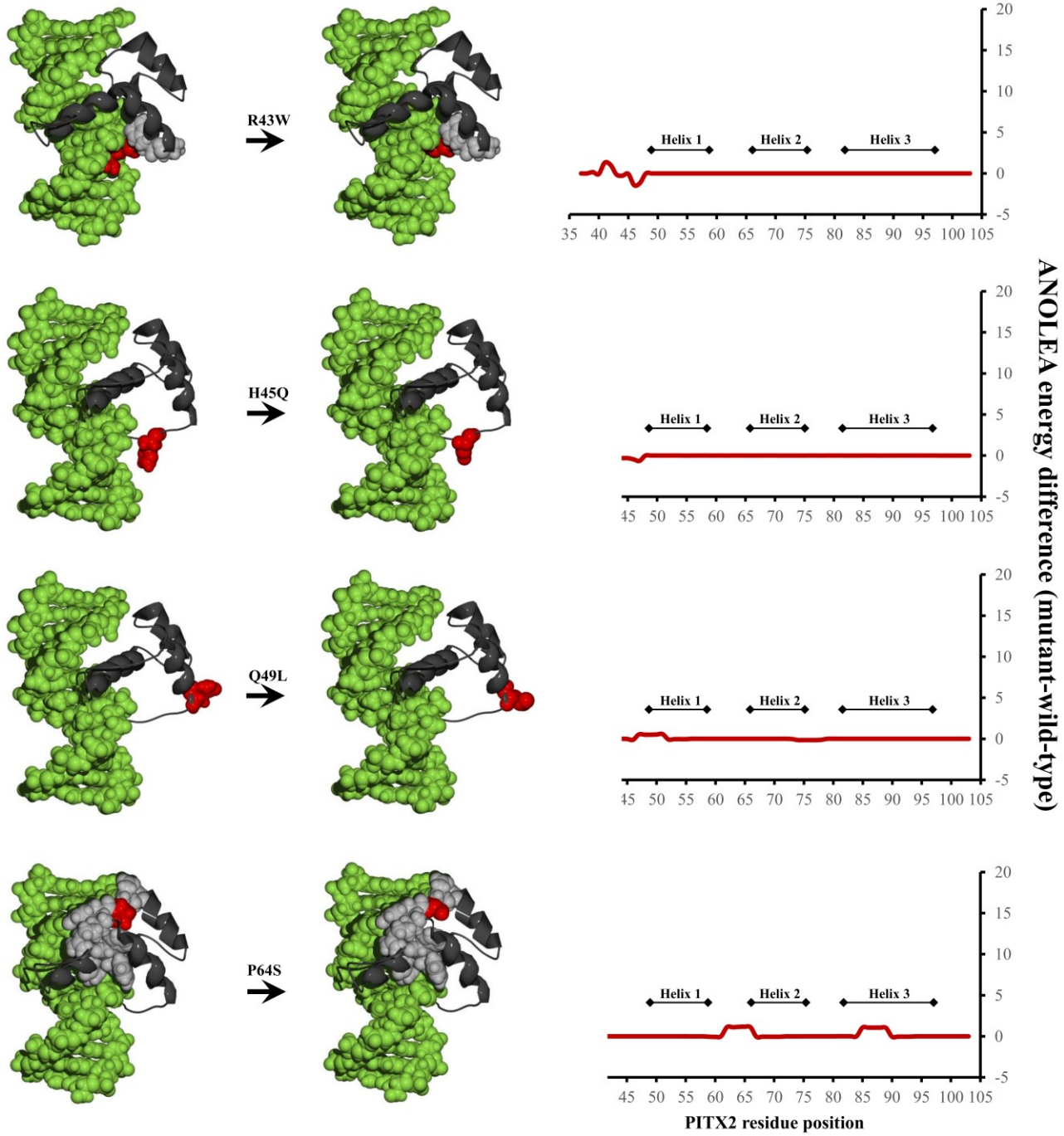
Zhou T, Chiang C-M. 2002. Sp1 and AP2 regulate but do not constitute TATA-less human TAF(II)55 core promoter activity. Nucleic acids research 30:4145–57.

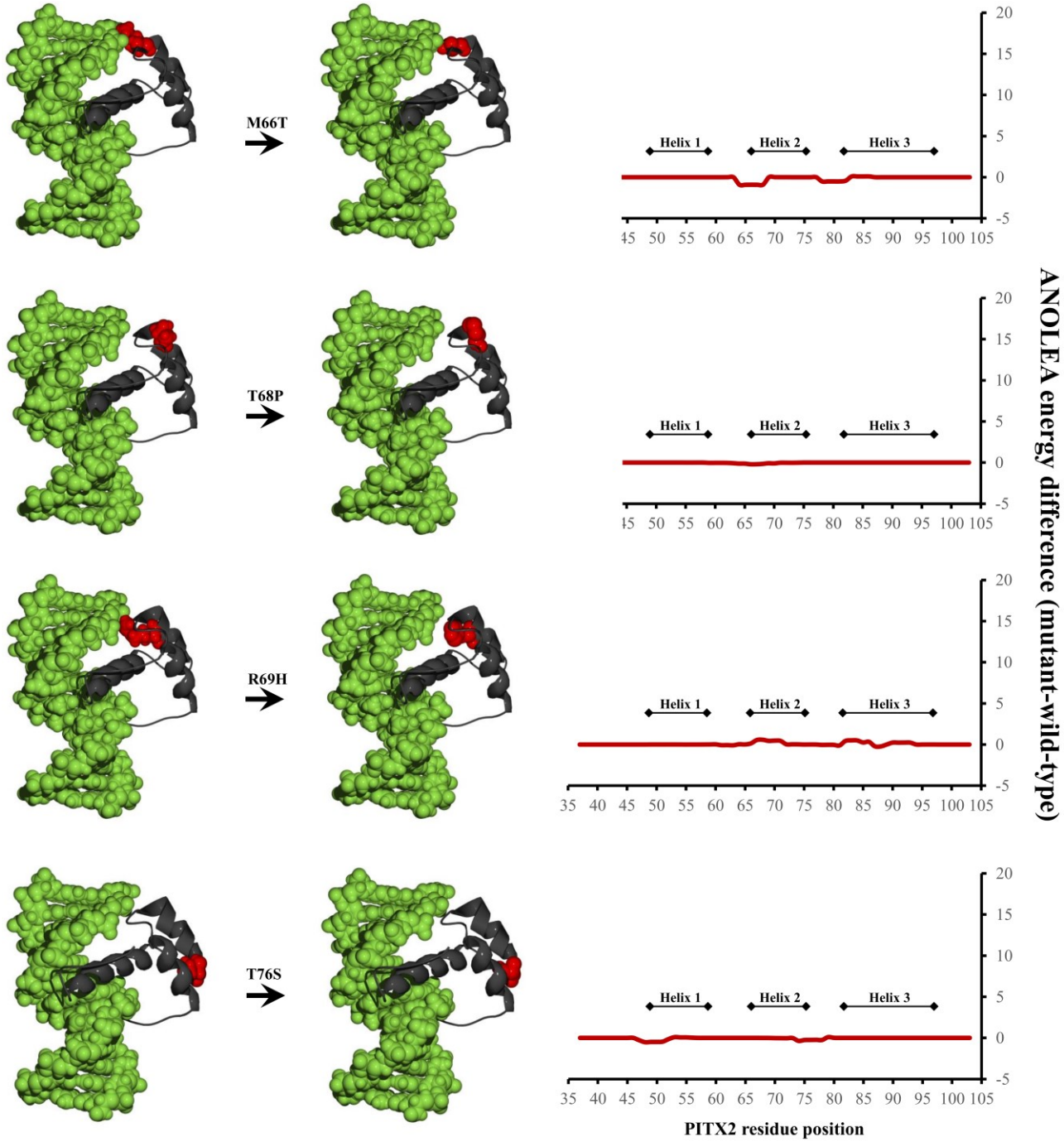
Ziakas NG, Chranioti A, Malamas A, Leliopoulou O, Mataftsi A. 2014. Congenital ectropion uveae presenting as acute glaucoma in a 3-year-old child. International Ophthalmology 34:97–98.

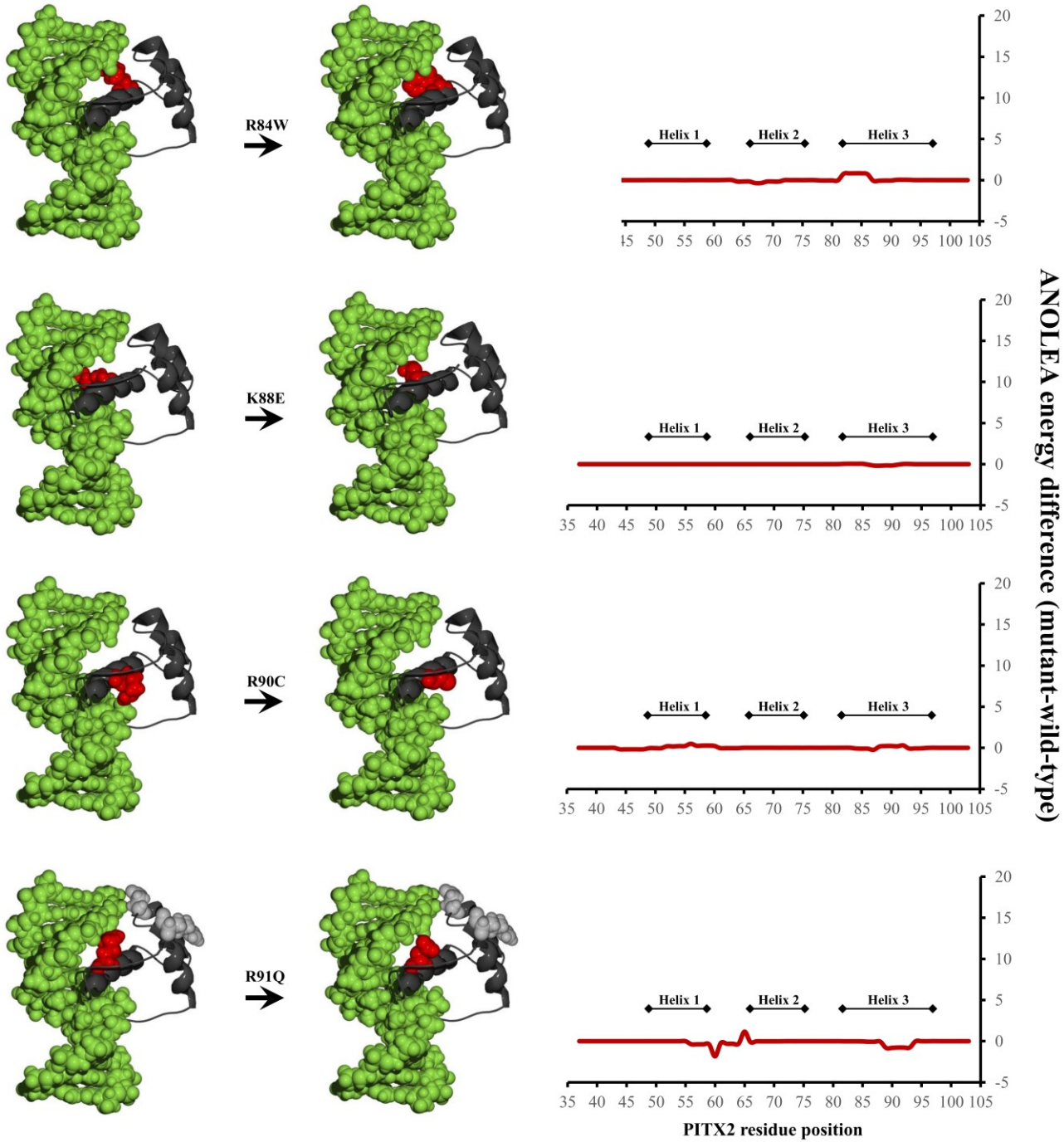
Zou C, Levine EM. 2012. Vsx2 controls eye organogenesis and retinal progenitor identity via homeodomain and non-homeodomain residues required for high affinity DNA binding. PLoS genetics 8:e1002924.

Appendix

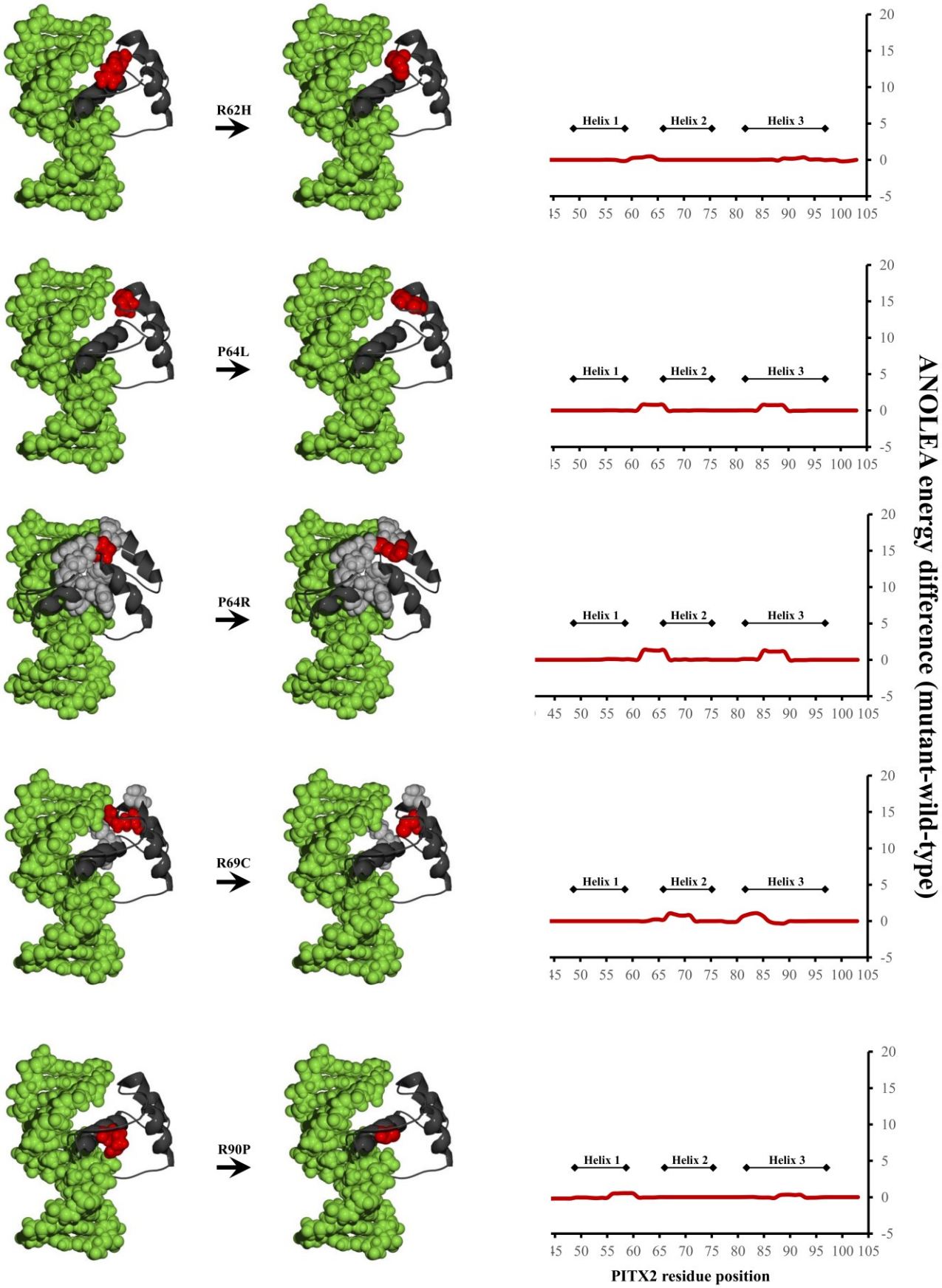
Supplementary Figure 1. Homology models (left) and scatterplots (right) of *in silico* analyses of functionally characterized variants in the *PITX2* gene. The 3D model of *PITX2* is presented with the protein backbone depicted in black ribbon, the co-crystallized DNA binding target in space-filled green model and the mutants positions in red. The wild-type and mutant-equivalent models were analyzed by the atomic nonlocal environment assessment (ANOLEA) server. Peaks on the scatterplots show the positions of amino acids that changed their pseudoenergy state, as a consequence of the mentioned variants.


R43W
H45Q
Q49L
P64S
Helix 1
Helix 2
Helix 3
20
15
10
5
0
-5
35 40 45 50 55 60 65 70 75 80 85 90 95 100 105
45 50 55 60 65 70 75 80 85 90 95 100 105
ANOLEA energy difference (mutant-wild-type)
PITX2 residue position


M66T
T68P
R69H
T76S
Helix 1
Helix 2
Helix 3
20
15
10
5
0
-5
45 50 55 60 65 70 75 80 85 90 95 100 105
35 40 45 50 55 60 65 70 75 80 85 90 95 100 105
ANOLEA energy difference (mutant-wild-type)
PITX2 residue position


R84W
K88E
R90C
R91Q
Helix 1
Helix 2
Helix 3
20
15
10
5
0
-5
35 40 45 50 55 60 65 70 75 80 85 90 95 100 105
ANOLEA energy difference (mutant-wild-type)
PITX2 residue position

Supplementary Figure 2. Homology models (left) and scatterplots (right) of *in silico* analyses of functionally uncharacterized variants in the *PITX2* gene. The 3D model of *PITX2* is presented with the protein backbone depicted in black ribbon, the co-crystallized DNA binding target in space-filled green model and the mutants positions in red. The wild-type and mutant-equivalent models were analyzed by the atomic nonlocal environment assessment (ANOLEA) server. Peaks on the scatterplots show the positions of amino acids that changed their pseudoenergy state, as consequence of the mentioned variants.


R62H
P64L
P64R
R69C
R90P
Helix 1
Helix 2
Helix 3
20
15
10
5
0
-5
45 50 55 60 65 70 75 80 85 90 95 100 105
ANOLEA energy difference (mutant-wild-type)
PITX2 residue position