

ABSTRACT

MARGARET MCDOUGAL

Investigating Aniridia, a Human Genetic Eye Disorder
(Under the Direction of DR. JAMES D. LAUDERDALE)

Aniridia is a rare congenital eye disorder typically diagnosed postnatally by partial or full iris hypoplasia. Associated foveal hypoplasia, indicated by early infancy nystagmus, causes reduced visual acuity. The progressive nature of the disease frequently leads to multiple ocular abnormalities such as glaucoma, keratopathy, cataracts, corneal vascularization and corneal opacification. Two-thirds of aniridic patients have heterozygous mutations in the *PAX6* gene. The *PAX6* protein is a highly conserved transcription factor crucial for normal eye development and cornea homeostasis, however the proteins exact molecular mechanism is still uncertain. Understanding the phenotypic variation caused by different *PAX6* mutations helps explain the molecular mechanism of the mutated protein and the complex role of *PAX6* in the eye. In this project, we screened the *PAX6* gene of 157 individuals, representing 58 aniridic families, by direct gene sequencing and conducted detailed ophthalmologic evaluations for each aniridic patient. A total of 38 mutations were identified, including 20 novel mutations. I compiled our results with the Human *PAX6* Allelic Variant Database to create a total of 565 independently ascertained variants in *PAX6*. Of these variants, 525 are associated with ocular malformations and 459 are specifically causal for isolated aniridia. Further analysis of mutation type frequencies and distributions indicated that nonsense (39%), frame-shift (29%), and splice junction (18%) mutations are predominately associated with aniridia, whereas the majority of non-aniridia phenotypes are caused by missense mutations (76%). This compiled mutation spectrum continues to yield important insight into the molecular mechanism of the mutated protein and the likely phenotypic defects.

INDEX WORDS: Aniridia; *PAX6*

INVESTIGATING ANIRIDIA, A HUMAN GENTETIC EYE DISORDER

by

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A Thesis Submitted to the Honors Council of the University of Georgia
in Partial Fulfillment of the Requirements for the Degree

BACHELOR OF SCIENCE

with HIGHEST HONORS.

and CURO SCHOLAR DISTINCTION

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Athens, Georgia

2010

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DEDICATION

This thesis is dedicated to my father, Dr. John S. McDougal. Thank you for not telling me how to live my life, but rather letting me watch you live yours. You're who I look to and inspire to be. You were the first person to ever have an influence in my life and the one person who will have it for eternity.

ACKNOWLEDGEMENTS

I want to express my most sincere thanks to Dr. Jim Lauderdale, who goes far beyond the role of a mentor and advisor. His devotion to each of his students, colleagues, and projects is nothing short of amazing. Without his unwavering patience, approachable personality, and innate teaching ability this thesis would not have been possible. I cannot thank him enough for challenging and encouraging me to achieve far beyond what I thought capable. His impact has shaped my undergraduate years, as well as my hopes for the future. He has instilled in me a passion that may very well carry me over to the “dark side” of a career in research.

A special thanks as well goes to my thesis reader, Dr. Mary Bedell. I thank her for introducing me to the wonders of the genetic world. Her contagious enthusiasm spun a web of curiosity and possibility that has motivated me to write this thesis.

I am indebted to my fellow lab members who worked tediously on this project, especially Brittany Fox, Amita Nawathe, Vijay Kalaskar, and Kelly Brothers. Each of you inspire me and keep me smiling. In addition, the fellow collaborators of this project are greatly appreciated, especially Jill Nerby, founder of AFI, and researchers at the Hamilton Eye Institute.

I want to express my sincere gratitude to John Duncan for patiently explaining his wealth of knowledge and constant guidance over the year. Also, a special thanks to Andrew Sornborger for his mathematical genius and helpful insights.

Finally, my heart and admiration goes out to each of the members of Aniridia Foundation who have united together with tremendous courage, bravery, and hope to fight this disease.

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CHAPTER 1 INTRODUCTION

1.1 Overview of Mammalian Ocular Development

The eye is a truly remarkable organ, composed of many different tissue types. Light that passes through the corneal tissue is focused by the lens tissue, and then converted into nerve signals by the multi-layered retinal tissue. Optic function critically depends on the correct assembly of these intricate tissue layers during development [1].

Mammalian ocular development occurs through a series of inductive signaling between the posterior outpocket of the forebrain neuroectoderm, the surrounding mesenchyme, and the anterior surface epidermis. Successive signals between these three embryonic tissues coordinate their development and differentiation. In early neurula stage embryos, an extension of the forebrain diencephalon forms the optic vesicle, which induces head ectodermal cells to pseudostratify and thicken into the lens placode [2-3]. The lens placode is the source of two separate signals. The first is a reciprocal induction back to the optic vesicle, causing it to fold inwards to form the optic cup. The second signal is to the overlying ectoderm to induce cornea formation. The initial signal to the anterior ectoderm layer causes a thickening and a secretion of a collagen matrix [4]. Then, the mesenchymal cells, derived from the neural crest, fill in through this collagen matrix and signal further differentiation through epithelial-mesenchymal interactions. Finally, the release of the thyroxin hormone causes dehydration and enables the cornea to become transparent and avascular [5-6]. Development will continue via induction

pathways as shown in **Figure 1** until the pigmented retina, neural retina, lens, and cornea have fully developed [7]

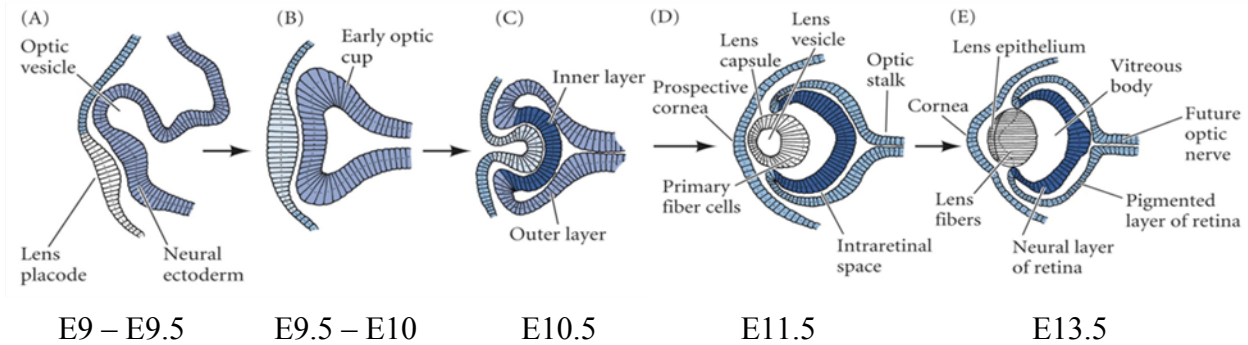


Figure 1: Early Eye Morphogenesis in Mouse Embryo.

At embryonic day 9 to 9.5 (E9-E9.5), an outpocket of the neural ectoderm forms the optic vesicle (OV). Upon contact with the surface ectoderm, the OV induces the formation of the lens placode. The OV and lens placode further develop into the optic cup and lens vesicle, respectively. Next, the anterior surface ectoderm thickens and forms a collagen matrix. At which point mesenchymal cells fill in through the matrix and cause further cell dehydration to form the avascular and transparent cornea. This figure was obtained from A.P. Monaghan [7].

In addition to the cornea, lens, and retina, visual perception depends on the development of the iris, ciliary body, and trabecular meshwork. The iris muscle tissue regulates the light influx into the eye and better focuses light onto the retina. The ciliary body secretes the aqueous humor that fills the posterior chamber and flows out through the pupil opening into the anterior chamber. The aqueous humor maintains intraocular pressure and provides nutrients for the avascular ocular structures. Also, the fluid provides waste management and is constantly drained through the trabecular meshwork into the venous system. Similar to the lens, cornea, and retina, these anterior ocular structures develop from induction pathways that involve epithelial-mesenchymal interactions [1].

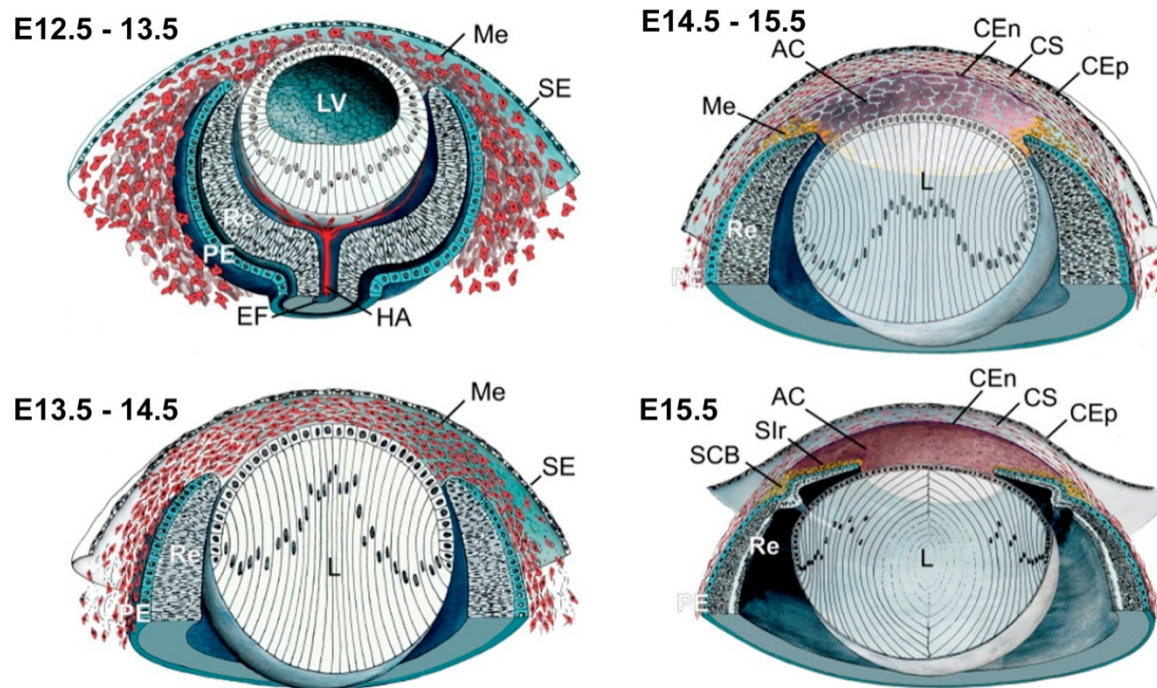


Figure 2: Mesenchyme-Ectoderm Interactions in the Eye of a Mouse Embryo.

Tissue differentiation in eye development occurs through successive mesenchyme-ectoderm interactions. Embryonic day (E); lens vesicle (LV); surface epithelium (SE); mesenchymal cells (Me); neural retina (Re); retinal pigmented epithelium (PE); embryonic fissure (EF); hyaloid artery (HA); lens (L); corneal endothelium (CEn); corneal epithelium (CEp); anterior chamber (AC); stroma of the iris (Sir); stroma of the ciliary body (CB) This figure was obtained from Cvekl and Tamm [1].

Figure 2 shows the mesenchyme-ectodermal interactions in the mouse eye between embryonic day 12.5 (E12.5) – E15.5. The above schematic diagram shows the mesenchyme-ectodermal interactions in the eye between embryonic days E12.5 - E15.5. As described previously, the mesenchymal cells (Me, shown in red) enter into the cornea ectoderm after the lens vesicle (LV), pigmented epithelium (PE), and neural retina (Re) have developed. The mesenchymal cells create an anterior layer called the surface ectoderm (SE) by E14.5. The single cornea ectoderm layer will further differentiate into the posterior corneal endothelium (CEn) and

the anterior epithelium (CEp). Mesenchyme cells between the corneal epithelium and endothelium differentiate into corneal stroma (CS). The lens (L) will then detach from the cornea layers and an aqueous fluid will fill the developing anterior chamber (AC) and separate the lens from the cornea. Around E15.5, mesenchyme cells (Me) will migrate towards the edge of the optic cup and developing cornea. Here the mesenchyme cells will cause the optic cup to enlarge to form the iris and ciliary body. Unlike other muscle tissues, which are fully derived from mesenchymal cells, the iris is made up of both mesenchymal and ectodermal cells. The two cell types continue to further develop into the stroma of the iris (SIr) and stroma of the ciliary body (SCB), which are shown in yellow [1].

1.2 Transcription Factor PAX6

The complex development pathway of the eye would not be possible without the transcription factor, PAX6. The PAX6 protein is one of the most highly conserved transcription factors throughout metazoan evolutionary lineage. In humans, the pre-mRNA extends approximately 23kb and gives rise to a 2.7kb cDNA, which is translated into a 422 amino acid sequence. As seen in **Figure 3**, the PAX6 protein consists of an N-terminal paired domain (PD), connected to the homeodomain (HD) through a linker region (LNK), and a C-terminal proline-serine-threonine (PST)-rich domain. The paired domain and homeodomain each have DNA binding activities, while the PST domain has a transactivation function [8]. **Figure 4** is a schematic representation of the PAX6 transcript. The 16 exons (represented by boxes) that make up the PAX6 gene are labeled 0 through 13 with the addition of the α promoter between exons 4

and 5 and the 5a alternative spliced exon. The shaded boxes indicate the exons that undergo translation.

Interestingly, the *PAX6* gene encodes several different isoforms, with canonical *PAX6*, *PAX6(5a)*, and *PAX6ΔPD* being the most common in vertebrates. Canonical *PAX6* and *PAX6(5a)* have two alternative promoters (P_0 and P_1), and begin translation at the ATG start codon in Exon 4. *PAX6(5a)* is an alternative splice form of the canonical *PAX6* and includes an additional 14 amino acid sequence (the 5a exon) at the end of exon 5. The third isomeric protein, *PAX6ΔPD*, lacks the paired domain (PD) found in the first two isomers. *PAX6ΔPD* has the P_α promoter and is suspected to begin translation at the ATG site found in Exon 7 [9].

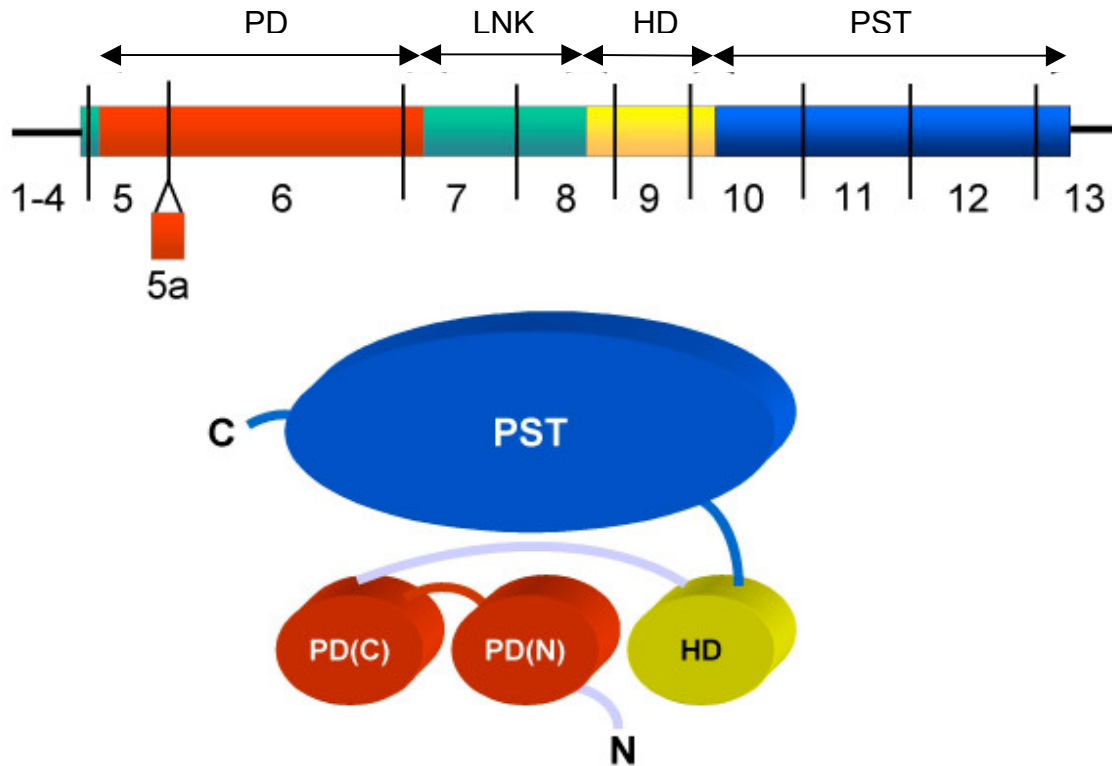


Figure 3: *PAX6* cDNA and protein. The paired domain is shown in red, the linker region in teal, the homeodomain in yellow, and the PST domain in blue. [8].

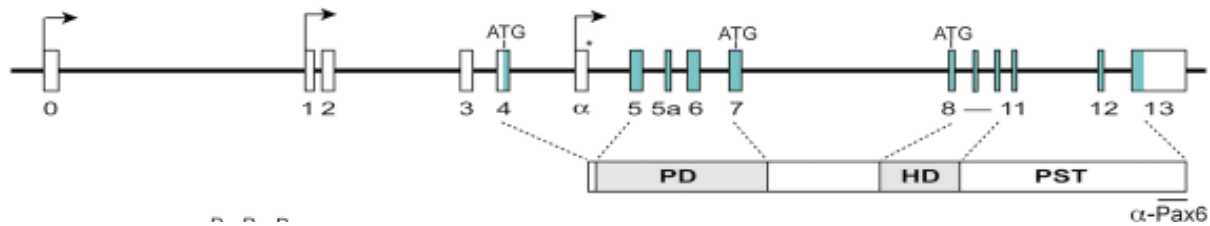


Figure 4: Schematic representation of the PAX6 transcript [9].

1.2.1 Role of PAX6 in Ocular Development

Pax6 plays a crucial role in eye induction and has additionally been implicated in a variety of events throughout eye morphogenesis. Detailed analysis of its cellular functions and expression patterns suggests involvement in processes such as cell differentiation, proliferation, and adhesion/migration [10].

Recent transgenic technology has revealed the specific requirements for PAX6 in initial lens development [11]. In the eye, PAX6 is a known transcription factor that is expressed in both the optic vesicle and head ectoderm. The protein is necessary to make the surface ectoderm competent to receive signals from the optic vesicle, enabling differentiation into lens tissue. [12]

In addition to this lens-forming competence role, PAX6 further differentiates the lens placode by signaling crystallin gene transcription. Crystallin genes encode for the soluble proteins that are responsible for lens transparency. PAX6 has the capability to bind to the promoter region of alpha-A-crystallin and alpha-B-crystallin gene in mice, and several other crystallin genes in guinea-pigs and chicks [13-16]. In addition, PAX6 is sufficient to induce an ectopic lens in *Drosophila* and *Bufo americanus* (frogs) upon misexpression [17-18]. These and

other findings, indicate the major role PAX6 plays in initial lens induction and crystallin expression.

The lens is not the only ocular structure that depends on PAX6 for normal development. PAX6, which is present in both mesenchymal and ectodermal cells, synchronizes the complex interactions between cells types of different origins to further coordinate anterior eye morphogenesis (Describe in **Figure 2**). The transcription factor also controls the expression of other regulators, including PAX2, SOX2, and a series of retinal helix-loop-helix transcription factors. PAX6 has been shown to bind to the promoter of the keratin-12 gene, which is essential for corneal epithelium integrity [19-20].

In addition, the PAX6(5a) isoform has shown DNA binding ability for specific target sites [21]. The phenotype of a knockout PAX6(5a) mouse showed severe iris hypoplasia and retinal malformations, but there were no alterations in the expression patterns of the other PAX6 isoforms. With this information, and the fact that the PAX6(5a) isoform is only present in vertebrates, it was proposed that PAX6(5a) isoform is crucial for iris development [22].

1.2.2 Role of PAX6 in Maintaining Ocular Homeostasis

Not only is PAX6 crucial for normal eye morphogenesis, but it is also required for maintaining eye homeostasis. After embryonic development, PAX6 is still expressed in the adult cornea, conjunctival, and limbal epithelium and is predicted to be involved in cornea maintenance [23-27]. The cornea's transparent and refractive properties provide 80% of the eye's optical power. Therefore, people would be functionally blind if the cornea was damaged and lost its transparency or became vascular. This damage can occur as the result of infection, injury, or

genetic disease.

Like the skin, the cornea is an epithelial layer that must constantly be renewed. With every blink, corneal cells are sloughed. The limbal stem cells (LSCs) allow for the continual turnover of the stratified epithelium cells of the cornea [28-29]. A sufficient amount of stem cells from the corneo-scleral limbus must produce a proliferative population of transient amplifying cells (TACs) in the basal epithelium which can further differentiate into post-mitotic terminally differentiated (TD) cells and renew the cornea [30-36].

PAX6, which is expressed in TACs from the limbal stem cell populations, is hypothesized to play a role in TAC differentiation into center corneal epithelium [37]. In addition, recent evidence indicates multiple roles for PAX6 in cornea wound healing. Corneal matrices undergo constant slow remodeling under normal conditions and rapid remodeling during wound healing. Remodeling is mediated by matrix metalloproteinases (MMPs) produced by the corneal epithelium cells and stromal fibroblasts. PAX6 directly regulates expression of MMP-9 and several cell adhesion molecules and keratin-12 [38].

1.3 PAX6 Mutations

The Pax6 amino acid sequence and expression pattern is highly conserved amongst all metazoans [39]. In fact, ectopic expression of the mouse Pax6 gene in *Drosophila* is capable of inducing a complete fly eye, indicating the highly conserved molecular mechanism of Pax6 throughout evolutionary lineages [40]. This high degree of structural conservation argues for the functional importance of every amino acid.

Polymorphism in *PAX6* are very rare and if one does occur it is likely to be a mutation that impairs ocular development. In fact, the Human *PAX6* Allelic Variant Database [41] contains 565 records, 525 of which are associated ocular malformations. Each of these 525 mutations were classified into one of the seven categories according to the apparent effect of each genetic change. The seven categories are nonsense, splicing, frame-shifting insertion or deletion (indel), in-frame indel, missense, frame-shifting indel extension, and run-on. The description of each of these mutation types are listed in **Table 1** (the color coding of mutation type seen in this table will be used throughout this report).

Table 1: Categories of Mutations in the *PAX6* Allelic Variant Database. Each of the 525 ocular disease-associated mutations in the database was assigned to one of these seven categories. The color coding seen here will be used throughout this report.

Category	Definition
Nonsense	Single nucleotide substitution creates a stop codon in the open reading frame
Splicing	Nucleotide substitution, deletion, or insertion in consensus splice site
Frame-shifting insertion or deletion	Deletion or insertion of nucleotides in the open reading frame – total number not divisible by three
In-frame insertion or deletion	Deletion or insertion of nucleotides in the open reading frame – total number divisible by three
Missense	Single nucleotide substitution changes one amino acid codon to another in the open reading frame
Frame-shifting insertion or deletion extension	Deletion or insertion of nucleotides in the open reading frame – total number not divisible by three. Frame-shift changes the termination codon to an amino acid codon. Translation predicted to continue into the 3' UTR.
Run-on	Nucleotide substitution, insertion, or deletion in the termination codon that causes it to change into an amino acid codon. Translation is predicted to continue into the 3' UTR.

1.3.1 Aniridia

Mutations in the *PAX6* gene can result in multiple eye abnormalities in humans, the most common being aniridia [1, 42-43]. Aniridia is a panocular, progressive, and congenital eye

disease typically diagnosed postnatally. It occurs in 1/64,000 to 1/96,000 live births [44]. The classical definition of aniridia is simply lack of an iris (shown in **Figure 5A**). However, the disease is also associated with additional eye abnormalities.

Presenting in early infancy, aniridia is characterized by iris hypoplasia with associated foveal hypoplasia. These underdeveloped anterior and posterior eye structures will cause reduced visual acuity, light sensitivity, and nystagmus. The progressive nature of the disease frequently leads to multiple ocular abnormalities such as glaucoma, keratopathy, cataracts, corneal vascularization and corneal opacification (shown in **Figure 5B**) [45].

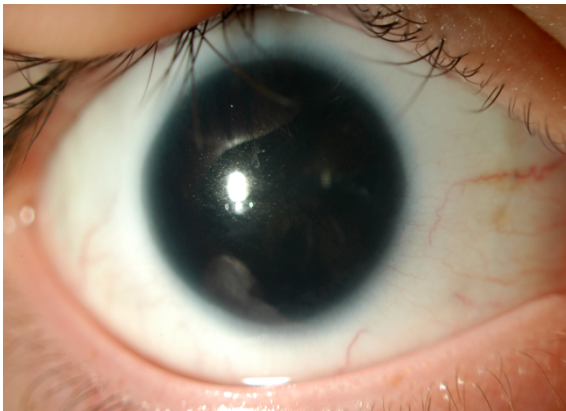


Figure 5A: Classic aniridia
(These pictures are patients in Aniridia Foundations International who participated in a *PAX6* mutation screen done by the Lauderdale lab.)

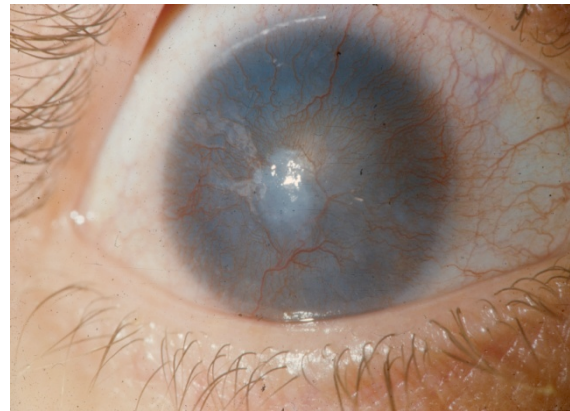


Figure 5B: Progressive stage of aniridia

Aniridia can be isolated or syndromic. Isolated aniridia is associated with only ocular defects resulting from a mutation in the *PAX6* gene or a regulatory region that controls the protein's expression. Syndromic aniridia is known as WAGR syndrome (Wilm's tumor, Aniridia, Genitourinary defects, and Retardation) and is associated with deletions at the 11p13 chromosome resulting in the deletion of the *PAX6* locus and the neighboring *WT1* locus [45].

Seventy percent of isolated aniridia cases are familial (f), and the mutation is inherited in

an autosomal dominant manner from an affected parent. The remaining thirty percent of cases are sporadic (s), or de novo, gene mutations [46]. Due to the environmental influence on the developing eye, even familial aniridia patients with the same mutation can display varying phenotypes [47].

1.4 Purpose of the Study

Two-thirds of patients diagnosed with aniridia have heterozygous loss-of-function mutations in the PAX6 protein. Sequence analysis of the *PAX6* gene and its regulatory elements can be used to identify the causal mutation for isolated aniridia [48]. The Lauderdale lab conducted a PAX6 mutational screen of patients in Aniridia Foundation International. The identified mutations were compiled with the Human PAX6 Allelic Variant Database to create a total of 565 independently ascertained variants in PAX6, 525 of which are associated with ocular disorders. To gain a better understanding of PAX6 and aniridia, we documented the frequency of mutation types, phenotypes associated with different mutations types, and hotspots throughout the coding region. Through mutational studies that determine the function of the mutated protein, insight can be made about the complex nature of PAX6.

CHAPTER 2 METHODS

2.1 Patients

The DNA samples were collected at the 2007 and 2009 Aniridia Foundation International Conference held in Memphis and Chicago, respectively. The PAX6 sequence analysis was performed by the Hamilton Eye Institute at the University of Memphis in Memphis, Tennessee. All participants gave informed consent, and the study protocol followed the tenets set by the Declaration of Helsinki. The phenotype of each individual was documented through pictures of the oculus sinister (left) and dexter (right) eye, as well as foveal pictures.

2.2 Mutation Detection

Figure 6 shows the flow chart method used to screen for PAX6 mutations. The blood tissue of the 157 individuals was collected (10mL for adults and 2mL for infants). Their genomic DNA (gDNA) was isolated and aliquoted in EDTA using whole genome amplification. The coding and noncoding exons of the PAX6 gene (exons 1-13), the alternatively spliced exon 5a, and splice junctions were directly sequenced and analyzed. All sequences were bidirectional and will be confirmed by repeat PCR. The mutation is reported in the new nomenclature system recommended by the Human Genome Variation Society [49].

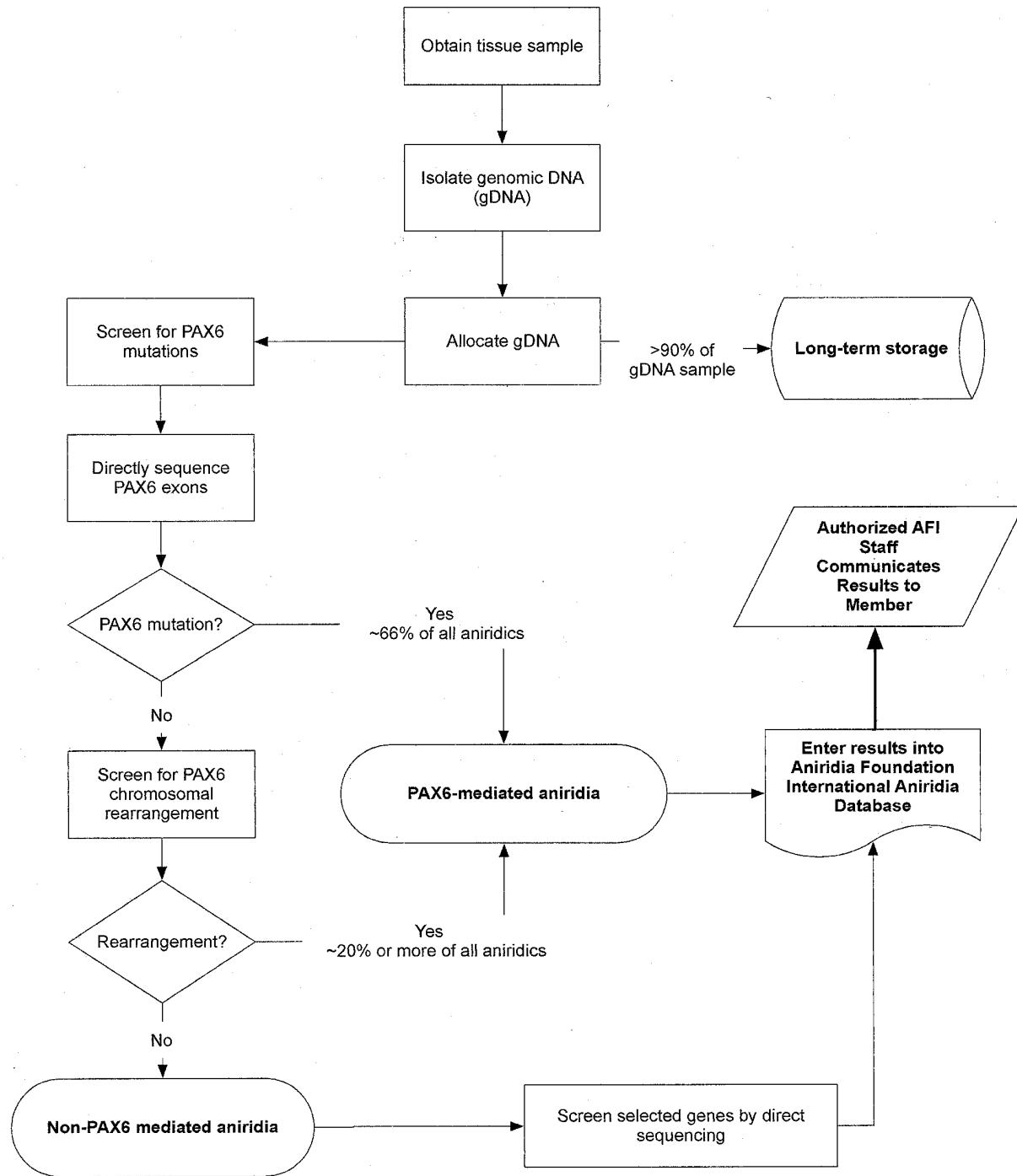


Figure 6: Aniridia Foundation International Mutational Screen. (Lauderdale 2010)

Direct gene sequencing is achieved first through polymerase chain reaction (PCR) amplification. PCR is the thermal cycling of denaturing, annealing, and elongating used to produce millions of copies of a short DNA strand. The denaturing step is achieved by raising the blood sample temperature to 94-98°C, which will break the hydrogen bonds in the double helix and generate two single DNA strands. Next, the temperature is lowered to 50-65°C to allow for primer annealing. Two primers were prepared to complimentary base pair to the 3' end of the sense (forward) and anti-sense (reverse) strand of each desired DNA region. The final step of elongation was achieved by raising the temperatures back to 72°C, an optimal thermal environment for Taq polymerase DNA replication. The polymerase used the primers as a starting point and continued elongating in the 5' to 3' direction a new complementary strand through the addition of either normal deoxynucleotides (dNTPs) or fluorescently labeled dideoxynucleotides (ddNTPs). Dideoxynucleotides will arrest chain elongation by eliminating the 3' hydroxyl reducing end of the nascent strand, thereby preventing further condensation elongation reactions [45].

Through this cycling, different sizes of fluorescently tagged DNA strands are created. The labeled strands are assorted according to length by being placed in a gel matrix. Since shorter strands will travel through the gel faster they will be detected sooner, creating a direct relationship between the length of the DNA strand and the time of detection. The DNA sequencing machine will read the fluorescent wavelengths at the 3' end of each fragment as the strands pass through the gel. Sequencing data is recorded electronically with colored peaks associated with the corresponding nucleotide [45].

The following figures show real patient data with a nonsense and frame-shift mutation.

The sequencer records both alleles of the patient's DNA and since isolated aniridia is caused by a heterozygous mutation, one of the alleles is wild type for PAX6 and the other is the mutant deleterious allele. For example, in **Figure 7A** the single peaks represents homozygous alleles encoding the same nucleotide, whereas the two peaks marked by the arrow show a heterozygous read of an adenine (A) and a thymine (T). Using tools such as the NCBI Basic Local Alignment Search Tool (BLAST), the correct wild type nucleotide can be determined to be an adenine at this point mutation. Therefore, the mutated allele has an A>T substitution that creates a nonsense mutation in Exon 5.

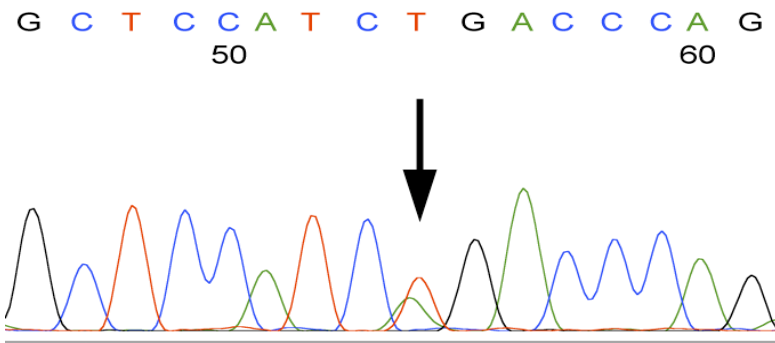


Figure 7A: Electrical recording of a nonsense mutation
c.199A>T; Ex6 (PD); p.Arg67X

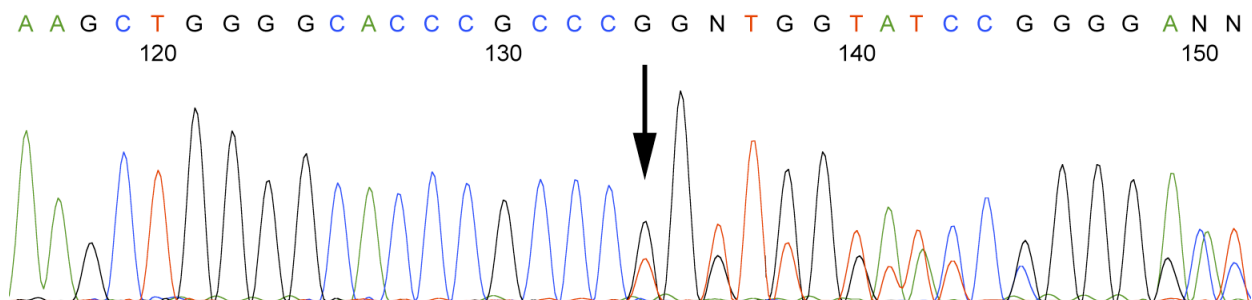


Figure 7B: Electrical recording of a frame-shift mutation
c.480delT; Ex7 (LNK); p.Gly161ValfsX66

CHAPTER 3 RESULTS

3.1 Aniridia Foundation International *PAX6* Screen

One-hundred-and-fifty-seven-individuals, representing 58 families, participated in this study. Eighty-one of the participants were diagnosed with aniridia and the remaining 87 participants were unaffected family members used for mutation confirmation. Of the 58 families, a total of 38 independent *PAX6* mutations were identified, including 20 novel mutations (**Table 2**).

Table 2: The 38 Independent *PAX6* mutations identified in the Aniridia Foundation International screen.

The source ID column is the numeric number given to each of the patients in our study, as well as indicated if their aniridia is sporadic (s) or familial (f). Note that if a mutation was identified in more than one family, it has only been listed once. However, the number of affected families is written in brackets in the Source ID column. In addition, the number of times the mutation has been independently reported in the *PAX6* Allelic Variant Database is in brackets in the DNA change column.

Source ID	Exon	Location	Domain	DNA change	Predicted Protein Change	Mutation Type
s-118	05	Exon	PD	c.57del1 (G) [novel]	p.Pro20HisfsX11	Frame shift; PTC in Ex5
f-108	05	Exon	PD	c.63_70del8 (GCCGGACT) [novel]	p.Pro22HisfsX31	Frame shift; PTC in Ex6
s-133	05	Exon	PD	c.112del1 (C) [reported 4 times]	p.Arg38GlyfsX16	Frame shift; PTC in Ex6
s-101	05	Exon	PD	c.112_116del5 (CGGCC)	p.Arg38ValfsX16	Frame shift; PTC in Ex6

				[novel]		
s-135	05	Exon	PD	c.123_126ins4 (GCGG) [novel]	p.Asp41GlufsX16	Frame shift; PTC in Ex6
s-137	05	Exon	PD	c.128C>A [novel]	p.Ser43Tyr	Transversion missense affects invariant Ser in the 3rd alpha helix of the N-terminal PD
s-138	05	Intron	PD	c.141+1G>T [novel]		Possible splice defect; transversion.
s-152	05	Intron	PD	c.141+2T>C [reported 2 times]		Splice defect. Transition
f-151, s-150	06	Exon	PD	c.179_185delATACGA insCTGAT [novel]	p.Tyr60SerfsX19	Frame shift; PTC in Ex6
f-113, f-112, f-114, f-128	06	Exon	PD	c. 199A>T [reported 3 times]	p.Arg67X	Transversion nonsense
f-146, f-147, f-148, f-149,	06	Exon	PD	c.204del1 (C) [novel]	p.Arg69GlyfsX10	Frame shift; PTC in Ex6
s-155	06	Exon	PD	c.332insG [novel]	p.Val111GlyfsX5	Frame-shift; PTC in Ex6
s-163	06		PD	c. 352_357+2del CCAAGC/gt [novel]	p.Pro117LysfsX93??	Possible in-frame deletion or possible splice mutation, deletion of donor codon (Further RNA analysis needed)
s-100, s-129 [2 families]	06	Intron	PD	c.357+1G>A [reported 9 times]	p.84_119del ??	Possible splice defect; Transition at a CpG mutable hotspot. G>A. (By analogy with a RT-PCR test run on another patient with the same mutation, probably activates

						cryptic splice site in exon 6, creating an in-frame deletion of last 36 amino acids of exon 6)
u-105	06	Intron	PD	c.357+1G>T [reported 2 times]	p.84_119del ??	Possible splice defect; Transversion at a CpG mutable hotspot. G>T (By analogy with other splice junction mutation, may use cryptic splice site in exon 6, creating an in-frame deletion of last 36 amino acids of exon 6)
s-169	06	Intron	PD	c.358-3_361delCAGG TGT [novel]		Splice Junction mutation. Possibly causes a PTC in Ex. 7 or abolishes the start of Ex. 7. Patient shows partial iris retention, typical of a splice junction mutation.
s-168	07	Exon	LNK	c. 365 C>A [reported 1 time]	p. Ser122X	Transversion nonsense
s-141 f-140	07	Exon	LNK	c.401delA [novel]	p.Lys134SerfsX13	Frame-shift deletion, PTC in Ex. 7
s-126	07	Exon	LNK	c. 467G>A [reported 1 time]	p.Try156X	Transition nonsense
s-127	07	Exon	LNK	c.480delT [novel]	p.Gly161ValfsX66	Frame-shift deletion. PTC in Ex8
s-117	08	Exon	LNK	c.524-? [novel]		~111 bp deletion of 5' end (uncertain b/c starts w/ primer and extends through splice junction)
s-102, s-160 [2 families]	08	Exon	LNK	c.607C>T [reported 23 times]	p.Arg203X	Transition nonsense at hypermutable CpG location

s-124	10	Exon	HD	c.771delG [novel]	p.Try257Cysfs X15	Frame-shift deletion, PTC in Ex10
f-144, f-167, s-164 [2 famili es]	10	Exon	HD	c.781C>T [reported 15 times]	p.Arg261X	Transition nonsense at hypermutable CpG location
s-110	10	Exon	HD	c.794G>A [reported 1 time]	p.Try265X	Transition nonsense
s-103	10	Exon	HD	c.795_799del5 (GAGAA) [novel]	p.Glu268Lysfs X13	Frame-shift deletion; PTC in Ex10
f-120, s-153, f-154 [2 famili es]	11	Exon	PST	c.949C>T [reported 25 times]	p.Arg317X	Transition nonsense at hypermutable CpG
f-130, f-170 (no pic)	11	Intron	PST	c.1032+6T>G [novel]		Transversion Splice Junction mutation
s-122	11	Intron	PST	c.1033-2A>T [reported 4 times]		Transversion Splice Junction mutation
s-143	12	Exon	PST	c.1160-1165del6 (GCACCT), ins6(TTCAAC) c.1172- 1183+5del17 [novel]	Unknown, 2 types of mutations detected. Could be an in-frame deletion of Ex12 or a splice junction mutation.	Predicted out-of- frame translation into 3'UTR. Protein with abnormal C- terminus predicted
f-123	12	Intron	PST	c.1183+5G>A [reported 1 time]		Transition Splice Junction mutation
s-119	12	Intron	PST	c.1183+1G>A [novel]	p.Pro345Aspfse xtX64??	Transition Splice Junction mutation, possible frame-shift deletion of Ex. 12 that could cause a termination in 3'UTR

	13	Exon	PST	c.1256del1 (C)	p.Pro419GlnfsExtX106	Frame shift extension; Termination in 3'UTR, C-terminal extension of 101 AA
s-115	13	Exon	PST	c.1267dupT [reported 20 times] or c.1268A>T [reported 4 times] Difficulty in sequencing this region	p.X423LeuextX*108 if dupT or p.X423LeuextX*15 A>T	Run-On mutation

3.2 The PAX6 Allelic Variant Database

The results from the mutational screen were compiled with the Human PAX6 Allelic Variant Database to create a total of 565 independently ascertained variants of PAX6. Of these variants, 525 are associated with ocular malformations and 459 are specifically causal for isolated aniridia. The following tables show the exon-by-exon distribution of mutations in the PAX6 Allelic Variant Database. **Table 3** shows the exon-by-exon distribution of pathological ocular mutations, **Table 4** shows mutations that cause aniridia, and **Table 5** shows mutations that cause phenotypes other than aniridia. **Figure 9** shows the distribution of mutation types in PAX6 Allelic Variant Database. **Figure 9A** shows mutations of all ocular abnormal phenotypes, **Figure 9B** shows mutations causing aniridia, and **Figure 9C** shows mutations causing non-aniridia phenotypes.

Table 3: Exon-by-exon distribution of 525 ocular disease-associated mutations in the PAX6 Allelic Variant Database

All Ocular Disease Muations														
Exon	2	3	4	5	5a	6	7	8	9	10	11	12	13	
Mutation Type														Total
Nonsense				10		18	18	36	38	30	27	3		180
Splicing	4	5	4	13		26	2	5	5	5	11	6		86
Frame-shift indel	1	1	2	29		37	28	6	5	14	6	8		137
In-frame indel			1	3		2								6
Missense			4	26	6	22	5	3	3	2		7	2	80
Frame-shift extension												6	4	10
Run-On													26	26
Total	5	6	11	81	6	105	53	50	51	51	44	30	32	525

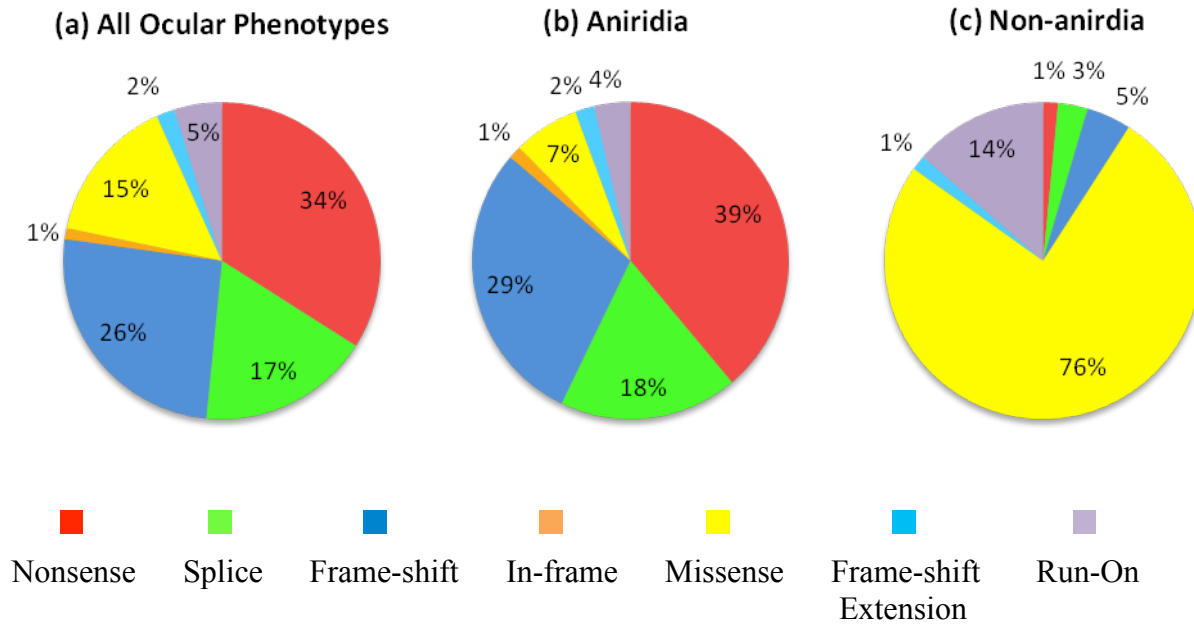
Table 4: Exon-by-exon distribution of 459 *PAX6* mutations that are causal for aniridia

Mutations Casual for Aniridia														
Exon	2	3	4	5	5a	6	7	8	9	10	11	12	13	
Mutation Type														Total
Nonsense				10		18	18	35	38	30	27	3		179
Splicing	3	5	4	13		26	2	5	5	4	11	6		84
Frame-shift indel	1	1	2	28		37	28	6	5	13	6	7		134
In-frame indel			1	3		2								6
Missense			3	11		8		3	2			2	1	30
Frame-shift extension												6	3	9
Run-On													17	17
Total	4	6	10	65		91	48	49	50	47	44	24	21	459

Table 5: Exon-by-exon distributions of 66 *PAX6* mutations that cause phenotypes other than aniridia

Non-Aniridia Mutations														
Exon	2	3	4	5	5a	6	7	8	9	10	11	12	13	
Mutation Type														Total
Nonsense								1						1
Splicing	1									1				2
Frame-shift indel				1						1		1		3
In-frame indel														
Missense			1	15	6	14	5		1	2		5	1	50
Frame-shift extension													1	1
Run-On													9	9
Total	1		1	16	6	14	5	1	1	4		6	11	66

Figure 9: Distribution of different mutation types in the PAX6 Allelic Variant Database. **A)** Distribution of 525 mutation causal for all ocular phenotypes. **B)** Distribution of 469 mutations directly casual for isolated aniridia. **C)** Distribution of mutation type casual for a non-aniridia phenotype: microphthalmia, optic nerve defects, and foveal hypoplasia.



3.2.1 Mutation Hotspots in the PAX6 coding region

Of the 459 mutations causal for aniridia, 449 fall within the protein coding region. **Figure 10**, on the following page shows the spread of the 449 aniridia mutations within exonic regions of PAX6. In addition, **Figure 11** shows a close up of all missense and in-frame mutations throughout the coding region, and **Figure 12** shows a close up of these mutations in just the paired domain.

Figure 10: Aniridic Mutations in the PAX6 Coding Regions

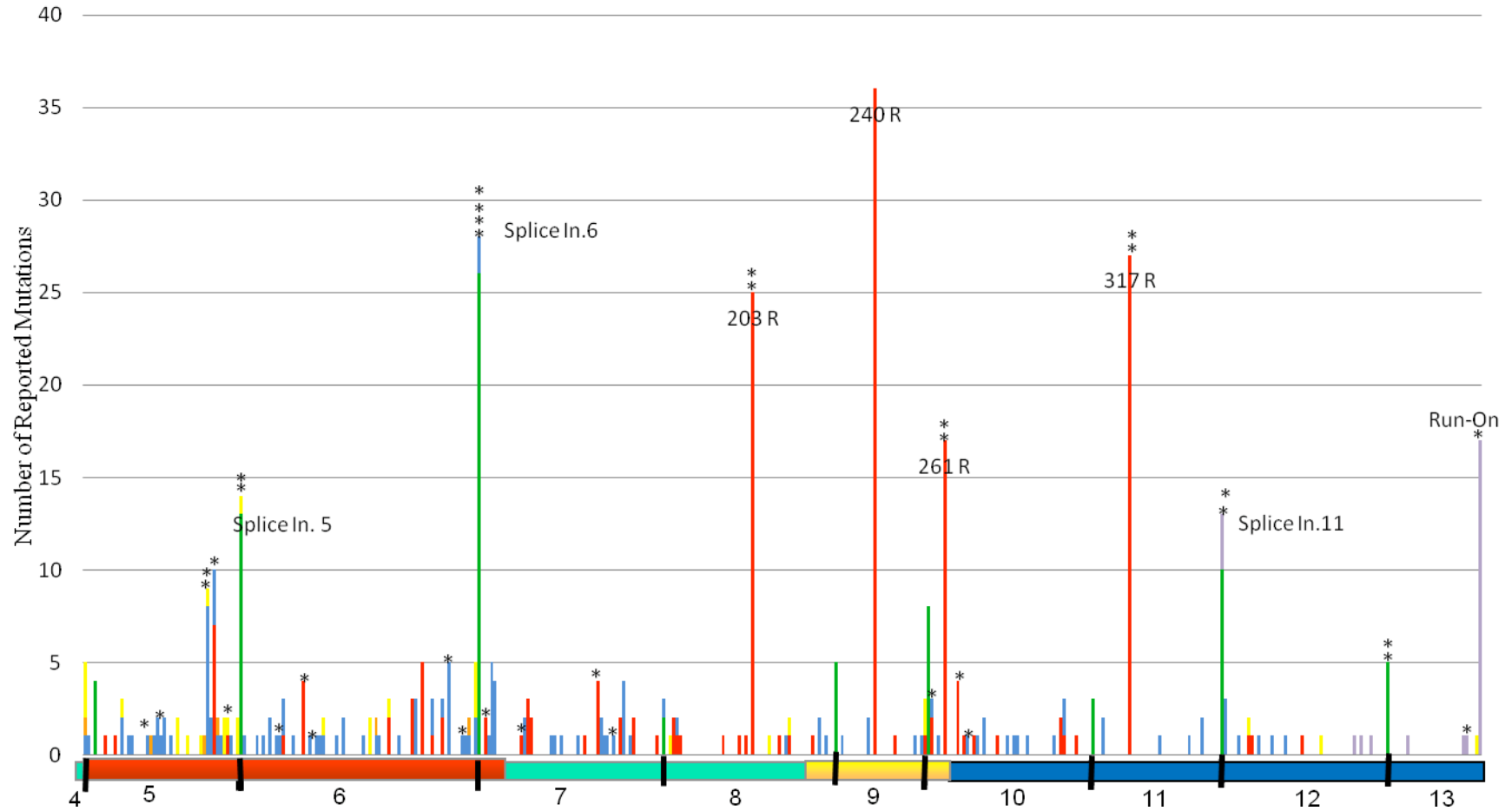


Figure 10: The 449 mutations that are casual for aniridia within the canonical PAX6 coding region. The asterisks (*) mark the mutations found in our Aniridia Foundation International cohort. Note the amino acids with a largest reported mutation rate are labeled. The highly mutable CpG sites of exon 8,9, 10, and 11 (R203 R240, R261, and R317) are the major source of nonsense mutations (red). In addition, the most common splice junction mutation (green) occurs between exon 6 and 7.

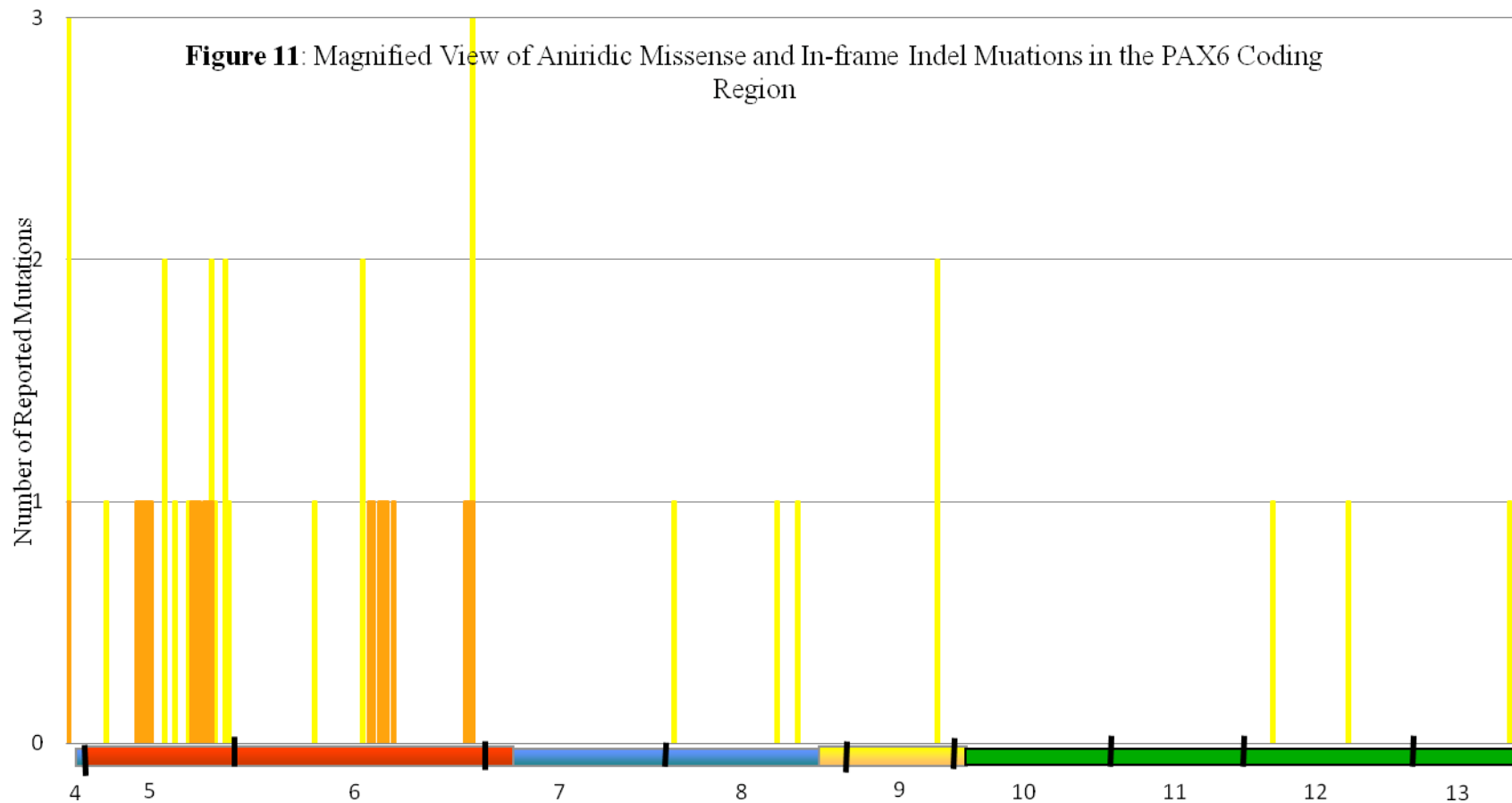


Figure 11: This is a close up view of the 30 missense (yellow) and 6 inframe deletion mutations (orange) in the canonical PAX6 coding region that are casual for aniridia. Note the change in scale used for the Number of Reported Mutations (y-axis) from Figure 10. Both missense and inframe deletions are rarely reported to cause aniridia, and typically result in a less severe phenotype. Notice that the majority of aniridia causing missense (63%) and all the inframe deletion (100%) mutations are located in the highly conserved paired domain (shown in red).

Figure 12: Magnified View of Aniridic Missense and In-frame Indel Mutations in the PAX6 Paired Domain

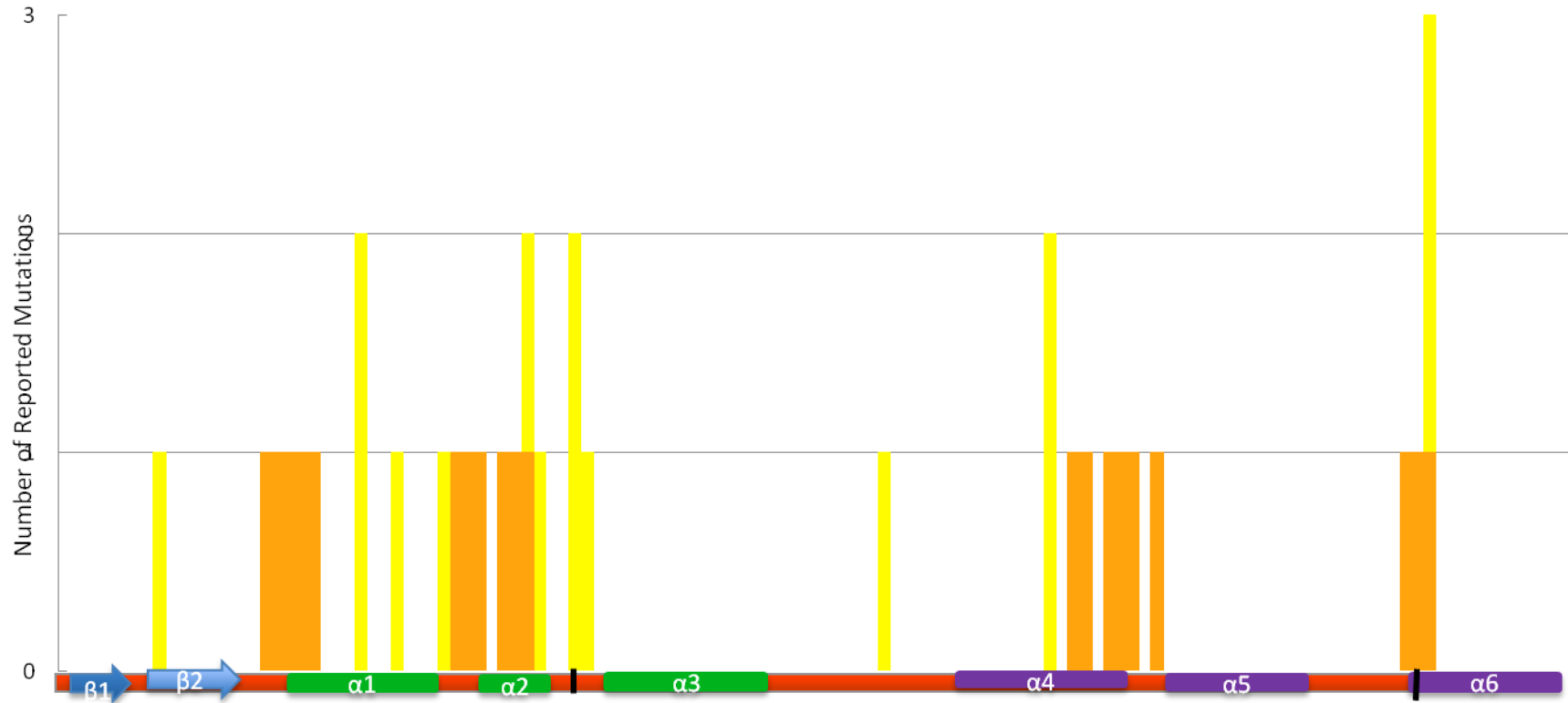


Figure 12: Close up view of the 19 missense (yellow) and 6 inframe deletion (orange) mutations in the bipartite paired domain that are casual for aniridia. N-terminal subdomain consists of two β turns and three α helices and the C-terminal subdomain consists of three more α helices. The three α helices of each subdomain are predicted to form a helix-loop-helix binding domain with the minor and major DNA grooves. Most of these mutations disrupt invariant amino acids that have been conserved over millions of years of evolutionary time, and are likely to be essential for normal PAX6 function. Note that the figure shows all the amino acids that are deleted in an inframe deletion.

CHAPTER 4 DISCUSSION

4.1 Analysis of the Aniridia Foundation International *PAX6* Screen

A deleterious variation was not found in all aniridic individuals in our study. Out of 58 surveyed aniridic families, only 38 mutations were identified in *PAX6* (roughly 65.5%). Although the detection rate is roughly two-thirds, as to be expected, a slightly lower detection rate could be due to mutations that lie in the regulatory regions of *PAX6*, as our focus was only on the exons 1 – 13 (including 5a). In addition, as clear from our results, we experienced a technical pitfall in screening exon 9. After resequencing with the new primer sets, we expect to add to our list of deleterious mutations, considering exon 9 is the home of 240R, the amino acid with the highest mutation rate in the *PAX6* Variant Database.

4.2 Frequency of Mutation Types Causal for Aniridia

Of the 525 ocular pathological mutations, 449 (87.4%) are associated with aniridia and 66 (12.6%) are associated with other phenotypes, including microphthalmia, optic nerve defects, and foveal hypoplasia. This data shows that the majority of non-aniridia phenotypes are caused by missense mutations (76%), whereas the aniridia phenotype is predominantly caused by nonsense (39%), frame-shift indel (29%), and splice junction (18%) mutations. The proportion of missense mutations has decreased from 15% of all cases to 7% of aniridia cases, while mutations

that introduce a premature stop codon (nonsense, splicing, and frame-shift mutations) have increased from 77% of all cases to 86% of aniridia cases.

4.3 Mutation Hotspots in the PAX6 coding region

As **Figure 10** indicates there are several residues in the open reading frame that are highly susceptible to mutation. The location of these hotspots may be explained by the distribution of CpG dinucleotides across the *PAX6* open reading frame, a structure known for its high mutability in the human genome [50]. Single nucleotide base changes at a CpG can give rise to either a TpG or a CpA, and result in either a missense, nonsense, or neutral polymorphism. The *PAX6* gene contains a total of 45 CpG dinucleotides in the open reading frame. Currently, single nucleotide polymorphisms (SNPs) have been at reported 26 of the total 45 CpG dinucleotides (58%).

Even though they have the same probability of hypermutation, it is no surprise that the remaining 19 CpG dinucleotides go unreported because a transition or transversion at these CpG locations is likely to cause either a nondeleterious missense or neutral polymorphism. This same ascertained bias explains why nonsense mutations make up the largest percentage of reported variants in the *PAX6* database [42].

In addition, this bias implies that 26 reported polymorphisms at the CpG dinucleotides sites are more likely to cause a deleterious mutation. For example, CpG locations that create an arginine CGA codon are the most highly reported mutations because sense-strand deamination of CGA would cause a CGA>TGA change, thus creating a nonsense mutation. The *PAX6* open-reading frame contains six CGA codons, all of which have been mutated at least once to give a

nonsense mutation. As **Figure 10** clearly shows, the CGA sites in exon 8, 9, 10, 11 (R203X, R240X, R261X, and R317X) are the major source of nonsense mutations. The CpGs in the last third of the *PAX6* open reading frame tend to be highly methylated and thus more inclined to undergo spontaneous 5'-cytosine deamination, causing a C>T transition [50]. These four CGA locations together account for a total of 105 variants in the database, all of which cause aniridia. Together they make up 22.9% of aniridia causing mutations, and 58.3% of total nonsense mutations.

Another significant hotspot indicated in **Figure 10** is the 3' splice junction site of Exon 6. Again, this hotspot can be explained through the hypermutability of CpG dinucleotides. This is the only splice junction site throughout the *PAX6* coding region that has a CpG dinucleotide. Cytosine is the last coding base of Exon 6 (c.357) and guanine is the first base of intron 6 (c.357+1). A total of 20 mutations have been reported in this CpG (c.371/357+1) location. All of these mutations are causal for aniridia, three are missense transversion mutations in c.371 and 17 are splice junction mutations in c.357+1. This CpG site contributes 20 of the 26 total splice junction mutations in between exon 6 and 7, which accounts for 30.2% of all splice junction mutations.

4.4 Aniridic Mutations: Theories of Molecular Mechanisms of Mutated *PAX6* Protein

The aniridia phenotype is shown to result from mutations in all seven categories. However, the different mutation types have different effects on the protein. The variation in phenotypes (**Figure 9**) caused by the different mutations in the *PAX6* gene can often be defined

by the different molecular mechanisms of the mutated protein. The seven mutations are group together based on predicted effect on the PAX6 transcription factor.

4.4.1 Nonsense and Frame-Shift Indel Mutations

Nonsense and frame-shift indel mutations, which introduce a premature termination codon (PTC) into the open reading frame, account for 86% of aniridic mutations. It is predicted that nonsense mediated decay (NMD) acts on these truncated mRNA strands and causes complete loss-of-function of the mutant allele and therefore haploinsufficiency of the PAX6 protein. NMD is a fairly recently discovered mRNA surveillance system that degrades mRNA transcripts with premature termination codons (PTCs) to prevent translation of truncated proteins that could cause deleterious dominant-negative or gain-of-function effects [51-52].

These mutation types almost always result in the classic aniridia phenotype (shown in **Figure 13A**). In fact, of the 180 reported nonsense mutations, all but one is causal for aniridia. (This one rare case has a nonsense mutation in exon 8 (p.Q205X) and displays severe bilateral optic nerve hypoplasia [53]). Likewise, of the 137 reported frame-shift indel mutations, all but three are associated with the aniridia phenotype. These three reported non-aniridic frame-shift mutations fall in exon 5, 10, and 12 and have closely related phenotypes such as partial aniridia and iris hypoplasia.

4.4.2 Splice Junction Mutations

Of the 86 reported splice junction mutations, only two are not associated with aniridia. However, the exact effect a splice junction mutation has on PAX6 is varies. It is possible that a

PTC could result and the mRNA transcript is degraded through NMD, similar to nonsense and frame-shift indel mutations. However, this theory does not apply to all splice junction mutations. For example, some splice junction mutations will cause only a single exon to be skipped in the mature mRNA strand because the spliceosome cannot recognize the mutated splice junction site. Since these mutation types escape NMD surveillance, a different molecular mechanism could explain the phenotypic variability amongst these aniridics. The partial iris retention as seen in **Figure 13B** results from some splice junction mutations.

4.4.3 Frame-Shift Indel Extension and Run-On Mutations

Frame-shift extension and run-on mutations account for roughly 5.6% of aniridic mutations. Both a frame-shift indel extension, occurring in exon 12 or 13, and a run-on mutation in the stop codon will result in a C-terminal extension (CTE) of the PAX6 mRNA strand. A run-on or extension mutation that does not undergo NMD could cause a dominant negative or gain-of-function effect and thus result in a more severe phenotype as shown in **Figure 13C**. This atypical phenotype explains why 34.6% of individuals with a run-on mutation are not diagnosed with aniridia.

Figure 13A: Mutation resulting in a PTC (Specifically this patient had a nonsense mutation)



Figure 13B: Splice Junction mutation. Shows partial iris retention in the left peripheral.

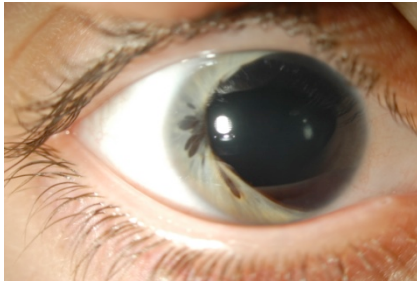
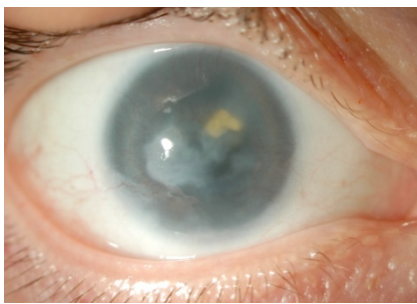


Figure 13C: C-terminal extension caused by a frame-shift mutation in exon 13 that abolished the stop codon.



4.4.4 Missense and In-frame Deletion Mutations

The phenotypes of missense and in-frame indel mutations are difficult to predict because the outcome depends on the activity of each specific mutant protein, which will vary from mutation to mutation depending on the location and function of the substituted or shifted amino acid residue. The phenotype of missense mutation carriers is normally milder than that of a loss-of-function mutation. Only a small percentage (8%) of aniridic cases are caused by non-loss-of-function mutations. Functional studies using these aniridic PAX6 missense or inframe indel mutations are useful in understanding the amino acids that are crucial for normal PAX6 function.

As the close up **Figure 11** indicates, many of these mutations are hypothesized to disrupt the start (1), stop (423), or a splice junction codon. Therefore, an aniridic phenotype is to be expected by these mutations due to failure to initiate translation, terminate translation, or piece

the protein together correctly. However, a large majority of these mutations populate the highly conserved DNA binding motifs, and therefore are predicted to disrupt an invariable DNA binding or transactivation domain.

Specifically, **Figure 12** indicates where these mutations fall within the highly conserved paired domain. The N-terminal subdomain consists of two β turns and three α helices and the C-terminal subdomain consists of three more α helices. The three α helices of each subdomain are predicted to form a helix-loop-helix binding domain with the minor and major DNA grooves. Most of these mutations disrupt invariant amino acids that have been conserved over millions of years of evolutionary time, and are likely to be essential for normal PAX6 function [54-55]. In fact of the 19 reported missense mutations reported in the paired domain 11 disrupt an invariant amino acid.

Interestingly, no missense or inframe indel mutations have been reported in alpha helices 3 and 5. Suggesting that mutations in these regions may not be as deleterious and therefore under represented [42].

4.5 Genotype – Phenotype Correlations

The expression pattern of the PAX6 gene indicates its multiple roles in ocular morphogenesis. Therefore, each PAX6 mutation can give rise to a variety of ocular disorders. Genotype-phenotype correlation must be carefully examined in order to determine the multiple functions of the PAX6 protein.

As discussed earlier, classical aniridia is caused by heterozygosity for a deficiency in the *PAX6* gene which results in a haploinsufficiency of the PAX6 transcription factor. During

development, a reduction in PAX6 dosage affects all three embryonic eye tissues: the surface ectoderm, the neuroectoderm, and the periocular mesenchyme [56]. Therefore, it is unclear which of the multiple interacting tissue components are directly and indirectly affected by reduction in PAX6 levels. Initial studies using Small-eye mice, which are heterozygous Pax6 mutant carriers ($Pax^{+/-}$) that serve as a model for human aniridia, indicated that lens induction is directly or cell-autonomously affected by haploinsufficiency of Pax6 and the resulting lens phenotype of $Pax^{+/-}$ mice caused further anterior ocular structure malformations [57-58]. This initial study indicated that the lens is directly affected by PAX6 deficiency and the remaining anterior ocular structures are indirectly affected.

However, in a recent study, tissue-specific deletion of one copy of Pax6 using the Cre/loxP system was conducted first in only the lens tissue and second in only the distal optic cup. As to be expected, *Pax6* heterozygosity in lens tissue mimicked the Small-eye mouse phenotype, with reduced lens size and adhesion between the lens and cornea. However, heterozygosity in only the lens mildly affected iris development. Interestingly, the second exclusive deletion of one Pax6 allele in only the distal optic cup mimicked most aspects of the Small-eye phenotype, without affecting the lens or the cornea. This study demonstrated for the first time that a considerable amount of the iris phenotype observed in Small-eye and human aniridia is due to the cell-autonomous dosage PAX6 dependency of the distal optic cup for normal iris development [59].

After eye development is complete, PAX6 continues to be expressed in multiple ocular structures including the lens, cornea, and retina. Therefore, PAX6 deficiency in many dosage dependent ocular structures that are involved in ocular homeostasis could explain the progressive

nature of aniridia. Specifically, the renewal of the cornea is impaired by PAX6 haploinsufficiency. The term aniridia-related keratopathy (ARK) is used to describe the progressive deterioration of the cornea observed in 90% of aniridic individuals [60-61]. Corneal changes in ARK include corneal vascularization, conjunctival invasion, and corneal epithelial abnormalities that will eventually lead to corneal clouding and vision loss.

The underlying mechanisms that lead to ARK are poorly understood. Traditionally, limbal epithelial stem cell (LESC) deficiency is the presumed pathogenic mechanism behind ARK [60, 62-64]. However, a definitive LESC marker has yet to be identified. Therefore, true limbal deficiency in aniridic individuals cannot be assumed. However, the expression of PAX6 in transient amplifying cells (TACs) suggests a pivotal role in corneal epithelium differentiation [37]. Moreover, additional studies suggest that the underlying causes of ARK could be multifactorial. Studies using heterozygous Pax6^{+/-} mice suggest that other factors in addition to LSCD could lead to corneal changes observed in the Sey-eye model including abnormally thin corneal development [65-66], abnormal epithelial migration [25], reduced corneal adhesion molecules [65], and abnormal wound-healing responses [67]. Further studies are needed to understand the exact pathophysiology of ARK and the etiology of aniridia as a whole.

CHAPTER 5 CONCLUSION

The *PAX6* gene is the most intensely study gene of the *PAX* family. The protein's highly conserved sequence throughout evolution, high homology in diverse organisms, and its ability to cause ocular tissue differentiation in ectopic locations has fascinated scientist for years. However, the exact molecular role of this protein is still uncertain. Through mutational studies that determine the fate of the mutated protein, insight can be made about the complex nature of *PAX6*.

This mutational screen found an additional 38 mutations causal for aniridia, 20 of which are novel mutations. Our data were congruent with previously published genotype-phenotype correlations and further validated molecular theories such as nonsense mediated decay. In addition, the *PAX6* Allelic Variant Database was updated. The previous comprehensive review was published five years ago when the database only contained 309 records. The current update includes 565 records, and is thus more valuable for further *PAX6* study.

Furthermore, the conducted genetic screen not only gives answers to aniridic individuals in our study, but also sheds light on possible treatment methods. Observing genotype variability in aniridics and understanding the ocular components susceptible to *PAX6* halpoinsufficiency provides important insight into the etiology of aniridia and further unveils the underlying complexity that leads to this multi-factorial human phenotype.

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