Unravelling the genetics of inherited retinal dystrophies: past, present and future

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Abstract

The identification of the genes underlying monogenic diseases has been of interest to clinicians and scientists for many years. Using inherited retinal dystrophies as an example of monogenic disease we describe the history of molecular genetic techniques that have been pivotal in the discovery of disease causing genes. The methods that were developed in the 1970's and 80's are still in use today but have been refined and improved. These techniques enabled the concept of the Human Genome Project to be envisaged and ultimately realised. When the successful conclusion of the project was announced in 2003 many new tools and, as importantly, many collaborations had been developed that facilitated a rapid identification of disease genes. In the post-human genome project era advances in computing power and the clever use of the properties of DNA replication has allowed the development of next-generation sequencing technologies. These methods have revolutionised the identification of disease genes because for the first time there is no need to define the position of the gene in the genome. The use of next generation sequencing in a diagnostic setting has allowed many more patients with an inherited retinal dystrophy to obtain a molecular diagnosis for their disease. The identification of novel genes that have a role in the development or maintenance of retinal function is opening up avenues of research which will lead to the development of new pharmacological and gene therapy approaches. Neither of which can be used unless the defective gene and protein is known. The continued development of sequencing technologies also holds great promise for the advent of truly personalised medicine.

Keywords:

Retina; photoreceptors; inherited retinal dystrophies; molecular genetics; next-generation sequencing;

1. Introduction

The human eye detects light for both visual and non-visual functions via photoreceptors located in the retina, the 0.5mm layer of tissue that lines the back of the eye. The retina consists of three distinct cell body layers separated by synaptic plexiform layers that contain the neuronal processes and synaptic contacts of the cells. There are six major neuronal cell types, the rods and cones located in the outer nuclear layer, the bipolar, horizontal and amacrine cells in the inner nuclear layer and the ganglion cells in the ganglion cell layer (Kolb et al., 1995). The rod and cone cells are the visual photoreceptors and are highly specialised neurons with a polarized structure. They consist of an outer segment with flattened membrane discs which house the proteins required for phototransduction including the visual pigment molecules and an inner segment which contains the cell machinery required for protein synthesis and energy production. Rods account for 95% of the photoreceptors in the human retina and enable vision in dim light. Cones are responsible for colour vision and central fine vision and are concentrated at the macula, although are present throughout the retina (**Figure 1**). There are approximately 120 million rods and 6 million cones in the human retina (Kolb et al., 1995).

Each of the photoreceptor outer segment discs contains millions of visual pigment molecules and it is these visual pigments molecules that enable the photoreceptors to respond to light. The visual pigment consists of an opsin molecule, rhodopsin in rods and one of three types of cone opsins in cones, and a chromophore derived from vitamin A, called retinal. The three cone types (L-cones, M-cones and S-cones) express opsin pigments sensitive to different wavelengths of light 560nm (L-opsin), 530nm (M-opsin) and 420nm (S-opsin) which enable them to differentiate between colours (Kolb et al., 1995). Opsins are seven transmembrane domain G-protein coupled receptors with the N-terminus and the C-terminus in the extracellular and cytoplasmic regions of the cell membrane respectively. Phototransduction is initiated when the absorption of a photon of light by the chromophore retinal, located in the binding pocket of the opsin, causes its photoisomerization from 11-cis retinal to all-trans retinal. This leads to a conformational change in opsin structure which in turn triggers a signalling cascade resulting in changes in cyclic guanosine monophosphate (cGMP) and calcium levels (Ca^{2+}) that act as second messengers to generate electrical signals (Palczewski, 2014).

Inherited retinal dystrophies (IRD) are a heterogeneous group of disorders associated with the dysfunction or death of the photoreceptors. Collectively they have an incidence of 1 in 2000-3000, affecting an estimated 2 million people worldwide (Hartong et al., 2006). These disorders exhibit a wide range of phenotypes with significant overlap but they can be broadly divided into three main groups: those that principally affect the periphery such as retinitis pigmentosa (RP) and choroideremia; those that primarily affect the macula (**Figure 1**), known as ‘macular’ or ‘central’ dystrophies; and those that affect both the centre and periphery as seen in cone-rod or rod-cone dystrophies. Other terms used to describe the IRDs often refer to the Ophthalmologist who first described the condition, for example Stargardt disease. This eponymous classification, although still in use has been either superseded or enhanced by a more detailed description of the disease using sophisticated imaging and clinical functional testing. The use of optical coherence tomography (OCT), autofluorescence imaging (AF) and colour imaging together with psychophysical and electrophysiological measurements have enabled much more detailed phenotyping to be performed.

Diseases that primarily affect the peripheral or mid peripheral vision have historically been labelled as Retinitis Pigmentosa (RP), a specific term originally coined by the Dutch ophthalmologist Franciscus Donders (Donders, 1855). RP is the most common form of IRD with an estimated frequency of 1 in 3500 individuals (Hartong et al., 2006). The classic form of RP is characterised by nyctalopia (night blindness) and progressive peripheral vision loss which worsens over time leading to "tunnel" vision.

In contrast macular dystrophies are characterised by loss of central vision, inability to see detail, abnormal colour vision and sometimes delayed dark adaptation. Macular diseases may have both rod and cone involvement. If limited in extent or relatively benign the photoreceptor loss may be restricted to the macula, and not affect global cone or rod function.

The conditions in which both cones and rods are affected, resulting in both central and peripheral vision loss at a similar time in the disease. Cone-rod dystrophies may show temporal differences in cell populations being affected. Categorising monogenic IRD into these three groups is a broad brush approach and does not allow for change over time. Many disorders start by affecting one area but can often progress and affect both centre and periphery or vice versa.

There are already over 60 genes described as causing RP; so not only is there phenotypic heterogeneity but there is also significant genetic heterogeneity. These genes can be inherited in an X-linked, autosomal dominant or autosomal recessive manner. When considering all types of IRD, there are now over 280 genes listed in RetNet (https://sph.uth.edu/retnet/home.htm) and pathogenic mutation(s) in these genes may be associated with different phenotypes depending on the type of mutation and its effect. The phenotype may be also affected by gene modifiers which will become clearer in the ensuing years with the bioinformatics assessment of whole genomes. There may also be significant variability in expressivity for some conditions, even in the same family with the same mutation in the same gene. Although in a few conditions for example Sorsby Fundus dystrophy, the condition appears fully penetrant with a similar phenotype in all affected individuals. Syndromic features may be present in addition to the IRD. This is seen in Usher syndrome where the visual loss is accompanied by either congenital deafness as seen in Usher type I, or partial hearing loss as seen in Usher type II. In addition to monogenic disorders, complex or polygenic disorders such as age-related macular degeneration (AMD) and diabetes contribute to the number of patients with visual impairment. These conditions are usually associated with an onset in later life. AMD, either in the early or late form, affects 1 in 3 people over the age of 85 (Evans et al., 2004). According to Retina International, a consortium of worldwide eye charities, the global cost of these debilitating disorders is estimated to be as "high as $20 billion annually underscoring the need for swift actions to raise awareness of prevention, diagnosis and treatment options" (http://www.retina-international.org/).

Sophisticated phenotyping and genotyping in patients with IRD have enabled a much more focussed approach to diagnosis, prognosis and counselling, with clear benefit to patients and their families as well as informing clinicians and researchers in the field. Determining the genetic basis of the condition is a major goal both in the National Health Service (NHS) and in research. Understanding the function of the gene both in its wild type and mutant state; analysing the deleterious effects of the mutation(s), and unravelling the mechanism of disease, are all dependent on identifying disease causing mutations. The advent of gene therapy and with gene editing techniques such as the

CRISPR/Cas9 system (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9) on the horizon, identification of the underlying disease gene will be the key step in being able to utilise these promising therapeutic avenues.

In the last 30 years the approaches used to identify retinal disease causing genes have evolved rapidly from the very laborious positional cloning methods in the mid-1980's to next generation sequencing (NGS) techniques that are becoming routine. This review will summarise these different approaches and show that each method has its merits. It is clear that even in this age of "big data" and in the post-Human Genome Project era, accurate and detailed clinical phenotyping is still vital to arriving at a molecular diagnosis for the patients.

2. Molecular genetic techniques: a short history

When Watson and Crick proposed the structure of DNA as a double helix in their seminal paper in Nature in 1953 (Watson and Crick, 1953b) and noted that:

> "*It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material.*"

The foundations of modern molecular genetics were laid. All of the molecular methods developed over the next 50 years or so utilise the complementary base-pairing (adenine with thymine and guanosine with cytosine) postulated in this and a subsequent paper (Watson and Crick, 1953a, b). Further work over the next few years included the proposal of the central dogma of molecular biology (DNA makes RNA, RNA makes protein) by Crick (Crick, 1970) and the deciphering of the genetic code, that three bases (or a codon) define an amino acid (Nirenberg and Matthaei, 1961). However the major methodological breakthrough in the 1960's and 1970's was the development of DNA sequencing techniques which enabled the linear order of the nucleic acids to be determined in turn allowing the primary amino acid sequence to be predicted (see 2.2). Nevertheless it was the development of several recombinant DNA technologies during the late 1960s and 70s that meant that scientists rapidly gained the tools that enabled them to cut and join DNA molecules, monitor these reactions and to grow millions of copies in bacterial cells (see **Table 1** for the timeline and 2.1).

2.1 Recombinant DNA methods

2.1.1. Type II restriction enzymes

It had been possible to isolate DNA from cells for many years but it was not until the discovery of type II restriction enzymes by Hamilton Smith and colleagues in 1970 (Kelly and Smith, 1970) that it could be cut in a controlled way. The finding that an enzyme "R" from *Haemophilus influenzae* cleaved DNA at a specific sequence meant that DNA could now be cut up into smaller pieces that had specific ends and they could be visualised using gel electrophoresis (Danna and Nathans, 1971) (**Table 1**). More enzymes recognising different sequences, usually 4-6bp long, were then subsequently isolated from different bacterial strains.

2.1.2 Cloning and transformation

The next major milestone was the development of plasmids as vectors for carrying these DNA fragments into bacterial cells. Plasmids are small circular DNA molecules found in bacterial cells that can replicate independently from the genome of the host. In 1972 Stanley Cohen was studying the

role that plasmids played in antibiotic resistance and devised a method that allowed bacteria to take up purified plasmid DNA, a process called "transformation" (Cohen et al., 1972). At the same time Paul Berg had also developed a method using the SV40 virus as a vector (Jackson et al., 1972). Cohen and colleagues then used the restriction enzyme *Eco*RI and DNA ligase, an enzyme that catalyses the formation of phosphodiester bonds at the end of two DNA molecules that had been discovered by several groups in 1967 (Cozzarelli et al., 1967; Gefter et al., 1967; Gellert, 1967; Olivera and Lehman, 1967; Weiss and Richardson, 1967), to construct a plasmid *in vitro* (Cohen et al., 1973). The plasmid they constructed contained the two antibiotic resistance genes and the cells that were transformed were resistant to both antibiotics. This meant that all the necessary tools were now in place to be able to manipulate DNA molecules *in vitro* (**Table 1**).

2.1.3. Southern blotting

Southern blotting was first described by Ed Southern in 1975 (Southern, 1975) and enabled DNA fragments visualised on an agarose gel to be transferred and immobilised on a nitrocellulose or nylon membrane providing a semi-permanent copy of the pattern obtained via electrophoresis. This meant that for the first time specific DNA fragments could be detected using hybridisation techniques with labelled DNA or RNA probes in a background of many other fragments. This method opened the door to many other applications including Northern blotting (immobilised RNA fragments) and Western blotting (transfer of proteins) as well as techniques such as restriction fragment length polymorphism (RFLP) analysis (see 3.1.2). Southern blotting is still used for the diagnosis of diseases such as the haemoglobinopathies and Facioscapulohumeral muscular dystrophy (FSHD).

2.2. First generation sequencing

After the description of the double helical structure of DNA by Watson and Crick in 1953 (Watson and Crick, 1953b) scientists wanted to be able to read the linear order of the four bases in order to be able to infer the sequence of the encoded protein. The search for the methods to do this began before the molecular biology techniques outlined above were described. Initially the limiting step for DNA sequencing was obtaining the starting material. Holley and colleagues overcame this by using transfer RNA molecules as they were small and could be purified. This enabled them to publish the first sequence of a nucleic acid molecule, *E.coli* alanine tRNA, they inferred the sequence from the fragments they generated using different ribonucleases (Holley et al., 1965). Advances in the methods for purifying viral DNA molecules enabled Wu and Kaiser working in Cornell in 1968 to publish the first successful DNA sequence from the short "sticky" ends of the bacteriophage lambda (Wu and Kaiser, 1968). They measured the incorporation of radioactive nucleotides by DNA polymerase to fill in the overhang however it took 3 years for them to complete the 12 base pair sequence.

The discovery of type II restriction enzymes and the advent of cloning processes (table 1 and 2.1) meant that smaller DNA fragments could be isolated and used as the starting material. This allowed the sequencing process to be developed further by two groups, Allan Maxam and Walter Gilbert in Harvard (Maxam and Gilbert, 1977) and Frederick Sanger in Cambridge (Sanger et al., 1977). The two groups utilised very different methodologies to achieve this and are summarised below (for a more detailed review see (Heather and Chain, 2016; Hutchison, 2007)).

2.2.1 Maxam and Gilbert

The method developed by Maxam and Gilbert in early 1977 was a chemical process that involved radioactively labelling the 5' end of the DNA strand, followed by chemical modification and subsequent cleavage (Maxam and Gilbert, 1977). The chemical treatment breaks the DNA at a proportion of one or two of the four nucleotide bases (G, A+G, C and C+T). Each reaction was run out in a single lane of a polyacrylamide gel which separates the fragments based on size. The gel is then exposed to X-ray film and the sequence of the fragment can be read. The method of Maxam and Gilbert was the first one to be widely used in laboratories.

2.2.2 Sanger sequencing

However it was the development of Frederic Sanger's "chain-termination" or dideoxy method in late 1977 that really revolutionised sequencing. The groundwork for this method was carried out in 1975 when Sanger together with Alan Coulson developed the so-called "plus and minus" system (Sanger and Coulson, 1975). This method utilised DNA polymerase to synthesise DNA from a primer by incorporating radiolabelled nucleotides. This was followed by a second round of DNA polymerase reactions where the "plus" reaction contained only one of the four deoxy nucleoside triphosphates (dNTPs) and the "minus" reaction contained the other three. The eight reactions were run out on polyacrylamide gels and exposed to X-ray film. Fragments differing in length by a single nucleotide could be distinguished. The biggest drawback of this method was in determining the length of runs of the same nucleotide (homopolymer runs). Over the next two years Sanger refined the technique and added dideoxynucleotides (ddNTPs) in to the reaction mix (Sanger et al., 1977). These ddNTPs are analogues of deoxynucleotides (dNTPs) and lack the 3' hydroxyl group required for chain formation (**Figure 2A**). By mixing a small amount of radiolabelled ddNTPs with the dNTPs required for chain synthesis a proportion of the fragments will "terminate" when the ddNTP is incorporated. By using four reactions each containing a separate ddNTP, running them out on a polyacrylamide gel followed by exposure to X-ray film the sequence can be read (**Figure 2Bi and ii**). This method was rapidly adopted in research laboratories and the technique was refined with ^{35}S being used instead of ^{32}P for labelling giving better resolution and smaller wells and thinner gels also helped to increase the read length (for review see (Heather and Chain, 2016; Hutchison, 2007).

The next step in the improvement of the technique was the development of cycle sequencing which uses a thermostable DNA polymerase and the polymerase chain reaction (PCR) (see 2.3) and enables repeated rounds of denaturation, annealing and DNA synthesis to take place (Carothers et al., 1989). This combination of PCR and Sanger sequencing reduces the amount of template DNA needed as the chain termination products are amplified but in a linear manner as only one primer is used.

A further refinement of the technique was the addition of different fluorescent dyes to each ddNTP replacing the radioactive labelling. This meant the reactions could now be carried out in one tube instead of four (see **Figure 2Biii**) and in 1986 the automation of the technique was reported (Smith et al., 1986). This first report from Leroy Hood's group at Caltech described how a computer could gather the sequence data directly. A consequence of this work was the development of the first commercial sequencers by Applied Biosystems (ABI) who launched the ABI 370A model shortly afterwards, which could generate 1000bp of sequence per day. This is the machine that was used by Craig Venter and colleagues to first sequence a gene using an automated system (Gocayne et al., 1987). Up until this point the only genomes that had been sequenced were those of viruses and organelles but the technological advances made the sequencing of more complex genomes

achievable. In 1995 Venter and others reported the complete genome sequences of two bacteria, *Haemophilus influenza* and *Mycoplasma genitalium* (Fleischmann et al., 1995; Fraser et al., 1995). The methodology adopted by the Venter group to determine the *Haemophilius influenza* genome was that of "shotgun" sequencing. This involved creating a library of random DNA fragments, cloning them and then sequencing each one. Computers were then used to assemble and align the sequences produced to give the complete genome. A refinement of this technique would later be used by Venter's team at Celera to assemble the human genome (see 3.3).

In 1996 ABI released the first commercial capillary electrophoresis DNA sequencer, the ABI PRISM 310, ending the need to load the reactions onto a slab gel. This could produce approximately 500-1000bp of sequence for each reaction. Two years later the ABI PRISM 3700 was released which had 96 capillaries and so had the capability of producing approximately 900,000bp sequence per day. Automated Sanger sequencing with dye terminators became the predominant sequencing technology and was used to generate the draft of the human genome (see 3.3).

2.3 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was invented by Kary Mullis in 1985 and its impact on molecular genetic research as well as forensic science cannot be overstated (Saiki et al., 1985). PCR is a method of generating millions of copies of a fragment of DNA without the need to grow them in bacteria. Some sequence information was needed to be able to design primers flanking the region of interest but the advances in sequencing technology (see 2.2) had made this possible. The original description of the PCR method used 1 microgram of template DNA, two 20bp long primers that flank the region of interest, a mix of all four dNTPs and buffer. The reaction mix was then heated to 100^{o}C for 1 minute (denaturation), cooled to 25^{o}C for 30 seconds (annealing) and then 5 units of the Klenow fragment of DNA polymerase was added and the reaction allowed to proceed for 2 minutes (extension). This was then repeated for 9 more cycles (Mullis and Faloona, 1987). This was a time-consuming process with the addition of Klenow in each cycle but in 1988 Erlich and colleagues including Mullis described the use of a thermostable DNA polymerase that had been isolated from the bacterium *Thermus aquaticus* (*Taq*) that lives in the hot springs in Yellowstone National Park (Saiki et al., 1988). The enzyme has evolved to withstand the extreme temperatures in which the bacteria live and therefore remains active during the high temperatures used in PCR. This meant that annealing and extension could now be done at higher temperatures and the polymerase did not need to be replenished in each cycle. This paved the way for the automation of the process and PCR machines became commercially available and subsequently the work horse of the modern molecular biology laboratory. Mullis was subsequently awarded the Nobel Prize for Chemistry in 1993.

Since then PCR has been developed for many uses including quantitative (or real time) PCR (qPCR). Previously attempts at measuring gene expression relied on methods such as Northern blotting (for RNA) or Southern blotting (for PCR products) which do not allow precise quantitation (see 2.1.3). Quantitative PCR overcomes these issues by monitoring the amount of a PCR fragment produced during each cycle of PCR allowing an accurate measurement to be obtained.

2.4 Next generation sequencing

Although Sanger sequencing (described in 2.2.2) is considered to be the gold standard for accuracy with reported error rates consistently less than 1% (Ewing et al., 1998) it has the major disadvantage of being slow. Only 500-1000bp of sequence is produced in a single reaction in 2 hours and larger

fragments have the added disadvantage of needing to be cloned. As a result the human genome project cost $2.7 billion to produce a draft sequence. It would therefore be prohibitively time consuming and expensive to use this technology for routinely sequencing genomes for uses such as clinical diagnostics. The process therefore needs to be both cheaper and quicker and this has led to the development of several new approaches known as “next-generation sequencing” (NGS) or “second generation sequencing”. These approaches utilise different technologies, outlined below, but share a common feature that they are “massively parallel”, that is they can sequence huge numbers of samples at one time.

2.4.1 Pyrosequencing

Pyrosequencing was first described in the laboratory of Pål Nyrén in Stockholm in 1996 (Ronaghi et al., 1996) and is based on the detection of pyrophosphate release when a nucleotide is incorporated into a growing DNA strand. The reaction mix contains template, a primer and DNA polymerase so when the correct dNTP is added to the reaction and incorporated into the DNA strand it is accompanied by the release of pyrophosphate (PPi) (shown schematically in **Figure 3**). If this reaction is carried out in the presence of two other enzymes, ATP sulphurylase and luciferase, each incorporation of a nucleotide results in light being produced. ATP sulphurylase converts PPi to ATP in the presence of adenosine 5' phosphosulphate (APS). In turn ATP drives the conversion of luciferin to oxyluciferin by luciferase that generates visible light. The light produced is detected by a charge coupled device (CCD) chip and seen as a peak in the raw data output (Pyrogram). The height of each peak (light signal) is proportional to the number of nucleotides incorporated. Apyrase, a nucleotide-degrading enzyme, is also present and continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added. Addition of dNTPs is performed sequentially. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace (**Figure 3**). This technique has the added advantage that sequencing is performed in real time; there is no need for electrophoresis. Like Sanger sequencing pyrosequencing is a “sequence-by-synthesis” (SBS) method as it utilises a DNA polymerase to incorporate nucleotides into a DNA strand.

2.4.2 454 (Roche 454)

Although pyrosequencing had been used for genotyping it was not until Jonathan Rothberg set up a company, 454 Life Sciences, in 1999 that the technique was exploited for massively parallel sequencing. Rothberg’s approach was to prepare shotgun libraries by fragmenting the DNA with nitrogen gas followed by the addition of adapters to each end of the fragments. These adapters are then used to attach the fragments to beads. The beads then undergo a water-in-emulsion PCR which coats each bead in a clonal DNA population. The DNA-coated beads are then arrayed in the 1.6 million wells of a picolitre reaction plate, one bead per well. Nucleotides are allowed to flow sequentially over the plate along with the required enzymes (see 2.4.1) and pyrophosphate release is measured using a charged coupled device below the wells (Margulies et al., 2005). This set up allowed reads of 400-500bp per well and given that there are usually a million wells that contain suitably clonal beads this increased the amount of sequence data generated enormously.

This technology was used to sequence James Watson’s genome which was finished in four months and was estimated to cost less than US$1.5 million (Wheeler et al., 2008). Compared to the estimated cost of $100 million of the first individual human genome, that of Craig Venter, published the previous year, which used Sanger sequencing the saving is considerable (Levy et al., 2007).

However pyrosequencing does have issues with homopolymer tracts as the run length is estimated from the amount of PPi released resulting in a relatively high error rate.

Roche bought 454 in 2007 and developed the second generation 454 Genome Sequencer FLX which was reported to be able to produce 100 Mb with 99.5% accuracy (for review see (Heather and Chain, 2016; Hutchison, 2007).

2.4.3 Solexa/Illumina

Solexa, subsequently bought by Illumina who are now considered to be the leaders in the field with >70% of the market, developed a different approach for massively parallel sequencing. Their chemistry originally developed by Shankar Balasubramanian and David Klenerman at the University of Cambridge bears a much closer resemblance to Sanger sequencing than the method used by 454. It uses ddNTPs to terminate the synthesis of a strand but the chain termination is reversible, allowing synthesis of a complementary strand to be performed one nucleotide at a time (Bentley et al., 2008). The identity of the ddNTP is determined by fluorescent labelling and detection by a laser, because each nucleotide is added sequentially the accuracy of the sequence data generated is increased but the run times are lengthened to several days, as opposed to several hours with 454. However it does mean that homopolymer tracts can be identified precisely. Illumina sequencing is much cheaper than the 454 and involves less hands-on time for preparation of samples. The sequencing process starts with the ligation of specific adapters to the DNA fragments, which are then immobilised on a solid surface called a flow cell. The flow cell surface is also coated with the adapters and the complementary adapters. Each single strand fragment has one immobilised to the surface will create a “bridge” by hybridising its free end to the complementary adapter on the flow cell surface. Clonal amplification then takes place creating up to 1000 identical copies (called DNA “polonies”). Up to 600 Gb of bases per run and 55 Gb per day can be generated.

2.4.4 Sequencing by Oligonucleotide Ligation and Detection (SOLiD)

A third methodology was launched by ABI in 2007, the Sequencing by Oligonucleotide Ligation and Detection (SOLiD) system is based on an approach developed in George Church’s laboratory in 2005 (Shendure et al., 2005). The preparation of fragments shares some similarities with that used for the 454 (see 2.4.2) so the sheared fragments are ligated to adapters, attached to beads and clonal amplification using emulsion PCR is carried out. However the beads used are much smaller which allows more data to be generated. The difference is that DNA ligase not DNA polymerase is used for incorporation making it a “sequencing-by-ligation” approach, as opposed to “sequencing-by-synthesis”. Sequencing by ligation involves the hybridisation and ligation of labelled probes and primer sequences to the DNA strand to be sequenced. The probes encode either one or two known bases and a series of degenerate bases that contain all the possible variations of the complementary bases of the template. Sequences that anneal perfectly are ligated to the primer, these are imaged and then the fluorophores are cleaved and a new cycle is initiated 5bp upstream. This is repeated 7 times and then a new primer starting at n-1 is used. Five sequencing primers, n, n-1, n-2, n-3 and n-4 are used sequentially. Each 35bp insert is sequenced twice increasing the accuracy but the algorithm for interpreting the data is complicated. So although the reads are short, 35bp, approximately 2-3Gb data per run can be generated (McKernan et al., 2009).

2.4.5 Ion Torrent

Ion torrent technology is a method of DNA sequencing that is based on the pH change that occurs when a hydrogen ion is released when a nucleotide binds to the template. The methodology is similar to that of 454, with emulsion PCR used to amplify molecules on a bead. Nucleotides are allowed to flow one at a time over the plate along with the required enzymes (see 2.4.1) and pH is measured. A decrease in pH indicates a hydrogen ion has been released which signifies that the dNTP has been incorporated. The advantages of this technique are that cost of sample preparation is low and that sequence read time is fast. However, it is limited to short read lengths (400bp) and long homopolymer repeats are difficult to count accurately (Rothberg et al., 2011).

3. Identification of human disease genes

The way molecular geneticists search for the causal genes underlying human disease has changed enormously since the identification of the gene for chronic granulomatosis disease (CGD) by Stuart Orkins' laboratory in 1986 (Royer-Pokora et al., 1986). This was the first time that a gene had been identified based purely on its chromosomal localisation with no prior knowledge about its function. Although it took another year to determine that the CGD gene actually encodes the cytochrome b beta subunit (*CYBB*) (Teahan et al., 1987). Up until 1986 the only human disease genes to be identified were those for which the underlying biochemical basis was known for example abnormal haemoglobin in sickle cell anaemia (Ingram, 1957) and inborn errors of metabolism such as alkaptonuria which was first described by Archibald Garrod as early as 1902 as a Mendelian trait (Garrod, 1902).

The observations that an extra copy of chromosome 21 could lead to Downs Syndrome (Jacobs et al., 1959; Lejeune et al., 1959) and that chromosome 22 was abnormally short in cases of chronic myelogenous leukaemia (Nowell and Hungerford, 1960) provided evidence that larger scale changes in DNA could also cause disease. Subsequently the identification of chromosomal rearrangements has played a crucial role in ascertaining disease genes.

However it was in the mid-1980's that the search for the genes underlying diseases including retinitis pigmentosa, Duchenne Muscular dystrophy, Huntingdon's Disease and Cystic Fibrosis, for which there was no knowledge about function, really took off. This was made possible by the revolution in molecular biology techniques, described in section 2 above and shown in **Table 1**. The step by step process is illustrated in **figure 4** and described in more detail below (see section 3).

3.1 Identification of chromosomal location

The first step in the identification of a disease gene is to determine its location. Which chromosome is it on? The identification of a chromosomal rearrangement such as a deletion or balanced translocation can significantly aid this process.

3.1.1 Chromosomal deletions and rearrangements

Geneticists have been using DNA deletions in the fruit fly *Drosophila melanogaster* for many years as a method of determining the location of a gene. The use of ionizing radiation as a method of inducing chromosomal rearrangements is also widely used. However human geneticists have to rely on naturally occurring deletions. The occurrence of deletions in human chromosomes is estimated to be as high as 1 in 200 births according to Unique a charity set up to help families with rare chromosome disorders (http://www.rarechromo.co.uk/html/home.asp). The first step in

determining whether a deletion is present is for a cytogeneticist to stain the chromosomes to produce G-banding (Giesma staining). However, even with high resolution banding techniques only deletions and rearrangements of more than 3 Mbp (3,000,000bp) can be detected (for review see (Kearney, 2001)). The advent of fluorescence *in situ* hybridisation (FISH) in the late 1980's enabled the detection of smaller microdeletions and complex rearrangements and translocations. The isolation of specific probes enabled FISH to be used for diagnosis of various diseases for example DiGeorge syndrome (OMIM 188400), caused by a deletion on the long arm of chromosome 22 (Desmaze et al., 1993) (see 3.6.1).

The identification of the CGD gene in 1986 was facilitated by deletions in Xp21 in two patients, BB and NF (Royer-Pokora et al., 1986). The identification of deletions in these patients enabled the region in which the gene must lie to be narrowed down significantly.

3.1.2 Linkage analysis

If there is no evidence from chromosomal analysis as to where the gene may be located then linkage analysis is used (Figure 3). Genetic linkage analysis is a method of determining the chromosomal location of a disease gene. It is based on the observation that the closer two markers are to each other the more likely they will be to remain together during meiosis. The recombination fraction is a measure of the distance between two markers and is defined in centimorgans (cM), named after the geneticist Thomas Hunt Morgan. One cM is defined as the distance between two markers that have a 1% chance of being separated due to recombination (recombination fraction of 0.01) between them in a single generation. One cM corresponds to a highly variable physical distance but it is widely accepted that 1cM is approximately one million base pairs. Genetic linkage is measured as a LOD (logarithm of the odds) score and a value of 3 or more is now accepted as indicating that two loci are linked and was first demonstrated by Newton Morton in 1955 (Morton, 1955).

The earliest markers used were restriction fragment length polymorphisms (RFLPs) (Table 1). The discovery of type II restriction enzymes and the development of techniques such as Southern blotting and cloning enabled this development (listed in Table 1). An RFLP is where a base change in the DNA sequence either creates or abolishes a restriction enzyme site. This can be visualised on a gel and followed through a family. A study by Botstein and colleagues in 1980 showed that the use of RFLPs for linkage was possible but the coverage across the genome was very low (Botstein et al., 1980). Even so in 1983 a collaborative effort involving James Gusella and Nancy Wexler and colleagues in Venezuela used 12 RFLPs to screen two families with Huntington disease. Unexpectedly they found one of the markers, G8, that had been mapped to chromosome 4 showed linkage to the disease (Gusella et al., 1983) (Table 1). This showed it was possible to define disease loci using these markers. However successful use of this method relies on having markers whose localisation has already been determined. In 1985 Eiberg and colleagues demonstrated linkage between the cystic fibrosis gene and the enzyme paroxonase (PON) (Eiberg et al., 1985) unfortunately the chromosomal localisation of PON was unknown. It took several more months before linkage was finally demonstrated with markers on chromosome 7 (Knowlton et al., 1985; Wainwright et al., 1985; White et al., 1985). The first linkage map of the human genome was developed by Donis-Keller and colleagues and consisted of 403 polymorphic loci, including 393 RFLPs which provided linkage to 95% of the human genome (Donis-Keller et al., 1987).

The use of RFLPs was a laborious process and the density across the genome was not high enough to be able to refine the disease locus to a manageable size. However the identification of polymorphic short interspersed tandem repeats (microsatellite markers) that consisted of 2-10bp of simple repeats that could be amplified using the polymerase chain reaction (PCR) (Saiki et al., 1985) provided more coverage and led to a second generation linkage map of the human genome containing 814 markers (Weissenbach et al., 1992) (see 3.3.1).

3.2 Positional cloning

In the post-human genome project age it is hard to imagine how difficult it was to identify a disease gene before there was sequence information. Positional cloning or "reverse genetics" as it was also called is a process of identifying a gene based only on its position in the genome. This is a time-consuming and cumbersome process and is illustrated using the identification of the gene for choroideremia (CHM) in 1990 as an example (Cremers et al., 1990). Nothing was known about the function of the CHM gene, so there was no *a priori* knowledge about what type of protein was defective. The strategy was to identify DNA markers that were located close to the disease locus and so were "linked". However it was known that choroideremia was X-linked so scientists at least knew which chromosome to look at. Researchers looking at other genetic conditions such as cystic fibrosis and Huntingdon's disease did not have information about the localisation so had to examine markers on many different chromosomes before getting linkage. Nussbaum and colleagues demonstrated in 1985 that choroideremia was linked to a marker, DXYS1, and the gene was located on Xq13-21 (Nussbaum et al., 1985). Although it took two more years before Cremers et al identified a key marker, DXS165, which was shown to be deleted in some patients with choroideremia (Cremers et al., 1987). This was the breakthrough moment and the DNA around the marker was mapped and several single copy probes were identified. Two of these showed conservation across various species and one showed retinal expression, this turned out to be the gene responsible (Cremers et al., 1990). It had taken five years of intensive work by several groups to go from linkage to identification of the gene but it showed that a human disease gene could be identified in this way. It took two more years to show that the CHM gene encodes RAB escort protein 1 (REP1) and is a geranylgeranyl transferase (Seabra et al., 1992).

All of these steps were made considerably easier when the polymerase chain reaction (PCR) and Sanger sequencing started to be widely used in molecular genetics laboratories (Table 1). Although it was the instigation of the Human Genome Project that changed the way genes were identified.

3.3 Human Genome Project

The ultimate aims of the Human Genome Project were to determine the sequence of the entire human genome, all 3 billion base pairs, and the position of all the genes. The idea was conceived through a series of scientific meetings held during the years 1984 to 1986 initiated by Robert Sinsheimer at the University of California Santa Cruz and Charles DeLisi of the United States Department of Energy (DOE) (for review see (Berg, 2006; Davies, 2001)). Initially there was not much enthusiasm for the project with many scientists worried about the cost of such an undertaking and it took until 1988 for the US National Research Council (NRC) to endorse the idea. The NRC suggested a phased approach with a budget of up to $200 million a year. Later that year the National Institutes of Health (NIH) under the leadership of James Wyngaarden took the decision that the agency should be a major player in the Human Genome Project (HGP). The NIH and DOE signed a memorandum of understanding in October 1988 to collaborate on the HGP. It was proposed that a broader

programme to create genetic, physical and sequence maps of the human genome in parallel with other species be carried out. In 1988 the National Centre for Human Genome Research (NCHGR) (now called the National Human Genome Research Institute (NHGRI)) was established by NIH and James Watson was appointed as its head.

In 1990 the NIH and DOE published the first 5 year plan (fiscal year 1991-1995) of the proposed 15 year project and declared that 1st October 1990 was the start of the HGP (for review see (Cantor, 1990; Watson, 1990)). The goals outlined in this plan included construction of a high-resolution genetic map of the human genome, production of physical maps of all human chromosomes and selected model organisms, determination of the complete sequence of human DNA and of the DNA of selected model organisms. As well as the development of capabilities for collecting, storing, distributing, and analysing the data along with the creation of appropriate technologies necessary to achieve these objectives. In parallel the HGP set up an ethics, social and legal research framework to address potential issues for different populations and aimed at equitable access to data (Watson and Cook-Deegan, 1990).

In 1993 a new 5 year plan was proposed because it was felt that there was now a much clearer understanding of what needed to be done to bring the project in on time (Collins and Galas, 1993). The progress in each of the main areas, construction of genetic maps, construction of physical maps, identification of genes and sequencing is outlined in 3.3.1, 3.3.2 and 3.3.3.

3.3.1 Genetic maps

In the initial NIH and DOE 5 year plan one of the goals was to construct a genetic map at a resolution of 2-5cM. It was estimated that this would need to consist of between 600 and 1500 markers. The development of the first genetic map of the human genome using RFLPs (Donis-Keller et al., 1987) was a major breakthrough but as described above (3.1.2) the coverage was sparse. The second generation genetic map using microsatellite markers that could be easily detected using PCR was published in 1992 and contained 813 markers (Weissenbach et al., 1992). At around the same time Lander and colleagues published a genetic map of the mouse genome with a 4.3cM coverage (Dietrich et al., 1992).

One of the primary goals of the HGPs 1993 five-year plan was to complete a detailed genetic map by 1995. The ultimate goal was to achieve a map with markers 1cM apart and it was estimated that this would need 3000 well-spaced markers. The idea of using sequence tagged sites (STSs) was first introduced by Maynard Olson in 1989 (Olson et al., 1989). STSs are short single copy DNA sequences (200-500bp) that can be easily detected by PCR. Olson and colleagues recognised that STSs could serve as markers for both genetic and physical maps. STSs could be from all the classes of previously used markers including RFLPs and microsatellites as long as they could be amplified by PCR. The comprehensive genetic map of the human genome was completed a year ahead of schedule and was a much denser map than originally visualised consisting of almost 6000 markers spaced at an average density of 0.7 cM (Murray et al., 1994). This marked the completion of the first major goal of the HGP and provided the tools to map disease genes much more easily than previously. The Weissenbach group also published a final genetic map based on 5264 microsatellite markers (Dib et al., 1996). These resources meant that in theory linkage mapping for most monogenic diseases could now be carried out to the 1cM level and also provided many markers to identify clones for the physical mapping part of the HGP.

3.3.2 Physical maps

The construction of a physical map of the human genome was one of the aims of the first 5 year plan (1991-1995) (National Center for Human Genome Research (U.S.) and United States. Department of Energy. Office of Health and Environmental Research. Human Genome Program., 1990). A physical map is a basic requirement for sequence generation; unless clones covering the entire genome can be identified there is nothing to sequence. It was expected that this would be an STS-based map with markers approximately 100kb apart. Several advances in molecular genetic techniques made this aim achievable. The first was the development of yeast artificial chromosomes (YACs) by Maynard Olson's group in 1987 (Burke et al., 1987) (Table 1). Up until this time bacterial plasmids that could hold up to 50 kb had been used for cloning, Olson's group replaced this with a linear molecule that mimics a yeast chromosome. This allowed the cloning of up to several hundred kilobases of DNA that could be replicated in yeast. Building "contigs" (contiguous fragments), a set of overlapping DNA fragments, now became much easier especially with the development of PCR based STS analysis. In 1992 the first physical maps of whole chromosomes were published, chromosome 21 and the Y chromosome respectively (Chumakov et al., 1992; Vollrath et al., 1992).

This led to the publication of a first generation physical map of the human genome in 1993 (Cohen et al., 1993). This map consisted of 33,000 YAC clones identified using over 2000 genetic markers. YACs were not without their problems though and there were reports of non-contiguous fragments being cloned as well as some that contained unstable inserts. This prompted Melvin Simon's group at California Institute of Technology to modify bacterial plasmids for the cloning of large fragments of DNA. These bacterial artificial chromosomes (BACs) had a cloning capacity of approximately 300 kb but they were stable and easy to use (Shizuya et al., 1992). Since then BACs have been the vector of choice for genomic research.

The second advance was the development of radiation hybrid mapping panels based on a technique developed in the 1970's (Goss and Harris, 1975). Goss and Harris showed that irradiated human cells could be rescued by being fused to rodent cells. These rodent-human hybrid cells each of which contains a single copy of a human chromosome are irradiated using high dose X-rays which breaks them into fragments. Radiation hybrid mapping uses a similar principle to mapping genes by linkage analysis (3.1.2) in that the further apart two markers are the more likely they are to be on different chromosomal fragments. The development of radiation hybrid maps of whole genomes was a crucial tool in the production of physical maps (Walter et al., 1994).

In 1995 a physical map of the human genome was published (Hudson et al., 1995). The map was the result of a huge collaborative effort from laboratories in the USA and France and consisted of 15,086 STSs with an average spacing of 199 kb. It was estimated that 94% of the genome was covered.

Physical mapping of various model organisms had begun much earlier, Coulson et al. had started to assemble a physical map of the nematode *Caenorhabditis elegans* as early as 1986 using restriction enzyme mapping (Coulson et al., 1986). The 100 million base pair *C.elegans* genome had been split into 860 clones ranging in size from 35-350kb and estimated to cover approximately 60% of the worm's genome. The introduction of YAC cloning enabled the whole genome to be represented (Coulson et al., 1991). The physical map of the mouse was completed in 1999 (Nusbaum et al., 1999).

3.3.3 Expressed sequence tags (ESTs) and cDNAs

In the first 5 year plan produced by NIH and DOE there was no explicit mention of the identification of genes although it was considered to be an implicit part of the project. It had been estimated that the human genome contained between 50,000 and 100,000 genes (National Center for Human Genome Research (U.S.) and United States. Department of Energy. Office of Health and Environmental Research. Human Genome Program., 1990). In the revised 5 year plan it was explicitly stated that one of the main aims of the HGP was to identify all the genes in the human genome and in certain model organisms (Collins and Galas, 1993). Two years earlier J. Craig Venter then a biologist at NIH had announced a strategy to find expressed genes (Adams et al., 1991). Previously a view had been put forward by Sydney Brenner that as the coding regions of genes only represented 3% of the genome that they should take priority (Brenner, 1990). Opponents of this view argued that it was difficult to identify all the mRNAs from different tissue types and that important information from the intragenic regions of the genome would be missed. Venter proposed a strategy to use partial sequences from cDNAs, called expressed sequence tags (ESTs), which are similar to STSs in that they are short sequences of 300-500pb in length generated from cDNAs. In their paper in 1991 Venter's group reported the identification of more than 330 genes and showed that the use of automated sequencing to generate ESTs was feasible (Adams et al., 1991). Up until this point most groups worked on a single gene so this strategy was ground breaking. The generation of ESTs meant that genes could now be "tagged" and placed on the physical map. This allowed researchers interested in a particular part of the genome, usually from linkage studies (see 3.1.2), to determine what genes were present in their region of interest. This was a massive step forward in the identification of human disease genes. In July 1990 a huge row had erupted when it was announced at a congressional hearing that NIH was filing a patent application on these partial genes. In April 1992 James Watson resigned as the head of NCHGR over this patent application. Venter also left NIH to set up the Institute for Genomic Research (TIGR), a non-profit organisation, when his application for funding to expand the EST sequencing project was rejected. The patent application was ultimately rejected.

At this time GenBank (http://www.ncbi.nlm.nih.gov/Genbank), a publicly accessible database, contained less than 3000 sequences, Venter and colleagues increased this by ten percent in just a few months. The publication of a further paper in 1992 reporting an additional 2375 genes almost doubled this number (Adams et al., 1992). GenBank had been set up by Walter Goad and colleagues at the Los Alamos National Laboratory with funding from NIH. It was originally intended to be a pilot project but as more and more sequence data was generated it was moved to the National Center for Biotechnology Information (NCBI), a division of NIH's National Library of Medicine in 1992. GenBank is part of the International Nucleotide Sequence Database Collaboration, which is comprised of the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL) and GenBank at NCBI and data is exchanged on a daily basis. It currently contains over 190 million sequences (June 2016).

In 1993 the Integrated Molecular Analysis of Genomes (IMAGE) consortium was set up by four academic groups who wanted to share libraries of cDNA clones and ensure that any sequence, mapping or expression information would be placed in public domain databases and be freely available to the research community (Lennon et al., 1996). The work was split between the four groups with the Soares group generating libraries, the Auffray group responsible for sequencing, the Polymeropoulos group mapping the clones and the Lennon group responsible for arraying and

distribution. The clones generated by the IMAGE consortium were available free of charge and without restriction for further study. As well as generating libraries from human, mouse and non-primate tissues they also arrayed cDNAs from zebrafish (*Danio rerio*), the pufferfish (*Fugu ruprides*) and the frog (*Xenopus laevis* and *Xenopus tropicalis)*.

Several groups then used a variety of approaches for the generation of sequence data from cDNAs (for review see (Sikela and Auffray, 1993)). This led to the establishment of an international consortium to develop and map gene based markers and in 1996 a paper reporting the mapping of 16,000 genes relative to a framework map of 1000 polymorphic microsatellite markers was published (Schuler et al., 1996). This initial report was followed 2 years later by a paper describing the mapping of 30,000 genes relative to the genetic map (Deloukas et al., 1998).

3.3.4 Sequencing of model organisms and the human genome

The sequencing part of the HGP project was the slowest to get started mainly because the human genetic and physical maps were required for progress to be made (see 3.3.1 and 3.3.2). The first five year plan did include the goal of sequencing an aggregate of 20 Mb of DNA in model organisms by 2005. One of the goals of the second 5 year plan was to improve current sequencing methods and/or develop new methods that would allow large scale sequencing to be carried out at a cost of $0.50 per base pair.

The sequencing of the genomes of smaller organisms did get underway and in 1990 the NIH started large scale trials on a number of model organisms, the bacterium *Escherichia coli* and *Caenorhabditis elegans* (nematode worm) and *Saccharomyces cerevisiae* (yeast). These sequencing projects took the approach of cloning fragments of the genome and initially sequencing them using radioactive methods (see 2.2.2). However the development of new automated fluorescent sequencing techniques helped to speed up the process (see 2.2.2).

In July 1992 the Wellcome Trust announced an investment of $95 million into the HGP and the opening of the Sanger Centre in 1993 with John Sulston as director marked the entry of the UK into the HGP.

The publication in 1995 by Venter and colleagues of the first genome sequence of a free-living organism, *Haemophilus influenza*, again changed the field (Fleischmann et al., 1995). This paper demonstrated that it was possible to randomly sequence clones and compile the data generated using computational methods rather than having to laboriously map each clone first.

At a meeting in February 1996 the HGP consortium members agreed to release sequence data into public databases within 24 hours and in April the NIH funded six groups to start the large-scale sequencing of the human genome. Later that year an international consortium published the complete yeast genome which is comprised of 12 million base pairs and was the first eukaryotic organism to be sequenced (Goffeau et al., 1996). The publication of the *E.coli* genome 4,639,221bp genome demonstrated that using a combined approach of cloning and random or “shotgun” sequencing could be used successfully (Blattner et al., 1997). In 1998 the complete sequence of the 97 Mb *C.elegans* genome were published and the authors stated that they would employ the same clone-based physical mapping approach if they began again (Consortium, 1998).

Arguably the biggest turning point in the history of the HGP came in May 1998 when Craig Venter announced the formation of a new company called Celera. He declared that they would sequence the human genome within 3 years at a cost of $300 million. In response the Wellcome Trust increased its contribution to $330 million and took on the responsibility for sequencing one third of the genome. The sequences generated by Celera were not publicly available but those generated by the HGP consortium were. This competition meant that the HGP moved on at speed and a draft sequence of more than 90% of the genome was predicted to be available by 2001 (Collins et al., 1998). In 1999 the first complete sequence of a human chromosome, the acrocentric chromosome 22, was published (Dunham et al., 1999) which was followed by that of chromosome 21 in 2000 (Hattori et al., 2000). A further validation of Venter's "shotgun" approach came with the publication of the largest genome yet to be sequenced, the 180 Mb *Drosophila melanogaster* (Adams et al., 2000; Myers et al., 2000).

Finally in February 2001 both groups published their working draft sequence, the HGP consortium in Nature (Lander et al., 2001) and Celera in Science (Venter et al., 2001). The finished genome (99% complete) was announced in 2003 50 years after the discovery of the double helix by Watson and Crick (Collins et al., 2003; Watson and Crick, 1953b).

3.3.5 Tools

The advent of the Human Genome Project resulted in a vast quantity of sequencing data being deposited into Genbank (see 3.3.3). This information however was useless without the tools to analyse it. One of the goals of the Human Genome Project was the 'development of computational tools such as algorithms, software and databases for the collection, interpretation and dissemination of the vast quantities of complex mapping and sequence data generated by the Human Genome Project' (Pearson and Soll, 1991). Researchers needed to be able to interrogate the databases to find sequences of interest. In 1983 two NIH researchers Wilbur and Lipman published an algorithm that allowed sequence databases to be searched in a few minutes (Wilbur and Lipman, 1983). Further analysis tools were developed such as BLAST (Basic Local Alignment Search Tool) which can search sequences in GenBank for similarity in a few seconds (Altschul et al., 1990).

The generation of a large number of sequences from various EST sequencing projects prompted the creation of a new EST database, dbEST (http://www.ncbi.nlm.nih.gov/dbEST), as a division of GenBank (Boguski et al., 1993). This provided a valuable resource enabling researchers to get information on the expression profile of a sequence of interest as well as determining the existence of human homologues of functionally characterised genes. An extension of this was the development of UniGene (http://www.ncbi.nlm.nih.gov/unigene), which grouped ESTs into gene specific clusters. However as some of the sequence generated for ESTs was of poor quality sometimes ESTs from the same gene were not clustered together, but UniGene did provide useful information on the expression profile. Although this always needed to be validated, using techniques such as Northern blot or *in situ* hybridisation.

There were also large amounts of genomic sequence being deposited into GenBank. This meant that researchers looking at a particular region of a chromosome now had some useful data but they needed tools to analyse the sequence to determine what genes were within their region of interest. This led to the development of many programs designed to predict coding regions within a genomic fragment. These programs used splice site prediction, exon identification and full gene prediction

algorithms. None were 100% accurate but using more than one program increased the accuracy (for review see (Lichanska and Simpson, 2002)).

Finally the development of databases with user friendly web browsers to access the data was a big step forward. For example the development of the Ensembl database (Hubbard et al., 2002) and the UCSC genome browser (Kent et al., 2002) meant that sequence data, known genes and predicted genes as well as information from other sources such as protein family databases, the OMIM database (Online Mendelian Inheritance in Man) and expression data from SAGE (see 4.3.2) were now integrated and openly accessible. None of these bioinformatics tools though are a substitute for laboratory work and all the predictions need to be verified experimentally, but they have significantly reduced the amount of bench work required.

3.4 Candidate gene analysis

Positional cloning *per se* is a time-consuming and laborious process (as outlined in 3.2). It has been applied successfully to many projects but as the information from the HGP increased an approach called "candidate gene analysis" became the method of choice. The development of more detailed genetic and physical maps (see 3.3.1 and 3.3.2) meant that researchers now had the tools to narrow their linkage region to 1-2Mb. These regions, although smaller than previously, might still contain between 60 and 150 genes. However as the number of ESTs and sequenced genes increased it was possible to make an "educated guess" as to which genes might be good candidates for the disease. This could be based on some knowledge of the physiology of the disease and the biological pathways involved. This is discussed in 4.2 in more detail in relation to retinal degenerations.

3.5 Mutation analysis

Many different strategies have been adopted to identify disease-causing mutations within genes of interest. The choice of technique is often based on cost, speed and sample size and whether the sequence of the mutation is already known. The methods described below are those that are used for scanning a particular region of the genome for any changes in sequence. The methods used for genotyping are not described here. The techniques use the base-pairing properties of DNA to identify changes. Sanger sequencing, usually an expensive option for large cohorts is considered to be the "Gold-Standard" technique for verification of any changes detected. The analysis to determine whether the variants detected are pathogenic is described in 4.5.

3.5.1 Single Strand Conformational Polymorphism Electrophoresis (SSCP)

Single Strand Conformational Polymorphism Electrophoresis (SSCP) uses radiolabelled PCR products, amplified from specific regions of the gene of interest. These are denatured and electrophoresed on a non-denaturing polyacrylamide gel. The single strands fold in a sequence dependent manner and single nucleotide alterations within the strands can result in changes in secondary structure and lead to differing electrophoresis mobility. Autoradiographs are used to visualise the band shift pattern (Orita et al., 1989).

3.5.2 Heteroduplex Analysis

The genetic recombination of single complementary strands derived from different homologous chromosomes leads to the formation of a double stranded molecule or duplex. These molecules can be homoduplexes when the sequence matches exactly or heteroduplexes when a change has occurred in one of the strands. Single base pair substitutions can be detected by heteroduplex analysis. Radiolabelled PCR products are amplified from specific regions of the gene of interest.

These are heated to denature the products and then are slowly cooled to room temperature allowing renaturing. The heteroduplexed PCR samples are electrophoresed on nondenaturing polyacrylamide gels followed by visualisation by autoradiography. The heteroduplex samples run more slowly due to the open double stranded configuration surrounding the mismatched bases compared to their corresponding homoduplexes (White et al., 1992).

An automated system, the WAVE analyser, has been developed by Transgenomics (Kuklin et al., 1997). It uses denaturing high pressure liquid chromatography (DHPLC) to separate hetroduplexes and provides a rapid and economic method to analyse large numbers of samples.

3.5.3 Denaturing Gradient electrophoresis (DGGE)

Denaturing Gradient electrophoresis (DGGE) is a gel electrophoresis strategy where the DNA molecules are electrophoresed at a constant 60^{o}C with an increasing concentration of denaturing chemicals which force the DNA to unwind. The point at which the DNA unwinds is referred to as the melt point. Identical DNA sequences have indistinguishable melting patterns but any variation in sequence will generate different melting points and hence have a different pattern when visualised on a gel. Primers are designed to cover the complete protein coding region as well as the immediate intronic regions essential for splicing.

3.5.4 Direct Sequencing

Fragments, usually comprising the exon and approximately 50bp of flanking intronic sequence, of the gene of interest can be amplified using PCR and then directly sequenced with no need for a cloning step. This is expensive for a large number of samples but is considered the most accurate.

3.6 Detection of copy number variation (CNV)

The human genome consists of approximately 25,000 protein coding genes and it was generally thought that each were present in two copies, one copy inherited from each parent. However, recent studies have found that sections of the genome ranging from thousands to millions of base pairs can vary in copy number. Copy number variations (CNVs) can affect whole or part of genes and lead to imbalances in gene dosage. CNV can be created by meiotic recombination, homology-directed and non-homologous repair of double strand breaks, and errors in replication. Genes involved in extracellular biological process such as cell adhesion, recognition and communication have been found to contain greater numbers of CNV compare to those involved in biosynthetic and metabolic pathways (Conrad et al., 2010).

After the discovery that normal human cells contain 46 chromosomes, gross changes to chromosome number or structure could be detected by cytogenetics techniques including karyotyping and metaphase spreads (reviewed in (Trask, 2002)). These early techniques were labour intensive and time consuming and only able to detect large alterations in chromosomes (see 3.1.1). New techniques have been developed to identify smaller regions and include fluorescence *in situ* hybridisation (FISH), array-Comparative Genomic Hybridisation (CGH) and Multiplex Ligation-Dependent probe amplification (MLPA).

3.6.1 Fluorescence *in situ* hybridisation (*FISH*)

In the early 1980's, fluorescence *in situ* hybridisation (*FISH)* was developed to detect the presence or absence of a specific region of DNA on a chromosome using a complementary fluorescent probe (Van Prooijen-Knegt et al., 1982). *FISH* overcame some of the limitations of the early cytogenetic

techniques such as the need for dividing cells and allowed much smaller regions of variation to be detected. However, prior knowledge of the region of interest is required to design the probe, limiting this technique to known genes.

3.6.2 Comparative Genomic Hybridisation (CGH)

A decade after the conception of *FISH*, a new analysis technique was introduced, comparative genomic hybridisation (CGH) (Kallioniemi et al., 1992). The original CGH allowed the detection and mapping of CNVs by labelling patient genomic DNA with green fluorescence and a normal reference genomic DNA sample with red fluorescence and hybridising both samples to normal metaphase chromosomes whereby both labelled samples compete to hybridise to their target. Analysis of the fluorescence shows equal expression as yellow, deleted regions appear red and duplicated regions green. This technique gave a global view of gains and losses in one reaction, however could not detect mosaicism, balanced chromosome translocations, inversions and whole-genome ploidy changes. To address some of the challenges of conventional CGH, the technique was refined to give the resolution of *FISH* but retain the screening capacity of CGH and was called microarray CGH or array-CGH. This technique replaced the metaphase chromosome spreads with cloned fragments of 100-200kb of known chromosomal location, dotted onto glass slides (Pinkel et al., 1998; Pollack et al., 1999). The slides are hybridised with the labelled samples and the different intensity patterns compared. The increased resolution of the technique comes from the ability to determine the distance between consecutive clones and their relative size when fluorescence patterns are compared. Standard resolution varies between 1-1.5 Mb but can be enhanced further by supplementing the array with more clones (reviewed in (Oostlander et al., 2004)). The use of cDNAs and oligonucleotides as probes has also been explored. The resolution when cDNAs are used is 267kb but the sensitivity obtained is lower (Pollack et al., 1999). Using oligonucleotides, initially 60mers, as probes proved to be an efficient, rapid and cost-effective way of performing CGH (Carvalho et al., 2004). This approach has been developed by commercial companies and is now the method of choice.

3.6.3 Multiplex Ligation-Dependent probe amplification (MLPA)

Multiplex Ligation-Dependent probe amplification (MLPA) is an alternative approach to identifying CNVs (Schouten et al., 2002). This is a high-throughput technique that uses a single, multiplexed-PCR reaction to analyse up to 50 DNA samples. MLPA can detect heterozygous deletions or duplications of one or more exons which are difficult to detect by conventional screening due to the presence of the normal copy. MLPA can also be used for identifying microdeletions and subtelomeric deletions and duplications. The MLPA reaction uses an MLPA probe consisting of two probe oligonucleotides. The oligonucleotides recognise the sequence adjacent to the target DNA and when both hybridise to their target sequence they can be ligated to form a complete probe. The oligonucleotides, in addition to containing the specific DNA sequence, contain the sequence of a universal forward primer and the other probe contains the universal reverse primer sequence. This allows each complete probe to be amplified in a PCR reaction. Each complete probe has a unique length and can be separated by capillary electrophoresis. The forward primer used in the amplification step is fluorescently labelled and by comparing the peak pattern obtained deleted or duplicated regions can be identified when compared to a known reference sample. Usually probe ratios of less than 0.7 are regarded as indicative of a deletion while ratios above 1.3 are duplications. Prior knowledge of the regions of interest is required for this technique but it can be used to identify mosaicism, which was a limitation in the previously described techniques (Schouten et al., 2002). MLPA allows the

combining of several genes of interest to be analysed at the same time and can be used in a research environment to study experimental genes of interest with bespoke probe design, but commercially available kits are used in a clinical environment and are available for individual genes such as PAX6 as well as for more complex conditions such as autism (MRC-Holland).

4. Identification of inherited retinal dystrophy genes

4.1 Early history of the discovery of RP genes

From the mid-1980's many of the molecular techniques described in **table 1** were being used routinely. With the generation of a genetic linkage map using RFLPs as markers (Botstein et al., 1980) progress began to be made. The work on identifying genes causing inherited retinal degeneration illustrates how the techniques and approaches outlined in section 2 and 3 have been used practically. Much of the early work focussed on choroideremia (CHM) (described in 3.2) and retinitis pigmentosa (RP)

In 1984 a linkage study was used to localise a gene for RP on the proximal part of the short arm of the X chromosome (Bhattacharya et al., 1984). The linkage to an RFLP, L1.28, which had a LOD score of >7 seemed convincing. However several patients had also been identified who suffered from multiple X-linked diseases and had varying deletions of the short arm of the X chromosome as the basis for their disease. This included patient BB who also had CGD (chronic granulomatosis disease) and had been used to identify the gene (see 3.1.1). By comparing the deletions in three patients, BB (CGD, RP, DMD and lacked the antigen Kx), OM (lacked Kx and CGD), SB (CGD, RP and lack of Kx) a region that must be responsible for the RP in these boys was identified. However this region did not contain L1.28 which lay in a more proximal region several million base pairs away (for review see (Humphries et al., 1992). Over the next five years linkage data from many families were collected and it became apparent that there were actually two RP loci, RP3 defined by the deletion data and RP2 linked to L1.28 (Ott et al., 1990). However, it took another 6 years to identify the gene for RP3 (Meindl et al., 1996; Roepman et al., 1996b) and 9 years to determine the RP2 gene (Hardcastle et al., 1999; Mears et al., 1999).

The first linkage for autosomal dominant RP was reported in 1989 by McWilliam and colleagues who showed linkage in a large Irish pedigree, with more than 50 affected members, to markers on chromosome 3 (McWilliam et al., 1989). The linkage, with a marker D3S47, was to the long arm of chromosome 3, 3q21, a region where the gene encoding the photopigment rhodopsin was located (Nathans and Hogness, 1984). Dryja and colleagues screened rhodopsin in 20 unrelated patients with autosomal dominant RP using PCR and sequencing. In five out of the 20 patients they found a heterozygous C→A change in codon 23 of the gene which changed the normal proline residue to histidine (P23H) (Dryja et al., 1990b). They confirmed this mutation in a large family by showing segregation in all affected members.

It was at this point that it became apparent that there was not just one adRP locus as several other families did not show linkage to markers on chromosome 3. Further work on one of these families, another large Irish pedigree, showed linkage to markers on chromosome 6 (Farrar et al., 1991a). This region contained the human orthologue of the peripherin RDS (PRPH2) gene, a photoreceptor specific gene that had been shown to be the cause of the mouse retinal degeneration slow (*rds*) mutant. The *rds* mutant was first described by van Nie and colleagues in 1978 (van Nie et al., 1978).

The mice show abnormal development of the outer segments followed by the slow degeneration of the photoreceptors with rods and cones equally affected. The *rds* gene was identified in 1989 as a photoreceptor specific transcript although its function was unknown (Travis et al., 1989). It was subsequently shown by two groups to be peripherin RDS, a structural component of the rod photoreceptors (Connell et al., 1991; Travis et al., 1991). So all of the evidence pointed to the fact that peripherin RDS seemed to be an excellent candidate gene. Farrar and colleagues screened all 3 exons of the human peripherin gene using PCR and SSCP (see 2.3 and 3.5.1) in 60 unrelated probands and identified a three base pair deletion that results in the loss of a highly conserved cysteine residue in one patient. Screening of the affected family confirmed segregation (Farrar et al., 1991b). Subsequently a serine to glycine substitution at codon 212 was identified in the original family used for linkage (Farrar et al., 1991a; Farrar et al., 1992). At the same time Kajiwara and colleagues also screened the peripherin gene in 106 adRP patients and found three different variants, a deletion of a proline residue, a proline to leucine substitution and a leucine to proline change that they found in two unrelated patients. Further analysis in the families confirmed the changes segregated with disease (Kajiwara et al., 1991). Since then there have been multiple mutations described in this gene.

At around the same time another locus for adRP was described in a large seven generation family with linkage to the pericentric region of chromosome 8 (Blanton et al., 1991). This report further confirmed the genetic heterogeneity in autosomal dominant RP.

The identification of rhodopsin and peripherin was made easier by some knowledge of the biology of the photoreceptors and the process of human phototransduction. In a “News and Views” article in Nature in 1990 Thaddeus Dryja talked about using a “candidate-gene” approach rather than using positional cloning (Dryja, 1990). He argued that although positional cloning had been used successfully, a better approach might be to identify genes known to have a role in the physiology of the disease and search for mutations in patients with that disease. He thought that the technological advances for mutation screening (see 3.5) made this a viable proposition (Dryja, 1990).

4.2 Candidate gene approaches

Many of the laboratories searching for the genes responsible for retinal dystrophies did start out using a positional cloning approach but as techniques and methodologies improved the majority moved over to a candidate gene approach as suggested by Dryja (Dryja, 1990). However the first example of a candidate gene approach was actually shown a few years earlier by Nathans, Hogness and colleagues (Nathans et al., 1986a; Nathans et al., 1986b). They reported the identification of the long-wave sensitive (LWS) (also known as red, L opsin or *OPN1LW*), medium-wave sensitive (MWS) (also known as green, M opsin or *OPN1MW*) and short-wave sensitive (SWS) (also known as blue, S opsin or *OPN1SW*) opsin genes (Nathans et al., 1986b) and went on to demonstrate that structural rearrangements of the LWS and MWS gene array on Xq28 were responsible for red-green colour blindness in approximately 8% of Caucasian males (Nathans et al., 1986a). This was the first example of the identification of a gene responsible for an inherited retinal disorder. In a further study of twelve families with blue cone monochromacy, a rare X-linked disorder characterised by the absence of both red and green cone sensitivities, Nathans and colleagues showed that mutations/rearrangements in both the LWS and MWS genes caused the disease (Nathans et al., 1989). They also reported a patient who had a slow progressive central retinal dystrophy and suggested that:

"*some peripheral retinal dystrophies may be caused by mutations in the genes encoding rhodopsin or other rod proteins*".

It was only a year later that Dryja and colleagues identified mutations in rhodopsin (Dryja et al., 1990b). As dysfunction or death of rod and cone photoreceptors is the primary cause of blindness in the vast majority of retinal degenerative diseases the obvious candidate genes were those involved in the phototransduction cascade or structural components of the photoreceptors (e.g. peripherin (PRPH2)). So as more genes involved in photoreceptor structure and function were identified, mapped and cloned this process became easier.

4.2.1 Retinal disease genes involved in photoreceptor maintenance and function

In the years between the identification of rhodopsin as a cause of autosomal dominant RP in 1990 (Dryja et al., 1990a) until the announcement of the completion of the human genome project (99% complete) in 2003 (Collins et al., 2003) most of the genes identified encoded proteins involved in the structure or function of photoreceptors (**Table 2**). Candidate gene screening as a methodology increased during the Human Genome project. It suddenly became easy to look in the region defined by linkage analysis and determine which genes were present and assess their role in the retina and photoreceptors. Sanger sequencing was then used to screen the linked family and to confirm segregation. Several groups chose to screen panels of unrelated patients with the same disease, without any linkage information, for changes in candidate genes using the methodologies described in 3.5. Examples include the screening of rod cGMP-gated channel alpha subunit (*CNGA1*) (Dryja et al., 1995) and Retinal Pigment Epithelium Specific Protein 65kDa (*RPE65*) (Marlhens et al., 1997). Dryja and colleagues screened 94 unrelated ad RP and 173 unrelated ar RP patients using SSCP (see 3.5.1) and Sanger sequencing (see 2.2.2) and identified mutations in *CNGA1* in 4 families with ar RP (Dryja et al., 1995). Marlhens and colleagues also used SSCP and Sanger sequencing to screen *RPE65* in 12 unrelated patients with Lebers Congenital Amaurosis (LCA) and identified two nucleotide changes in one family that each lead to premature termination of the RPE65 protein. Showing this method could be also be used successfully.

Table 2 lists the genes causing IRD that were identified between 1990 and 2003, this is the period spanning from when the first gene, rhodopsin, was identified as a cause of autosomal dominant RP (Dryja et al., 1990b) until the announcement of the completion (99% complete) of the Human Genome Project (Collins et al., 2003). Of the 48 genes identified during this time 40 of them were identified using candidate gene screening as part of the process. In 1998 the Retinal Information Network database (RetNet) (http://www.sph.uth.tmc.edu/Retnet/) was established as a repository for information being generated about retinal degeneration genes (Daiger et al., 1998). In 1995 RetNet listed 55 retinal disease loci which has increased to 293 (as of November 2016) however the proportion of unknown causal variants has remained relatively steady at approximately 15-20% (http://www.sph.uth.tmc.edu/Retnet/) indicating that there are still many genes to find.

However the identification of the splicing factor *PRPF31* (pre mRNA processing factor 31) as the causal gene for ad RP (RP11 on 19q13.4) is an example of an unforeseen gene causing RP (Vithana et al., 2001). *PRPF31* is a ubiquitously expressed gene that would be expected to play a central role in mRNA processing in most tissues but it is only in rod photoreceptors that mutations cause a disease phenotype. The subsequent demonstration that *PRPC8* (pre-mRNA processing factor 8) and *PRPF3* (pre mRNA processing factor 3) also caused ad RP on chromosome 17p13.1 (RP13) and 1q21.2

(RP18) respectively confirmed the role of these splicing factors in retinal degeneration and indicated a crucial role in the normal maintenance of rod function (Chakarova et al., 2002; McKie et al., 2001).

Over the next few years many causal genes were identified with no previous known role in the retina and the rod and cone photoreceptors. This process was facilitated by the use of animal models and the analysis of the retinal transcriptome to identify functional candidate genes.

4.3 Identification of functional candidate genes

During the 1990s and early 2000s the process of linkage analysis and candidate gene screening was the methodology widely in use to identify disease genes. However several groups, including ours, used various approaches to try and identify genes that had a ***functional*** role in the retina. These methods have the advantage that they produce a catalogue of candidate eye disease genes that play a role in the development, maintenance and normal functioning of the retina. Various approaches have been taken including microarray (see 4.3.3), serial analysis of gene expression (SAGE) experiments (see 4.3.2) and the use of several different model systems (see 4.3.1 and 4.3.5). The completion of the Human Genome Project has also led to several bioinformatic approaches utilising expression data generated as part of the project. Sohocki and colleagues identified retina/pineal expressed genes as potential candidate genes for inherited retinal disorders (Sohocki et al., 1999). This approach proved fruitful with the isolation of the gene AIPL-1 (aryl-hydrocarbon interacting protein like-1), which has been shown to be mutated in Leber congenital amaurosis (LCA4) (Sohocki et al., 2000). The identification of this gene also implicated a previously unknown pathway as essential for the normal functioning of the retina and illustrates the usefulness of these types of approaches.

4.3.1 Animal models

Animal models, both naturally occurring and genetically engineered, have played an important role in understanding the development, functioning and maintenance of the mammalian retina. Models of retinal degeneration in mouse have been known about since the first description of the Keeler rodless (*r*) retina over 90 years ago (Keeler, 1924). Subsequently this mutant was shown to be the same as *rd* and *rd1* and had a mutation in the rod cGMP phosphodiesterase β-subunit (*PDE*) (Bowes et al., 1990; Pittler et al., 1993). However it was the identification of peripherin as the gene underlying the *rds* mutant in 1989 (Travis et al., 1989) and the subsequent identification of mutations in both peripherin (Farrar et al., 1991b; Kajiwara et al., 1991) and PDE (McLaughlin et al., 1993) in RP patients that demonstrated the usefulness of these naturally occurring mutants.

The mouse has been the model system of choice for several reasons, the existence of many naturally occurring mutants (see **Table 3**) as well as the ability to manipulate the mouse genome *in vitro* to generate transgenic mice. The development of the techniques described in section 2 and the tools developed as part of the human genome project (see 3.3) made this easier. The identification of the gene underlying the naturally occurring *shaker-1* (*sh-1*) mouse mutant which was a model of inherited deafness illustrates this (Gibson et al., 1995). Gibson and colleagues identified the *sh-1* gene using a traditional positional cloning approach and showed that a mutation in the unconventional myosin gene, *myosin 7a*, was responsible. The human *myosin 7a (MYO7A)* gene mapped to a region on chromosome 11 that contained a locus for Ushers Syndrome type 1, sequencing of the *MYO7A* in nine unrelated USH1B families identified five mutations, two different premature stop codons, a 6bp deletion and 2 missense mutations (Weil et al., 1995). USH1B patients

exhibit profound congenital hearing loss, absent vestibular function and RP with an onset pre-puberty. Interestingly there was no evidence of retinal abnormalities in the *shaker-1* mice but subsequent analysis showed that *Myo7A* was localised to the connecting cilium of the rod photoreceptors and that Myo7a participates in the transportation of the opsin through the cilium (Liu et al., 1999). These findings provided the first direct evidence that opsin travels along the connecting cilium en route to the outer segment and again underlies the usefulness of mouse models in the analysis of retinal genes. **Table 3** describes many of the models that have been used in retinal research including larger mammals such as dogs, cats and sheep.

4.3.2 Serial analysis of gene expression (SAGE) experiments

Serial analysis of gene expression (SAGE) is a method that was developed to enable the analysis of a large number of transcripts from a specific tissue (Velculescu et al., 1995). It involves isolating short sequence tags (10-14bp) from the transcripts in any particular tissue of interest. These tags can then be concatenated, cloned and sequenced. The number of times a particular tag is identified can provide some quantification information.

Connie Cepko's group used this approach to identify genes expressed in mammalian rods (Blackshaw et al., 2001) and in human peripheral retina, macula, and retinal pigment epithelium (Sharon et al., 2002). Blackshaw and colleagues generated SAGE libraries from developing retina from embryonic day 12.5 (E12.5) to post-natal day 6.5 (P6.5) and adult retina (Blackshaw et al., 2001). They sequenced 50,000-60,000 tags from each library which they estimated would enable them to detect transcripts with an abundance of <0.01% of the total mRNA. By using a library constructed from microdissected outer nuclear layer (ONL) they were able to distinguish rod specific and inner retina genes. They identified many of the genes already known to be expressed in the retina but detected 264 uncharacterised retinal specific genes. They also generated SAGE libraries from P10.5 $crx^{-/-}$ mice which had been used previously for microarray studies (see 4.3.3) (Sohocki et al., 1999).

Using a similar approach with libraries prepared from human peripheral retina, macula and RPE they identified 89 retina-specific or enriched genes of which 14 were already known to cause a retinal disease (Sharon et al., 2002). Both studies generated a database of genes expressed in the retina and provided a useful resource of candidate genes for human retinal disease. An extension of this work in 2004 incorporating *in situ* expression analysis of 1051 genes enabled them to be grouped according to retinal cell type in which they were expressed (Blackshaw et al., 2004).

The integration of genome sequence with gene expression data of this type is a useful resource for the identification of human disease genes and for this reason when the Ensembl database was being developed SAGE data was also incorporated (see 3.3.5 and (Hubbard et al., 2002).

4.3.3 Microarray and transcriptome analysis

The development of microarrays, a technology first developed in the late 1980's (Augenlicht and Kobrin, 1982) enables the simultaneous analysis of thousands of transcripts. The precursors to the modern microarrays were developed in the 1970's when the colony hybridisation technique was developed. DNA randomly cloned in to plasmids was transferred from agar plates to nitrocellulose membranes before radioactively labelled probes were used to identify clones containing DNA complimentary to the probe from the 1000's of colonies on the filters (Grunstein and Hogness, 1975). However it was in the 1990's that modern arrays were conceived. Three types of arrays emerged; *in situ* synthesised arrays, spotted arrays and self-assembled arrays.

Foder and colleagues generated the first *in situ* synthesised arrays which used light directed chemical synthesis on a solid substrate (Fodor et al., 1991). This technique was refined by Affymetrix to generate eight-mer oligonucleotide probes of known sequence, attached to the slides in a known conformation (Pease et al., 1994). The pattern generated from the array provided the sequence of the target. Affymetrix has subsequently developed this technology to develop a range of DNA arrays for genotyping (Chee et al., 1996; Hacia et al., 1996) and expression analysis (Lockhart et al., 1996; Wodicka et al., 1997).

Mark Schena and colleagues developed the first high capacity spotted array system using *Arabidopsis thaliana* as their model organism (Schena et al., 1995). They developed a system of robotic printing of cDNA clones onto glass slides in a known configuration. These were generated from either a full length cDNA clones or cloned Expressed Sequence Tags (ESTs) (see 3.3.3) and they could be up to 1000bp in length. Fluorescent probes were generated from total *Arabidopsis* mRNA by reverse transcription. The probes were hybridised to the slides and then scanned with a laser to detect the probe signal, by reducing the intensity of the scan, quantitative measurements of gene expression could be achieved. At this time, verification of results was performed using densitometry on Northern blots; now verification is performed using quantitative PCR (qPCR).

The third type of array, self-assembly, emerged in 1998 (Michael et al., 1998). This method uses small polystyrene beads on which different DNAs are synthesised. The beads assemble in wells at the ends of fiber optic arrays. As each well can only hold one bead the result is a randomly assembled array. Illumina developed this technology to use a glass surface instead of fibres (for review see (Bumgarner, 2013).

Microarrays can be custom made for specific research need or several companies such as Affymetrix, Illumina and Agilent supply off the shelf arrays. For example the Affymetrix GeneChip Mouse Exon 1.0 ST Array contains over 1.2 million probe sets with approximately four probes per exon and 40 probes per gene. There have been numerous studies which have employed microarray technology to elucidate the genes expressed in the retina through development and also in health and disease. The three main strategies that have been used are; using normal retina to find all genes differentially expressed at different time points through development to maturity; comparing healthy retina to that of a specific disease state; and finally *in silico* analysis using published data sets.

In 2004 two studies used wild type mice to assess global gene expression in the retina, however their approaches differed (Dorrell et al., 2004; Hackam et al., 2004). Dorrell et al used eight postnatal time points to assess gene expression as the retina matures using the Affymetrix Mu74Av2 gene chip which contained 6000 known genes and 6500 ESTs. They found that 2635 genes and 2794 ESTs were expressed threefold higher than background levels in at least one time point during retinal development (Dorrell et al., 2004). Hackam and colleagues compared retina with brain and liver to find differentially expressed genes using a custom array containing 5376 genes and ESTs. They found 733 genes were preferentially expressed in the retina (Hackam et al., 2004). Both groups found upregulation of genes you would expect in the retina, such as *PDE6*, arrestin, recoverin, peripherin and *Rom*1. Both studies identified genes that were not previously known to play a role in the retina as well as novel genes. Dorrell and colleagues aimed to identify novel genes involved in the maturation of the retina whereas Hackam and colleagues aimed to identify retinal genes that mapped to disease loci.

The second approach that has been used is to compare the gene expression pattern found in mouse models of eye disease, both naturally occurring and transgenic, to the pattern found in wild-type age matched controls. This process can identify genes that are specific to a particular cell type and can also identify gene networks and candidate genes for disease. This method was shown to be fruitful in studies by two groups (Bowne et al., 2002; Kennan et al., 2002). Both groups used transgenic mouse models, Kennan et al. used *Rho*$^{-/-}$, carrying a targeted disruption of the rhodopsin gene and Bowne and colleagues used *Crx*$^{-/-}$, carrying a targeted disruption of the *Crx* gene (see **table 3**). They compared the transgenic animals to wild type littermates and identified differentially expressed retinal genes. Both groups found that the gene *Impdh1* was strongly down regulated in their study. Human *IMPDH1* mapped into a region containing the locus for retinitis pigmentosa 10 (RP10). Screening of the Spanish adRP family by Kennan and colleagues, originally used to localize the RP10 gene, revealed an Arg224Pro substitution in *IMPDH1* which co-segregated with the disease phenotype (Kennan et al., 2002). Bowne et al screened three RP10 families, two American and one British, and identified Asp226Asn substitution. Illustrating how the combination of linkage mapping and generation of functional candidate genes can result in the successful isolation of disease causing genes.

The majority of studies that have used animal models aimed to find new pathways and novel genes and have used models of key development or functional or structural proteins such as, *Rho* (Kennan et al., 2002), *Nrl* (Yoshida et al., 2004), *Crx* (Livesey et al., 2000), *Nr2E3* (Chen et al., 2005; Corbo and Cepko, 2005) or *Pde and Prph2* (Demos et al., 2008). Krisnan and colleagues examined several models including rhodopsin kinase (*Rhok*$^{-/-}$), arrestin (*Sag*$^{-/-}$) and transducin (*Gnat1*$^{-/-}$) (Krishnan et al., 2008).

We have also used animal models to identify rod and cone specific genes (Holt et al., 2015). We used retinas from P80 *rd* mice, *cl*$^{-/-}$and *rd/cl* and age matched wild-type mice to compare gene expression. The *rd* mutation is autosomal recessive cause of retinal degeneration in mice and occurs in *Pde6B* gene (Bowes et al., 1990; Pittler and Baehr, 1991). The mice carrying this mutation have a rapid degeneration of the rods with total loss achieved by P65. The cone cells are also affected, but to a lesser extent. The cone cells can be specifically lesioned using the *cl* transgene, which is comprised of a partial human red cone opsin promoter combined with an attenuated diphtheria toxin gene (Soucy et al., 1998). The mice lose >85% of UV cones and total loss of green cones, while the rods are unaffected (Freedman et al., 1999). Mice carrying both *rd/rd* mutation and the *cl* transgene show virtual loss of all photoreceptors (Lucas et al., 1999). By using both models we hypothesised that we could identify genes involved in both rod and cone function. The Affymetrix Exon 1.0 array was used for this study which contains 1.2 million probe sets. We found, as expected, many of the known photoreceptor genes were down-regulated. However we found 418 genes that were differentially expressed in our study. These genes are currently being investigated and assessed as candidate genes (Broadgate and Halford, unpublished results).

Two studies have used *in silico* approaches to identify novel genes from data that had been previously collected (Qian et al., 2005; Zhang et al., 2005). Zhang et al. used mouse retinal EST data to define over 33,700 retina transcript clusters (RTCs). These RTCs may represent different parts of the same gene and they estimated that approximately 19,000 genes may be expressed during mouse development. They verified their *in silico* approach using microarrays and total RNA from mouse P21 retina compared to RNA from pooled organs including heart, lung, spleen, liver and

kidney (Zhang et al., 2005). Qian and colleagues gathered their data from Unigene database (see 3.3.5) (http://www.ncbi.nlm.nih.gov/UniGene), the human microarray study by Chowers et al. (Chowers et al., 2003) and SAGE and EST data from the NCBI databases. The result was the identification of retina-specific genes that contained binding sites for the retinal transcription factors *CRX*, *NRL* and *NR2E3*.

All studies, regardless of which commercial or custom array is used, generate a catalogue of genes that lead to a better understanding of the complex retinal environment.

4.3.4 RNA sequencing (RNA-seq)

RNA sequencing (RNA-seq) or whole transcriptome shotgun sequencing (WTSS) utilises NGS methods to quantify the amount of RNA in a sample at a given time. This technique was developed to overcome some of the disadvantages of microarray analysis sometimes caused by hybridisation artefacts and because it provides a simpler and more comprehensive method of determining the transcriptome composition (Mortazavi et al., 2008).

There have been several studies utilising this approach to examine the retinal transcriptome. An initial study by Brooks and colleagues aimed to compare NGS retinal transcriptome profiling to microarray and qPCR methods (Brooks et al., 2011). They used retinas from wild type and $Nrl^{-/-}$ mice and identified over 16,000 transcripts and demonstrated that this methodology was applicable to studying the retinal transcriptome (Brooks et al., 2011). A further study by Gamsiz and colleagues using adult murine retina showed that over 15,000 genes were expressed (Gamsiz et al., 2012). One of the big advantages of RNA-seq is that alternatively spliced genes are also identified and indeed Gamsiz and colleagues identified 3655 alternatively spliced retinal genes.

Most of the studies described above have used mouse models and retinal tissue and whilst these approaches have generated many potential candidate genes it could be argued that the mouse retina is not the same as the human retina. Human studies are rare because of the difficulties in obtaining tissue but in a recent study by Farkas et al. RNA derived from three normal human retinae have undergone RNA-seq to produce a human retinal transcriptome (Farkas et al., 2013). They identified 116 potential novel human genes as well as thousands of potential novel exons (Farkas et al., 2013).

4.3.5 *Drosophila melanogaster* as a model system

The use of *Drosophila melanogaster* as a model system is well documented and has proved to be an indispensable tool in trying to understand the complexities of human disease. Mammalian homologues of several *Drosophila* genes involved in the development of the visual system, even though invertebrate and vertebrate eyes are structurally distinct, have been shown to be involved in human eye disease. Mammalian homologues of *Drosophila* developmental mutants such as *eyeless* and *eyes absent* (*eya*) have been identified and shown to be associated with disease. Mutations in the mammalian homologue of *eyeless*, *Pax6*, cause *Aniridia* and *Small eye* (*Sey*) in human and mouse respectively (Glaser et al., 1992; Hill et al., 1991; Jordan et al., 1992; Ton et al., 1991). Several *eya* homologues have been identified in vertebrates and haploinsufficiency for *EYA1* in humans is responsible for branchio-oto-renal (BOR) syndrome (Abdelhak et al., 1997). However it was earlier work by William Pak in the 1960's and 70's generating mutant flies that had defects in phototransduction that opened up this pathway to investigation (for review see (Pak, 2010)). The subsequent demonstration that the *ninaE* (neither inactivation nor after potential) mutant was

caused by a mutation in the *Drosophila* rhodopsin gene and showed homology to the vertebrate gene established this pathway as a useful model system (O'Tousa et al., 1985). Similarly, naturally occurring mutations in the human arrestin gene results in Oguchi disease characterised by an elevation of the rod threshold and nightblindness (Fuchs et al., 1995). Absence of either of the two arrestin genes in *Drosophila* results in light-induced retinal degeneration (Dolph et al., 1993). These similarities and an increasing amount of evidence to suggest that components of the pathway are present in the mammalian retina (for review see (Brockerhoff, 2011)) led several groups including ours to identify human homologues of *Drosophila* phototransduction genes as candidate genes for human eye diseases. Banfi and colleagues initiated the *Drosophila*-related expressed sequences (DRES) project, which identified human and murine transcripts homologous to *Drosophila* mutant genes (Banfi et al., 1996).

The initial steps of invertebrate and vertebrate phototransduction show many similarities, with both systems being initiated by photon absorption by rhodopsin, a visual pigment composed of an opsin protein and a chromophore derived from vitamin A_1, and subsequent activation of a G-protein (transducin in vertebrates and Dgq_{α} in *Drosophila*). This results in the stimulation of a cyclic-GMP phosphodiesterase which leads to closure of membrane cation channels and subsequent hyperpolarisation of the cell surface in vertebrates. However, in *Drosophila* the G-protein activates a phospholipase C which hydrolyses phosphatidylinositol-4-5-bisphosphate (PIP_2) into two second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is involved in the release of intracellular calcium which opens membrane channels and in turn leads to membrane depolarisation. DAG activates protein kinase C. The regeneration of PIP_2 for subsequent signalling is tightly regulated and involves a number of enzymes, several of which have mammalian homologues (Fitzgibbon and Hunt, 1995) A photoreceptor specific form of the enzyme CDP-diacylglycerol synthase (*eye-CDS*) is thought to be a key regulator of this pathway in *Drosophila*. It catalyses the formation of diacylglycerol from phosphatidic acid and regulates the amount of PIP_2 available for signalling. *cds* mutants develop light-induced retinal degeneration (Wu et al., 1995). Using the amino acid sequence of *eye-CDS* several related mammalian sequences were identified in dbEST. These clones were then used in more conventional experiments, as probes on retinal cDNA libraries to identify fuller sequences. Two mammalian CDP-diacylglycerol genes (*CDS1* and *CDS2*) were isolated by us, and others (Halford et al., 1998; Heacock et al., 1996; Saito et al., 1997; Volta et al., 1999; Weeks et al., 1997). We have shown that mouse *Cds1* shows a restricted expression pattern in the inner segments of the photoreceptors and *Cds2* is more ubiquitously expressed (Inglis-Broadgate et al., 2005). Initial screening of a panel of 130 patients with IRDs of unknown cause using heteroduplex analysis (see 3.5.2) and Sanger sequencing (2.2.2) has proved inconclusive and analysis is ongoing (Halford and Ocaka, unpublished results).

The same type of approach has been applied to another gene in the PI-PLC pathway in *Drosophila*, retinal degeneration B (*rdgB*), which was one of the first *Drosophila* retinal degeneration mutants to be identified (Hotta and Benzer, 1969). The gene encodes a membrane-bound phosphatidylinositol transfer protein. Flies defective for this gene also develop light enhanced retinal degeneration (Harris and Stark, 1977). Three mammalian homologues of *rdgb, PITPNM1, PITPNM*2 and *PITPNM3*, have been described (for review see (Ocaka et al., 2005)). The demonstration that the murine *PITPNM1* is able correct the visual defect in *rdgB* defective flies suggested that there are similarities between the invertebrate and vertebrate phototransduction pathways not previously appreciated

(Chang et al., 1997). The identification of a Q626H missense mutation in the PYK2-binding domain of *PITPNM3* in two Swedish families with autosomal dominant cone dystrophy (CORD5) (Kohn et al., 2007) supported this conclusion. Incidentally we had previously mapped *PITPNM3* to human chromosome 17p13 (Ocaka et al., 2005) and the CORD5 locus to 17p12–p13 (Balciuniene et al., 1995). The more widespread use of NGS technologies (see 2.4 and 4.4) may reveal more variants in the homologues of the *Drosophila* retinal degeneration mutants providing an insight into the role of this pathway in the mammalian retina.

4.4 Next Generation Sequencing

The next generation sequencing technologies (NGS) (outlined in 2.4) have improved both the efficiency and the cost of DNA sequencing. Over the last 15 years the National Human Genome Research Institute (NHGRI) has tracked the cost of sequencing an entire genome and their figures (see https://www.genome.gov/sequencingcostsdata/) show that in September 2001 the estimated cost was over US$95 million. A comparable amount to the estimated cost of generating the first individual human genome, that of Craig Venter, using Sanger sequencing which was estimated to be US$100 million (Levy et al., 2007) (see 2.4.2). From 2001 to mid-2007 the cost approximately halved every two years due to the development of increased throughput of Sanger sequencing methods with the cost reduced to US$40 million in October 2003. However the introduction of the first NGS technologies in mid-2007 was when the costs started to fall significantly. The first human genome to be sequenced using NGS technology (the Roche 454 platform) (see 2.4.2) was estimated to have only cost US$1.5 million (Wheeler et al., 2008). Since then improvements in the technologies have significantly reduced the cost of an entire genome which is currently estimated to be US$1245 (October 2015). It has therefore become a viable proposition to sequence the whole genome of an individual using these high-throughput NGS technologies.

4.4.1 Whole exome sequence (WES) versus whole genome sequencing (WGS)

As the NGS sequencing technologies have developed and improved vast amounts of data are being generated. The human genome consists of approximately 3×10^9 bases however the protein coding genes (the “exome”) only account for 1% of this (3×10^7 or 30Mb) with the remaining 99% consisting of intergenic and intronic regions. It is estimated that 85% of disease causing mutations are located in the exome or the splice sites flanking the exons. It was proposed that sequencing just the exome would be a more efficient methodology for finding rare variants in human disease (Ng et al., 2009). A study by Choi and colleagues demonstrated that it was possible to combine Roche whole exome arrays with Illumina sequencing and capture >95% of the exome (Choi et al., 2009). This approach of whole exome sequencing (WES) concentrates the sequencing power to the protein coding regions which ensures a good depth of coverage (in our pipeline 70-90% of covered bases having over 100x coverage) and achieves high quality genotyping of these regions. However the poor coverage on the remaining 99% of the genome results in the limitation of the discovery of regulatory region changes and large scale structural variation that might have significant phenotypic consequences. The second drawback is that as WES features an enrichment step that is primer based this can introduce bias against regions with poor primer annealing characteristics. These limitations can be overcome by whole genome sequencing (WGS), since it covers greater than 95% of the entire human genome, and it does not rely on primer-based enrichment. WES on the other hand is associated with a faster turn-around time for results. In addition our current relatively limited knowledge of the functions of the intergenic and intronic regions all currently point to WES as the best choice for patient genotyping at this point in time. However, the continued increase in computing power and our

growing knowledge of the human genome also mean that phasing in WGS for clinical diagnosis is probably only a matter of time (see 4.6).

4.4.2 From sequencing to diagnosis

The first step in the analysis of sequencing data produced by WES and WGS is alignment against a human reference genome before variants are called and annotated. The impact of a variant can be inferred from several sources (**Table 4**):

1. Literature support. If the variant has been reported to be causative in a set of phenotypes that match the patient, and this association is backed up by robust evidence, then the variant can be classified as damaging with a high confidence. Sanger sequencing is then used to confirm the variant and to check segregation and the patient's case is considered to be "solved".
2. Allele frequency. The allele frequency of a variant in a population can be retrieved from large population studies such as the Exome Aggregation Consortium (ExAC) (Lek et al., 2016). At the time of writing, ExAC contains the genotypes derived from WES of 60,706 unrelated individuals who were not affected by severe paediatric disease and which are segregated into six ethnic groups (African, East Asian, European (Finnish), European (Non-Finnish), Latino, South Asian and Other). Therefore, a typical autosomal position will have information from around 120,000 alleles. It has already proven a valuable resource in helping identify causative variants for rare Mendelian disorders (Lek et al., 2016). For retinal dystrophies that affect 1 in 3000 individuals in the UK, variants with allele frequency higher than 1/3000 in ExAC should be discarded for further investigation (for recessive cases, homozygote frequency should be considered instead). If the ethnicity of the patient is known, the variant's allele frequency can then be considered in its ethnic background. For variants not covered by ExAC, Kaviar (Known VARiants), a compilation of human single nucleotide variants collected from many diverse sources can be used instead for allele frequency queries (Glusman et al., 2011). Recently, the Broad Institute published a beta version of gnomAD (http://gnomad.broadinstitute.org/), which hosts 126,216 exome sequences and 15,136 whole-genome sequences. It can be foreseen that it will soon replace ExAC as the gold standard population genome reference database.
3. Variant consequence. The consequence of a nucleotide change ranges from a "benign" synonymous variant (a nucleotide change that does not lead to a protein sequence change, which was thought to be highly unlikely to cause a significant effect (see 4.5)) to a "probably damaging" gain of a stop codon (a nucleotide change that leads to a truncated protein product) variant. For missense variants (nucleotide changes that lead to a different amino acid sequence but where the length is preserved), their impacts are more variable and if there is no obvious evidence for pathogenicity in the literature, then their influence on the protein's function can usually be predicted by software such as Polyphen (Adzhubei et al., 2010), SIFT (Kumar et al., 2009) and CADD (Kircher et al., 2014).

4.4.3 Identification of retinal degeneration genes using WES and WGS

The first example of the use of WES in identifying variants in rare disease was reported in 2009 in a study by Ng and colleagues (Ng et al., 2009). They analysed four unrelated individuals affected with Freeman-Sheldon syndrome, (FSS; OMIM #193700) a rare autosomal dominant disorder for which they had no linkage data. Their initial analysis aimed at determining how many genes contained a

variant shared in multiple individuals. This produced over 2000 candidate genes. However, by applying filters that removed dbSNP variants they were left with only one gene, *MYH3* (myosin heavy chain 3) which was shown to segregate with FSS in these families. This proof of concept paper suggested that targeted capture and massively parallel sequencing of exomes is a powerful tool to identify the genetic cause of rare Mendelian disorders.

The first retinal disease gene to be identified using these NGS technologies was *TSPAN12* (tetraspanin 12) as a cause of familial exudative vitreoretinopathy (FEVR) in a paper by Nikopoulos and colleagues in 2010 (Nikopoulos et al., 2010). The authors reported two large Dutch pedigrees with FEVR showing linkage to a region on chromosome 7, one family showed linkage to a 40Mb region and the second family to a smaller region of 17Mb completely contained within the larger region. They used a targeted NGS approach to analyse 338 genes in the larger linked region using a Roche 454 GS FLX sequencer. Three potential genes were identified but two, *PTCD1* and *ZAN*, were excluded as they were not located within the smaller linkage region. The third gene identified, tetraspanin 12 (*TSPAN12*) did map into the smaller shared linkage region. Conventional Sanger sequencing confirmed that the missense variant detected (c.709G>C; p.Ala237Pro) was present in both the probands and in all the affected relatives of both families. Nikopoulos and colleagues then screened the *TPSPAN12* gene in nine more FEVR families. The p.Ala237Pro mutation was detected in 2 further families and a p.Gly188Arg change was found in a fifth family. These findings were confirmed in a study published back-to-back by Poulter and colleagues who used a candidate gene approach (Poulter et al., 2010). Three FEVR genes had previously been identified, Norrie disease protein (*NDP)* (Chen et al., 1993), Frizzled 4 (*FZD4)* (Robitaille et al., 2002) and Leucine repeat protein 5 (*LRP5*) (Toomes et al., 2004) and had been shown to be components of the same signalling pathway. Work by Junge and colleagues had demonstrated that *TSPAN12* was also part of this pathway (Junge et al., 2009). This prompted Poulter and colleagues to screen a panel of 70 FEVR patients; they identified seven heterozygous *TSPAN12* mutations (Poulter et al., 2010).

To date only two genes involved in IRD have been identified using WGS, *NEK2* (never in mitosis gene A-related kinase 2) as a cause of RP (Nishiguchi et al., 2013) and *PRDM13* (positive regulatory domain-containing 13) as the cause of North Carolina Macular Dystrophy (Small et al., 2016).

In a previous screen of 30 genes found commonly in European and North American cohorts pathogenic mutations were only found in 14% of 193 Japanese RP patients (Jin et al., 2008). This observation indicates that RP genes can be population specific so Nishiguchi and colleagues carried out a proof of concept study analysing the effectiveness of using WGS to identify retinal degeneration genes (Nishiguchi et al., 2013). They used WGS on 16 unrelated RP patients (8 North American and 8 Japanese) to determine if causal genes could be identified using this technology. Their initial analysis focussed on the known ar RP genes. They detected homozygous or compound heterozygous mutations in 7 genes previously associated with ar RP in three Japanese and five American patients. This study illustrates the power of WGS in that among the mutations detected there was a 2.3 kb deletion in *USH2A* and an inverted duplication of ~446 kb in *EYS* changes that would not have been detected by WES or conventional sequencing methods. Finally they identified a homozygous frameshift mutation (p.L206fs) in one Japanese patient in the ciliary gene *NEK2* (never in mitosis gene A-related kinase 2), which encodes a serine/threonine-protein kinase. This change was not detected in 1250 Japanese control samples. Subsequent screening of the *NEK2* gene in a cohort of 267 RP patients failed to find any disease causing mutations.

The second study using WGS to identify an IRD was led by Kent Small to finally identify the gene for the North Carolina Macular Dystrophy (NCMD) locus on chromosome 6 (MCDR1) (Small et al., 2016). They used a targeted capture approach to sequence 6 members from 3 families, 1 was unaffected. They identified a shared haplotype of 14 rare variants in the 5 affected individuals. One of the variants, V1, was absent from all published databases and 261 controls but was found in 5 other NCMD families. It lies upstream of two genes, *PRDM13* (PR domain containing 13) and CCNC (cyclin C), in a DNase 1 hypersensitivity site. The finding of a complete duplication of the PRDM13 gene in another NCMD family and the demonstration that the variants altered macular development led Small and colleagues to propose that this was the causal gene (Small et al., 2016).

In the years from 2011-2016 fifty three IRD genes have been identified using WES and WGS (**Table 5**). Comparison with **Table 2** which lists 48 genes but took thirteen years to identify illustrates the power of these new technologies. What WES and WGS are succeeding in doing is uncovering mutations in genes that would not have been on a candidate gene list because they have unknown functions in the retina.

4.5 How do we know the gene is the gene?

So how do we know the variant we have identified in a specific gene is the cause of disease, in other words how do we know that it is pathogenic? Especially if the variant is in a gene of previously unknown function in the retina and is only identified in a single patient. These are the questions asked by geneticists and clinicians regardless of the methodology used.

The identification of a pathogenic mutation (designated as pathogenic based on the lines of evidence listed in section 4.4.2 and **Table 4**) in an affected individual with confirmation of segregation of the disease in the family is usually enough evidence to consider the case "solved". However if the case cannot be solved at this stage, then genes that have "potentially damaging" (PD) variants will need to be prioritised for further investigation. Typically, WES will yield several hundred genes with PD variants in a patient. Among them, 10 to 50 genes will have at least two PD variants, providing a useful pool of candidate genes for recessive monogenic diseases. In order to efficiently prioritise the genes, several sources of knowledge need to be utilised. For retinal dystrophy, RetNet (https://sph.uth.edu/retnet/) (see 4.2.1) has a list of genes that are known to be causal in a wide range of abnormal retinal phenotypes, and is usually the first knowledge database to refer to. Online Mendelian Inheritance in Man (http://www.omim.org/) is a generic database that draws connections between genes and Mendelian diseases, including retinal dystrophies. Literature searches using resources such as PubMed (http://www.ncbi.nlm.nih.gov/pubmed) are also a reliable source of knowledge. All of this information is summarised in **Table 4**. Other useful information can include data ascertained from retinal microarray studies (see 4.3.3) (for example (Holt et al., 2015)) which we use in our pipeline (Yu and Halford, unpublished data).

However what is a pathogenic mutation? Obviously a frameshift leading to premature termination and truncated protein or a deletion or inversion which results in loss of functional domains would be expected to cause disease. However what about missense mutations? Many missense mutations will also result in loss of function of the protein but when we find a conservative change such as a glycine to a valine can this really be causing the disease? What about synonymous changes when the nucleotide change does not alter the amino acid? The use of several prediction programmes such as

Polyphen and SIFT among others (see 4.4.2 and **Table 4**) can help with this type of analysis. Databases such as ExAC (see 4.4.2) are also starting to provide useful metrics such as a set of conservation scores for most of the human genes (pLI scores). Depending on the size of a given gene's coding sequence and the number of alleles available, ExAC compares the expected number of loss-of-function variants and the actual observed number, and deduces a conservation score. This score ranges from 0 (not conserved) to 1 (conserved). It was shown that a dominant gene is likely to have a higher ExAC's pLI score than average (Lek et al., 2016).

Information from the literature and databases can also be useful because if it is clear the amino acid at this position in the protein is conserved across species then this may indicate its importance in protein structure or function. Historically however these prediction programmes are no more than 'predictions' and need confirmation in the laboratory. Synonymous nucleotide changes, which usually occur in the third base of a codon, were considered to be "silent" as the protein sequence is not altered. However, recent work has shown that there are over 50 human diseases that have been associated with synonymous changes which exert a biological effect. These can include changing splice sites or binding sites for transcription factors (for review (Hunt et al., 2014)).

When deciding whether the variant(s) of the gene under scrutiny are pathogenic, some knowledge of what the product of the gene does can be very helpful. For example if it codes for a protein which is known to play a role in the structure, function or maintenance of mammalian photoreceptors then that would lend weight to the argument.

Corroboration in a second unrelated family is usually seen as definitive evidence that the gene causes the disease. This is clearly illustrated by the identification of *NMNAT1* mutations by four groups in 49 families with LCA (see 4.4.3) (Chiang et al., 2012; Falk et al., 2012; Koenekoop et al., 2012; Perrault et al., 2012a). Changes in *NMNAT1* are now estimated to account for 5% of LCA. Yet in the era of NGS as a methodology for finding disease genes there are going to be more and more examples of singletons with variants in genes of previously unknown function exerting an effect on the retina or related tissues (**Table 5**).

The chance of finding a similar case increases through multi-centre collaborations. However a recent paper by Yen and colleagues (http://biorxiv.org/content/early/2016/05/19/054023) illustrated that using different pipelines for variant annotation is a source of unwanted variance. The un-automated downstream candidate genes identification, usually having little or no computerised process assistance, is even more variable in comparison. A valuable lesson learnt from the UK Inherited Retinal Disease Consortium (www.ukirdc.org/) is that as many things as possible should be done in one place, in a single computerised pipeline. All exome sequencing is performed at Leeds University, and alignment and variant calls are made in University College London. Pontikos and Yu have developed a web-based platform (Phenopolis) with the purpose of maximising the computerised proportion of a genetic diagnosis, minimising inconsistency in data interpretation and reducing the time and cost of the process (http://biorxiv.org/content/early/2016/10/31/084582).

This also highlights the need for functional analysis of the mutant protein. The identification of *NEK2* as a cause of RP in an isolated Japanese patient illustrates this point. Elegant work by Nishiguchi and colleagues demonstrated that inactivation of the gene in zebrafish caused retinal photoreceptor defects that could be rescued by the addition of human NEK2 mRNA (Nishiguchi et al., 2013). Similarly exome sequencing in one single generation Ashkenazi Jewish ar RP family by Zuchner and

colleagues identified a p.Lys42Glu in *DHDDS* (dehydrodolichyl diphosphate synthetase) (Zuchner et al., 2011), which is another previously unknown gene. They knocked down the gene in zebrafish using a morpholino approach and demonstrated that the photoreceptor outer segments were very short or completely missing in the retinas. Subsequent analysis by Zelinger and colleagues in 15 unrelated Ashkenaki Jewish families identified the same p.Lys42Glu missense mutation, confirming *DHDDS* as the causative gene (Zelinger et al., 2011).

4.6 Using NGS for clinical diagnostics

As described previously inherited retinal dystrophies (IRD) are a phenotypically and genetically heterogeneous group of disorders. For example to date over 60 genes causing retinitis pigmentosa (RP) have been described (see RetNet (https://sph.uth.edu/retnet/home.htm)). This provides a huge challenge for clinicians and it is recognised that genetic testing has an increasingly important role in IRD, not only for clinical diagnostics and for prognosis but also for potential therapeutic interventions such as gene therapy.

Most of the techniques and approaches outlined in sections 2 and 3 do not lend themselves to being used routinely in diagnostic laboratories because they are prohibitively time consuming or expensive. However in 1999 Asper Biotech introduced an ophthalmology microarray platform to screen for known IRD genes. This utilised a technology known as Arrayed Primer Extension (APEX) microarrays which allowed the simultaneous screening of hundreds of DNA variants. The APEX reaction involves hybridising target DNA to sequence specific oligonucleotides on a glass slide (Kurg et al., 2000; Tonisson et al., 2000). So the introduction of commercial Asper chips made some advances but only known mutations can be detected. The development of NGS technologies provided a potential new methodology. NGS allows multiple genes to be sequenced in parallel and this could be used to increase molecular diagnostic rates in IRD. This led several groups including ours to set up studies looking into the feasibility of NGS as a diagnostic tool in IRD. We set out to identify pathogenic mutations in a cohort of patients with IRD from an Ophthalmic Genetic clinic and decided to use 'phenotype based' gene panels as our model thus aiming to address the complexities of introducing NGS into a clinical diagnostic setting (Shanks et al., 2013). The approach of using phenotype targeted gene panels, rather than using one panel with all known IRD genes on it was aimed: 1) To make the testing 'user friendly' for clinicians, and 2) to save on bioinformatics analysis. For example by using a macular panel, if a patient whose phenotype was 'macular' and a mutation in *TIMP3* was identified, this would save time in the bioinformatics part of the pipeline as only 'macular genes' would be analysed. If the phenotype were simplex RP with no syndromal features, and the results of the RP, and RP-like phenotype Oxford panel (111 genes) tested negative, then mutations in rarer genes or new genes could be investigated using WES or WGS as part of a research initiative. In this way this basic initial gene panel testing can identify between 55-60% on the first pass, which is a high yield in genetic testing in a clinical diagnostic setting. It is clear that early onset and positive family history increase detection rate (Shanks et al., 2013). This detection rate can be higher for single gene assessment if the gene is associated with a distinct phenotype, which is recognised by the referring clinician (Downes et al., 2012) (Shanks et al., 2013).

Several other studies utilising NGS in the identification of IRD genes have reported identification of 57% (Audo et al., 2012b), ~50% (Neveling et al., 2012), 50-55% (O'Sullivan et al., 2012) and 55% (Glockle et al., 2014) respectively. However the limitations of gene panels include only being able to test the known genes on the panel; having to redesign the panel to incorporate new genes with

associated expense; the role of gene modifiers cannot be adequately explored by this approach. However for a monogenic IRD at this point in time, this is a cost effective pragmatic approach. Indeed stratified gene panels may be retained over the next few years while the complexities of whole exome and genome and the 'big data' generated requiring analysis are addressed, and may run in parallel, depending on the condition, population and rarity of the gene for some years to come. Panel Testing and progressing to WES and WGS have already demonstrated the need for close collaboration between scientists, bioinformaticians and clinicians in order to interpret all this new data in a meaningful way. Family studies investigating segregation of presumed pathogenic variants, a thorough knowledge of the phenotypes, inheritance patterns, mutation types, genes and a bioinformatics pipeline using all the available resources with access to functional studies may all be necessary to achieve an accurate genotype.

There are other issues that also need to be resolved before NGS technologies can be fully integrated into the clinic. There are ethical considerations, such as the reporting of incidental findings and variants of unknown certainty. The American College of Medical Genetics and Genomics (ACMG) had previously developed guidance on the interpretation of sequence variants for clinical laboratories (Richards et al., 2008). These guidelines have recently been updated to take into account the advent of NGS in clinical laboratories and provide clear guidelines on nomenclature (Richards et al., 2015). These findings may have consequences for other family members who may have been unaware genetic testing was being undertaken. Results may imply that genetic and social relationships within a family are not what they had thought. This all highlights the importance of careful genetic counselling pre-testing. This is also imperative in the context of WGS and WES where variants in genes which may confer risk for other systemic disorders may be identified. Also the identification of an accurate genotype does not take into account variable expressivity and penetrance which can complicate the diagnosis. In addition careful consideration of confidentiality, and data security and handling of big data, and storage and access to these data all have to be managed and monitored appropriately.

5. Conclusions and future directions

The identification of genes underlying rare Mendelian disorders has been of interest to clinicians and scientists for centuries. The advances in DNA technology during the 1970's and 1980's, particularly the development of Sanger sequencing and the polymerase chain reaction, made it possible to start to identify the genes causing monogenic disease. These were time consuming and laborious undertakings but actually were ultimately successful (see 3.2). The search for genes causing IRD began using positional cloning approaches with choroideremia being the first IRD gene identified using these methods (see 3.2).

Several groups had identified families with RP inherited in an autosomal dominant manner (ad RP) including a large Irish pedigree that showed linkage to a region on chromosome 3q21 (McWilliam et al., 1989). The gene for rhodopsin, the photopigment found in rod photoreceptors, had been mapped to the same region of chromosome 3 (Nathans and Hogness, 1984). This was too big a coincidence and indeed screening of twenty ad RP patients for changes in the gene by Dryja and colleagues identified a point mutation causing a proline to histidine change at position 23 in 5 patients (Dryja et al., 1990b). This finding resulted in the observation that ad RP was genetically heterogeneous, as not all families had changes in rhodopsin, and it prompted a new methodology

the so-called “candidate gene” approach (see 4.2). This method was based on the idea that genes involved in the structure or function of the photoreceptors would be excellent candidate genes. This approach became the method of choice and resulted in the identification of many IRD genes many of which do have a well-defined functional or structural role in the retina (**table 2**). Several groups also used a purely candidate gene approach, without performing linkage analysis first, to identify causal genes and used mutation analysis methods such as SSCP (see 3.5) to screen large cohorts of patients for variants in functional candidate genes. For example Dryja and colleagues screened 173 unrelated patients with ar RP for changes in the alpha subunit of the rod cGMP-gated channel (*CNGA1*) and identified 4 families with disease causing mutations (Dryja et al., 1995). The *CNGA1* gene was chosen as mutations in two proteins involved in the rod phototransduction cascade, rhodopsin (RHO) and the beta subunit of rod cGMP phosphodiesterase (*PDE6B*) had been implicated in causing RP (Dryja et al., 1993; McLaughlin et al., 1993).

However, the instigation of the Human Genome Project (HGP) in 1990 marked a step change in disease gene identification. Obviously the major aim of the project was to obtain the sequence of the entire human genome but during this process tools and databases were developed that made the identification of disease genes much easier. The first step of a positional cloning approach was to obtain linkage data, that is, to identify the region of the genome where the disease being studied was located (**figure 4**). The HGP made this process easier as there were a much larger number of informative markers than previously resulting in smaller linkage regions (see 3.3.1 and 3.3.2). The region could then be “mined” for genes, the development and mapping of expressed sequence tags (ESTs) facilitated the mapping of 30,000 genes relative to the genetic map (see 3.3.3) (Deloukas et al., 1998). The genes located in the linked region could then be prioritised based on putative function and expression profile. The development of transcriptome analysis tools such as microarrays, SAGE and more recently RNA-seq as well as the use of animal models meant that there was an increasing amount of knowledge about genes expressed in the retina adding to the pool of “candidate genes” (see 4.3).

Strictly speaking the identification of mutations and structural rearrangements in the red and green opsin genes on the X chromosome causing red-green colour blindness was actually the first example of candidate gene analysis (Nathans et al., 1986a). Although at the time it was thought that individuals with these variants had a stable condition and they were not thought to have an IRD. It was shown subsequently that some subjects develop a progressive disease with evidence of loss of visual acuity, and macular atrophy (Ayyagari et al., 1999). It is now apparent that mutations in the red and green opsin genes can result in a spectrum of cone photoreceptor defects (for overview see (Gardner et al., 2014)). This also serves to illustrate the importance of long term clinical follow up to identify those disorders that originally appeared to be stationery diseases but actually are progressive.

The development of NGS techniques has been a major milestone in the identification of disease genes and to date has resulted in the discovery of 53 genes many of which have no previously documented function in the retina (**table 5**). This illustrates the power of NGS in that it is now possible for the first time to identify disease genes with no prior knowledge about its location negating the need for linkage analysis. Currently the method of choice is whole exome sequencing (WES) which utilises targeted capture techniques to screen all known exons and their flanking splice sites. It is estimated that over 85% of all disease causing mutations are located in the exome. The

advantage of using WES is that as the sequencing is more focussed excellent coverage is obtained (usually 100x) but the coverage may not be consistent as various target enrichment strategies are used. WES also only analyses 1% of the genome so untranslated regions, promoters and other potentially functional regions are not analysed. On the other hand it is less expensive than whole genome sequencing (WGS) and provides a much smaller dataset and so is easier to interpret. But how do we establish which variants after filtering for SNPs are disease causing? The bioinformatician needs to take into account as much information about the consequence of any variant on the predicted protein, allele frequencies and if there is literature support (see 4.4.2, 4.5 and **Table 4**). The variant will then be confirmed using Sanger sequencing (still considered to be the gold standard) and family segregation studies will be performed where samples are available. The identification of changes in the same gene in other unrelated families is usually seen as definitive evidence. However functional analyses will need to be carried out if any of these indicators are not present.

It is clear that these new technologies, although not without their problems in terms of data management, are facilitating the identification of novel genes for IRD. These are exciting times for research on photoreceptors and the retina as previously unknown pathways are being discovered opening up new avenues for therapy. Underlying all of this work is that patients want to know the cause of their disease, the inheritance pattern and what can be done to cure it (Combs et al., 2013a; Combs et al., 2013b). These new technologies are already playing a considerable role in a clinical diagnostic setting. Currently a very useful clinical approach is to use gene panels comprising the known disease genes. Costs associated with analysis can be reduced if these panels are phenotype driven; for example a patient with a macular disease would be screened initially using a panel containing known macular genes. Samples with a negative result can then be analysed by WES and WGS, which is currently happening in a research setting. This can lead to the identification of a variant or variants in a novel gene, which appears highly likely to be the cause of disease but is of unknown pathogenicity. This can be further investigated using a combination of examining segregation of the variant within the family, functional studies of the gene and waiting for other families to be described in the literature. However the use of NGS and the establishment of collaborations between groups working on the same disease can be very helpful in identifying a second family with mutations in the same gene examples include the UK Inherited Retinal Disease Consortium (www.ukirdc.org/) and the European Retinal Disease Consortium (http://www.erdc.info/). This also highlights the importance of good phenotyping, ensuring that patients with the same clinical features are being compared. To this end there is ongoing work to try and develop a standardised “vocabulary” to describe phenotypes. The Human Phenotype Ontology (HPO) aims to provide a standardised classification (**Table 4**) which in turn can be used for clinical diagnosis (Kohler et al., 2014; Kohler et al., 2017). The UKIRDC have developed a web based platform, Phenopolis, which utilises HPO terms relevant to IRDs (see 4.5).

Even with these advances in technology and data interpretation leading to NGS technologies being utilised in clinical diagnostic laboratories it is estimated that approximately 40% of patients do not receive a confirmed genetic diagnosis (Audo et al., 2012b; Shanks et al., 2013). Many of these patients will have mutations in as yet undiscovered genes but many will have variants in gene promoters and tissue-specific regulatory regions which projects such as the ENCODE (Encyclopedia of DNA Elements) consortium are starting to decipher. This international collaboration of research groups is funded by the National Human Genome Research Institute (NHGRI) and aims to identify

the functional elements in the genome (Kellis et al., 2014), these data are all publicly available (https://www.encodeproject.org/).

Whilst the utility and accuracy of WES and WGS in a clinical diagnostic setting continues to be explored, technology also continues to advance resulting in the single molecule, so called third generation sequencing methods including nanopore and PacBio. The development of nanopore sequencing allowing the detection of a single molecule of DNA holds great promise (for review see (Feng et al., 2015). A nanopore is a hole with a diameter of a nanometre which can be set in an electrically resistant polymer membrane. An ionic current is passed through the nanopore and if a sample such as a molecule of DNA passes through or near the pore aperture it disrupts the current. Measurement of the current allows the identification of the molecule that caused the disruption. Nucleotides A, C, G and T can be identified by this method, allowing the sequence of the sample being analysed to be read. The advantages of this technology are that there is minimal sample preparation and the need for amplification or modification has been eliminated and long reads of 10-50 kbp can be generated. However, sequencing samples at the molecular level requires ultra-precise high speed detection and as yet the electrical and optical capabilities are not quite good enough, but these issues are being addressed.

Pacific Biosciences (PacBio) have developed an approach called Single Molecule Real-Time (SMRT) sequencing. One of the major disadvantages of the second generation sequencing technologies (outlined in 2.4) is they have short read lengths and hence are not very accurate in complex genomic regions. SMRT sequencing overcomes this by having both longer read lengths and faster run times but it has a lower throughput and so is currently more expensive (for review see (Rhoads and Au, 2015)). SMRT sequencing captures sequence data as a target DNA molecule is replicated. The template, SMRTbell, is created by ligating hairpin adapters to both ends of a double-stranded DNA molecule. This is then loaded onto a chip called a SMRT cell where it diffuses into a sequencing unit called a zero-mode waveguide (ZMW). Each ZMW contains an immobilised single DNA polymerase which can bind to either of the hairpin adapters and start replication. The four differently labelled fluorescent nucleotides are also added and as a base is held by the polymerase a distinct light pulse is generated for each base. These light pulses are recorded and then interpreted to produce the sequence generated in each ZMW. The first chemistry (C1) developed by PacBio enabled reads of approximately 1500bp to be generated but the newer chemistries (C4) can produce up to 10kb compared to the Illumina HiSeq reads of 250bp. These so-called third generation sequencing technologies are showing great promise but still have limitations both technological and cost to overcome. If they can be solved then the realisation of sequencing a whole genome for less than $1000 and maybe less than $100 becomes a reality.

As well as wanting to know their genetic diagnosis patients also want to know about treatment options (Combs et al., 2013a; Combs et al., 2013b). There have been major advances in the past few years in regard to treating Mendelian genetic diseases. The eye has been at the forefront of this research as it has proved to be an ideal target for therapeutic testing as it has an immune privileged status, is formed of discrete compartments, is accessible and can be monitored non-invasively. In addition the contralateral eye can be used as a control for optimal analysis. Many groups have used various approaches to treat genetic eye diseases these include cell (RPE) transplantation and stem cell therapies and both viral and non-viral gene therapy.

Adeno-associated virus (AAV)-based systems remain the most popular gene delivery method due to their high efficiency and successful clinical results (for review see (Schon et al., 2015)). The first demonstration that AAV could be used to deliver a gene to the retina was in a study by Bennett and colleagues who successfully used subretinal injection of the wild type phosphodiesterase β gene to delay photoreceptor degeneration in the *rd* mouse mutant (Bennett et al., 1996). The first successful large animal retinal gene therapy treatment was in the naturally occurring LCA2 (*RPE65*$^{-/-}$) canine model of Leber congenital amaurosis. The AAV vector was used to deliver a functioning copy of *RPE65* into the retina which resulted in long term restoration of vision in the dog (Acland et al., 2001). This provided proof of principle to take the therapy into safety studies first in non-human primates (Jacobson et al., 2006) and then into clinical trials in LCA patients (Bainbridge et al., 2008; Cideciyan et al., 2008; Maguire et al., 2008). The studies in the patients showed short-term rescue, which highlights the differences between animal models and human studies. Many animal studies deliver the treatment before the onset of the disease, whereas degeneration is often already advanced when patients are recruited to studies. There may be a therapeutic window in patients that after which success will be limited (Cepko and Vandenberghe, 2013). AAV therapy for several other inherited retinal dystrophies are currently being assessed in clinical trials including *REP1* for choroideremia (MacLaren et al., 2014) and *MERTK* for autosomal recessive retinitis pigmentosa (Conlon et al., 2013). Gene therapy using *ABCA4* for Stargardt disease, *MYO7* for Usher syndrome, *RS1* for X-linked Retinoschisis and *RPGR* for X-linked RP are still being analysed in animal models or in the early stages of clinical trials (for review see (Sengillo et al., 2017; Sengillo et al., 2016).

Non-viral approaches using nanoparticles (NPs) for the delivery of genes are also being developed and have been used to successfully deliver genes to the RPE and photoreceptors *in vivo* (for review see (Adijanto and Naash, 2015)). However none of the DNA carriers described and characterised to date can match the levels of transduction seen with virus systems.

The other major approach has been the use of cell based therapies to treat IRDs. There are two main methods, replacement or the preservation of cells; in both cases the underlying genetic defect is not addressed. Replacement usually involves the subretinal injection of wild type RPE cells derived from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). These new functional RPE cells supplement the defective and dying RPE cells. Preservation strategies involve the injection of cells that will support and help maintain the remaining photoreceptors. For a comprehensive review on these cell based approaches see (Jones et al., 2017).

However, probably the most exciting technology to emerge in the last few years as a potential method for treating monogenic disease is that of gene editing. This technique is revolutionising what can be done in a molecular biology laboratory in the same way that PCR did in the 1980's (**Table 1**). The approach utilises the fact that the cell is able to repair double stranded breaks in its DNA which it does either by joining the two ends of the DNA molecule together by a mechanism called non-homologous end-joining (NHEJ) or by using the sister chromatid as template a process called homology directed repair (HDR). The use of meganucleases, zinc finger nucleases (ZFN), CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9) and TALENS (transcription activator-like effector nucleases) can be used to target these breaks to specific regions (for reviews on their use in retinal disease see (Hung et al., 2016; Sengillo et al., 2017; Yanik et al., 2017)).

The advances in molecular biology techniques outlined here have enabled many genes causing IRDs to be determined. There are still many more to be found but the progress made in next generation sequencing technologies has made this a viable option for use in clinical diagnostic laboratories meaning that more patients will secure a genetic diagnosis. More work needs to be done on the best ways of analysing the large amounts of data generated by these methods and to ensure that the data is confidential and stored securely. Ethical issues also need to be discussed by the wider community especially the reporting of incidental findings. The prospects for therapies targeted to a specific gene defect are also on the horizon, with both gene therapy and stem cell replacement therapies showing great promise. However the prospect of gene editing techniques is one of the most exciting advances in molecular biology in the last 60 years but the ethical implications need to be debated widely.

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Internet resources

dbEST: http://www.ncbi.nlm.nih.gov/dbEST

dbSNP: http://www.ncbi.nlm.nih.gov/SNP/

ENSEMBL: http://www.ensembl.org/index.html

Exome Aggregation Consortium (ExAC): http://biorxiv.org/content/early/2015/10/30/030338

GenBank: http://www.ncbi.nlm.nih.gov/Genbank

NCBI: http://www.ncbi.nlm.nih.gov/

Online Mendelian Inheritance in Man (OMIM): http://www.omim.org/

PubMed: http://www.ncbi.nlm.nih.gov/pubmed

RetNet: http://www.sph.uth.tmc.edu/Retnet/

UCSC: https://genome.ucsc.edu/

UniGene: http://www.ncbi.nlm.nih.gov/unigene

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Table 1. Timeline showing the important techniques and milestones in the development of molecular genetics

Year	Technique/method	Reference	Uses	Milestone	Reference	Year
				Isolation of DNA by Frederick Meischer	For review see (Dahm, 2005)	**1869**
				Demonstration that DNA is the genetic material	(Avery et al., 1944)	**1944**
1952	Electrophoresis	(Markham and Smith, 1952)	Separation and visualisation of DNA fragments			
				Structure of DNA	(Watson and Crick, 1953b)	**1953**
1956	Discovery of DNA polymerase	(Kornberg et al., 1956)	Replication of DNA			
1967	Discovery of DNA ligase	(Cozzarelli et al., 1967; Gefter et al., 1967; Gellert, 1967; Olivera and Lehman, 1967; Weiss and Richardson, 1967)	Joining DNA fragments together			
1968-69	Discovery of type I restriction enzymes	(Arber and Linn, 1969; Meselson and Yuan, 1968)	Cutting DNA randomly			
1970-71	Discovery of type II restriction enzymes	(Danna and Nathans, 1971; Kelly and Smith, 1970)	Cutting DNA at specific sites and so can be used for mapping			
1972	Transformation of *E.coli*	(Cohen et al., 1972; Jackson et al., 1972)	Insertion of plasmids into bacteria for propagation			
1973	Cloning	(Cohen et al., 1973)	*In vitro* construction of plasmids			
1975	Southern blotting	(Southern, 1975)	Immobilisation of DNA on membranes			
1977	DNA sequencing	(Maxam and Gilbert, 1977; Sanger et al., 1977)	Determining the linear sequence			
				Genetic linkage map using RFLPs	(Botstein et al., 1980)	**1980**
				Huntington's disease linkage	(Gusella et al., 1983)	**1983**
1985	Polymerase Chain Reaction (PCR)	(Saiki et al., 1985)	Generation of thousands of copies of a fragment of DNA			
1986	Automated DNA sequencing	(Smith et al., 1986)	Automation of chain termination technique with introduction of Applied Biosystems AB370A sequencer			
1987	Generation of Yeast Artificial chromosomes (YACs)	(Burke et al., 1987)	*In vitro* manipulation of 200-2000kb DNA			
				First generation linkage map	(Donis-Keller et al., 1987)	**1987**
1988	PCR with *Taq*I	(Saiki et al., 1988)	Allowed automation of technique			

				Human Genome Project conceived	For review see (Cantor, 1990; Watson, 1990)	**1988**
				Cloning of the cystic fibrosis gene	(Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989)	**1989**
				Human Genome Project begins	(Cantor, 1990; Watson, 1990)	**1990**
				Second generation linkage map	(Weissenbach et al., 1992)	**1992**
				Human Genome Project review	(Collins and Galas, 1993)	**1993**
				Comprehensive linkage map	(Murray et al., 1994)	**1994**
1996	Pyrosequencing	(Ronaghi et al., 1996)	Enabled sequencing by synthesis			
				Sequence of human chromosome 22	(Dunham et al., 1999)	**1999**
				Draft human Genome sequence published	(Lander et al., 2001; Venter et al., 2001)	**2001**
				Completion of Human Genome Project	(Collins et al., 2003)	**2003**
2004-05	454 Genome Sequencer	(Margulies et al., 2005)	First commercial NGS machine			
2006	Genome Analyzer (Solexa)	(Bentley et al., 2008)	Could sequence 1 Gb data in a single run			
	Sequencing by Oligonucleotide Ligation and Detection (SOLiD)	(McKernan et al., 2009)				
				First complete genome of an individual by Sanger sequencing	(Levy et al., 2007)	**2007**
				First complete genome of an individual by NGS	(Wheeler et al., 2008)	**2008**

Table 2. Genes causing inherited retinal dystrophies (IRD identified) between 1990 and 2003 the time from the first IRD gene (RHO) being identified to the announcement of the completion of the Human Genome Project (HGP).

Year	Gene		Function	Method of identification	Disease	Reference
1990	*RHO*	Rhodopsin	Phototransduction	Linkage, candidate gene	ad RP	(Dryja et al., 1990b)
	CHM	Rab escort protein 1	Holoenzyme formation	Linkage, positional cloning	CHM	(Cremers et al., 1990)
1991	*PRPH2*	Peripherin	Photoreceptor OS structure	Linkage, candidate gene	ad RP	(Farrar et al., 1992) (Kajiwara et al., 1994)
1993	*PDE6B*	Rod cGMP phosphodiesterase beta subunit	Phototransduction	Candidate gene	ar RP	(McLaughlin et al., 1993)
1994	*ROM1*	Retinal outer segment membrane protein 1	Structural	Candidate gene	Digenic RP	(Kajiwara et al., 1994)
1995	*CNGA1*	Rod cGMP-gated channel alpha subunit	Phototransduction	Candidate gene	ar RP	(Dryja et al., 1995)
	MYO7A	Myosin VIIA	Structural	Linkage, candidate gene	USH 1A	(Weil et al., 1995)
	PDE6A	cGMP phosphodiesterase alpha subunit	Phototransduction	Candidate gene	ar RP	(Huang et al., 1995)
	SAG	Arrestin	Phototransduction	Linkage, candidate gene	CSNB	(Fuchs et al., 1995)
1996	*GNAT1*	Rod transducin alpha subunit	Phototransduction	Candidate gene	CSNB	(Dryja et al., 1996)
	GUCY2D	Retinal-specific guanylate cyclase	Phototransduction	Linkage, positional cloning, candidate gene	LCA	(Perrault et al., 1996)
	RPGR	Retinitis pigmentosa GTPase Regulator	Intraflagellar Transport	Linkage, positional cloning, candidate gene	XLRP	(Meindl et al., 1996) (Roepman et al., 1996a)
1997	*ABCA4*	ATP-binding cassette Subfamily A member 4	Retinal metabolism	Linkage, positional cloning, candidate gene	ar RP/STGD	(Allikmets, 1997)
	CRX	Cone-Rod Homeobox	Transcription Factor	Linkage, candidate gene	ad RP/LCA	(Freund et al., 1997)
	GRK1	Rhodopsin kinase	Phototransduction	Candidate gene	CSNB	(Yamamoto et al., 1997)
	RLBP1	Retinaldehyde Binding Protein 1	Retinal metabolism	Linkage, candidate gene	ar RP	(Maw et al., 1997)
	RPE65	Retinal Pigment Epithelium Specific Protein 65kDa	Retinal metabolism	Candidate gene	ad RP/ar RP/LCA	(Marlhens et al., 1997) (Gu et al., 1997)
1998	*BEST1*	Bestrophin 1	Anion channel	Linkage, positional cloning, candidate gene	ad RP/ar RP	(Petrukhin et al., 1998)
	CACNA1F	Calcium Voltage-gated channel subunit Alpha 1F	Calcium channel regulation	Linkage, positional cloning, candidate gene	XL CSNB	(Bech-Hansen et al., 1998) (Strom et al., 1998)
	CNGA3	Cone cGMP-gated channel alpha subunit	Phototransduction	Linkage, candidate gene	ACHM	(Kohl et al., 1998)
	GUCA1A	Guanylate cyclase activating protein 1A	Phototransduction	Linkage, candidate gene	ad COD	(Payne et al., 1998)
	TULP1	Tubby-Like Protein 1	Tissue development and	Linkage, positional cloning,	ar RP	(Banerjee et al., 1998)

			maintenance	candidate gene		(Gu et al., 1998) (Hagstrom et al., 1998)
	USH2A	Usherin	Structural	Linkage, positional cloning	USH 2A	(Eudy et al., 1998)
1999	*CRB1*	Crumbs 1	Tissue development and maintenance	Linkage, candidate gene	ar RP	(den Hollander et al., 1999)
	EFEMP1	Fibulin 3	Structural	Linkage, positional cloning, candidate gene	ad DHRD	(Stone et al., 1999)
	NRL	Neural Retina Leucine Zipper	Tissue development and maintenance	Linkage, candidate gene	ad RP/ ar RP	(Bessant et al., 1999)
	RDH5	Retinol dehydrogenase 5	Retinal metabolism	Candidate gene	FA	(Yamamoto et al., 1999)
	RGR	Retinal G-protein coupled receptor	Retinal metabolism	Candidate gene	ar RP	(Morimura et al., 1999)
	RP1	Retinitis pigmentosa 1	Tissue development and maintenance	Linkage, positional cloning, candidate gene	ad RP/ ar RP	(Pierce et al., 1999) (Sullivan et al., 1999)
	RP2	Retinitis Pigmentosa 2	Tissue development and maintenance	Linkage, positional cloning, candidate gene	XL RP	(Hardcastle et al., 1999) (Mears et al., 1999)
2000	*AIPL1*	Aryl hydrocarbon receptor interacting protein like 1	Transport, protein trafficking	Linkage, candidate gene	ad CORD/ar LCA	(Sohocki et al., 2000)
	CNGB3	Cone cGMP-gated channel beta 3 subunit	Phototransduction	Linkage, positional cloning,	ACHM	(Kohl et al., 2000)
	HRG4 (Unc119)	Unc-119 Lipid binding Chaperone	Neurotransmitter release	Candidate gene	ad COD	(Kobayashi et al., 2000)
	MERTK	MER proto-oncogen, tyrosine kinase	Transmembrane protein	Candidate gene	ar RP	(Gal et al., 2000)
	NYX	Nyctalopin	TBD	Linkage, positional cloning	XL CSNB	(Bech-Hansen et al., 2000)
	PROM1	Prominin 1	Structural	Linkage, candidate gene	ar RP	(Maw et al., 2000)
2001	*CNGB1*	Rod cGMP-gated channel beta subunit	Phototransduction	Linkage, candidate gene	ar RP	(Bareil et al., 2001)
	FSCN2	Fascin Actin Bundling protein 2	Cellular Structure	Candidate gene	ad RP	(Wada et al., 2001)
	LRAT	Lecithin Retinol Acyltransferase (Phosphatidyl choline-Retinol-O-Acyltransferase)	Retinal metabolism	Candidate gene	ar RP/LCA	(Thompson et al., 2001)
	PRPF31	Pre mRNA processing factor 31	Splicing	Linkage, positional cloning, candidate gene	ad RP	(Vithana et al., 2001)
	PRPF8	Pre mRNA processing factor 8	Splicing	Linkage, positional cloning, candidate gene	ad RP	(McKie et al., 2001)
	USH1C	Harmonin	Structural	Linkage, candidate gene	USH	(Bitner-Glindzicz et al., 2000) (Verpy et al., 2000)
2002	*GNAT2*	Cone transducin alpha subunit	Phototransduction	Candidate gene	ACHM	(Kohl et al., 2002)
	IMPDH1	IMP (Inosine 5'-monophasphate) Dehydrogenase 1	Regulates cell growth	Linkage, positional cloning, candidate gene	ad RP	(Bowne et al., 2002) (Kennan et al., 2002)

	PRPF3	Pre mRNA processing factor 3	Splicing	Linkage, candidate gene	ad RP	(Chakarova et al., 2002)
	RP9/PAP1	Retinitis Pigmentosa 9	Splicing	Linkage, positional cloning, candidate gene	ad RP	(Keen et al., 2002)
2003	*RIMS1*	Regulating synapse membrane exocytosis 1	Neurotransmitter release	Linkage, positional cloning, candidate gene	ad CORD	(Johnson et al., 2003)
	RPGRIP1	Retinitis pigmentosa GTPase Regulator Interacting protein 1	Interacts with RPGR	Linkage, candidate gene	ar CORD/LCA	(Hameed et al., 2003)

ACHM: achromatopsia; ad: autosomal dominant; ar: autosomal recessive; COD: cone dystrophy; CORD: cone-rod dystrophy; CSNB: congenital stationary night blindness; DHRD: Doyne Honeycomb retinal dystrophy; FA: fundus albipunctatus; LCA: Leber's congenital amaurosis; OS: outer segments; RP: Retinitis pigmentosa; USH: Usher's syndrome; XL: X-linked

Table 3. Animal models of human retinal diseases

Gene Symbol	Gene Name	Function	Disease	Natural or Transgenic	Species	Model name	Reference
Abca4	*ATP-Binding Cassette Subfamily A Member 4*	Retinoid cycle/Transport	**RP/STGDs**	Transgenic	Mouse		(Weng et al., 1999)
Adam9	*ADAM Metallopeptidase domain 9*	Cell matrix interactions	**CRD**	Transgenic	Mouse		(Parry et al., 2009)
				Natural	Canine	*GIT*	(Kropatsch et al., 2010)
Aipl1	*Aryl hydrocarbon Receptor Interacting Protein Like 1*	Nuclear transport	**CRD/LCA**	Transgenic	Mouse		(Ramamurthy et al., 2004)
Arl3	*ADP Ribosylation Factor like GTPase 3*	TBD	**RP**	Transgenic	Mouse		(Schrick et al., 2006)
Arl6	*ADP Ribosylation Factor like GTPase 6*	Ciliary Function	**RP**	Transgenic	Zebrafish		(Pretorius et al., 2010)
Atf6	*Activating Transcription Factor 6*	Transcription Factor	**ACHM**	Transgenic	Mouse		(Wu et al., 2007)
Bbs1	*Bardet-Biedl Syndrome 1*	TBD	**RP**	Transgenic	Mouse		(Kulaga et al., 2004) (Davis et al., 2007)
Bbs2	*Bardet-Biedl Syndrome 2*	TBD	**RP**	Transgenic	Mouse		(Nishimura et al., 2004)
Best1	*Bestrophin 1*	Transmembrane Chloride Channel	**RP**	Transgenic	Mouse		(Zhang et al., 2010)
				Natural	Canine	*CMR*	(Guziewicz et al., 2007) (Zangerl et al., 2010)
C2orf71	*Chromosome 2 open reading frame 71*	TBD	**RP**	Transgenic	Zebrafish		(Nishimura et al., 2010)
				Transgenic	Mouse		(Kevany et al., 2015)
Cabp4	*Calcium Binding Protein 4*	Voltage Channel Modulation	**CSNB/LCA**	Transgenic	Mouse		(Haeseleer et al., 2004)
Cacna1f	*Calcium Voltage-Gated Channel Subunit Alpha 1f*	Subunit of calcium channel	**CRD/CSNB**	Natural	Mouse	*Nob2*	(Chang et al., 2006b)
				Transgenic	Mouse		(Mansergh et al., 2005)
				Natural	Rat		(Gu et al., 2008)
				Transgenic	Zebrafish		(Jia et al., 2014)
Cacna2d4	*Calcium Voltage-Gated Channel Auxiliary Subunit Alpha2delta 4*	Subunit of calcium channel	**COD**	Natural	Mouse		(Wycisk et al., 2006)
				Natural	Canine		(Kondo et al., 2015)
Cdhr1	*Cadherin Related Family Member 1*	Calcium Dependent Cell-Cell Adhesion	**CRD**	Transgenic	Mouse		(Rattner et al., 2001)
Cerkl	*Ceramide Kinase Like*	Cell Survival	**CRD/RP**	Transgenic	Mouse		(Garanto et al., 2012)
Cep290	*Centrosomal Protein 290*	Ciliogenesis	**LCA**	Natural	Cat	*rdAc*	(Menotti-Raymond et al., 2007)
				Transgenic	Mouse		(Chang et al., 2006a)
Chm	*Choroideremia*	Holoenzyme formation	**CHM**	Transgenic	Mouse		(van den Hurk et al., 1997)
				Transgenic	Zebrafish		(Starr et al., 2004)
Clrn1	*Clarin 1*	Synaptic Transmembrane Protein	**RP/USH**	Transgenic	Mouse		(Geng et al., 2009)
Cnga3	*Cyclic Nucleotide-Gated Channel, Alpha3*	Visual Transduction	**ACHM**	Natural	Canine	*Erd*	(Acland and Aguirre, 1987) (Tanaka et al., 2015)
				Transgenic	Mouse		(Biel et al., 1999)
				Natural	Sheep		(Reicher et al., 2010)
Cngb1	*Cyclic Nucleotide Gated Channel Beta 1*	cGMP-gated ion channel subunit	**RP**	Transgenic	Mouse		(Zhang et al., 2009)
				Natural	Canine	*cd*	(Winkler et al., 2013)
Cngb3	*Cyclic Nucleotide Gated Channel Beta 3*	Modulation of channel function	**CRD/ACHM**	Natural	Canine		(Sidjanin et al., 2002)
				Transgenic	Mouse		(Ding et al., 2009)
Crb1	*Crumbs-1*	Retinal Development	**RP/LCA**	Natural	Mouse	*Rd8*	(Mehalow et al., 2003)
Crx	*Cone-Rod Homeobox*	Transcription factor	**RP/LCA**	Transgenic	Mouse		(Furukawa et al., 1999)
Cyp4v3	*Cytochrome P450 Family 4 Subfamily V member 3*	Fatty acid and steroid metabolism	**RP**	Transgenic	Mouse		(Lockhart et al., 2014)
Dhdds	*Dehydrodolichyl Diphosphate Synthase Subunit*	Enzymatic function	**RP**	Transgenic	Zebrafish		(Zuchner et al., 2011) (Wen et al., 2014)
Efemp1	*EGF-containing Fibulin-like Extracellular Matrix Protein 1*	Structural	**DHRD**	Transgenic	Mouse		(Fu et al., 2007)
Fam161a	*Family with Sequence Similarity 161 Member A*	TBD	**RP**	Transgenic	Mouse		(Karlstetter et al., 2014)
Fscn2	*Fascin Actin-Bundling Protein 2*	Crosslinks and bundles f-actin	**RP**	Transgenic	Mouse		(Yokokura et al., 2005)
Gnat1	*Guanine Binding protein, Alpha Transducing Activity Polypeptide 1*	Visual Transduction	**RP**	Transgenic	Mouse		(Hattar et al., 2003)
Gnat2	*Guanine Binding protein, Alpha Transducing Activity Polypeptide 2*	Visual Transduction	**ACHM**	Natural	Mouse	*Cpfl2*	(Chang et al., 2006a)
				Transgenic	Zebrafish		(Kennedy et al., 2007)
Gnb1	*Guanine nucleotide binding protein Beta*	Visual Transduction	**RD**	Natural	Mouse	*Rd4*	(Kitamura et al., 2006)
Gpr179	*G Protein-Coupled Receptor 179*	G-Protein Coupled Receptor	**CSNB**	Transgenic	Zebrafish		(Peachey et al., 2012)
				Natural	Mouse	*Nob5*	(Balmer et al., 2013)
Grk1	*G protein-Coupled Receptor Kinase 1*	Visual transduction	**CSNB**	Transgenic	Mouse		(Whitcomb et al., 2010)

Grm6	*Glutamate Metabotropic Receptor 6*	Glutamate Receptor	**CSNB**	Transgenic	Mouse		(Masu et al., 1995)
				Natural	Mouse	*Nob7*	(Qian et al., 2015)
Guca1a	*Guanylate Cyclase Activator 1A*		**COD/CRD**	Transgenic	Mouse		(Mendez et al., 2001) (Howes et al., 2002)
Gucy2d (Ret-GC)	*Guanylate Cyclase 2D, retinal*	Visual Transduction	**COD/LCA**	Natural	Chicken	*rd*	(Semple-Rowland et al., 1998)
				Transgenic	Mouse		(Yang et al., 1999)
			LCA	Transgenic	Zebrafish		(Stiebel-Kalish et al., 2012)
Ift140	*Intraflagellar Transport 140*	Cilia Activity	**RP/LCA**	Transgenic	Mouse		(Miller et al., 2013)
Ift172	*Intraflagellar Transport 172*	Intraflagellar transport	**RP**	Transgenic	Mouse		(Huangfu et al., 2003)
				Transgenic	Zebrafish		(Halbritter et al., 2013) (Bujakowska et al., 2015)
Impdh1	*Inosine Monophosphate Dehydrogenase 1*	Enzymatic	**RP/LCA**	Transgenic	Mouse		(Gu et al., 2003) (Aherne et al., 2004)
				Transgenic	Chicken		(Inglehearn et al., 2003)
Lrat	*Lectin Retinol Acyltransferase*	Visual Transduction	**RP/LCA**	Transgenic	Mouse		(Batten et al., 2004)
Lrit3	*Leucine rich Repeat, Ig-Like and Transmembrane Domains 3*	TBD	**CSNB**	Natural	Mouse	*Nob6*	(Neuille et al., 2014)
Mertk	*MER Proto-Oncogene, Tyrosine Kinase*	Disc Shedding	**RP**	Natural	Rat	*RCS*	(D'Cruz et al., 2000) (Bourne et al., 1938)
MyoVIIA	*Myosin VIIA*	Photoreceptor Structure	**USH**	Natural	Mouse	*Shaker*	(Gibson et al., 1995)
Nek2	*NIMA Related Kinase 2*	Cell division	**RP**	Transgenic	Zebrafish		(Nishiguchi et al., 2013)
Neurod1	*Neuronal Differentiation 1*	Transcription factor	**RP**	Transgenic	Mouse		(Pennesi et al., 2003)
Nr2e3	*Nuclear receptor Subfamily 2 Group E Member 3*	Transcription factor	**RP**	Natural	Mouse	*Rd7*	(Akhmedov et al., 2000) (Haider et al., 2001)
Nrl	*Neural Retina Leucine Zipper*	Transcription factor	**RP**	Transgenic	Mouse		(Mears et al., 2001)
Nyx	*Nyctalopin*	TBD	**CSNB**	Natural	Mouse	*Nob*	(Gregg et al., 2003)
				Transgenic	Zebrafish		(Bahadori et al., 2006)
Ofd1	*Ofd1, Centriole and Centriolar Satellite protein*	TBD	**RP**	Transgenic	Mouse		(Ferrante et al., 2006)
				Transgenic	Zebrafish		(Ferrante et al., 2009)
Pde6A	*α-subunit of rod cyclic GMP-PDE*	Visual Transduction	**RP**	Natural	Canine	*Rcd1*	(Suber et al., 1993)
Pde6B	*β-subunit of rod cyclic GMP-PDE*	Visual Transduction	**RP**	Natural	Mouse	*Rd1*	(Bowes et al., 1990) (Pittler et al., 1993)
				Natural	Canine	*Cpfl1*	(Petersen-Jones et al., 1999)
Pde6C	*Phosphodiesterase 6C, cGMP specific, Cone,*	Visual transduction	**COD**	Natural	Mouse		(Chang et al., 2009)
				Transgenic	Zebrafish		(Stearns et al., 2007)
Pde6g	*Phosphodiesterase 6G*	Visual transduction	**RP**	Transgenic	Mouse		(Tsang et al., 1996)
Poc1b	*POC1 Centriolar Protein B*	Ciliogenesis	**CRD**	Transgenic	Zebrafish		(Pearson et al., 2009) (Beck et al., 2014) (Roosing et al., 2014)
Pomgnt1	*Protein O-linked Mannose N-Acetylglucosaminyltransferase 1*	Transmembrane Protein	**RP**	Transgenic	Mouse		(Hu et al., 2010)
Prcd	*Progressive Rod-Cone Degeneration*	TBD	**RP**	Natural	Canine		(Zangerl et al., 2006)
Prom1	*Prominin 1*	Transmembrane Glycoprotein	**STGD/CRD/ RP**	Transgenic	Mouse		(Dellett et al., 2014)
Prpf3	*pre-mRNA processing factor 3*	Splicing factor	**RP**	Transgenic	Mouse		(Graziotto et al., 2011)
Prpf4	*pre-mRNA processing factor 4*	Splicing factor	**RP**	Transgenic	Zebrafish		(Linder et al., 2011) (Chen et al., 2014)
Prpf8	*pre-mRNA processing factor 8*	Splicing factor	**RP**	Transgenic	Mouse		(Graziotto et al., 2011)
Prpf31	*pre-mRNA processing factor 31*	Splicing factor	**RP**	Transgenic	Mouse		(Bujakowska et al., 2009) (Graziotto et al., 2011)
				Transgenic	Zebrafish		(Linder et al., 2011)
Prph2	*Peripherin 2 (rds)*	Photoreceptor Structure	**RP**	Natural	Mouse	*Rds/Rd2*	(Travis et al., 1991)
				Transgenic	Mouse		(McNally et al., 2002)
			CRD	Transgenic	Mouse		(Ding et al., 2004)
Rbp3	*Retinol-Binding Protein 3*	Visual transduction	**RP**	Transgenic	Mouse		(Liou et al., 1998)
Rd3	*Retinal Degeneration 3*	TBD	**LCA**	Natural	Mouse	*Rd3*	(Chang et al., 1993)
				Natural	Canine	*Rcd2*	(Kukekova et al., 2009)
Rdh5	*Retinol Dehydrogenase 5*	Vitamin A Cycle	**FA**	Transgenic	Mouse		(Driessen et al., 2000)
Rho	*Rhodopsin*	Visual Transduction	**RP**	Natural	Canine		(Kijas et al., 2002)
				Transgenic	Mouse		(Olsson et al., 1992) (Humphries et al., 1997) (Lem et al., 1999)
Rims1	*Regulating Synaptic Membrane Exocytosis 1*	synaptic vesicle exocytosis	**CRD**	Transgenic	Mouse		(Schoch et al., 2002)
Rlbp1	*Retinaldehyde Binding Protein 1*	Visual Transduction	**RP/RCD**	Transgenic	Mouse		(Saari et al., 2001)
Rom1	*Retinal Outer Segment Membrane Protein 1*	Photoreceptor Structure	**RP**	Transgenic	Mouse		(Clarke et al., 2000)
Rpe65	*Retinal Pigment Epithelium-Specific Protein 65KDa*	Retinoid Cycle	**LCA/RD**	Natural	Canine		(Aguirre et al., 1998)
			LCA	Natural	Mouse	*Rd12*	(Pang et al., 2005)
			RD	Transgenic	Mouse		(Redmond et al., 1998)
Rdh12	*Retinol Dehydrogenase 12*	Visual Transduction	**RP/LCA**	Transgenic	Mouse		(Maeda et al., 2006)

Rp1	*Retinitis Pigmentosa 1*	Outer segment disc stacking	**RP**	Transgenic	Mouse		(Gao et al., 2002)
Rp2	*Retinitis Pigmentosa 2*	Beta-tubulin folding	**RP**	Transgenic	Mouse		(Li et al., 2013)
Rpgr	*Retinitis Pigmentosa GTPase Regulator*	Protein Transport	**RP**	Natural	Canine	*XLPRA*	(Zhang et al., 2002) (Zeiss et al., 2000)
				Transgenic	Mouse		(Hong et al., 2000)
Rpgrip1	*Retinitis Pigmentosa GTPase Regulator Interacting protein 1*	Photoreceptor Structure	**LCA**	Natural	Canine		(Mellersh et al., 2006)
		Photoreceptor Structure	**LCA**	Transgenic	Mouse		(Zhao et al., 2003) (Won et al., 2009)
RS1	*Retinoschisin-1*	Cellular Organisation	**RS**	Transgenic	Mouse		(Weber et al., 2002)
Sag	*S-Antigen Visual Arrestin*	Phototransduction	**RP**	Transgenic	Mouse		(Xu et al., 1997) (Chan et al., 2007)
Sema4A	*Semaphorin 4A*	Enhance of T-cell activation	**CRD/RP**	Transgenic	Mouse		(Rice et al., 2004)
Slc24a1	*Solute Carrier Family 24 Member 1*	Modulator of Intracellular Calcium Levels	**CSNB**	Transgenic	Mouse		(Vinberg et al., 2015)
Slc7a14	*Solute Carrier Family 7Member 14*	Cationic transporter protein	**RP**	Transgenic	Zebrafish		(Jin et al., 2014)
Snrnp200	*Small Nuclear Ribonucleoprotein U5 Subunit 200*	Splicing Factor	**RP**	Transgenic	Zebrafish		(Liu et al., 2015a)
Spata7	*Spermatogenesis Associated 7*	TBC	**RP/LCA**	Transgenic	Mouse		(Eblimit et al., 2015)
Spp2	*Secreted Phosphoprotein 2*	TBC	**RP**	Transgenic	Zebrafish		(Liu et al., 2015b)
Topors	*TOP1 Binding Arginine/Serine Rich Protein*	TBC	**RP**	Transgenic	Zebrafish		(Chakarova et al., 2011)
Trnt1	*TRNA Nucleotidyl Transferase 1*	tRNA function for protein synthesis	**RP**	Transgenic	Zebrafish		(DeLuca et al., 2016)
Ttc8	*Tetratricopeptide Repeat Domain 8*	Ciliogenesis	**RP**	Transgenic	Mouse		(Tadenev et al., 2011)
			RP	Natural	Canine		(Downs et al., 2014)
Ttpa	*Tocopherol (Alpha) Transfer Protein*	Transport	**RP**	Transgenic	Mouse		(Leonard et al., 2002)
Trpm1	*Transient Receptor potential Cation Channel Subfamily M member 1*	Visual transduction	**CSNB**	Natural	Horse	*LP*	(Bellone et al., 2008)
			CSNB	Transgenic	Mouse		(Koike et al., 2010)
Tulp1	*Tubby Like Protein-1*	Transcription Factor	**RP/LCA**	Natural	Mouse	*Rd5*	(Noben-Trauth et al., 1996)
Unc119	*Unc-119 Lipid Binding Chaperone*	Synaptic Neurotransmitter release	**CRD**	Transgenic	Mouse		(Kobayashi et al., 2000) (Kubota et al., 2002) (Ishiba et al., 2007)
Ush2a	*Usherin*	Tissue development and maintenance	**RP/USH**	Transgenic	Mouse		(Liu et al., 2007)
Znf408	*Zinc finger Protein 408*	Transcription Factor	**RP**	Transgenic	Zebrafish		(Collin et al., 2013)
Znf513	*Zinc finger Protein 513*	Transcription Factor	**RP**	Transgenic	Zebrafish		(Li et al., 2010)

ACHM: achromatopsia; CHM: choroideremia; CMR: Canine multifocal retonopathy; COD: cone dystrophy; cpfl: cone photoreceptor function loss; CRD: cone rod dystrophy; DHRD: Doyne honeycomb retinal dystrophy; FA: fundus albipunctatus; GIT: Irish Glen of Imaal Terrier: LCA: Leber's congenital amaurosis; nob: no b-wave; rcd: rod-cone dysplasia; RCS: Royal College of Surgeons; RD: retinal dystrophy; RP: Retinitis pigmentosa; RS: retinoschisis; STGD: Stargardt disease; TBD: To Be Determined; USH: Usher's syndrome; XLPRA: X-linked progressive retinal atrophy

Table 4. Tools and resources used in the analysis of WES and WGS data

Tool/resource	Type	Description	URL	Reference
HPO	*Literature support*	The **H**uman **P**henotype **O**ntology (HPO) aims to provide a standardized vocabulary of phenotypic abnormalities encountered in human disease.	http://human-phenotype-ontology.github.io/	(Kohler et al., 2014)
OMIM	*Literature support*	**O**nline **M**endelian **I**nheritance in **M**an (OMIM) contains human genes and genetic disorders and traits, with particular focus on the molecular relationship between genetic variation and phenotypic expression	http://omim.org/	
PubMed	*Literature support*	A free search engine accessing a comprehensive list of references and abstracts on life sciences and biomedical topics	www.ncbi.nlm.nih.gov/pubmed	
RetNet	*Literature support*	Connects causative genes and loci to inherited retinal diseases using published reports	https://sph.uth.edu/retnet/	(Daiger et al., 1998)
dbSNP	*Allele frequency*	Contains polymorphisms including SNPs, small-scale multi-base deletions or insertions and short tandem repeats or STRs	http://www.ncbi.nlm.nih.gov/snp/	
ExAC	*Allele frequency*	Contains WES data from over 60,000 unrelated individuals who were not affected by severe paediatric diseases	http://exac.broadinstitute.org/	
ENSEMBL	*Allele frequency and variant consequence*	Contains annotated genes, computes multiple alignments, predicts regulatory function and collects disease data	http://www.ensembl.org/index.html	(Yates et al., 2016)
Kaviar	*Allele frequency*	Contains both WGS and WES data from over 70,000 individuals (13.2K WGS, 64.6K WES). It excludes cancer genomes but includes some data from cell lines and individuals affected by disease. Relatedness in the data has not been removed yet	http://db.systemsbiology.net/kaviar/	(Glusman et al., 2011)
UCSC	*Allele frequency and variant consequence*	A genome browser that displays tracks from assembly contigs and gaps, mRNA and EST alignments, multiple gene predictions, cross-species homologies, single nucleotide polymorphisms, sequence-tagged sites, radiation hybrid data and transposon repeats. It also includes data from The Encyclopedia of DNA Elements (ENCODE) that focuses on functions of non-coding regions.	https://genome.ucsc.edu/index.html	(Kent et al., 2002)
1000 Genomes	*Allele frequency*	Contains WGS data from over 1000 individuals. Due to poor depth, it only	http://www.1000genomes.org/	(Genomes Project et al., 2015)

		considers variants of at least 1% in the populations studied		
CADD	*Variant consequence*	Score variants based on simulated evolutionary changes. It takes annotations from the Ensembl Variant Effect Predictor, ENCODE, UCSC that span a wide range of data types including conservation metrics like GERP, phastCons, and phyloP; functional genomic data like DNase hypersensitivity and transcription factor binding; transcript information like distance to exon-intron boundaries or expression levels in commonly studied cell lines; and protein-level scores like Grantham, SIFT, and PolyPhen.	http://cadd.gs.washington.edu/	(Kircher et al., 2014)
pMut	*Variant consequence*	Predicts possible impact of an amino acid substitution based on the use of different kinds of sequence information to label mutations, and neural networks to process this information	http://mmb.pcb.ub.es/PMut/	(Ferrer-Costa et al., 2005)
Polyphen	*Variant consequence*	Predicts possible impact of an amino acid substitution on the structure and function of a human protein using straightforward physical and comparative considerations. It is now superseded by Polyphen-2	http://genetics.bwh.harvard.edu/pph2/	(Adzhubei et al., 2010)
SIFT	*Variant consequence*	Predicts whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids.	http://sift.bii.a-star.edu.sg/	(Kumar et al., 2009)
MutationTaster	*Variant consequence*	Predicts disease-causing potential of variants based on evolutionary conservation, splice-site changes, loss of protein features and changes that might affect the amount of mRNA. Unlike Polyphen, SIFT and CADD, it also evaluates indel changes.	http://www.mutationtaster.org/	(Schwarz et al., 2010)

Table 5. Retinal disease genes identified by whole exome sequencing (WES) or whole genome sequencing (WGS).

Gene symbol	Gene Name	Aliases	Chromosome	Disease	Identification Method	Original reference
ACBD5	Acyl-CoA-binding domain-containing protein 5		10p12.1	ar CORD with psychomotor delay	Homozygosity mapping and WES	(Abu-Safieh et al., 2013)
ADAMTS18	ADAM metallopeptidase with thrombospondin type 1 motif 18	*KNO2*	16q23.1	Knobloch syndrome ar RD early onset	Homozygosity mapping and WES	(Aldahmesh et al., 2011) (Peluso et al., 2013)
ADIPOR1	Adiponectin receptor 1	*PAQR1*	1q32.1	ar syndromic RP	WES	(Xu et al., 2016a)
AGBL5	ATP/GTP binding protein-like 1		2p23.3	ar RP	WES	(Kastner et al., 2015)
ARL3	ADP ribosylation factor like GTPase 3		10q24.32	ad RP	WES	(Strom et al., 2016)
ATF6	Activating transcription factor 6	*ATF6A*	1q23.3	ar ACHM	Homozygosity mapping and WES	(Ansar et al., 2015; Kohl et al., 2015; Xu et al., 2015a)
BBIP1	BBSome interacting protein 1	*BBIP10, BBS18*	10q25.2	ar BBS	WES	(Scheidecker et al., 2014)
CLUAP1	Clusterin associated protein 1		16p13.3	ar LCA	WES	(Soens et al., 2016)
CSPP1	Centrosome and spindle pole associated protein 1		8q13.2	ar Joubert syndrome	WES	(Akizu et al., 2014; Shaheen et al., 2014; Tuz et al., 2014)
CTNNA1	Catenin alpha 1	*MDPT2*	5q31.2	ad MD	Linkage mapping and WES	(Saksens et al., 2016)
DHDDS	Dehydrodolichyl diphosphate synthetase	*RP59*	1p36.11	ar RP	Homozygosity mapping and WES	(Zelinger et al., 2011; Zuchner et al., 2011)
DRAM2	DNA-damage regulated autophagy modulator 2	*TMEM77*	1p13.3	ar MD with adult onset	Homozygosity mapping and WES	(El-Asrag et al., 2015)
DTHD1	Death domain containing protein 1		4p14	ar LCA with myopathy	Homozygosity mapping and WES	(Abu-Safieh et al., 2013)
EMC1	ER membrane protein complex subunit 1		1p36.13	ar RP	Homozygosity mapping and WES	(Abu-Safieh et al., 2013)
EXOSC2	Exosome component 2		9q34.12	ar RP and hearing loss	WES	(Di Donato et al., 2016)

GNB3	G protein subunit beta 3		12p13.31	ar CSNB	WES	(Vincent et al., 2016)
GNPTG	N-acetylglucosamine-1-phosphate transferase gamma subunit		16p13.11	ar RP with skeletal abnormalities	WES	(Schrader et al., 2011)
GPR125	G protein-coupled receptor 125	*PGR21, TEM5L*	4p15.2	ar RP	Homozygosity mapping and WES	(Abu-Safieh et al., 2013)
GPR179	G protein-coupled receptor 179	*CSNB1E*	17q12	ar CSNB complete	Animal model and WES	(Audo et al., 2012a; Peachey et al., 2012)
HarS	Histidyl-tRNA synthetase	*HRS, USH3B*	5q31.3	ar USH	Homozygosity mapping and WES	(Puffenberger et al., 2012)
HGSNAT	Heparan-alpha-glucosaminide N-acetyltransferase	*MPS3C, TMEM76, RP73*	8p11.21	ar RP	WES	(Haer-Wigman et al., 2015)
HK1	Hexokinase 1		10q22.1	ad RP	Linkage mapping and WES	(Sullivan et al., 2014; Wang et al., 2014)
IFT140	Intraflagellar transport 140	*MZSDS, SRTD9, WDTC2*	16p13.3	ar RP and ar LCA ar Mainzer-Saldino syndrome	WES	(Xu et al., 2015b) (Perrault et al., 2012b) (Schmidts et al., 2013)
ITM2B	Integral membrane protein 2B	*ABRI*	13q14.2	ad RD	WES	(Audo et al., 2014)
KIAA1549	KIAA1549		7q34	ar RP	Homozygosity mapping and WES	(Abu-Safieh et al., 2013)
KIZ	Kizuna centrosomal protein	*RP69*	20p11.23	ar RP	WES	(El Shamieh et al., 2014)
LAMA1	Laminin alpha 1	*PTBHS*	18p11.3	ar RD and cerebellar dysplasia	Homozygosity mapping and WES	(Aldinger et al., 2014)
LRIT3	Leucine-rich repeat, Ig-like and transmembrane domains 3	*FIGLER4*	4q25	ar CSNB	WES	(Zeitz et al., 2013)
LZTFL1	Leucine zipper transcription factor like-1	*BBS17*	3p21.3	ar BBS	Homozygosity mapping and WES	(Marion et al., 2012)
MAK	Male germ-cell associated kinase	*RP62*	6p24.2	ar RP	WES	(Ozgul et al., 2011; Tucker et al., 2011)
MAPKAPK3	Mitogen-activated protein kinase-activated protein kinase 3		3p21.2	ad RD	WES	(Meunier et al., 2016)
miR204	Micro RNA 204		9q21.12	ad RD and iris coloboma	Linkage mapping and WES	(Conte et al., 2015)
MFSD8	Major facilitator superfamily domain	*CLN7, CCMD*	4q28.2	ar MD	Linkage mapping and WES	(Roosing et al., 2015)

	containing 8					
MVK	Mevalonate kinase		12q24.11	ar RP	WES	(Siemiatkowska et al., 2013)
NBAS	Neuroblastoma amplified sequence	*NAG, ILFS2, SOPH*	2p24.3	ar OA and syndromic RP	WES	(Segarra et al., 2015)
NEK2	Never in mitosis gene A-related kinase 2	*NLK1, RP67*	1q32.3	ar RP	WGS	(Nishiguchi et al., 2013)
NEUROD1	Neuronal differentiation protein 1	*MODY6*	2q31.3	ar RP	WES	(Wang et al., 2015)
NMNAT1	Nicotinamide nucleotide adenylyltransferase 1	*LCA9, PNAT1*	1p36.22	ar LCA	Linkage mapping and WES	(Chiang et al., 2012; Falk et al., 2012; Koenekoop et al., 2012; Perrault et al., 2012a)
PCYT1A	Phosphate cytidylyltransferease 1 choline alpha		3q29	ar CORD and skeletal disease	WES	(Hoover-Fong et al., 2014; Yamamoto et al., 2014)
PNPLA6	Patatin-like phospholipase domain-containing protein 6	*BNHS, OMCS, SPG39*	19p13.2	ar LCA	WES	(Kmoch et al., 2015)
POMGNT1	O-linked acetylglucosaminyltransferase 1	*MDDGA3, MDDGB3, MDDGC3*	1p34.1	ar RP	WES	(Xu et al., 2016b)
PRDM13	Positive regulatory domain-containing 13	*MCDR1, NCMD, PBCRA1*	6q16.2	ad MD North Carolina	Linkage mapping and WGS	(Small et al., 2016)
RCBTB1	RCC1 And BTB Domain Containing Protein 1	*RCBT1*	13q14.2	ar syndromic RD, ar RD, ad FEVR and Coats disease	Homozygosity mapping and WES	(Coppieters et al., 2016; Wu et al., 2016)
RDH11	Retinol dehydrogenase 11		14q24.1	ar RP	WES	(Xie et al., 2014)
SLC7A14	Solute carrier family 7 member 14	*RP68*	3q26.2	ar RP	WES	(Jin et al., 2014)
SPP2	Secreted phosphoprotein 2	*SPP24*	2q37.1	ad RP	Linkage mapping and WES	(Liu et al., 2015b)
TMEM237	Transmembrane protein 237	*ALS2CR4, JBTS14*	2q33.1	ar Joubert syndrome	Homozygosity mapping and WES	(Huang et al., 2011)
TRNT1	CCA adding tRNA nucleotidyl transferase 1	*SIFD*	3p26.2	ar RP and erythrocytic microcystosis	WES	(DeLuca et al., 2016)
TSPAN12	Tetraspanin 12	*EVR5, NET2*	7q31.31	ad FEVR	Linkage mapping and WES	(Nikopoulos et al., 2010; Poulter et al., 2010)
TTLL5	Tubulin tyrosine ligase-like family member 5	*CORD19, STAMP*	14q24.3	ar COD and CORD	WES	(Sergouniotis et al., 2014)
TUBGCP4	Tubulin gamma complex-associated protein 4		15q15.3	ar chorioretinopathy and microcephaly	WES	(Scheidecker et al., 2015)

WDR19	WD repeat domain 19 protein	*ATD5, CED4, NPHP13, IFT144, PWDMP*	4p14	Ciliopathies with RP	WES	(Bredrup et al., 2011)
ZNF408	Zinc finger protein 408		11p11.2	ad FEVR ar RP	Linkage mapping and WES	(Collin et al., 2013) (Avila-Fernandez et al., 2015)

ad: autosomal dominant; ar: autosomal recessive; BBS: Bardet-Biedl syndrome; COD: cone dystrophy; CORD: cone-rod dystrophy; CSNB: congenital stationary night blindness; FEVR: familial exudative vitreoretinopathy; LCA: Leber's congenital amaurosis; MD: macular dystrophy; OA: optic atrophy; RD: retinal dystrophy; RP: Retinitis pigmentosa; USH: Usher's syndrome; WES: whole exome sequencing; WGS: whole genome sequencing

Figure legends

Figure 1. Fundus image of a normal retina.

The macula contains a high proportion of cone cells, which are most densely clustered in the fovea. The posterior pole marks the area of the retina between the optic disc (the bright yellow oval on the left of the image) and the macula.

Figure 2. Schematic representation of radioactive and fluorescent Sanger sequencing.

A) The structure of a deoxynucleotides (dNTP) and a dideoxynucleotides (ddNTP), the ddNTP lacks the hydroxyl group required for chain elongation during DNA synthesis and so addition of a ddNTP results in the termination of synthesis.

B) (i) Shows the principle of Sanger sequencing. Sanger sequencing (also known as dideoxy or chain termination sequencing) requires template DNA (single stranded), an oligonucleotide primer, DNA polymerase, dNTPs and four ddNTPs (either radioactively labelled or each labelled with a different coloured fluorescent tag). The primer hybridises to the DNA template and chain elongation will occur until the incorporation of a ddNTP. A mixture of DNA chains each of different lengths will be generated and these can be separated based on size using gel electrophoresis. (ii) Schematic representation of the autoradiograph produced when the synthesised DNA chains labelled using radioactivity are separated by size and exposed to X-ray film. Note a separate lane is used for each ddNTP. The sequence is read from the gel from smallest to largest. (iii) Schematic representation of the sequence generated using fluorescently labelled ddNTPs. As the ddNTPs are labelled with different fluorescent dyes they can all be added to one tube and separated using capillary electrophoresis and an electropherogram generated.

Figure 3. Schematic representation of pyrosequencing.

Pyrosequencing (or “sequencing-by-synthesis”) involves the hybridisation of a sequencing primer to a single stranded DNA template. This is then incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates, adenosine 5´ phosphosulphate (APS) and luciferin. The first of four deoxynucleotide triphosphates (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxynucleotide triphosphate into the DNA strand, if it is complementary to the base in the template strand. The incorporation of dGTP into the strand releases a molecule of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. In the presence of adenosine 5´ phosphosulphate (APS) the enzyme ATP sulphuylase converts PPi to ATP. In turn the ATP drives the conversion of luciferin to oxyluciferin by luciferase to generate visible light proportional to the amount of ATP present. The light is detected by a charge coupled device (CCD) camera and seen as a peak in a pyrogram. Apyrase, a nucleotide degrading enzyme, continuously degrades unincorporated dNTPs and excess ATP and when degradation is complete the cycle starts again.

Figure 4. Schematic figure showing the steps in the identification of human disease genes.

The first step in the identification of a disease gene is the ascertainment of families and/or patients with the disease being studied. The DNA is then examined for any gross chromosome aberrations such as translocations or large detectable deletions. The presence of a rearrangement will allow the chromosomal location of the gene to be identified. If not the families will need to undergo linkage analysis to determine the chromosomal rearrangement. After the genetic locus has been identified there are two ways forward, either a positional cloning

approach or candidate gene analysis. Positional cloning was the methodology of choice before the Human Genome Project (HGP) when there was little information about the genes in a particular region. Post HGP candidate gene analysis is the preferred method which involves identifying the genes in the region and prioritising their screening based on their putative function. Once a candidate gene has been identified then a mutation screen in the affected patients is undertaken, this is followed by segregation analysis in the family. If the gene does not segregate with the disease it is back to candidate gene analysis if it does the gene has been identified and needs to be followed by functional analysis.

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Figure 1

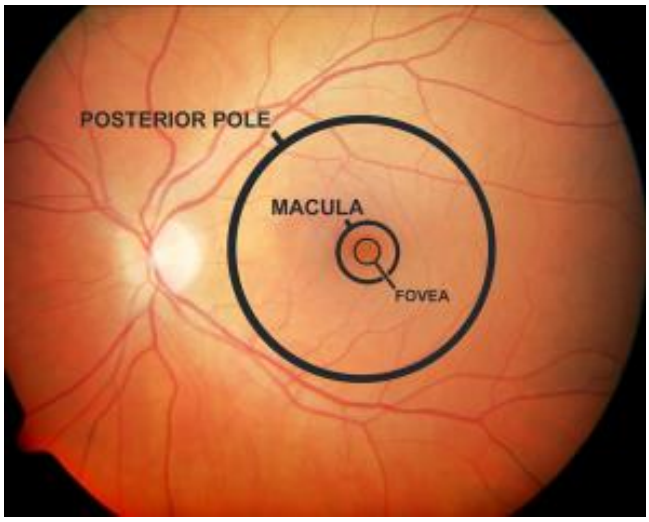
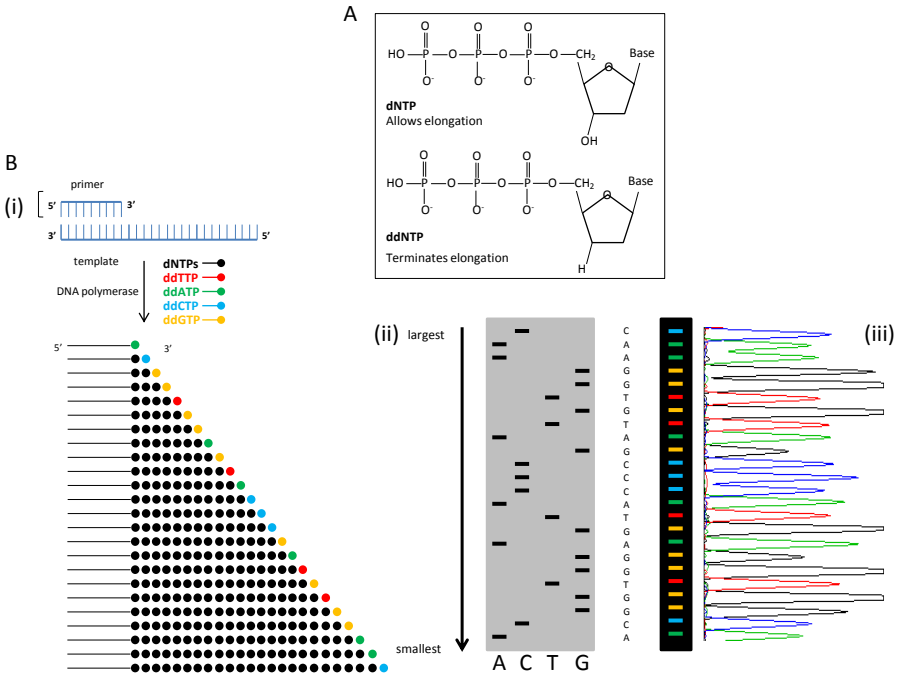


Figure 2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Allows elongation
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Terminates elongation
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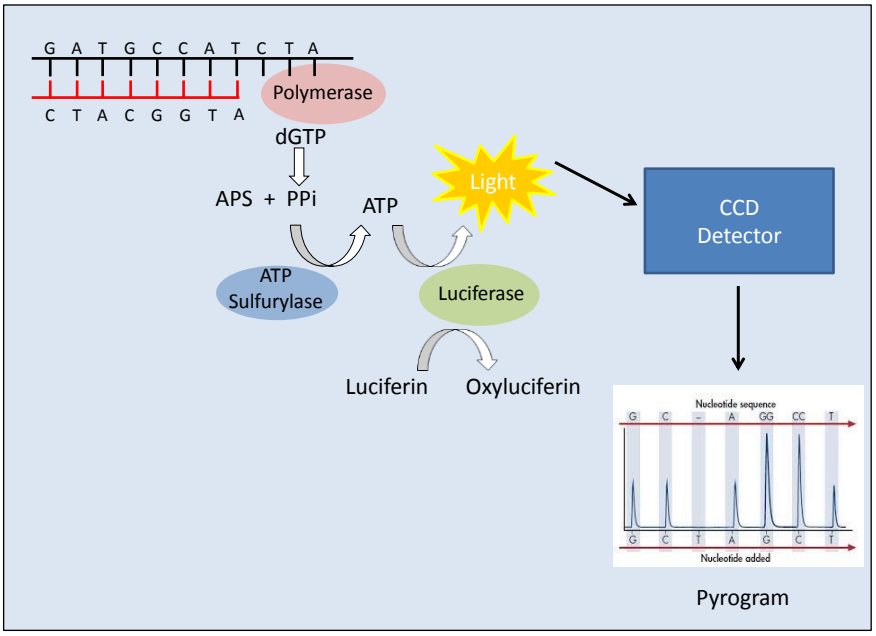
Figure 3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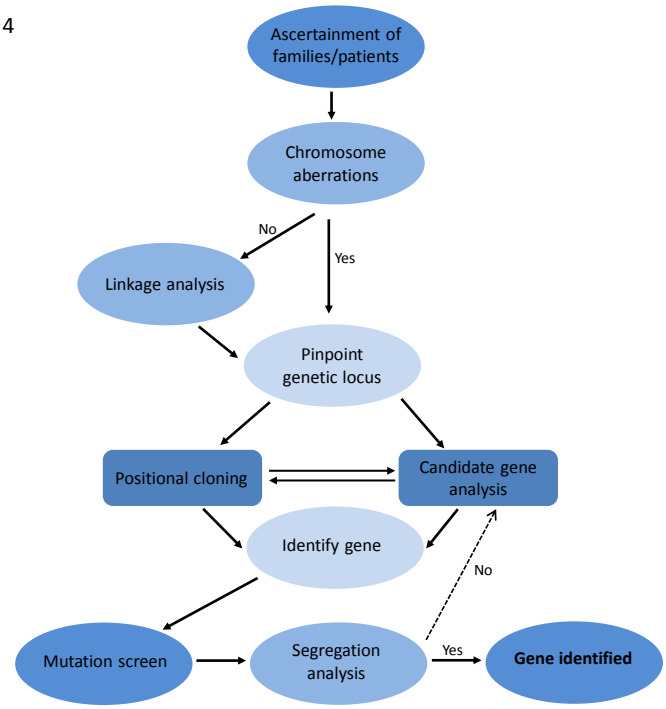
Nucleotide added
Pyrogram

Figure 4


Ascertainment of families/patients
Chromosome aberrations
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Yes
Linkage analysis
Pinpoint genetic locus
Positional cloning
Candidate gene analysis
Identify gene
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Mutation screen
Segregation analysis
Yes
Gene identified