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# Pax6 3′ deletion results in aniridia, autism and mental retardation

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## Abstract

The *PAX6* gene is a transcription factor expressed early in development, predominantly in the eye, brain and gut. It is well known that mutations in *PAX6* may result in aniridia, Peter's anomaly and kertatisis. Here, we present mutation analysis of a patient with aniridia, autism and mental retardation. We identified and characterized a 1.3 Mb deletion that disrupts *PAX6* transcriptional activity and deletes additional genes expressed in the brain. Our findings provide continued evidence for the role of *PAX6* in neural phenotypes associated with aniridia.

## Introduction

The Paired box 6 (*PAX6*) gene encodes a transcription factor that is involved in several developmental pathways and is expressed early in the development of the eye, numerous regions of the brain, and the pancreas. The 22 Kb *PAX6* gene contains 14 exons, including an alternatively spliced exon 5a, and encodes a 422 aa protein. The Pax6 protein contains two DNA binding domains—a paired domain and a homeodomain—and one proline/serine/threonine-rich (PST) transactivation domain.

Mutations in *PAX6* primarily cause aniridia, though different types of mutations in *PAX6* lead to different ocular phenotypes. For example, while most *PAX6* nonsense mutations lead to aniridia (MIM 106210), many missense mutations result in Peter's anomaly (MIM 603807) (Azuma et al. 1996, 1998; Azuma and Yamada 1998; Azuma et al. (1999). It is thought that while nonsense mutations result in haploinsufficiency due to nonsense mediated decay (NMD), missense mutations may affect the ability of Pax6 to bind to specific targets thereby leading to differing phenotypes (Azuma et al. 2003; Chao et al. (2003; Hanson (2003).


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Additionally, *PAX6* is a key gene in a contiguous gene deletion syndrome termed 'WAGR', so named for 'Wilm's tumor, aniridia, genital or urinary tract abnormalities, and mental retardation/developmental delay' (MIM 194072). This syndrome is usually the result of a deletion encompassing all or part of *PAX6* and Wilm's tumor 1 (*WT-1*). However, with the development of more specific fluorescent in situ (FISH) probes it is apparent that smaller deletions in this region can result in WAGR syndrome. WAGR syndrome also varies in its presentation. It is thought that mutations and deletions in *WT-1* are likely responsible for the Wilm's tumor phenotype while mutations and deletions in *PAX6* are likely responsible for the aniridia phenotype (van Heyningen et al. 2007; Fischbach et al. (2005). It is still unclear which genes in the WAGR locus are responsible for the mental retardation phenotype associated with the syndrome.

However, beyond eye phenotypes, there is emerging evidence for the role of *PAX6* mutations in human behavioral and neurodevelopmental phenotypes. It is well known that *PAX6* influences development of the nervous system and brain through regulation of proneural genes such as neurogenin 2 (*Ngn2*) and achaete-scute complex homolog-like 1 (*Mash-1*) (van Heyningen and Williamson 2002; Scardigli et al. (2003). In human populations, recent studies have identified individuals with *PAX6* mutations who present only with mental retardation and aniridia (Malandrini et al. 2001; Ticho et al. (2006; Graziano et al. (2007). MRI studies of patients with aniridia and no obvious intellectual deficits have shown subtle brain abnormalities including a lack of the anterior commisure and pineal gland (Mitchell et al. 2003). The *Pax6* heterozygous mutant mouse, *small eye*, shows both ocular and neuronal phenotypes including an absent olfactory bulb, a decrease in cortical neurons and cortical plate thickness, as well as altered dorso-ventral patterning of the forebrain.

Here we describe molecular genetic characterization of a male with autism, moderate mental retardation, and familial aniridia. The genetic investigation of individual 3A was performed with both targeted fluorescent in situ hybridization probes and a high-density oligonucleotide microarray. These tests identified a deletion 3′ to *PAX6* that adds to the growing number of genetic abnormalities implicating *PAX6* not only in eye phenotypes, but also in neurodevelopmental and behavioral disorders such as autism.

## Phenotypic characterization

The patient was the 5 lb 12 oz result of a 37-week gestation. He is the only child of non-consanguineous parents (Fig. 1). His mother also has aniridia and a history of major depression, anxiety, and social awkwardness but was not diagnosed with autism. The mother's quality and quantity of social relationships is significantly diminished as reported by family members. She is currently estranged from her son and other members of her immediate family.

Pregnancy and birth history were unremarkable, though the patient was brought back to the hospital three days after birth because he had lost weight and had a decreased temperature. Bilateral complete aniridia was present from birth. As an infant he was described as colicky. He sat at 5 months, crawled at 8–9 months and walked at 12–13 months. He first spoke at 18 months and developed roughly a dozen words. His language regressed at age two and by 36 months he was nonverbal, was diagnosed with autism at the University of Washington, Seattle, and remains nonverbal to date.

His behaviors were and continue to be consistent with a diagnosis of autism. As a toddler and young child he displayed arm flapping, toe walking, spinning, showed little to no eye contact, and did not engage in pretend play. Instead he engaged in extensive ritualistic lining and organizing of toys and objects. He did not engage his peers or adults socially, though he was affectionate with his parents. He is now described as rigid and routine bound. He has periods of hyperactivity and constant vocalization, most often humming Christmas tunes and

inappropriate laughter. He currently takes Abilify (10 mg daily) for disruptive and self-injurious behavior. He is not described as aggressive towards others. He has severe compulsions including hair pulling, and is impulsive without regard to consequences. He takes Clonidine (0.2 mg nightly) and Trazodone (75 mg nightly) for severe sleep disturbance and sleeps an average of 6 h a night. He also displays a remarkably high pain tolerance, strong preference for soft and extremely spicy foods, and Pica behaviors. His intellectual development was significantly delayed. Behavior problems have prevented valid IQ testing, though adaptive behaviors as reported by teachers and parents are consistent with moderate mental retardation. He also presents with a sebaceous nevus on the scalp midline. The patient also underwent a brain MRI at the University of Iowa Hospitals and Clinics (UIHC) at age 13 years, which was unremarkable for gross cortical malformations.

## *PAX6* deletion detection

### Cytogenetic characterization in patient 3A

Cytogenetic testing was conducted through UIHC to determine the cause of the aniridia, developmental delay and autism in patient 3A. Karyotyping and fragile X testing were both unremarkable. Testing of the Prader-Willi/Angelman locus (15q11-13) for deletions and abnormal methylation was also normal. The patient was also evaluated for WAGR syndrome. Renal ultrasound at age 13 years was negative, and FISH analysis of the 11p12-14 WAGR region showed a deletion only of probe FO2121 ish del(11)(p13p13)(FO2121-), 11p13(FATx2, D11S324x2, WT-1x2). The location of the deleted probe is approximately 100 kb distal to the 3′ prime end of *PAX6*. This region has been shown to harbor regulatory elements for *PAX6* expression (Lauderdale et al. 2000; Kleinjan et al. 2006; Kim and Lauderdale 2006).

### Deletion detection in patient 3A

The deletion boundaries were further delineated using Affymetrix GeneChip® Human Mapping 250K Microarrays. DNA from patient 3A and his father 2A (mother was unavailable for testing), was hybridized according to the manufacturer's instructions to affymetrix *NspI* arrays, which contain probes for ~260,000 SNPs scattered across the genome. The assay uses 250 ng of genomic DNA digested with *NspI* and restriction enzyme (New England Biolabs, Boston, MA), ligated to an adaptor using T4 DNA ligase (New England Biolabs), and amplified by PCR using Titanium Taq (Clontech). PCR products were then purified from excess primer and salts by a DNA amplification cleanup kit (Clontech) and a 90 μ aliquot was fragmented using DNase I. An aliquot of the fragmented DNA was separated and visualized in a 3% agarose gel in 1X TBE buffer to ensure that the bulk of the product had been properly fragmented. The fragmented samples were end-labeled with biotin using terminal deoxynucleotidyl transferase before each sample was hybridized to the *NspI* arrays for 16 h at 49°C. After hybridization the arrays were washed and stained using an Affymetrix Fluidics Station 450. The most stringent wash was 0.6 × SSPE, 0.01% Tween-20 at 45°C, and the samples were stained with R-phycoerythrin (Molecular Probes). Imaging of the microarrays was performed using a GCS3000 (Affymetrix) high-resolution scanner. To detect genomic duplications and deletions, we used a publicly available program, CNAG, developed at The University of Tokyo (Nannya et al. 2005).

For patient 3A, analysis of the microarray data identified six putative CNVs (Table 1). The CNVs seen on chromosomes 10q11, 15q11 and 17q12 were present in the asymptomatic father and are known to be common copy number polymorphisms according to publicly available databases (Nannya et al. 2005;Conrad et al. 2006;Sebat et al. 2004). Of the remaining three CNVs, the two on chromosomes 4q21 and 11q21, have been identified in control populations and are listed in the Database of Genomic Variants (Iafrate et al. 2004). The 11p14.1-p13 deletion was novel and contained the *PAX6* enhancer elements (Fig. 2) and thus was the focus

of further characterization. We narrowed the deletion region by assessing heterozygosity of the genotypes generated from the SNP array. The 1.3 Mb deletion begins approximately 35 Kb distal to the last exon of *PAX6* (Fig. 3). The deletion includes the 3′ enhancer regions characterized by Lauderdale and Wilensky (2000). Additionally, the coding regions of *PAX6* were sequenced in the proband and his father to rule out the possibility of a compound heterozygous mutation. No additional mutations were identified.

## Follow-up molecular studies

A panel of 400 independent autism probands was screened for mutations in the last exon of *PAX6*, (the 14th exon including exon 5a) and the 3′UTR ($N = 200$) of *PAX6* using single strand conformation polymorphism (SSCP) (Sheffield et al. 1993). We chose to screen only the 14th exon and 3′UTR at this time because of the potential increase in liability toward autistic-like impairments associated with mutations toward the 3′ end of *PAX6*. We screened these regions using amplicons less than 250 bp in length. PCR products were electrophoresed on 6% nondenaturing polyacrylamide gels at 20W for approximately 3 h at room temperature while being cooled by a fan. The gels were then treated with silver nitrate to visualize the amplified DNA fragments. Any amplicons showing SSCP shifts were then forward and reverse sequenced to determine if a base pair change had occurred. The sequence data were analyzed using the Sequencher gene analysis computer program (Gene Codes, Ann Arbor, MI). We identified only one novel SNP (3′UTR + 314[T/C]) in the 3′UTR that did not segregate with autism in the affected family and is likely a rare polymorphism.

## Discussion

We thus report a deletion of the 3′ region of *PAX6* that is causing aniridia and, we believe, autism in the same individual. The mutation was likely inherited maternally, as this patient's mother also had sporadic aniridia and a lifelong history of anxiety, social awkwardness and depression that required at least one psychiatric hospitalization (per report of father).

Aniridia is almost exclusively caused by *PAX6* mutations, which include nonsense (37%); frame shift (23%); and splice site, missense, anti-termination mutations, and in-frame deletions or insertions (39%) (Hanson 2003). Nonsense mutations are presumed to produce truncated transcripts that activate NMD, resulting in haploinsffiency of *PAX6*. Missense mutations of *PAX6* are a less frequent cause of aniridia than expected, and while it is known that missense variants often result in different ocular conditions (e.g. keratisis), some have speculated that missense variants may also result in more severe neural phenotypes that are thus not enriched in the aniridia population (Tzoulaki et al. 2005). Based on earlier findings, it has been suggested that the 3′ end of the gene is of special interest in this vein for a number of reasons. First, aniridia-causing nonsense mutations of *PAX6* rarely occur near the 3′ end of the gene. While such variants may simply be less deleterious, it is also the case that mutations near the end of transcripts often escape NMD, and thus these mutations may produce more severe dominant negative phenotypes (Tzoulaki et al. 2005). In keeping with this, two previously cited *PAX6* anti-termination mutations that resulted in translation into the 3′ untranslated region produced autism or autism spectrum phenotypes in addition to aniridia (Chao et al. 2003; Heyman et al. 1999). Also, a de novo nonsense mutation in exon 10, which falls within the 3′ end of the gene containing the PST transactivation domain, was recently found in an individual with aniridia and mental retardation (Graziano et al. 2007). It was for this reason that the last exon of *PAX6* was screened for mutations in our sample of 400 autism probands. While none of the probands showed evidence of a mutation, our screening technology cannot detect deletions that extend the length of the amplicon, nor did any of the probands in this set have aniridia.

Our finding, while located near the 3′ end of *PAX6* likely results in haploinsufficieny as previously seen in similar deletions and rearrangements (Lauderdale et al. 2000; Fantes et al. 1995). Deletions in this region have been shown to abolish *PAX6* expression and cause aniridia in patients due to loss of enhancers and a downstream regulatory region (DRR) (Lauderdale et al. 2000; Crolla and van Heyningen 2002). These enhancers have shown compelling evidence of tissue specificity as well as cooperativity (Kleinjan et al. 2006; Kim and Lauderdale 2006). To our knowledge, ours is the first cryptic deletion of this kind in a patient with aniridia, autism and mental retardation. Figure 3 illustrates the locations of some of the emerging enhancer regions and reported patient breakpoints.

While the aniridia in our subject is almost certainly caused by the *PAX6* deletion, it is possible that his autism is due to a different genetic defect, or is caused by a combination of the *PAX6* deletion and deletion of one or more of the other genes in this region. Six other genes lie in the deleted region: metalophosphoesterase domain containing 2 (*MPPED2*), doublecortin domain containing 5 (*DCDC5*), doublecortin domain containing 1 (*DCDC1*), IMP1 inner mitochondrial membrane (*IMMP1L*), zinc finger CLS domain containing 3 (*DPH4*) and elongation factor protein 4 (*ELP4*). Very little is known about these genes. *MPPED2*, *DCDC1* and *DCDC5* are expressed in fetal brain but their specific function is unclear. *ELP4* is a ubiquitously expressed member of a complex of proteins that associates with histone acetyltransferases and RNA polymerase II to aid transcriptional elongation, while *IMMP1L* has peptidase activity and is localized to the mitochondria. Aside from *PAX6*, the doublecortin genes are the most compelling candidates for an autism/mental retardation susceptibility gene in this region. Mutations in doublecortin (*DCX*) are known to cause lissencephaly, a disorder of neuronal outgrowth that results in mental retardation (Kerjan and Gleeson 2007). However, we note that our subject had a clinically normal brain MRI and his autism is not likely due to unregulated neuronal outgrowth as in the case of *DCX* mutations, although it is possible that more subtle changes in brain morphology were not detected.

Further support for *PAX6* as the autism disease gene, however, comes from studies showing that a variety of cognitive and brain phenotypes are associated with *PAX6* mutations. Indeed Heyman et al. (1999) noted a peculiar behavioral phenotype that segregated with a *PAX6* mutation in a large, three-generation pedigree. Family members showed impulsivity, social ineptness, and disinhibition (Heyman et al. 1999). Subsequent fMRI studies of this family resulted in identification of both structural and functional brain abnormalities (Ellison-Wright et al. 2004). Additionally, children with *PAX6* anirida and no obvious cognitive delay were found to have difficulty processing auditory information despite normal audiograms (Bamiou et al. 2007a, b). These children also had significantly smaller corpus callosi and anterior commissures—structures that contain interhemispheric fibers—compared to children without *PAX6* mutations. It is also interesting to note that over half of this small sample of children with *PAX6* mutations also showed difficulty with understanding prosody and extrapolating meaning from spoken language (i.e., didn't “get a joke” as well as peers). This impairment is also often seen in children with Asperger Syndrome or high-functioning autism. Another recent report identified a missense mutation in exon 6 that segregated with aniridia and varying degrees of cognitive impairment and affective dyscontrol in 14 members of a large five generation pedigree (Dansault et al. 2007). Some affected family members also showed brain dysmorphology as assessed by MRI. Taken together, these impairments in auditory and language processing, cognition, and social perception show significant overlap with the core symptom domains of autism. While still tentative, the connection between aniridia, autism, and 11p14.1-p13 is reinforced by rates of autism in WAGR syndrome as high as 20–25% (Fischbach et al. 2005; Xu et al. 2007). Though these reports do not implicate *PAX6* specifically, they do offer a broader rational for the existence of an autism gene in the 11p14.1-p13 region and suggest that WAGR syndrome may also cause a rare syndromal form of autism, much like neurofibromatosis (Zafeiriou et al. 2007; Williams and Hersh 1998).

In conclusion, our study complements a growing body of work showing increased liability toward a spectrum of cognitive and behavioral abnormalities that include autism in individuals with *PAX6* mutations (Table 2). Additionally, our data and suggests that 11p14.1-p13 may harbor liability toward autism in a small number of cases. We suggest that *PAX6* should be prioritized as a candidate gene in this region and investigated more thoroughly. Additionally, the Pax6 downstream targets *Ngn2* and *Mash-1* should be considered as possible candidate genes for autism. This is not necessarily surprising given that many of the key regulatory genes that influence eye development, such as *PAX6*, are also involved in early neural development. Muscle-eye-brain disease, for example, is caused by mutations in O-mannose beta-1,2-N-acetylglucosaminyltransferase, while mutations in *CEP290* can cause both Leber's Congenital Amaurosis and Joubert Syndrome, which is characterized by autistic-like features (Cideciyan et al. 2007). It may in fact be the case that eye and brain phenotypes arising from dysfunction of the same gene will become recognized as a common occurrence.

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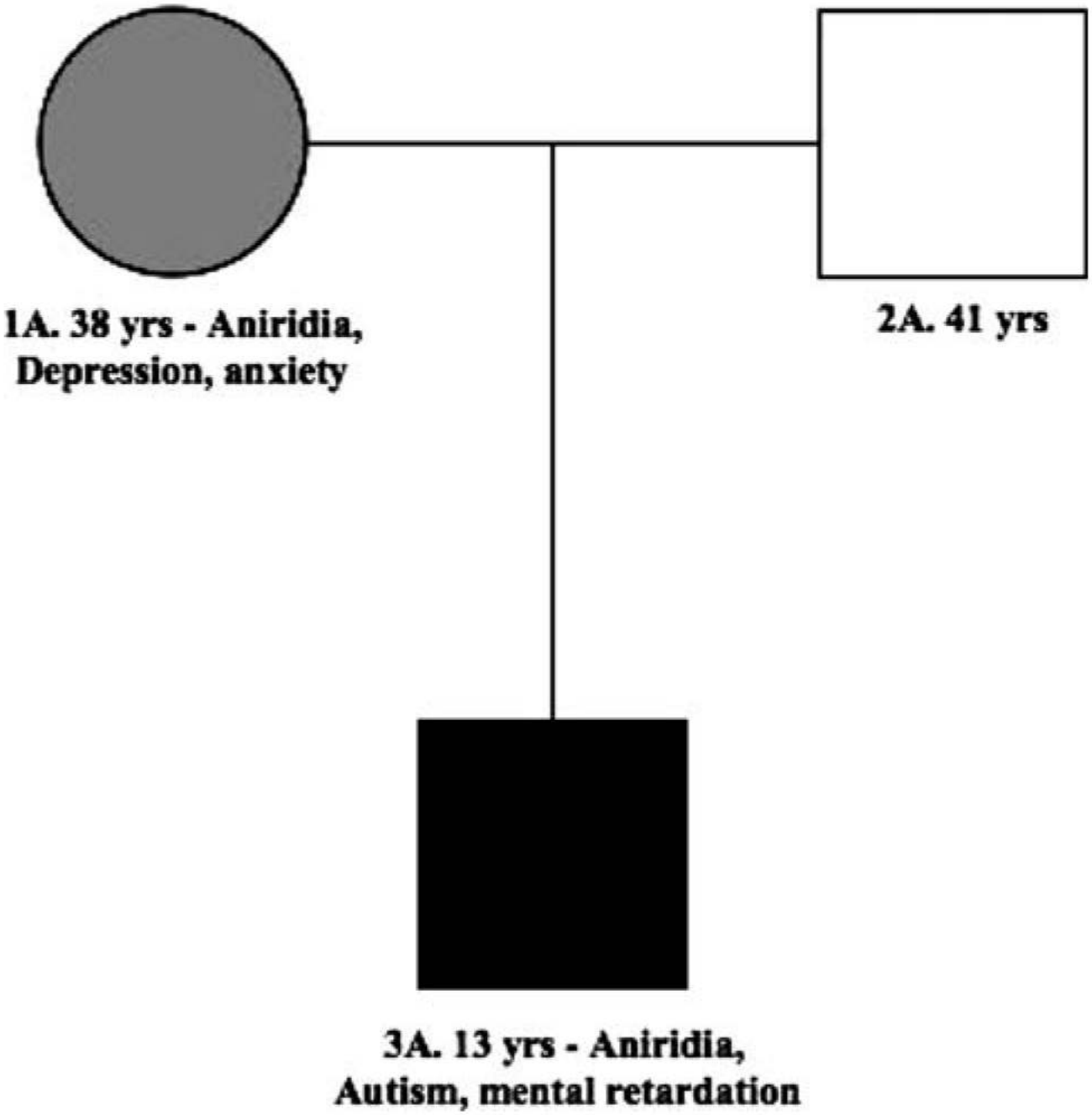


**Fig. 1.**
Pedigree of family A

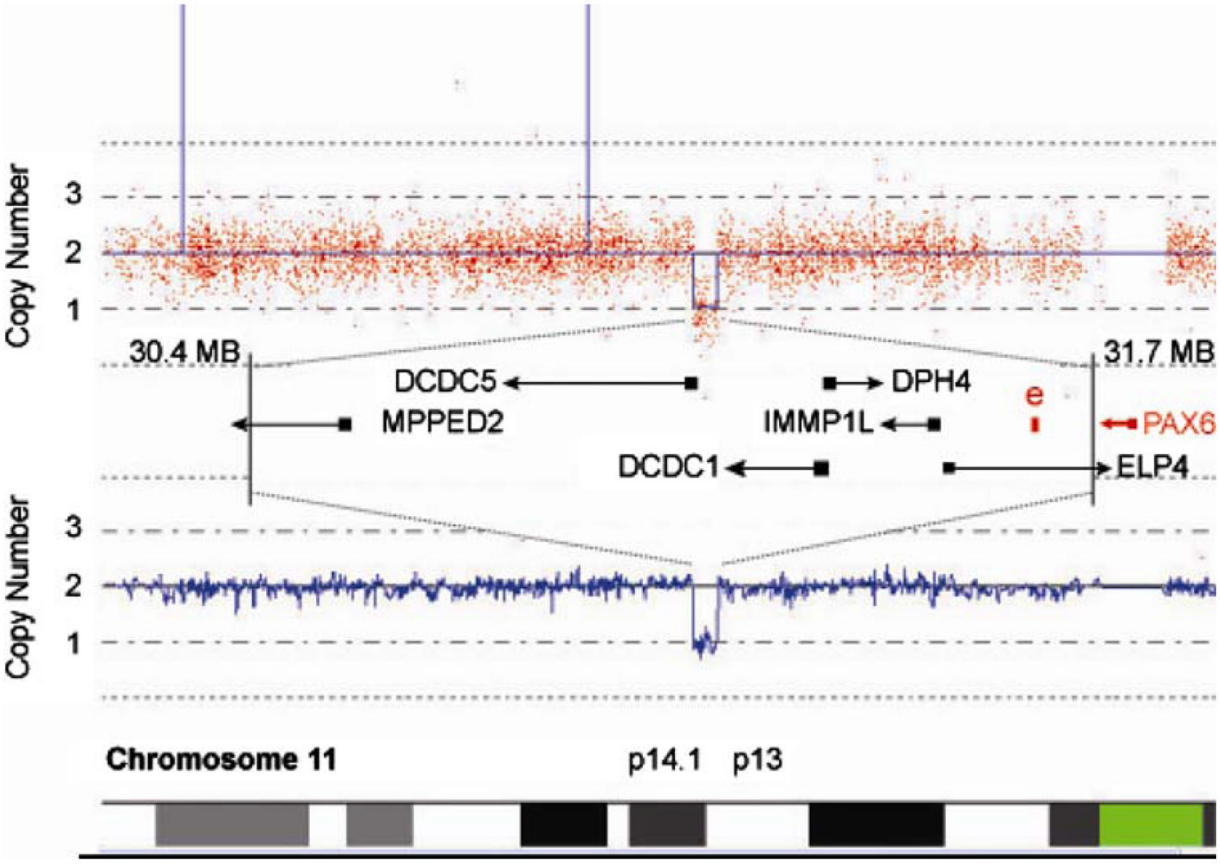


**Fig. 2.**

Deletion region in patient 3A. The deletion is 1.3 MB and contains enhancer elements ("e") for PAX6, as well as a number of other potential autism candidate genes. The dots in the upper part of the ideogram indicated individual signal intensity values for SNP probes, the line within the *dots* is a Hidden Markov Model prediction of copy number. The lower part of the CNAG ideogram is a smoothed statistical average of signal intensity. The CNAG output is scaled to the chromosome key below it, indicating the chromosome band of the deletion

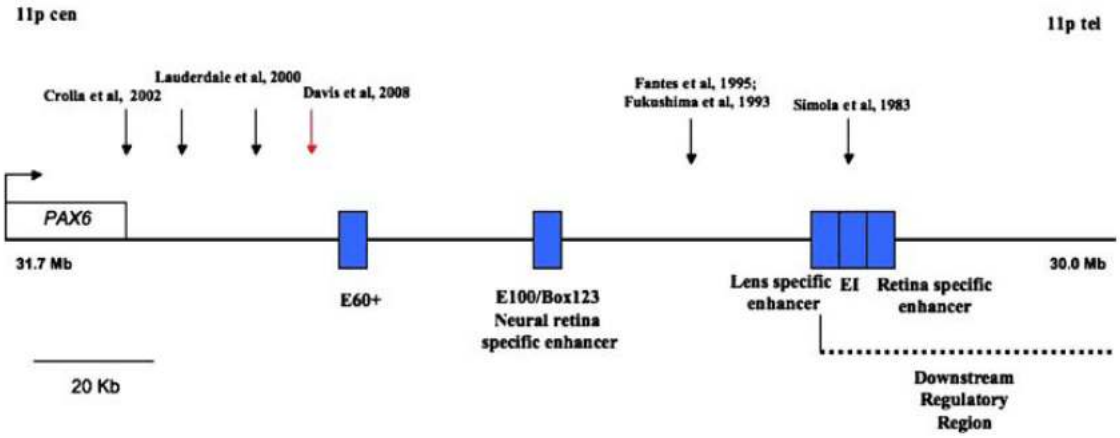


**Fig. 3.**

Deletion breakpoints in relation to some defined 3′ enhancer regions. *Arrowheads* represent patient deletion/rearrangement breakpoints. It should be noted that Crolla and van Heyningen (2002) identified more breakpoints than those depicted here, however this is the most proximal breakpoint that may leave PAX6 transcription unit in tact. Six children shared this breakpoint and all were under the age of 1 year when studied. The *boxes* represent enhancer regions (Griffin et al. 2002; Kleinjan et al. 2001, 2004, 2006; Kim and Lauderdale 2006). The downstream regulatory region is denoted with a *dashed line*. This region is critical to basal levels of *PAX6* transcription. *ELP4*, which is not shown, is located approximately 27 Kb telomeric to *PAX6*

**Table 1**

Copy number variations detected in the autism/aniridia proband

| Chromosome band | Type | Begin (bp position) | End (bp position) | Length (bp) | SNPs | Novel | Genes in Region |
|---|---|---|---|---|---|---|---|
| 4q21.21 | del | 81,829,520 | 82,069,628 | 240,108 | 23 | No | C4orf22 “provisional status” |
| 10q11.21 | dup | 45,392,878 | 47,987,627 | 2,594,749 | 27 | No | Many genes |
| 11p14.1-p13 | del | 30,404,574 | 31,758,933 | 1,354,359 | 125 | Yes | *PAX6* enhancer, *ELP4*, *DCDC1*, *DCDC5*, *IMMP1L* |
| 11q21 | dup | 96,187,042 | 96,195,684 | 8,642 | 11 | No | No genes |
| 15q11.2 | del | 18,427,103 | 20,329,239 | 1,902,136 | 66 | No | Many genes |
| 17q12 | del | 31,473,221 | 33,318,471 | 1,845,250 | 104 | No | Many genes |

Sequence locations are from NCBI Build 36.1. SNPs describes the number of SNPs deleted or duplicated across the interval. Novel describes whether CNVs have been previously identified in the interval in normal controls. Several publicly available databases were consulted to determine if a CNV was novel including UCSC Genome Browser “CNV track”, the database of genomic variants, and the autism-related chromosome rearrangement database

**Table 2**

Cases of *PAX6* mutations associated with developmental anomalies including autism, mental retardation and aggressive phenotypes (Brown et al. 1998)

| Number of patients | Mutation | Exon/intron location | Type of mutation | Ocular phenotype | Neural phenotype | References |
|---|---|---|---|---|---|---|
| 1 | IVS2 + 2T>A | Intron2 | Possible splice site error | Iris anomalies, foveal hypoplasia, | Mild learning disability | Ticho et al. (2006) |
| 1 | IVS2 + 9G>A | Intron2 | Pathogenicity not determined | Micropthalmia, microcornea, powdered cataract, nystagmus, scerocornea, keratisis | Cognitive impairment | Dansault et al. (2007) |
| 1 | c.492C>T | Exon 5 | R44X | Aniridia; Cataract; Glaucoma; Keratitis; Nystagmus | One individual in family has moderate mental retardation | Neuner-Jehle et al. (1998) |
| 1 | c.20T>A | Exon 5a | V7D | Cataract; Nystagmus; Peters' Anomaly | Adhesion of cervical bones 1 and 2. Vascular anomaly of the brain stem. | Azuma et al. (1999) |
| 1 | c.572T>C | Exon 5 | L46P | Micropthalmia, cataract, glaucoma, nystagmus | Cognitive impairment | Dansault et al. (2007) |
| 1 | c.622T>G | Exon 6 | I87R | Aniridia | Microcephaly, developmental delay | Tang et al. (1997) |
| 14 | c.655A>G | Exon 6 | S74G | Foveal Hypoplasia; Nystagmus, macular coloboma | 4 have epilepsy, 2 have mental retardation 8 have cognitive impairment with “abnormal emotivity” | Dansault et al. (2007) |
| 2 | c.719C>A | Exon 6 | S119R | Anirida | 2 family members have mild mental retardation, 1 with speech impairment, 1 with aggressive behaviors | Malandrini et al. (2001) |
| 1 | c.719C>A | Exon 6 | S119R | Aniridia | Mental retardation, behavioral anomalies | Williamson and van Heyningen, unpublished, Human PAX6 Allelic Variant Database |
| 1 | c.765C>T | Exon 7 | Q135X | Aniridia | Learning disabilities | Love et al. (1998) |
| 1 | c.1080C>T | Exon 10 | R240X | Aniridia | Mild developmental delay | Hever et al. (2006) |
| 1 | c.1133G>A | Exon 10 | W275X | Aniridia | Ataxia and mental retardation | Graziano et al. (2007) |

| Number of patients | Mutation | Exon/intron location | Type of mutation | Ocular phenotype | Neural phenotype | References |
|---|---|---|---|---|---|---|
| 1 | c.1135T>C | Exon 10 | F258S | Iris anomaly, coloboma of optic nerve, retina, and choroid | Mental retardation | Azuma et al. (2003) |
| 1 | c.1237G>T | Exon 10 | S292I | Optic nerve hypoplasia | Mental retardation | Azuma et al. (2003) |
| 5 | c.1615del10 | Exon 13 | Out of frame extensior | Aniridia | 5 family members with unusual neurobehavioral phenotype | Heyman et al. (1999) |
| 1 | c.1630T>A or c.1629insT | Exon 13 | Run-on mutation | Aniridia | Developmental delay, possible autism | Chao et al. (2003) |

Cases were identified in the Human *PAX6* allelic variant database and through a search of current literature (Ton et al. 1991). Base pair positions are numbered according to conventional *PAX6* cDNA numbering