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ANNUAL REVIEWS

Annual Review of Genomics and Human Genetics

A Journey Through Genetics to Biology

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Annu. Rev. Genom. Hum. Genet. 2022. 23:1–27

First published as a Review in Advance on May 13, 2022

The *Annual Review of Genomics and Human Genetics* is online at genom.annualreviews.org

https://doi.org/10.1146/annurev-genom-010622-095109

Keywords

autobiography, gene mapping, eye development, genes in disease, gene regulation, gene networks, model organisms

Abstract

Although my engagement with human genetics emerged gradually, and sometimes serendipitously, it has held me spellbound for decades. Without my teachers, students, postdocs, colleagues, and collaborators, I would not be writing this review of my scientific adventures. Early gene and disease mapping was a satisfying puzzle-solving exercise, but building biological insight was my main goal. The project trajectory was hugely influenced by the evolutionarily conserved nature of the implicated genes and by the pace of progress in genetic technologies. The rich detail of clinical observations, particularly in eye disease, makes humans an excellent model, especially when complemented by the use of multiple other animal species for experimental validation. The contributions of collaborators and rivals also influenced our approach. We are very fortunate to work in this era of unprecedented progress in genetics and genomics.

HOW I CAME TO STUDY GENETICS

Genetics studies particularly, but perhaps most research, is like tackling a giant puzzle. I love problem-solving, so it is not surprising that genetics research became my fate. There were, however, other relevant factors channeling me toward this path.

I was born in 1946 in Békéscsaba, a city in southeast Hungary, to Holocaust survivor parents. My father, Laszlo, had been in the Mauthausen labor camp. My mother, Anna, was in Auschwitz, Ravensbrück, and Buchenwald, including a death march shortly before liberation. Sadly, everyone else in both of their families was murdered in Auschwitz, including their respective spouses.

At the time of my birth, Laszlo was running the family textile manufacturing firm, but soon afterward, the factory was nationalized by the incoming Communists. Because of his excellent education as a textile engineer in Germany, before World War II, he was invited to work at the textile research institute in Budapest despite his capitalist background. My younger sister, Judy, and I were made very aware that education is life's major portable asset. A solid training in science was deemed more robust than most arts subjects, though becoming broadly cultured was an important side aim. Living in Budapest in the 1950s, our parents were always stressed but worked hard to maintain a calm atmosphere. Though hidden from us at the time, there were always tense political undertones. Anna took a risk by not attending the public mourning following the death of Stalin in 1953—one of my earliest memories. Not surprisingly, Anna particularly was very keen to leave Communist Hungary, though of course she never mentioned it in front of us children. Later, she said that Hungarians will always be anti-Semitic—and as a nation, they still are today. Because Laszlo was out of the country on a lecture tour during the 1956 uprising, we failed to get out while the borders were open. But sponsored by Anna's uncle, who had lived in Britain since 1919, our family was granted an immigrant passport. We arrived in Worthing, Sussex, on January 30, 1958.

Judy and I went to school immediately. By August 1958, Laszlo had a job as technical director at a knitwear company in Loughborough, and we had joined him there. With some help, we moved to a 1950s semidetached house by October. Having missed the 11-plus exam in February, I took, and passed with flying colors, a similar test at the Leicestershire Education Authority in August, but by then all grammar-school-equivalent places had been assigned, so I was placed in an avant garde streamed comprehensive school. It was a large school with a broad curriculum. The top stream was pushed hard, taking 11 subjects at O level. Even before O level, at age 15, a Nuffield project introduced us to *Drosophila* breeding experiments (using school milk bottles in a cupboard heated by a light bulb), and we read articles about the structure of viruses (33). Good O-level results in 1962 bestowed the freedom to choose any subjects for A level, and without hesitation I took chemistry, physics, and biology, with mathematics self-taught the following year. The exam results and my teachers encouraged me to take the Oxford and Cambridge entrance exams. I was invited to interviews at both places; the Cambridge one, which included lab practicals, was in the bitterly cold January of 1965. At the interviews, I met girls who are still dear friends.

In 1963, at the earliest possible time after our arrival in Britain, the family applied for British citizenship, and we were all interviewed by local Criminal Investigation Department (CID) officers over three consecutive evenings. My parents said that it might seem immodest to mention my ambition to go to Cambridge, but I did mention it. They also had parental concerns that Cambridge might be too grandiose and impractical; however, when the offer of a place arrived by telegram from Girton (Lady Margaret Hall in Oxford had already offered me a place by then), I persuaded my parents that Cambridge was the best choice for biochemistry, which, at that time, I aimed to study.

CAMBRIDGE 1965–1968

Representing the postwar bulge, my cohort regularly encountered new courses and syllabuses. Cambridge in 1965 was no exception. We were guinea pigs for two new courses, Biology of Cells and Biology of Organisms, and for a redesigned chemistry course. It was an exciting time—molecular biology was just evolving and not yet fully married up with organismal diversity. Ernest Gale, one of the architects of the new Biology of Cells course, said he liked to teach in a helical manner, presenting topics at increasing complexity, so everyone could ascend the ladder some of the way. In year two, we returned to older courses, in my case chemistry, biochemistry, and pathology. Small group supervisions, a hallmark of the college-centered teaching system, allowed us to discuss topics both broadly and minutely. Often keen PhD students, sometimes from other colleges, were asked to supervise Girton students. Marion Bramley and I were assigned for biochemistry supervisions to second-year PhD student Simon van Heyningen, from King's College. The first of our weekly essays was to write on the evidence for the citric acid cycle. That year, we learned a lot about marshaling evidence for statements and, conversely, what type of evidence is required to support a hypothesis, and hence how to design experiments. At the end of the second term, in early 1967, I succumbed to infectious mononucleosis (Epstein–Barr virus). This debilitating condition played havoc with my revision schedule, and I failed to achieve the 2.1 grade required for admission to the oversubscribed Biochemistry Part II (honors equivalent) class. However, my old love for genetics reemerged, and I was happy to join Genetics Part II, with just nine others (all male, reflecting the general Cambridge male/female ratio in those days). The department (119), which had land for growing crops, was then located away from other departments and quite a long bike ride from Girton.

In June 1967, when our supervisions with Simon van Heyningen had ended, I invited him to a tea party with friends at Girton. He reciprocated with a picnic in Norfolk. Later, just after the Six-Day War, we fortuitously met up in Israel, where I was visiting relations and Simon was touring with a friend. In October, just as the term began for Genetics Part II, we got engaged, and we married in June 1968. Meanwhile, the genetics course covered many exciting areas of molecular and evolutionary genetics as well as nice examples of gene regulation, mechanisms, and networks in *Escherichia coli*, *Drosophila*, and mouse. David Cove and Michael Ashburner were memorable teachers and discussants—we really got to delve into details with our lecturers in this advanced course. I also recall a visit and discussion—actually more of a soliloquy—from Sydney Brenner, when he told us that he was setting out to study nervous system development using genetics in a nematode worm comprising only approximately 1,000 cells. Sydney has always been one of my heroes. His mind was amazing, though his often humorous sharp pronouncements could be extremely wounding (never to me). Genetics Part II gave us insight into scholarly thinking and discourse, and for me at least, the course kindled my desire to become a research scientist.

AN EXCITING PhD DESPITE THE TWO-BODY PROBLEM

Once you have a long-term partner with the desire for each to develop their career, extra criteria come into play. Simon was awarded his PhD in August 1968, and we flew to Chicago in early September so that he could join David Shemin's lab at Northwestern University in Evanston, Illinois, as a postdoc. I was pleased to be offered a teaching assistant post for a year and the possibility of enrolling as a graduate student. The following year, still pursuing graduate studies, I joined the lab of Frank Neuhaus as a research assistant, working on bacterial cell wall synthesis. After Cambridge-style teaching, the more fact-and-memorization-focused course at Northwestern felt constraining, but in year two we started wide-ranging journal club discussions that were

stimulating. Altogether, we enjoyed our time at Northwestern and the opportunity to absorb US culture and mores at what was quite a turbulent time politically. Chicago is also a mecca for modern architecture, one of Simon's contagious interests. We decided, however, to return to the UK after two years in Evanston. Obviously, Simon would look for a lectureship or similar post, and as a potential PhD student, I would surely find a place at, or near, his destination.

The first offer he accepted was from the Department of Biochemistry at University College London (UCL), so I arranged a PhD place at the UCL Galton Laboratory with Harry Harris, whose work in biochemical genetics (48) was fascinating. But then Simon received a more desirable offer from Oxford, leading me to approach Walter Bodmer (9), the arriving Professor of Genetics (a newly established chair). Luckily, although he had been determined not to have any students in his first year, he agreed to take me on, supported by a Medical Research Council (MRC) three-year doctoral training grant. When we arrived in autumn 1970, the labs were freshly painted empty rooms. With departmental demonstrator Ian Craig, our first task was to equip the lab—a useful lesson in itself. The interlude also provided an opportunity to read and discuss the broader literature on the science and art of somatic cell genetics, the Bodmer lab topic that I was most excited to embark on (9). Fortuitously, the Sendai virus cell fusion technology had been developed in the nearby Oxford School of Pathology by Henry Harris and John Watkins (49). Following fusion of rodent and human cells, selection is applied so that only hybrids survive. Human chromosomes are lost randomly, permitting the assignment of human markers to specific cosegregating chromosomes. Before the era of DNA-level analysis, we studied the protein products of genes—identifying the electrophoretically distinguishable human and rodent isoforms of enzymes by functional staining. Much of the original expertise in enzyme electrophoresis was developed in the Galton Laboratory, led by Harry Harris (48), so I ended up collaborating with Sue Povey and David Hopkinson (121), key members of the lab that I had so nearly joined. My major specific aim was to explore the genetics of mitochondrial enzymes. How were these proteins encoded, given preliminary evidence that the hybrid cell mitochondria carried only rodent-derived DNA?

Distinguishing rodent and human mitochondrial enzyme isoforms by electrophoretic means was found to be impossible in many cases. Surrounded by superb immunologists in the Oxford Department of Biochemistry, I was inspired to make rabbit antibodies to purified human mitochondrial malate dehydrogenase and alanine aminotransferase and used the classical Ouchterlony diffusion technique to distinguish human and mouse isoforms (124). This allowed us to confirm that these enzymes were nuclear-encoded and to assign the malate dehydrogenase gene to human chromosome 7 (124). Citrate synthase, the next gene in the citric acid cycle to malate dehydrogenase, mapped to chromosome 12, and the cytoplasmic form of malate dehydrogenase mapped to a third chromosome. Thus, unlike with bacterial operons, consecutive genes in a pathway and homologous genes did not map to the same human chromosome. Our 1973 *Nature* paper (124) led swiftly to an invitation, directly to me, to a meeting on mitochondrial function in Bari, Italy, which included luminaries like Giuseppe Attardi and Igor Dawid as participants. A major lesson I learned was not to pack my slides into checked luggage, as my lost luggage only reached Bari just in time for my talk.

Life in the Bodmer lab was never dull: There were constant discussions, joint meetings with other illustrious labs, and a string of eminent visitors (**Figure 1**). Janet Rowley from Chicago accompanied her immunologist husband on sabbatical. With four children, she worked part time, developing quantitative measurement of fluorescent quinacrine banding intensity, allowing her to identify chromosomes, and even fragments, unequivocally. This was immediately useful for gene mapping (100), but more famously, she defined the precise composition and abnormal function of different leukemia-associated translocation chromosomes. Other visitors included

Figure 1

Happy memories of Oxford: members of the Bodmer lab in 1971, soon after its establishment. Back row, from left: Harry Dovey, Karl Ytterborn, Irving Goldberg, Walter Bodmer, David Roberts, and Markus Nabholz. Third row: Ian Craig, Sally Craig, Janet Rowley, Mrs. Cresser, Bengt Olle Bengtson, Veronica van Heyningen, Elinor Tolley, and Paul Handford. Second row: Mrs. Fletcher, Maria Guida Boavida, and Julia Bodmer. First row: Sue Black, David Buck, and Liz Burns.

Barry Blumberg, who was awarded a 1976 Nobel Prize for work on hepatitis B, and Bill Sly, who characterized many metabolic diseases.

MOVING TO EDINBURGH

In October 1973, Harry Harris (external) and Henry Harris (internal) examined my DPhil thesis, written in six weeks—the only speedy writing I have ever done. Simon soon began the search for a tenured lectureship, as the Oxford demonstratorship was finite. When he was offered a university lectureship in biochemistry in Edinburgh, we were very happy to move there. Fortuitously, I had just been awarded a Beit Memorial Fellowship, a prestigious and portable award that allowed me to choose which Edinburgh lab to work in. After wide consultations, I was pleased to join the MRC Mammalian Genome Unit (MGU) led by Peter Walker. The MGU had fantastic expertise in DNA methods and early studies of the genome (an obscure new term then). Ed Southern was a senior member, having just invented his eponymous DNA blotting technique. Adrian Bird, another colleague, was pondering DNA methylation and the clustering of protected CpG

dinucleotides in islands at gene promoters. A graduate scientist prepared and aliquoted restriction enzymes full time.

My planned role at the MGU was to use somatic cell hybrids for DNA-level gene mapping with Southern blotting. The technology was not quite ready then. However, Simon and I decided that this was a good time for some practical human genetics, and in July 1975 our son, Paul, was born, soon after an MRC site visit to the MGU when I was 38 weeks pregnant. The visiting panel comprised Sydney Brenner, Anne McLaren, Walter Bodmer, and Alan Williamson. Brenner and Bodmer spent a lot of time in heated discussion on many scientific points. As I stood waiting to give my presentation, Brenner said, "Hurry up, girl, speak before you pop." Luckily, I thought this was funny. Over tea, Anne McLaren encouraged me to return to work after the child was born, as it was perfectly possible to manage science and motherhood—she did not add "as I have done," but she was obviously a fabulous role model. Of course, there were others, too: Simon's mother, Ruth van Heyningen, was a working research scientist.

My part-time return happened sooner than planned, when Peter Walker asked me after a few weeks to keep the cell lines going—cell culture being one of my skills. I walked there with the pram and did cell work while Paul slept in the common room, which would probably not be permitted now. In January 1976, when Paul went to the splendid University Day Nursery, I returned to work. Somewhat to Walker's surprise and disapproval, Eleanor was born 18 months after her brother, just as the extended Beit Fellowship ended. When she was four months old, I applied for and was offered an advertised postdoctoral post on the other side of town, at the MRC Clinical and Population Cytogenetics Unit (CAPCU), led by John Evans. In June 1977, I started what turned out to be 35 years in that unit. The MRC allowed me to work 30 hours a week, which was perfect—just enough, with some evening reading, for serious research, and it worked well with the 9–5 nursery schedule. What luck that the Edinburgh biological community was so accommodating!

MAKING MONOCLONALS AS ANALYTICAL TOOLS

The new post at CAPCU, later to become the MRC Human Genetics Unit (HGU), was in the group of Michael Steel, working on lymphocyte interactions. My interest turned to exploring these cellular encounters with newly produced monoclonal antibodies. I readily set up the new technology that had been described in detail by Georges Köhler and César Milstein. The process was very similar to the cell fusion experiments I had been doing in Oxford, though to get single-cell clones from the outset, the fusion products were diluted immediately into multiple 96-well plates. Success depended significantly on robust, selective screening assays. Soon, we were using our own monoclonal antibodies—probably the first made in Edinburgh—to recognize nonpolymorphic regions on subunits of human leukocyte antigen (HLA) class 2 molecules. We were able to subclassify lymphocyte populations (41, 125). Antibody DA6.147, recognizing the alpha subunit of HLA-DR, was freely shared and widely used. Later, the MRC sold numerous hybridomas nonexclusively to several companies. They are still available. Soon after successfully gaining MRC tenure in February 1981, I began my own projects, working with just one and later two technicians—no postdocs or PhD students. At that time, MRC staff scientists were not permitted to apply for outside funding.

As hybridoma making was taking off, a common-room conversation with David Brock from the University of Edinburgh Department of Human Genetics led to a significant collaboration. David had shown that elevated maternal serum alpha-fetoprotein could be used to diagnose anencephaly and spina bifida in pregnancy. To secure a constant source of suitable antibodies for screening, we agreed to make a set of monoclonals from mice immunized with purified alpha-fetoprotein. Better assay sensitivity and specificity are possible with sandwich immunoassays using two antibodies that bind noncompetitively to different epitopes on the analyte (10). The antibodies produced were further developed for commercial use by a new MRC-promoted company, Celltech.

Following this success, we produced several sets of monoclonals to protein and peptide antigens, mostly serum components. Our collaborators, the MRC Immunoassay Team, had highlighted the need for high-affinity antibodies to permit accurate quantitation of thyroid-stimulating hormone (TSH). It was important to distinguish normal euthyroid TSH levels from the ultralow levels seen in hyperthyroidism. A simple way to select for high-affinity antibodies suggested itself, and I developed the idea with help from Simon, leading to our only joint publication (123).

Our final adventure with serum-protein studies focused on an anonymous protein that David Brock and others had found was elevated in cystic fibrosis patients. David's group had prepared a guinea pig antibody that we used to define granulocytes as a major source of the antigen. Mouse–human hybrids expressing the protein showed unequivocal cosegregation with chromosome 1 (126), while shortly afterward the chromosome 7 assignment of the cystic fibrosis disease locus was published (74, 130). Nevertheless, we made monoclonals recognizing this protein, initially dubbed cystic fibrosis antigen. We set up an enzyme-linked immunosorbent assay for serum quantitation and, with Cambridge collaborators, partially sequenced the antibody-purified protein. Using that information, Julia Dorin, a brilliant first PhD student in my lab, identified a matching cDNA clone, encoding a putative calcium-binding protein, one of two linked paralogous subunits, S100a and S100b (25). Officially named S100A8 and S100A9, they are now known as calprotectin (61) and also as alarmins (131), with pro- and anti-inflammatory roles. They are major serum markers of inflammation and induced in some abnormal epithelial cells, including in some cancers (134). Recently, high serum levels have been associated with severe SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) infection (107).

GENE MAPPING AND POSITIONAL DISEASE GENE IDENTIFICATION

The arrival of Nick Hastie at CAPCU triggered a great discussion on beginning a wider collaborative project to study genetic diseases. With John Evans (CAPCU director) and Ed Southern (MGU director), many CAPCU scientists participated in a June 1983 meeting convened to discuss plans for mapping and identifying disease genes. This was long before the formal human genome era. Encouraged by CAPCU's renowned cytogeneticists, we decided to aim for the genes implicated in the contiguous gene syndrome Wilms tumor, aniridia with genitourinary abnormalities, and mental retardation (WAGR syndrome). It was already known that this complex phenotype was often associated with cytogenetically visible deletions of chromosome 11p (34). Aniridia and Wilms tumor predisposition were expected to be caused by mutations in different genes since familial aniridia in a large family with no Wilms tumor incidence had been assigned to the same chromosomal region (79). Through long-standing collaborations with the University of Edinburgh Department of Ophthalmology, we had immediate access to five independent cases of WAGR syndrome. It was agreed that a reliable, if laborious, way to analyze and map the deletions would be to segregate the deleted and normal human chromosome 11 into separate rodent–human hybrid cell clones. With the help of talented research technician Anne Seawright and skillful cytogenetics technician Judy Fletcher, the hybrids were made, fusing lymphoblastoid cells from the deletion patients with the mouse myeloma cell line. Generous colleagues donated monoclonal antibodies recognizing human-specific cell-surface markers encoded on chromosome 11 (115). Specific rearranged chromosome 11 fragments could be selected (104, 122). The locally devised fluorescence cell sorting system was soon replaced by the more efficient use of anti-mouse immunoglobulin magnetic beads. Selection of hybrid lines stabilized them for analysis of human markers. The position of the WAGR region was progressively refined as more cases were studied with accumulating new probes. Several international groups collaborated, with some friendly rivalry, on this project.

Wilms tumor, a childhood developmental kidney tumor, was predicted to be caused by tumor suppressor gene loss: Inactivation of one copy predisposes to tumor development, but the second

copy is lost as the tumor forms (75). *WT1*, encoding a DNA-binding zinc-finger transcription factor, was identified as the candidate gene for Wilms tumor predisposition in the Housman lab at the Massachusetts Institute of Technology (12). They immediately sent us the cDNA clone, and in situ analysis in human fetal tissue quickly confirmed disease-relevant expression in early kidney and genital development (90). Full confirmation of *WT1* as the causative gene in Wilms tumor came with identification of the first intragenic mutation (42). Subsequently, many *WT1* mutations identified were defined as loss-of-function changes (89), but in Denys–Drash syndrome, cryptorchidism, and pseudohermaphroditism associated with a strong Wilms tumor risk, we identified dominant-negative *WT1* changes (78).

In 1991, the aniridia candidate gene emerged in Grady Saunders's lab in Texas. Aniridia deletion mapping defined a 70-kb smallest region of overlap, in which the gene *PAX6* mapped. These collaborators also instantly shared the cDNA clone and early data with us, and authorship of their initial paper (114). Like WT1, PAX6 was predicted to be a transcription factor, a member of the paired-homeodomain family. Complex expression was revealed in neuronal and surface-ectoderm components of the developing human eye (114). The first two human mutations were soon identified (60) by nested reverse transcription PCR of presumed illegitimate *PAX6* expression (65) in lymphoblastoid cells. These early studies on *PAX6* heralded a new era for my lab.

THE EMERGENCE OF INDEPENDENCE AND CEMENTING OF COLLABORATIONS

In early 1992, John Evans suggested that both Nick Hastie and I should submit proposals to the new International Research Scholar program of the Howard Hughes Medical Institute (HHMI). The timeline was extremely tight, and we worked away independently—in my case to the very last minute, with no sleep on the last day. By this time, we had both decided that we were keen to pursue the genetic mechanisms and biology of Wilms tumor and aniridia. Our skills were complementary, with Nick a molecular biologist and I with genetics and cell and tissue expertise. We worked well together. I was still engaged in gene mapping, interacting closely with Claudine Junien from Paris to oversee the generation of a detailed and accurate map for chromosome 11 (17, 62). In late 1992, after a chromosome 11 workshop in Baltimore, a family airport call revealed (though it was meant to be a surprise for my return) that I had been awarded an HHMI International Research Scholar grant. Around this time, our MRC minders stipulated that, as independent principal investigators, Nick and I should run separate programs. Of course, our strong and fun collaboration continued, but from this stage Nick officially focused on *WT1* functions and kidney development, while I delved into *PAX6* and aniridia. Subsequently, both programs broadened excitingly and gratifyingly. With collaborators, my group extended genetic exploration to other developmental eye anomalies and more fundamentally to the dissection of genetic mechanisms highlighted by our *PAX6* studies. The concept that well-phenotyped disease studies make humans a great biological model system became a firm conviction (120).

From 1992, I headed what came to be the Medical and Developmental Genetics Section. Suddenly, there was much more people management to deliver. But exploring *PAX6* in health and disease remained the main focus and professional passion in life, with some continued chromosome 11 mapping. Bridging the two, a set of tiling cosmids was identified for the *PAX6* region, as well as several human yeast artificial chromosome (YAC) clones carrying *PAX6* with large flanking DNA regions (30).

With HHMI funding, I was able to expand my lab a little. Initially, it ran with talented technicians, and then Julia Dorin continued from PhD student to postdoc, followed by the arrival of a series of excellent postdocs, including Isabel Hanson, Dieter Englekamp, Dirk-Jan Kleinjan,

Penny Rashbass, Marie Fernandes, Pedro Coutinho, Patricia Yeyati, and Shipra Bhatia, to explore different aspects of *PAX6* function and some of the general concepts raised by the initial *PAX6* findings. Kathy Williamson, originally a PhD student, returned to run eye gene mutation analysis. Reciprocal collaborative work with Nick Hastie's group remained an important aspect of our progress and enjoyment. In 2000, clinical geneticist David FitzPatrick joined the MRC HGU as a group leader, allowing us to develop a hugely enjoyable and successful collaboration in disease gene identification, always followed by dissection of the underlying biological mechanisms.

FOCUS ON EYES

In retrospect, I can articulate the strategy we followed, but it was developed piecemeal as we progressed. My attempt to summarize the overlapping components of the program is, inevitably, shown as a Venn diagram (**Figure 2**), demonstrating my well-known fondness for this format. As usual, several research paths were initiated, and some produced results to be followed up. Initially, we identified *PAX6* mutations in as many human cases as possible—we collected aniridia and phenotypically related cases continuously for years, before and after *PAX6* identification. Detailed clinical phenotypes were recorded for comparative analysis. Most cases represented classical and variant aniridia, but subsequently related anterior segment anomalies were included, along with, eventually, cases of microphthalmia and anophthalmia. This broad sweep was suggested partly by the *Small eye* mouse heterozygote and homozygote phenotypes. The heterozygous mouse has

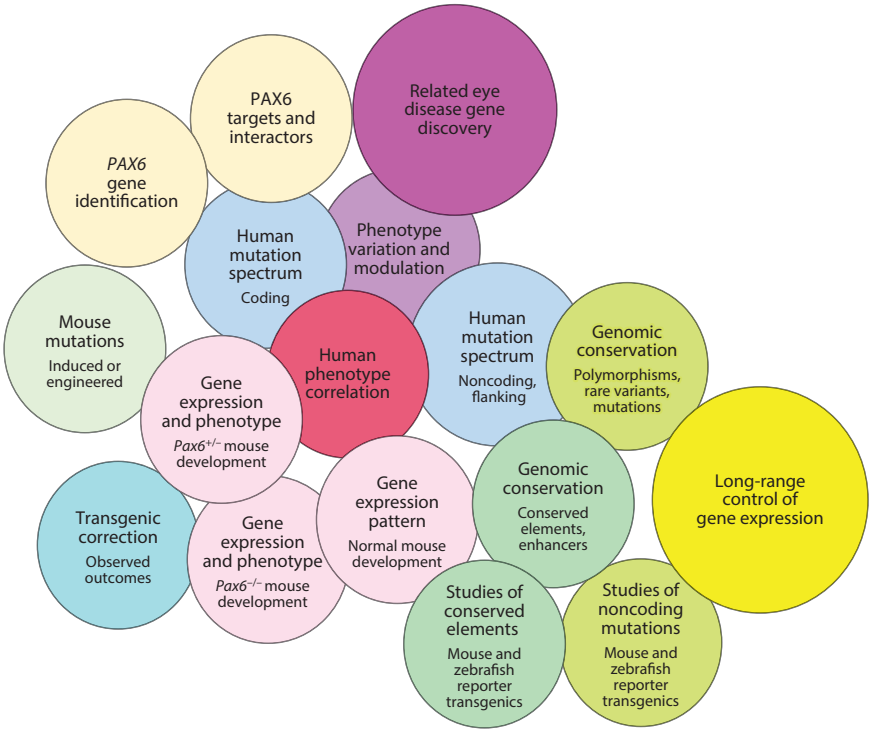


Figure 2

The evolution of a long-term project, showing how different aspects of our group's work interlinked and grew from studying human developmental anomalies, the implicated genes, and the different mechanisms of disease causation, gene interactions, and networks.

reduced eye size (microphthalmia) and cataracts (32, 54, 60). Homozygotes die neonatally with no eyes (anophthalmia) (54), no olfactory bulb (102), brain anomalies (59, 81), and no pancreatic endocrine cells (111).

Over the years, many ophthalmologists—among them Tony Moore, Françoise Meire, Birgit Lorenz, Elise Héon, Nicky Ragge, Christina Gerth-Kahlert, and Michael Clark—and several clinical geneticists sent blood and diagnostic information from interesting cases. Hundreds of lymphoblastoid lines from patients and their families were immortalized, permitting wide-ranging analysis. More recently, special meetings with talks from clinicians, scientists, and patients have proved highly informative for all participants. In 2012, when I stopped running a lab, I became patron of a UK patient organization, Aniridia Network (**https://aniridia.org.uk**). Combining information from patients' experiences with experimental data reveals a more complex picture of *PAX6*-associated congenital developmental anomaly and progressive disease.

WIDE-RANGING EXPLORATION OF *PAX6* FUNCTION

Evolutionary Conservation of *PAX6*

From the outset, it was clear that *PAX6* is a highly conserved gene, with complete human–mouse amino acid identity. The paired and homeobox regions are ~90% identical to those of *Drosophila* and *Caenorhabditis elegans*. There is also significant vertebrate synteny conservation in the genomic region (67, 69, 86, 96). With the availability of accurate genomic sequence from multiple species, conservation of many noncoding elements, as well as the *Pax6* gene itself, emerged (71, 86). Functional conservation was sensationally illustrated when the *Drosophila eyeless* gene was found to be a *pax6* ortholog/paralog that also acts as a master regulator of fly eye development (43, 92). Expression of *Pax6* from multiple species (flies to mammals) in selected *Drosophila* imaginal discs leads, astonishingly, to the development of ectopic eyes (43). Eye phenotypes have been associated with *PAX6* ortholog mutations in *sunrise* zebrafish at the *pax6b* locus (67, 138), as well as in multiple mutants in mice (32, 54), humans (45, 46, 60), *Drosophila* (92), and *C. elegans* (14) (**Figure 3**). These findings led Gehring (35) to declare *PAX6* the master gene for eye development and to propose that the evolution of all forms of eyes was monophyletic. This is a contentious area (129) where the answer depends partly on how the eye is defined. In my view, the underlying functional unit may have evolved from one ancestral system, but complex eyes were then assembled multiple times, using these components.

As a transcription factor, PAX6 is expected to bind DNA target sequences in a specific manner, though variation is conferred by co-binding with different partner proteins. Pedro Coutinho, a talented mathematician/biologist postdoc, gathered published PAX6 target sequences and used hidden Markov modeling and sequence conservation to define further PAX6 targets genome-wide (18). The most convincing target genes have multiple predicted flanking noncoding sites, and predicted binding loci often encode well-established targets of PAX6, such as *MEIS1*, *MAB21L2*, *PAX6*, *DACH1*, *SIX3*, *DLX5*, *PTF1A*, and *TCF7L2*. Targets include eye-, brain-, and pancreas-development genes, as summarized by Cvekl & Callaerts (22).

Autoregulation of PAX6 has been repeatedly documented (4, 68, 80). As noted, PAX6 probably always co-binds with other transcription factor partners. One well-documented partner and target is SOX2 (and *SOX2* is also a major eye anomaly gene; see below). The interactions are diverse (63, 64), and the predicted PAX6-SOX2 co-binding sequences are quite distinct and fulfill different SOX2 enhancer functions.

Expression

Developmental and adult gene expression is most readily studied in model organisms, classically by RNA in situ hybridization. For an evolutionarily highly conserved gene, like *PAX6*, it is

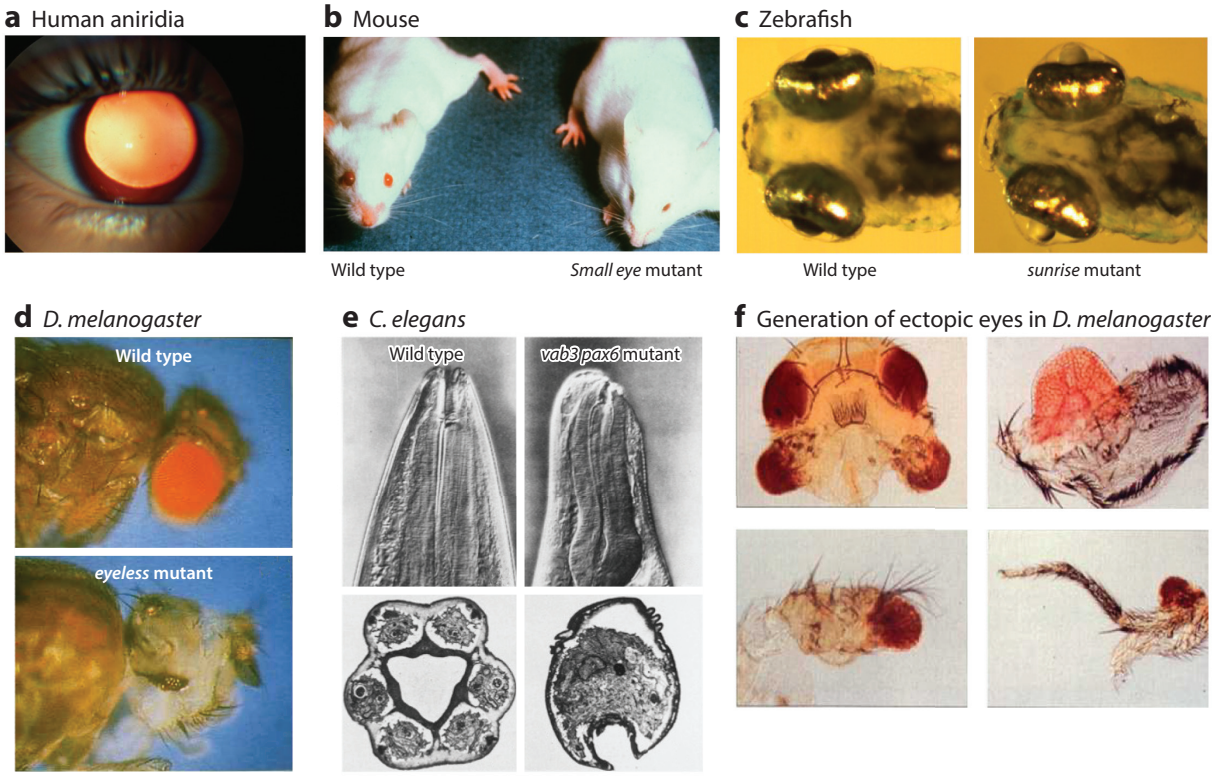


Figure 3

Model organisms with *PAX6* mutations. The evolutionarily highly conserved *PAX6* gene has been studied in multiple model organisms. Eye phenotypes are most obviously associated with *PAX6* mutations in human aniridia (panel *a*), the *Small eye* mouse mutant (panel *b*), the *sunrise* zebrafish mutant (panel *c*), and the *eyeless Drosophila melanogaster* mutant (panel *d*). In the *pax6* mutant *vab3* of *Caenorhabditis elegans* (which has no eyes), microscopy reveals nervous system disorganization in the head region (panel *e*, with Nomarski optics above and transverse section electron micrographs below). In *D. melanogaster*, directed (mis)expression in certain imaginal discs generates ectopic eyes on the legs, wings, and antennae (panel *f*). Panel *a* provided by Professor David Taylor. Panel *e* adapted with permission from Reference 77; copyright 1977 The Wistar Institute Press. Panel *f* adapted with from Reference 43 with permission from AAAS.

relatively safe to extrapolate from one species to another. Knowing the spatiotemporal expression pattern helps in devising a thorough phenotypic study in mutation carriers. Dissecting the mutation-associated changes provides insight into gene function. For dosage-sensitive developmental regulators like *Pax6*, both monoallelic and biallelic phenotypes are informative. Not all expressing tissues are equally dosage sensitive. In fact, subtle variations in gene expression levels may play a key role in how, where, and when the differentiation of different cell types occurs (101). The full phenotypic spectrum, including the variation seen within and among aniridia families, is still being explored after 30 years.

We studied different tissues, in some instances with expert collaborators, in *Small eye* mouse and/or in aniridia patients, observing obvious and subtle phenotypes. Johan Ericson and James Briscoe from Tom Jessell's lab came to study aberrant motor-neuron specification in the developing neural tube of the $Pax6^{Sey/Sey}$ mouse (27). With the aid of a set of anti-PAX6 monoclonal antibodies that he and Anne Seawright had made (and that are commercially available), Dieter Engelkamp studied the abnormal development of the cerebellum and precerebellar nuclei in the *Small eye* mouse (26). He showed, using $Pax6^{Sey/Sey}$ mice, that migration of neurons from the

rhombic lip requires expression of *Pax6*. Neurologist Sanjay Sisodiya wanted to study the forebrain of a cohort of volunteer aniridia patients. He identified cerebral malformation, including hypoplasia or absence of the anterior commissure and olfactory system dysgenesis (108), which was confirmed and expanded later with a more powerful magnetic resonance imaging (MRI) scanner (139) and suggested that the severity of the brain phenotype correlates with predicted mutational severity. Cognitive function, particularly working memory capacity, was affected (113). Pineal gland absence or hypoplasia was also revealed by these imaging studies (87), and the sleep difficulties were subsequently recognized in additional patients, some of whom were later shown to respond to treatment with melatonin.

One member of the MRI-scanned cohort, with hearing problems, saw audiologist Doris Bamiou, after which further study in other cases, including young children with aniridia, demonstrated auditory interhemispheric transfer deficiency in aniridia (2, 3). Help with classroom hearing comprehension can be offered to young patients. In addition to neural system anomalies, the role of pancreatic islet cell dysfunction (50, 112) in later-onset obesity and diabetes is now being assessed. The most obvious effects of PAX6 deficiency are revealed in the congenital and progressive eye phenotype of aniridia, which reflects the complex eye expression pattern: partial or complete absence of the iris, foveal hypoplasia, likely nystagmus, possible cataract and glaucoma, and probable progressive corneal keratopathy (45–47, 55, 56, 98, 118, 136). Many groups have contributed to defining the eye phenotype caused by *PAX6* mutation.

I have been frustrated that mouse work was almost always carried out in homozygous mutant or knockout mice and therefore, because of *Pax6*-null lethality, only during prebirth development. The subtleties of the murine heterozygous state remain mostly unexplored.

Lessons from Mutations

As we approach the possibility of targeted therapy, knowing the precise molecular change will be critical. It is also informative for understanding function. In 1998, we set up a mutation database (11) that was skillfully curated by Isabel Hanson until 2017 (40), with *PAX6* mutation information on more than 1,000 cases (with 491 of those being unique, mostly single-nucleotide, intragenic changes).

Classical aniridia is typically associated with predicted protein-truncating mutations (47, 56, 91) that are expected to result in haploinsufficiency, as a consequence of nonsense-mediated decay of the prematurely terminating mRNA (118). Missense changes, mostly expected to be deleterious, are associated with variant phenotypes (45, 46). In 2009, we summarized the mutation data and correlated them with the phenotypic information (56). More recently, genotype–phenotype comparisons were collated from multiple sources by the group of current colleague and clinician scientist Mariya Moosajee (21, 66). The effects of missense mutations are variable, and some are milder than classical aniridia (56). However, over many years, we accumulated several recurrent, predominantly de novo, missense mutations predicted to disrupt the DNA-binding role of the paired domain, leading to severe microphthalmia-like phenotypes (136). The two dominant or dominant negative mutations associated with the most severe phenotypes (S54R and N124K) are at homologous positions in the equivalent duplicated halves of the paired domain. They are sites that are also highly conserved in other *PAX* paralogs (136).

Structural Genomic Changes and Dosage Associated with Eye Anomalies

As discussed from the outset, there are many chromosomal rearrangements associated with aniridia—these genomic changes are not in the database, though many of our deletion cases are described (19, 20, 99). Judy Fantes's elegant investigation of the original SIMO translocation

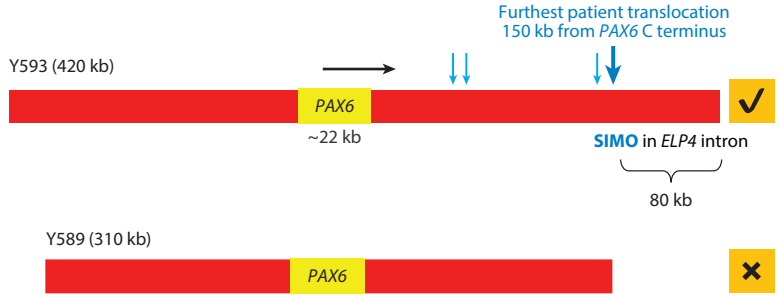


Figure 4

Large regulatory regions flanking *PAX6* are needed for full functional rescue. The mouse *Small eye* mutation (*Pax6* stop codon) was rescued (102) by a human 420-kb *PAX6*-containing YAC, Y593, which carries the 22-kb, 13-exon *PAX6* gene (the *black arrow* indicates the direction of transcription) flanked by an approximately 200-kb regulatory element containing DNA on each side. The light blue arrows indicate patient translocation breakpoints; the large darker blue arrow indicates the furthest downstream patient breakpoint, SIMO, which is present in Y593. YAC Y589, which is missing 80 kb of the Y593 downstream region, including the SIMO breakpoint site, fails to rescue the *Small eye* mutation. Abbreviation: YAC, yeast artificial chromosome.

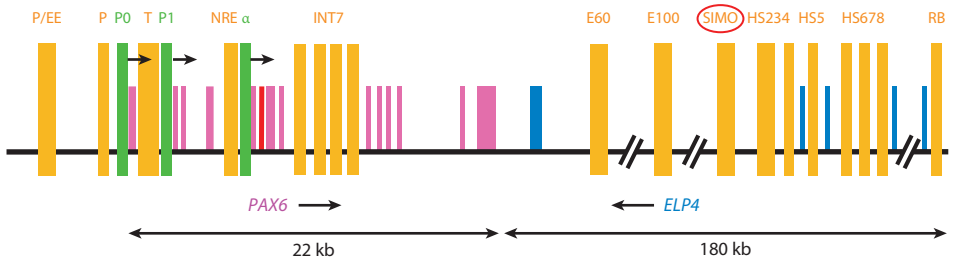
and an inversion breakpoint, using fluorescence in situ hybridization, with our *PAX6* YACs and cosmid contig mapping clones revealed that gene function can be disrupted by interruption of the proposed regulatory region as much as 150 kb distal to the polyA addition site for *PAX6* (29). The existence of distant, and particularly downstream, regulatory elements was a relatively new concept. At the time, promoters were considered the major control elements, often dissected by so-called promoter bashing (24).

We also demonstrated the importance of the intact downstream regulatory domain of *PAX6* by showing that the larger YAC Y593 (which includes the SIMO translocation breakpoint), but not the shorter YAC Y589, can rescue the *Small eye* mouse mutant phenotype (71, 102) (**Figure 4**). The YAC transgenic mice also demonstrated that overexpression of *PAX6*, with too many copies of the rescuing YAC, leads to severe eye malformation (102).

In long-standing collaboration with David Price's group, we inserted tau-GFP in frame into the *PAX6* gene of YAC Y593 and produced YAC Y593_*PAX6*–GFP reporter transgenic mice, recapitulating *PAX6* expression in a spatiotemporally and quantitatively controlled manner (70, 80, 116). Bred to mice with different levels of *PAX6* expression, the reporter provides insight into the developmental effects of *Pax6* dosage and demonstrates positive and negative Pax6 autoregulation (80). Autoregulation of many, if not all, transcription factors is a critical property, essential for feedback and feed-forward control, and other instances were also demonstrated for PAX6 (4, 68).

Regulatory Function and Dysfunction

Exploring the complex regulation of *PAX6* has been a major part of my group's program. Dirk-Jan Kleinjan was a key driver of this project from his arrival in 1997 until my retirement in 2012 and has actually continued the exploration of *PAX6* long-range control as a side project ever since. We set out our early ideas in a review written very soon after his arrival (72). Before the Human Genome Project or the Encyclopedia of DNA Elements (ENCODE) Project had even begun, we started to use DNase hypersensitivity to reveal open chromatin regions in *PAX6*-expressing cells, which were absent from nonexpressing cells. Probes from the SIMO breakpoint region downstream of *PAX6* indicated accessible chromatin (71). Enhancer activity was studied using reporter transgenesis in mice, establishing that spatiotemporally distinct expression patterns are



Upstream and intragenic enhancers

- P/EE: pancreas, lens
- P: pancreas, retina
- P0: promoter
- T: telencephalon, retina, lens
- P1: promoter
- NRE: retina
- α: internal promoter
- INT7: diencephalon P1 and P2, hindbrain

Downstream enhancers

- E60A: early telencephalon, optic cup, diencephalon P3
- E60B: late telencephalon, pontine nuclei and migratory stream, olfactory bulb, (R2, R6–8)
- E100: neuroretina, diencephalon P1, olfactory bulb and epithelium
- SIMO: lens, diencephalon P1, rhombencephalon
- HS234: retina, nasal placode
- HS5: diencephalon P1, retina
- HS8: retina (ganglion cell layer), olfactory bulb and epithelium, trigeminal
- RB: diencephalon P2, telencephalon, (olfactory bulb), pineal

Figure 5

Heritage image of the *PAX6* downstream regulatory region. Numerous noncoding *PAX6*-regulatory elements (*orange bars*) are displayed diagrammatically in the *PAX6* gene introns (*pink exons*) and in downstream regions in the introns of the neighboring *ELP4* gene (*blue exons*). Most have been tested for enhancer function in reporter transgenic mice (67–71), and some have also been tested in zebrafish (4, 5, 7). Promoters P0, P1, and α are in green, and the downstream SIMO enhancer is circled. The observed tissue-specific expression patterns are detailed in the color-coded table at the bottom, illustrating that individual enhancers fulfill a distinct spectrum of functions and that tissues in different regions are regulated by multiple different enhancers in time and space.

driven by neighboring conserved elements (39, 71) (**Figure 5**). A decade later, we still found that this approach yielded useful information on novel enhancers (82). Sequence comparison helped to identify conserved noncoding regions that could be tested as potential enhancer elements. PipMaker (103), now probably obsolete, was an easy-to-use program with clear, visually appealing outputs. Enhancer functions were demonstrated in multiple ways using reporter transgenesis; for example, a pineal enhancer was identified through loss of reporter expression, when the most distal end of the YAC Y593_*PAX6*–GFP construct was deleted in one transgenic line (70).

When Shipra Bhatia joined the lab, she began making fluorescent reporter transgenic lines in zebrafish—most suitable for studies of early development, with its transparent embryos. In two collaborative studies with an eminent Singapore lab (7, 96), the evolution and sequence identity of upstream and downstream *PAX6* noncoding elements from ancient gnathostomes (jawed vertebrates) to humans was revealed using comparative sequence analysis (103, 128). In addition, *PAX6* gene duplications, distinct from the well-known duplications in zebrafish, were identified (96, 97). Comparison of conserved syntenies was useful in defining true orthologs. The tissue specificity of enhancers was compared using reporter transgenic analysis in mouse and zebrafish. The stability and reproducibility of the observed patterns are remarkable.

We identified *PAX6* coding region mutations and genomic rearrangement mutations in many aniridia cases but had a few cases where no mutation was found. Sequencing of highly conserved enhancers revealed one instance where a de novo single-nucleotide change in the well-defined SIMO element was identified. The disrupted sequence had been defined as an autoregulatory site for *PAX6*. Bhatia et al. (4) demonstrated in both mouse and zebrafish reporter transgenics that the mutated element had lost the ability to drive expression in the developing lens, while diencephalon and hindbrain expression was preserved. PAX6 binding to the mutated element was also almost abolished. The zebrafish study used the dual fluorescence reporter system, where

the wild-type and mutant enhancers were linked to different-color fluorescent reporters, and the expression patterns driven by each simultaneously in cotransfected stable embryos were compared. Similarly altered expression patterns were also observed in several cases with Pierre Robin sequence with mutations in a distant *SOX9* craniofacial element (5, 38). In the Pierre Robin cases, there was evidence for incomplete penetrance. This is not at all surprising in anomalies driven by transcription factor haploinsufficiency, as precise threshold values are not robust.

There is not much discussion in the literature about why reporter transgenesis can be used across large evolutionary distances: Why can a developmental enhancer element direct an intricate, spatiotemporally correct, gene-relevant expression pattern in mouse or zebrafish and show specific deviation from that pattern with just a single-nucleotide change in some cases (4, 5)? I believe that the 1–4-kb elements are turned on by very precise binding of specific transcription factor complexes whose stoichiometry is highly conserved through the evolution of homologous developmental systems. Conservation may be even wider and more ancient in some cases. The well-defined chicken delta-crystallin enhancer that binds a PAX6- and SOX2-containing complex may produce a conserved pattern all the way to *Drosophila* (8).

The zebrafish dual fluorescent reporter system is elegant and informative on enhancer function. Comparisons can be made across species over evolutionary distance as well as between wild-type and mutant enhancers. Bhatia et al. (6) recently refined the system by creating a single-vector construct that can accommodate both wild-type and mutant enhancer sequence, each with distinct (usually a red and a green) fluorescent reporter attached, with an insulator sequence separating them. The construct is targeted to a predefined neutral docking site in the zebrafish genome (6).

Although clearly very important, a level of regulation we have not discussed or worked on is alternative splicing control. The role of mammalian *PAX6* exon 5a is not clear. The use of alternative promoters, some of which may lead to the production of the paired-less isoform of the PAX6 protein, has also not been explored in detail, though we mention the concept (68, 96). Similarly, we have not explored the role of noncoding RNAs encoded in the *PAX6* region and implicated in the regulation of the gene. Mechanisms of genomic organization and chromatin arrangement into topologically associating domains have not been explicitly addressed for the *PAX6* flanking regulatory regions, but we were early proponents of enhancer–promoter interactions by looping, as discussed in our well-cited 2005 review of how long-range control of gene expression may be disrupted in disease (74). The *PAX6* regulatory domain is, however, one of the most explored complex multienhancer systems and has been adopted for detailed dissection in a collaboration by my long-term colleague Wendy Bickmore with the Boeke lab in their Dark Matter Project (13).

Exploring noncoding regulatory variation for a specific gene is informative for understanding disease mechanisms in general. More than 90% of variants linked to common multifactorial diseases fall in the noncoding parts of the genome. Developing techniques to demonstrate the biological effects of such variation will help to understand these common diseases.

NETWORKING: GENES IN THE *PAX6* NETWORK IMPLICATED IN OTHER EYE ANOMALIES

Interactions and dialogue with clinicians have been fruitful, instructive, and enjoyable throughout our studies. Most of the developmental eye anomalies we have studied are thankfully rare, so we needed to collect cases worldwide. Our collaborators offered us typical cases and related phenotypes. Given the mouse phenotypes for both heterozygous and homozygous (lethal) *Pax6* mutations, we were offered and requested cases of microphthalmia, anophthalmia, and coloboma (grouped together as MAC cases) and eye plus nasal malformations (arrhinia), as well as some atypical cases of iris anomalies, such as Gillespie syndrome. This seemed a logical approach when we noted early on that classical aniridia was strongly associated with *PAX6* null mutations

(47, 118). *PAX6* missense changes accounted for a proportion of milder and more severe or atypical cases, including some colobomas and microphthalmias (45–47, 88, 118, 136).

When extensive analysis failed to identify *PAX6* mutations, other than a few deletions (1), in many of our collected cases, we began to look for other genes, particularly for cases of microphthalmia and anophthalmia. These studies were initiated before the completion of even the initial phase of the Human Genome Project, and certainly before exome or whole-genome sequencing was available. Most cases either were sporadic or came from very small families, so linkage mapping was not feasible. As before, we were guided to candidate genes by rare genomic rearrangements. Research led by our collaborators, the FitzPatrick group, identified the now well-studied *SOX2* high-mobility group (HMG) DNA-binding transcription factor gene as a key anophthalmia gene (28, 36, 94, 137). Monoallelic mutations of the single-exon *SOX2* gene are generally associated with severe syndromic, often bilateral, anophthalmia with no vertical transmission. Seizures and often endocrine anomalies, sometimes with genital or gonadal phenotypes, accompany many *SOX2* anophthalmias (31, 109, 117). A milder missense mutation, with features overlapping those of aniridia, was reported in a four-generation family (85), and we also found the same mutation in a similarly mild mother and child pair (36). The mutation just C-terminal to the HMG box may interfere with SOX2 protein interaction with the PAX6 paired domain as they co-bind to their specific DNA dual targets (58, 76). The phenotypic spectrum associated with *SOX2* mutations shows variable expressivity, some of which may be due to allelic differences (137). Some functions, such as its early role in maintaining multipotency, may not be affected by monoallelic mutations.

Soon after defining the role of *SOX2*, we identified *OTX2* as a major gene mutated in anophthalmia and microphthalmia (93). The evolutionarily conserved role of *OTX2* in neural and eye development and later retinal differentiation was well established. In the initial paper, key features associated with *OTX2* mutation included observation of highly variable phenotypic severity within families, including a surprisingly high frequency of unaffected mutation carrier parents and two gonosomal mosaic mothers (93). Milder or no malformation in one eye was also frequent. This was confirmed in a larger cohort and MAC cases reviewed by Williamson & FitzPatrick (135). Extraocular features were also seen: Pituitary deficiency is commonly associated with *OTX2* mutation, leading to growth retardation (52), and seizures, sometimes with later onset, are also observed. In some cases, the otocephaly–dysgnathia craniofacial phenotype, similar to the phenotype of the mouse heterozygous knockout, was seen (36).

PAX6, *SOX2*, and *OTX2* are all part of the regulatory network for eye development (53). Exploring and extending this network, which exhibits complex cross-regulatory features, provides a rich source of potential eye disease genes. Defining such networks is therefore a rewarding task, explored in many studies (22, 23), including our earlier work to identify PAX6 targets (18). Because of the deployment of multiple feedback and feed-forward controls and coregulation, the network is not hierarchical (**Figure 6**).

The many other genes implicated in MAC were discussed in the 2014 review by Williamson & FitzPatrick (135). Several other transcription factor genes with key roles in eye development have been repeatedly identified. Some, like *VSX2* (also known as *CHX10*), *RAX*, and *FOXE3*, cause disease through biallelic mutations. Some enzymic components of the retinoic acid pathway are also involved in biallelic, sometimes lethal disease (135). Additional well-established transcription factors and signaling factors in eye development have been documented, but in only a few cases for each (22, 135).

VAGARIES OF HUMAN GENETICS

A notable, if rare, feature of genetic eye disease surfaced (not for the first time) in our original *OTX2* study (93). In one family with a severely affected daughter, there was also a much more

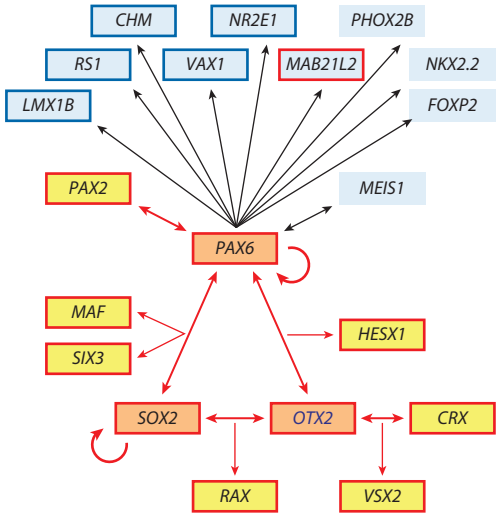


Figure 6

Network of *PAX6*-, *SOX2*-, and *OTX2*-interacting genes implicated in eye disease, showing the red-bordered interacting genes, where we and others have identified recurrent, mostly haploinsufficient, mutations in aniridia, microphthalmia, coloboma, anophthalmia, and other developmental eye conditions. *PAX6*, *SOX2*, and *OTX2* (*orange boxes*) are early-acting and at the top of the hierarchy. The genes in yellow boxes are variously identified targets and interactors of the top genes and also established disease-associated genes. Red arrows denote regulation, reciprocal regulation, and additional target interactions, identified in multiple labs (18, 22, 53, 58, 64, 135, and many other works not cited here). Curly arrows show autoregulation (58, 64, 76, 80). Genes in boxes with red borders are associated with eye diseases. The blue-gray boxes toward the top are predicted PAX6 target genes identified by hidden Markov modeling (18). The blue borders around some of these *PAX6* interactors indicate genes previously identified to carry eye disease–associated mutations.

mildly affected son. This man was married to a woman with neurofibromatosis, which disguised the fact that she also had a congenital iris anomaly. *NF1* and *PAX6* mutations were identified in the wife. Remarkably, their young daughter—who at the time was just starting normal education, although with some visual impairment—was shown to carry her mother's *NF1* and *PAX6* mutations and her father's *OTX2* mutation (51). Individuals with visual impairment frequently have partners who are also visually impaired, and the genetic consequences can be complex. We have not been able to follow up on this child with three different dominant/haploinsufficient mutations. Her mild phenotype is puzzling, but one possibility is that eliminating the function of one *PAX6* and one *OTX2* allele helps to restore the stoichiometry of these two proteins where they interact.

DISEASE-ASSOCIATED GENES FROM GENOME SEQUENCING

David FitzPatrick is a talented and insightful clinician and a highly skilled molecular biologist and bioinformatician. When exome and later whole-genome sequencing started to become feasible on a large scale, he was a key mover in the group that planned and realized the genomic study of difficult unsolved congenital anomaly cases in two projects run at the Wellcome Sanger Institute: the UK10K project (**https://www.uk10k.org**) and the Deciphering Developmental Disorders project (132). These were UK-wide collaborative projects with recruitment of carefully phenotyped genetically unsolved cases. David ensured that many of the unsolved cases we still had in our developmental eye disease collection in 2010, when the first of the projects began, were included in one of these programs. Several additional gene identifications emerged from these projects after my 2012 retirement from the MRC HGU.

One of the first successes was the identification of *MAB21L2* as the gene mutated in a spectrum of eye malformations, including colobomas and microphthalmia, with skeletal involvement in some cases (57, 95). MAB21L2 and its close paralog MAB21L1 are repeatedly defined targets and interactors of PAX6 (18, 22). On the return journey from a meeting in Italy that we organized in 2002 to kick-start the MAC gene-finding project, David FitzPatrick, Kathy Williamson, and I had pinpointed *MAB21L1* and *MAB21L2* as likely implicated genes. Identifying these initial mutations was gratifying. The biochemical function of MAB21L2 is not understood, though structural prediction suggests nucleic acid interaction (95). The name is derived from the original *C. elegans* mutation (*male abnormal 21*; one of the *pax6* mutations is *mab18*). This single-exon gene is highly conserved and located within an intron of a membrane-trafficking BEACH domain protein. The *mab21*-like genes and their genomic relationship to the BEACH domain gene are conserved from *C. elegans* to the mammalian orthologs. The monoallelic disease mutations in *MAB21L2* produce recurrent changes in closely clustered amino acids and are thought to be dominantly acting, though functionally the gene is not understood. Biallelic disease is caused by a suggested inactivating missense change in a different domain.

Genome sequencing led to two further interesting gene discoveries: one in Gillespie syndrome, which causes a distinct iris anomaly accompanied by ataxia, and one in arhinia, which leads to absent or severely malformed nasal structures and developmental eye anomalies. The small cohort collections of individuals with very rare diseases were begun as part of our search for possible extended *PAX6* phenotypes based on expression pattern, but extensive analysis did not identify *PAX6* mutations in either case (1).

In Gillespie syndrome, monoallelic and biallelic mutations were identified in the gene *ITPR1* (encoding inositol 1,4,5-trisphosphate receptor type 1, an ITP-responsive calcium channel) (44, 83). Interestingly, similar and sometimes overlapping mutations, both monoallelic and biallelic, had previously been identified in *SCA15* (spinal muscular atrophy type 15). The monoallelic mutations are predominantly disruptive missense changes, arising mostly de novo. The biallelic changes are mainly stop codons. No *ITPR1* mutations were found in any remaining unsolved aniridia cases, demonstrating that aniridia is distinct from Gillespie syndrome, with its scalloped-edge iris anomaly.

The final disease for which a gene was identified, by several groups simultaneously, was Bosma arhinia—a very rare malformation of nasal, ocular, and facial structures. Once more, both monoallelic dominant missense and biallelic loss-of-function changes were found in *SMCHD1* (structural maintenance of chromosomes flexible hinge domain–containing 1), which encodes a protein engaged in chromatin modulation by methylation (105). *SMCHD1* is also implicated in a different disease: facioscapulohumeral dystrophy type 2.

Identifying implicated genes for these rare, often severe—and therefore not transmitted—diseases is really only feasible with the power of genome sequencing and bioinformatics. It is unexpected to see monoallelic and biallelic forms of more or less the same phenotype in three different cases and, in two instances (with *ITPR1* and *SMCHD1*), the existence of a different phenotype associated with the same gene, though I agree with Andrew Wilkie (133) that, knowing how genes fulfill multiple functions in different tissues and at different times, we should not find such pleiotropy surprising.

PHENOTYPE VARIATION AND POSSIBLE MODULATION

Throughout my studies, I have been observing and discussing phenotype variation, which is a critical concept in clinical genetics for several reasons. Understanding why and how phenotypes vary within and among families is important for providing counseling. If the mechanism is understood,

then there may be opportunities to modify the disease burden using drugs or environmental changes. With Patricia Yeyati, we explored the role of the chaperone HSP90 in modifying the eye phenotype in zebrafish (67, 138). As an aside, what better example of serendipity is there than for my lab to find that a requested zebrafish with an unidentified eye mutation (*sunrise*) carries a novel missense change in *pax6b* (67)? That eye phenotype was readily modulated by manipulating HSP90 levels (138). Amino acid substitutions are ideal HSP90 targets, as beautifully illustrated in *C. elegans* experiments where repeat expansion mutants are titrated against different temperature-sensitive variants (37). Mutations at different loci, each requiring chaperone help, will modify each other's severity by competing for the limited chaperone supply. Phenotypes may be modulated by genetic variation at other loci, even where they are functionally unrelated. Environmental stress can confer similar negatively modulating effects, and environmental amelioration should improve phenotypic severity. These are interesting lessons to contemplate during a pandemic (127).

UNFINISHED ENDING

Following my retirement and move to London to be nearer to family, I was delighted to be able to join the UCL Institute of Ophthalmology as an honorary professor. This institute is a great center for eye research, attached to the famed Moorfields Eye Hospital, where many of my clinical colleagues work. I have no lab or grants—just welcoming colleagues to interact with. I was a coapplicant on a Fight for Sight PhD grant with Andrew Webster, a prominent ophthalmologist and genomic scientist who works mainly on retinal diseases and also serves as the lead on sensory disease genomics in the UK 100,000 Genomes Project. We set out to pursue the genetics underlying North Carolina macular dystrophy (NCMD), a relatively mild, mostly nonprogressive autosomal dominant retinal condition with a developmental defect of the fovea.

Several large families were available for analysis. Disease loci with indistinguishable phenotypes had already been assigned by linkage to two loci: *MCDR1* on chromosome 6q16 and *MCDR3* on chromosome 5p15 (84). Just as new PhD student Raquel Silva was starting her project, a paper was published by colleagues from Iowa (110) identifying possible implicated genes. On chromosome 6, a large duplication was found to include the gene *PRDM13*, and, in addition, two different closely juxtaposed single-nucleotide variants (SNVs) in a noncoding genomic region were shown to be markers for different shared disease-associated haplotypes. The existence of founder haplotypes in *MCDR1*-linked disease had been established previously. In the *MCDR3* locus region on chromosome 5, a large duplication was found to include the *IRX1* gene in all affected individuals of one large NCMD family (110). Whole-genome sequencing of selected cases from our NCMD cohort identified two small, independent, overlapping duplications downstream of *IRX1* (16). This suggests that genomic perturbation over this much smaller region is associated with *MCDR3* pathology. Both duplications can be identified by PCR, and the UK-arising duplication is a founder event seen in several additional families. Three of the UK cohort were found to carry one of the *MCDR1* founder SNVs, V2, described by Small et al. (110). The mechanism of disease causation is unclear, though the *MCDR3* duplicated region displays active open chromatin status during fetal retinal development. A complex *IRX1*-region deletion–duplication family with limb anomalies in addition to NCMD has also been seen (15) but is not easy to fit into the puzzle. However, a more severe macular condition, progressive bifocal chorioretinal atrophy, was closely linked to the *MDCR1* locus, and sequencing identified two novel SNVs (106) close to the classical *MDCR1* SNVs (110) in the region upstream of *PRDM13*. A de novo, mosaic SNV, identified in the mildly affected mother, is also present in her more severely affected full mutation carrier son (106).

The mechanisms leading to NCMD are elusive, but there has been progress. The finding of ancestral haplotypes at both monoallelic loci was unexpected. Induced pluripotent stem cells have

been set up from key patients, but it is doubtful whether retinal differentiation in culture can recapitulate foveal development, and mice do not have foveae. For the moment, the story is unfinished.

REFLECTIONS ON THE PATHS TRAVELED

Writing this review brought home to me anew how much I enjoy biology and what a great tool genetics is for exploring mechanisms. The process also reminded me that it is not possible to plan or map out your career in advance. For example, I am glad that I did not wait to have children until I was fully established. But there is no way to be certain of outcomes in advance—much of life inside and outside the lab is experimental, and it is not always possible to repeat the experiment more carefully, but it is important to take good notes and learn lessons. Though I enjoyed being a group leader and section head, managing people is more complex than doing experiments. I aim to lead from the front, meaning probably that I want to illustrate how things should be done, but I have tried not to expect more from others than I do from myself. I know that I have failed and disappointed some people and think that we should develop effective mechanisms for regular, not just annual, 360° appraisals.

There have been quite a few lucky turns in my life and career. There has not been serious sex discrimination, aside from perhaps the occasional failure to be lead author. I have enjoyed and worked hard at leadership roles—even minor ones, such as seminar organizer or meeting organizer. I recommend such activity to women, as involvement in such organizing provides opportunities to receive seminar and meeting invitations in return. Being an extremely slow reader made grant committees very hard work, but they are rewarding learning opportunities, both at the scientific concept level and to demonstrate what good grants look like.

It is interesting to note here that when I joined the MRC, we were entirely core funded and not allowed to apply for outside grants, but that rule disappeared pretty early on, though we still had significant basic core support. Becoming an HHMI International Research Scholar was a great step forward. We had an opportunity to attend one of the subject-relevant annual meetings with regular HHMI scientists. Other career achievement highlights include becoming an honorary professor at the University of Edinburgh and a nicely spaced series of elections as a fellow of the Royal Society of Edinburgh, a fellow of the Academy of Medical Sciences, a member of the European Molecular Biology Organization, and ultimately a fellow of the Royal Society—in the same year that our first grandchild was born. Being a trustee of National Museums Scotland was a wonderful, different experience, followed immediately by membership of the Human Genetics Commission, which included many eminent and interesting people. Thinking about ethical and societal issues has provided critical insights. I have enjoyed serving on various committees and councils, including the Academy of Medical Sciences, the European Molecular Biology Organization, and the Royal Society.

Over the past 10–15 years, supporting and mentoring younger scientists and helping them take important steps in their careers has been a privilege. The mentees are mostly brilliant, and I have learned a lot from them. As I have enjoyed being honored (including receiving a CBE for services to science), it is obvious that putting others forward for such honors, though it can be quite an onerous task, is very pleasing when it works out. Being president of the European Society of Human Genetics and of the (UK) Genetics Society was interesting. The latter was hard but rewarding work over three years. Both societies organize wonderful and affordable meetings where young scientists can network and meet peers and senior colleagues in a relaxed atmosphere. Chairing the Royal Society's Diversity Committee for the past three years was one of the most thought-provoking roles I have taken on, and I certainly aim to continue delivering equity and inclusion.

My progress and achievements have rested on the shoulders of many giants. The fantastic support from family, friends, and colleagues has been, and always will be, essential. The interactive spirit and diversity of the people I have worked with have given me joy and sustenance.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

My parents' determination to come to the UK and start from scratch after their awful experiences was heroic. I am grateful to my husband, Simon, for encouraging and supporting my scientific and other endeavors, and to Paul and Eleanor for their early self-sufficiency and continuing engagement in family life.

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