Systematic mutation analysis and functional characterization of candidate genes for primary open angle glaucoma
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There is one thing that gives radiance to everything. It is the idea of finding something around the corner.
- Gilbert Keith Chesterton

When you’ve got it, you’ve got it. When you haven’t, you begin again. All the rest is humbug.
- Edouard Manet
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1. Summary

**Background and objectives.** Glaucoma is a clinically and genetically heterogeneous group of ophthalmologic disorders leading to visual impairment and a major cause of blindness worldwide. The most common form is primary open angle glaucoma (POAG), which is inherited as a complex trait. Therefore, multiple genetic and environmental susceptibility factors play a role in the disease, each with a small contribution to its aetiology. Several loci have been linked to POAG, but only three genes (myocilin, WDR36 und optineurin) have been identified until now, accounting for about 5% of the cases. The aim of this thesis was to identify new glaucoma predisposing genes through systematic mutation screening of functional candidate genes located on chromosomal regions previously linked to POAG. Functional characterization of mutations found in RPGRIP1 was also performed.

**Methods.** The initial study population used in the mutation screening consisted of 399 unrelated German patients with POAG and 376 control subjects without any signs of glaucoma upon ophthalmologic examination. For the replication study, additional 383 POAG patients and 104 controls were used. The functional studies comprised yeast two-hybrid assays, coimmunoprecipitation, expression of fluorescent proteins and RT-PCR.

**Results.** Association of RPGRIP1 variants with POAG was found in both cohorts of German patients. Most of these mutations were located in or near the C2 domains of the protein. Yeast two-hybrid experiments demonstrated that some of these amino acid alterations located in the C2 motif of RPGRIP1 impaired the interaction between this protein and nephrocystin-4. Coimmunoprecipitation and colocalization studies of both proteins corroborated also these results. RT-PCR led to the discovery of three novel RPGRIP1 isoforms. These isoforms were detected in cDNAs from whole blood, retina and sclera from a healthy donor, but not in choroid or cornea.

**Conclusions.** Replicated association of heterozygous RPGRIP1 variants with POAG in German patients was found. Amino acid variants located in the C2 domain of RPGRIP1 were functionally validated and characterised as *bona fide* mutations. Thus, carrying mutations in RPGRIP1 increase the susceptibility of one individual to develop glaucoma. However, the complete molecular pathway and the role of the protein in the pathological mechanisms leading to glaucoma still need to be clarified. Additional functional studies should be also
performed in order to validate and characterize the newly identified transcripts and its possible relevance in the aetiology of POAG.
2. Zusammenfassung

**Hintergrund und Ziele.** Das Glaukom ist eine klinisch und genetisch heterogene Augenerkrankung, die zur Beeinträchtigung des Sehvermögens führt und weltweit eine Hauptursache der Erblindung darstellt. Die häufigste Form ist das Primäre Offenwinkelglaukom (POWG), welches als komplexes Merkmal vererbt wird. In der Krankheitsentstehung spielen eine Reihe von genetischen und Umweltfaktoren eine Rolle, wobei jeder dieser Faktoren zur Ätiologie beiträgt. Einige Genloci sind mit POWG gekoppelt, bis jetzt wurden jedoch nur in drei Genen (Myocilin, WDR36 und Optineurin) Sequenzvarianten identifiziert, die etwa 5% der Fälle erklären. Ziel dieser Arbeit war es, durch systematisches Mutationsscreening funktioneller Kandidatengene in mit POWG gekoppelten chromosomalen Regionen neue Glaukomgene zu identifizieren. Des Weiteren wurden die in *RPGRIP1* gefundenen Mutationen funktionell charakterisiert.


**Schlussfolgerungen.** In deutschen Patienten wurde eine Assoziation von heterozygoten *RPGRIP1*-Varianten mit POWG gefunden und repliziert. Aminosäureaustausche innerhalb der C2-Domäne von RPGRIP1 wurden als *bona fide*-Mutationen funktionell validiert und charakterisiert. Folglich erhöht sich dadurch für Mutationsträger die Suszeptibilität für die
Entwicklung eines Glaukoms. Der komplette molekulare Mechanismus sowie die Rolle des Proteins in der Pathologie des Glaukoms müssen noch geklärt werden. Außerdem sollten zusätzliche funktionelle Studien durchgeführt werden, um die neu identifizierten Transkripte sowie ihre mögliche Relevanz in der Ätiologie des POWG zu bestätigen und zu charakterisieren.
3. Introduction

Genetics has become a central strand in medical research. Since the completion in April 2003 of the Human Genome Project (www.ornl.gov) and public availability of the entire human genome sequence, gene discovery dramatically speeded up and the haploid human genome has been estimated to contain three billion nucleotides and close to 23,000 genes (Pennisi 2003), far fewer than had been expected before. The focus is now centred on the identification of disease genes. Approximately 4,000 Mendelian disease phenotypes are currently known in man, but for no more than 2,500 of these the fundamental molecular defect has been identified at the DNA level (Hamosh et al. 2005). Different methods are currently used to establish links between genetic disorders and specific genes; however, identifying the factors conferring susceptibility to common complex diseases, such Primary Open Angle Glaucoma (POAG), remains exceedingly difficult. Among the promised benefits of human genetics research are better understanding of disease, personalised preventive medicine, gene therapy, and pharmacogenetic drug therapy tailored to our individual genetic profiles.

3.1. Genetics of complex diseases

3.1.1. Monogenic versus complex diseases

Most genetic disorders discovered to date are monogenic and follow a simple Mendelian inheritance pattern. In a monogenic disease the phenotype is caused by an abnormality in one single gene, which may contain a point mutation or an insertion/deletion that changes the coding sequence or promoter, and hence the amino acid sequence of the protein, thereby triggering the disease. Although environmental factors, age of onset and/or allelic heterogeneity (several different alleles of the same gene, giving rise to the same disease phenotype) can complicate the picture, monogenic disorders usually have a high correlation between genotype and phenotype i.e. there is a high penetrance. The penetrance is a measure of the probability that a person carrying a specific genotype (variant allele) also expresses the disease (displays the phenotype) (Smith and Lusis 2002).

By contrast, in a complex disease susceptibility is controlled by multiple genetic and environmental risk factors, and potentially by interactions in and between them, where each of these risk factors has only a modest effect on susceptibility (Cardon and Abecasis 2003). Complicating the picture is the fact that the allelic variants predisposing for a disease are often common variants found in a large part of the population. These people may live their full life span without being affected by the disease, despite carrying the susceptibility allele.
The reduced or incomplete penetrance in these individuals is influenced by age of onset, sex, environmental factors and other genetic variants referred to as genetic background (Lander and Schork 1994).

### 3.1.2. Methods for genetic dissection of complex diseases

Among the factors that contribute to the difficult challenge of discovering complex disease genes are the low heritability of most complex traits, the presence of incomplete penetrance, phenocopies, underlying molecular heterogeneity and epistasis (Weeks and Lathrop 1995), imprecise definition of phenotypes (Levy et al. 2000), inadequately powered study designs (Blangero 2004), and the inability of standard sets of markers (single nucleotide polymorphisms (SNPs), copy number variations (CNVs), or microsatellites) to extract complete information about inheritance (Wiggs 2007). The full array of genetic approaches should be used, including linkage analysis, association studies, and candidate gene analysis.

#### 3.1.2.1. Linkage analysis

Linkage analysis is based on the co-inheritance of genetic markers and phenotypes in families over several generations, and identifies haplotypes that are inherited intact over them (Laird and Lange 2006). A haplotype is a combination of alleles found at neighbouring loci on a single (haploid) chromosome, sufficiently close together such that their alleles tend to cosegregate within families (Borecki and Province 2008). In order to estimate the evidence for linkage, the LOD (logarithm (base 10) of an odds ratio) score, a statistical test developed by Newton E. Morton in 1955, is used. The odds ratio (OR) is the probability of observing the specific genotypes in a family given linkage at a particular recombination fraction versus the same probability computed conditional on independent assortment (Borecki and Province 2008). A LOD score of 3 is usually taken as statistically significant evidence for linkage, meaning that the linkage hypothesis is $10^3$ times more likely than the hypothesis that the two loci are not linked; values close to 1 favor independent assortment.

This approach has good power for detecting uncommon genes with major effect, but its power to detect the modest effects of common genetic variants on disease is more limited due to the lack of clear genetic segregation of some DNA variants in multigenerational family material, and by the modest contribution to disease made by individual genetic variants (Cardon and Bell 2001).

#### 3.1.2.2. Association studies

Association studies are based on the retention of adjacent DNA variants over many generations in specific populations. Thus, they can be regarded as very large linkage studies
of unobserved, hypothetical pedigrees (Cardon and Bell 2001). In association studies, allele frequencies are compared to assess the contribution of genetic variants to phenotypes either with a case-control design (allele frequencies between affected and healthy unrelated controls are compared), or using a family-based approach (transmitted versus untransmitted parental alleles) (Laird and Lange 2006).

Association studies are easier to conduct than linkage analysis, because no multicycle families or special family structure are needed, and they are also more powerful for detecting weak susceptibility alleles (Strachan and Read 2004).

3.1.2.3. Candidate-gene approaches

Candidate genes are selected for further study either by their location within a previously determined region of linkage, or on the basis of other biologic hypotheses, like appropriate expression pattern, appropriate function, or homology to other disease genes (Strachan and Read 2004).

The most comprehensive analysis of candidate genes is obtained by resequencing the entire gene in patients and controls, and searching for variants that are enriched or depleted in disease genes. However, because such studies are laborious and expensive, they are usually limited to the coding regions of the candidate genes (Tabor et al. 2002).

3.2. Glaucoma, general aspects

The glaucomas are the principal cause for optic nerve degeneration and the second cause of irreversible blindness worldwide, after cataract (Resnikoff et al. 2004; Quigley and Broman 2006). This medical condition refers to a heterogeneous group of disorders characterized by degeneration of the optic nerve, specific loss of visual field, and chronic painless progression, usually (but not invariably) associated with an elevated intraocular pressure (IOP) (Shields, et al., 1996).

There is some controversy relating to the true derivation of the word “glaucoma”. It goes back to the Ancient Greeks in 400 B.C., meaning clouded or blue-green hue, but also owl. In the Hippocratic Aphorisms the term glaucoma (γλαύκωμα) was used to describe blindness coming on in advancing years associated with a glazed appearance of the pupil. There were no clear distinction between cataracts and glaucoma and it is highly likely that the only type of glaucoma recognised in ancient times was symptomatic acute glaucoma (Fronimopoulos and Lascaratos 1991). The first association of the disease with a rise in intraocular pressure occurs in the Arabian writings “Book of Hippocratic treatment”, of At-Tabari (10th century).
In European writings, it is Dr Richard Bannister (1622) who makes the first original and clear recognition of a disease with a tetrad of features: eye tension, long duration of the disease, absence of perception of light and presence of a fixed pupil. Dr Drance (1973) provided for the first time the definition of glaucoma as a disease of the optic nerve (an optic neuropathy) caused by numerous factors, called risk factors (Grewe, 1986).

It is estimated that 4.5 million persons globally are blind due to glaucoma (World Health Organization data) and that this number will rise to 11.2 million by 2020 (Quigley and Broman 2006). It is noteworthy that due to the silent progression of the disease, at least in its early stages, up to 50% of affected persons in the developed countries are not even aware of having glaucoma. This number may rise to 90% in underdeveloped parts of the world (Sommer et al. 1991).

3.2.1. Diagnostics

Although the definition of glaucoma has not been consistent across studies, it is generally referred to as a progressive optic neuropathy involving characteristic excavation of the optic disc with corresponding loss of visual field (Foster et al. 2002). Since the optic nerve transmits visual images to the brain, damage to parts of it correspondingly reduces vision. To estimate the damage of the optic nerve, the diameter of the eye's cup is compared to that of its disc to obtain a physical gauge of the likelihood of glaucoma. Estimates are made vertically along an imaginary line drawn through the center of the disc from the 12 o'clock to the 6 o'clock position. The normal optic nerve has a cup-to-disc ratio of less than 0.5, indicating a low probability of glaucoma. Moderately advanced cupping, with a cup-to-disc ratio of 0.6 to 0.8 and a neural rim starting to thin, increases the suspicion of glaucoma. Almost total cup-to-disc ratio of 0.9, exhibiting a very thin neural rim, creates a high level of glaucoma suspicion (Figure 3.1.). Scanning laser polarimetry, optical coherence tomography, or confocal scanning laser ophthalmoscopy are some of the methods used for monitoring glaucoma by imaging of the eye's optic nerve and internal structures.
Figure 3.1. **Ophthalmoscopy of the optic nerve head.** Numbers represent the cup-to-disc ratio. A ratio of 0.3 indicates no glaucoma; 0.9 confirms glaucoma. From South Texas Retina Consultants (www.strc.cc).

### 3.2.2. Classification, prevalence and risk factors

Glaucoma is subdivided depending on the presence of primary and secondary characteristics. Primary characteristics include the status of the iridocorneal angle and the age of onset. Secondary characteristics include IOP, pseudoexfoliations and developmental abnormalities:

- **Primary Open Angle Glaucoma (POAG):** primary type of glaucoma with late age of onset, open iridocorneal angle and elevated IOP.
- **Normal Tension Glaucoma (NTG):** primary type of glaucoma with open iridocorneal angle and normal IOP. This type of glaucoma is usually sorted under POAG.
- **Juvenile Open Angle Glaucoma (JOAG):** primary type of glaucoma with age of onset between 33-40 years of age, open iridocorneal angle and elevated IOP.
- **Primary Close Angle Glaucoma (PCAG):** primary type of glaucoma with closed iridocorneal angle and elevated IOP. Acute form of glaucoma.
- **Congenital Glaucoma:** primary type of glaucoma with onset prior to 3 years of age, open iridocorneal angle and elevated IOP.
- **Secondary Glaucoma:** a conglomerate of forms with a secondary cause as pseudoexfoliations, developmental abnormalities or trauma.

Primary open angle glaucoma (POAG) is the major primary type of glaucoma in most populations worldwide, while Asian populations have a high frequency of closed angle glaucoma (PCAG) (Foster and Johnson 2001; Lai et al. 2001). The prevalence of POAG varies between ethnic populations. POAG is five times more common in African Americans than in Caucasians (Tielsch et al. 1991). The prevalence in populations in predominantly black Barbados (12.8%) and St. Lucia (8.8%) is much higher than that of most other populations (Mason et al. 1989; Leske et al. 1994). In addition, glaucoma is six times more...
likely to cause blindness in blacks than in whites. Blacks have thinner corneas than whites (by about 23 microns) and this may well be the factor that puts blacks at a higher risk for glaucoma progression (www.nei.nih.gov).

In many cases, POAG is accompanied by an elevation of intraocular pressure (IOP), but whether this should be considered a diagnostic criterion or a risk factor is under debate. Most of the times the elevation of IOP results from impaired drainage of aqueous humour. The aqueous humour is produced by the ciliary body in the posterior chamber of the eye and enters the anterior chamber through the pupil, then drains out through the trabecular meshwork into Schlemm’s canal, which drains into the bloodstream (Figure 3.2.). If the aqueous humour cannot drain properly, either because the drainage canals become clogged (as in chronic glaucoma) or because the iris is pushing against the cornea (as in angle-closure glaucoma), it backs up, exerting pressure on the gel in the vitreous cavity at the center of the eye. Eventually the building pressure affects the optic nerve at the rear. During routine eye exams, a tonometer is used to measure IOP, and a value over 24 mmHg can indicate glaucoma level, but not always, as these measures are not absolute and some individuals tolerate higher pressures than others.

![Aqueous humour production and outflow](image)

**Figure 3.2. Aqueous humour production and outflow.** Image modified from National Eye Institute, National Institutes of Health (www.nei.nih.gov).

The risk for POAG increases rapidly after age 40. People aged 70 and older are about four to seven times more likely to develop glaucoma than people 40 to 50 years old (Coleman 1999). A family history of the disease has long been recognized as a major risk factor for developing glaucoma (Wolfs et al. 1998) (see also 3.3. Genetics of POAG).
The relation between primary open-angle glaucoma and gender is not clear. In the Baltimore Eye Survey (Tielsch et al. 1991), the Beaver Dam Eye Study (Klein et al. 1992), and the Blue Mountains Eye Study (Mitchell et al. 1996), no significant difference was found between prevalence of POAG in men and women. However, in the Framingham Eye Study (Kahn et al. 1977), the Barbados Eye Study (Leske et al. 1994), and the Rotterdam Study (Wolfs et al. 2000), up to a twofold higher prevalence was found in men.

Other reported risk factor for glaucoma include hypertension (Bonomi et al. 2000), corneal thickness (www.nei.nih.gov), high myopia (Mitchell et al. 1999), diabetes (Ellis et al. 2000) and cigarette smoking (Brandt 2008).

### 3.2.3. Pathogenesis

Many theories have surfaced regarding the exact mechanisms behind glaucomatous damage, but the complex nature of the disease and the inaccessibility of the internal structures of the human eye have limited current knowledge. The primary pathologic event in the disease, the apoptotic death of retinal ganglion cells (RGC), is thought to be initiated by damage to their axonal fibers at the optic-nerve head. Ischemia, excitotoxicity, autoimmunity, axonal injury and glial activation are some of the insults that may contribute to retinal ganglion cell death (Libby et al. 2005), as represented in Figure 1.3. The relative importance of specific damaging processes may differ between patients.

![Diagram of diverse factors contributing to apoptotic retinal ganglion cell (RGC) death in glaucoma](image)

Figure 1.3. **Diverse factors contributing to the apoptotic retinal ganglion cell (RGC) death in glaucoma.** *(From Libby et al. 2005).*
The ganglion cells die as a result of apoptosis (Dreyer et al. 1996) that can be caused by mechanical compression, nutritional insufficiency, or toxicity of intrinsic molecules affecting neurons or blood vessels (Tielsch et al. 1994). The mechanical theory suggests that physical alterations in the optic nerve head lead to the obstruction of the axonal fibres (Gupta 2004). This theory tries to correlate the characteristic pattern of glaucomatous optic nerve damage with the anatomy of the lamina cribosa and the fact that the regions with the greatest damage corresponds to those of the lamina cribosa that have the thinnest laminar beams.

Several lines of evidence suggest that chronic oxidative stress is important in glaucoma pathogenesis, as reflected in some of its features: age-dependent clinical onset, constant exposure of the trabecular meshwork to H₂O₂ in the aqueous humor, and altered cellular and molecular responses to H₂O₂ exposure in vitro (Green 1995). The trabecular meshwork (TM) is the main target tissue of glaucoma in the anterior chamber. The development and progression of glaucoma is accompanied by cell loss and functional impairments in this tissue (Tian et al. 2000). In addition, the effect of reactive oxygen species (ROS) on the adhesion of TM cells to extracellular matrix proteins results in a rearrangement of cytoskeletal structures that may lead to TM disruption (Zhou et al. 1999). Oxidative stress can also influence biological reactions of TM cells (Tamm et al. 1996) and may contribute to the changes observed in ageing and in primary open-angle glaucoma such as trabecular thickening and trabecular fusion (Hogg et al. 2000). Results of more recent investigations provide also convincing evidence that reactive oxygen species play a key role in the pathogenesis of POAG (Izzotti et al. 2009).

### 3.2.4. Treatment

Glaucoma causes irreversible blindness due to death of retinal ganglion cells that can only be prevented by therapeutic intervention in the early stages of the disease. Since peripheral visual damage occurs first, and because the disease is typically pain free with no obvious symptoms, substantial visual damage can occur before diagnosis. Treatment for glaucoma is effective and in the vast majority of cases useful sight can be retained for life if the treatments are used properly and the agreed management regime followed.

#### 3.2.4.1. Classical treatments

The mainstay of glaucoma treatment is to lower the eye pressure, either with eye drops or surgery. Eye drops usually form the first stage of treatment for glaucoma, and can work in different ways: most commonly the drops act to reduce the amount of aqueous humour being produced by the ciliary body (beta-blockers, alpha-adrenergic agonists, carbonic anhydrase
inhibitors), some increase the outflow of aqueous humour from the eye (prostaglandins, parasympathomimetics, hyperosmotic agents), and some do some of each (epinephrine).

There are also a variety of types of surgery for glaucoma (trabeculectomy, aqueous shunt procedure) depending on the individual needs of the patient. Lasers are also used to treat both open and closed angle glaucomas with different lasers and different techniques (iridectomy, cyclophotocoagulation, scatter panretinal photocoagulation, trabecuoplasty, peripheral iridotomy) used according to need (Detry-Morel et al. 2008).

3.2.4.2. Investigational Glaucoma Treatments

While some experimental glaucoma medications explore new ways of controlling IOP, other treatments are directed at protecting the optic nerve (neuroprotection) to prevent eye damage, potential vision loss or even blindness.

Many ongoing clinical studies are trying to find neuroprotective agents that might benefit the optic nerve and certain retinal cells in glaucoma (Levin and Peeples 2008). In all optic neuropathies including glaucoma, the initial site of injury is the axons of retinal ganglion cells. Axonal injury triggers apoptotic mechanisms that ultimately culminate in retinal ganglion cell death. Neuroprotective approaches are varied: 1) the prevention of apoptosis by inhibiting TNF-α and caspase activity (Tezel 2008); 2) blocking excessive Ca²⁺ overload due to overactivation of NMDA receptors (Dong et al. 2008); or 3) blocking nitric oxide toxicity (Stefan et al. 2007). Many of the neuroprotective agents were developed from the results of work done on other central nervous system diseases such as Parkinson's and multiple sclerosis. Examples of neuroprotective agents under investigation for treatment of glaucoma include Namenda (memantine), Copaxone (glatiramer acetate) (Cheung et al. 2008), and *Gingko biloba* (Quaranta et al. 2003).

Other investigational treatments for glaucoma, aimed at controlling high IOP, include Retane (anecortave acetate), and nanoparticles (Zimmer et al. 1994).

Some people with glaucoma use marijuana because research conducted in the 1970s found that it had a small, short-term effect in lowering intraocular pressure. However, no research has found that marijuana is anywhere near as effective as legal glaucoma medications (Tomida et al. 2006; Kogan and Mechoulam 2007).

3.2.5. Animal models

Animal models with either induced or spontaneous diseases often permit extensive and invasive investigations not usually possible in human patients. A variety of animal models to understand the mechanisms of formation and evacuation of the aqueous humor as well as
homeostasis maintenance of intra-ocular pressure has been proposed in different animal species like rabbits (Kolker et al. 1963), dogs (Gelatt et al. 1977), monkeys (Dawson et al. 1993), rats (Shareef et al. 1995) and pigs (Ruiz-Ederra et al. 2005). The appearance of the DBA/2J mouse, which develops a progressive increase of IOP that induces the death of ganglionary cells (John et al. 1998), has given rise to a large amount of studies to establish the existence of homologies with some type of glaucoma in humans. The increase in IOP in these animals appears at 8 months of age and remains chronically high until their death. However, some factors such as the reduced size of the ocular globe and the absence of lamina cribosa in mice and rabbits, together with the diversity of structures and differences in function of drainage angles specific to each animal species limit the use of these animal models for some type of studies. Subsequently, the use of animal models in the glaucoma research until now has not been highly fruitful.

3.3. Genetics of POAG

Several lines of evidence support the fact that POAG may have a genetic basis. Family history has been revealed to be one of the most important risk factors for POAG development (Tielsch et al. 1994). The Rotterdam Eye Study investigated the familial aggregation of POAG and found a tenfold increased relative risk of the disease in first degree relatives of affected compared with the general population (Wolfs et al. 1998). In the Barbados population family study (including persons of African ancestry), 10% of living relatives examined had open angle glaucoma (Nemesure et al. 2001). Racial differences in prevalence of POAG exist, further supporting a genetic predisposition for glaucoma. The prevalence in Africans is estimated to be six times higher than in Caucasians in certain age groups (Racette et al. 2003). Further evidence for a genetic basis of POAG stems from twins studies. It has been reported that POAG was found to be significantly more concordant in monozygotic twin pairs (98.0%) than their spouses (70.2%) (Gottfredsdottir et al. 1999). The genetic basis of POAG is also supported by the fact that some non-human animal species also develop heritable forms of POAG. Inherited spontaneous POAG has been identified in rhesus monkeys (Macaca mulatta) and both autosomal dominant and recessive POAG is present in dog breeds (in particular the beagle and miniature poodle) (Gelatt and MacKay 1998).
3.3.1. Inheritance and implicated loci

Primary open angle glaucoma is not a mendelian disease caused by a single susceptibility allele, but a trait with a more complex mode of inheritance. Some families with glaucoma appear to present autosomal dominant inheritance. Also, many of the individual signs of POAG are heritable, including cup-to-disk ratio, IOP, aqueous outflow facility, and the steroid response (Alward et al. 1996), but no single Mendelian mode of inheritance can adequately describe POAG as a whole. Consequently, it has been proposed that POAG has a complex or multifactorial aetiology (Newell 1986). In such a model, the interaction of several genes and environmental factors contribute to the pathology of glaucoma, and therefore a single underlying susceptibility gene cannot be assumed even in a single pedigree. Alternatively, POAG may represent a collection of clinically indistinguishable simple Mendelian disorders. Within a population, the genetic characteristics of one form of Mendelian POAG would be obscured by the presence of others (Johnson et al. 1996).

Since the first description of a heritable form of POAG by Benedict in 1842, more than 10 glaucoma loci have been identified through linkage analysis, although the disease-causing gene is only known for three of these loci. These three known glaucoma genes are myocilin (MYOC), optineurin (OPTN) and WD repeat domain 36 (WDR36). Of these, only MYOC is established as directly glaucoma causative, while the roles of OPTN and WDR36 are still unclear due to conflicting evidence. Genomewide scans using families (mainly sibpairs) demonstrating clustering of the disease have led to the identification of a larger number of genetic intervals containing many possible candidate genes (Wiggs et al. 2000; Nemesure et al. 2003; Wiggs et al. 2004). Taken together, these two strategies have revealed at least 20 POAG loci (Table 3.1.). Among them, 14 loci have been designated GLC1A to GLC1N by the HUGO Genome Nomenclature Committee (www.gene.ucl.ac.uk/nomenclature).
Table 3.1. **POAG susceptibility loci identified.**

### 3.3.2. Known glaucoma genes

Until now, only three genes (myocilin, WDR36 and optineurin) have been classified as POAG-causing genes.

#### 3.3.2.1. Myocilin (MYOC)

Myocilin (formerly referred to as the trabecular meshwork-induced glucocorticoid response protein or TIGR) was the first POAG gene to be identified (Stone et al. 1997). It mapped to chromosomal region 1q, where the locus for the juvenile form of POAG had previously been identified (GLC1A) (Sheffield et al. 1993). MYOC mutations are found in 1.1-4% of late onset POAG patients (Allingham et al. 1998; Lam et al. 2000; Pang et al. 2000; Mataftsi et al. 2001; Michels-Rautenstrauss et al. 2002; Bruttni et al. 2003; Kanagavalli et al. 2003; Melki et al. 2004).
et al. 2003; Aldred et al. 2004; Sripriya et al. 2004; Weisschuh et al. 2005; Rose et al. 2007), and in JOAG patients, MYOC mutations frequencies range from 6% to 36% in different populations (Wiggs et al. 1998; Shimizu et al. 2000; Alward et al. 2002). To date more than 70 disease-associated mutations in MYOC have been identified (Human Gene Mutation Database), with the p.Q368X mutation being the most common known individual glaucoma causing variant worldwide (Fingert et al. 1999), and a founder effect has been revealed for this frequent mutation (Faucher et al. 2002).

Myocilin is a secreted 55-57 kDa glycoprotein that forms dimers and multimers. The protein has an amino terminal signal sequence, a myosin like domain, a leucine zipper domain, and an olfactomedin domain. Most of the known mutations occur in the olfactomedin domain, which is highly conserved among species (Tamm 2002).

Myocilin protein is expressed in high amounts in the trabecular meshwork, sclera, ciliary body, and iris, and at considerable lower levels in retina and optic nerve head (Tamm 2002). Although myocilin is found ubiquitously in the eye, it is also expressed in many extraocular tissues, suggesting that it may not have an eye-specific function (Karali et al. 2000; Fingert et al. 2002).

The function or functions of myocilin in the eye remain unknown. It has been postulated that MYOC facilitates aqueous humour outflow, or that it has a protective role against stress (Johnson 2000). However, early truncations and deletions are not pathogenic in humans (Lam et al. 2000; Wiggs and Vollrath 2001) and mice with null alleles do not develop high IOP or glaucoma (Kim et al. 2001). These two observations suggest that MYOC is not necessary for normal IOP homeostasis, and that mutations in the gene do not cause the disease by a loss of function effect. Different groups have shown in vitro that mutant MYOC forms insoluble aggregates that are not secreted and accumulate in the intracellular space (Zhou and Vollrath 1999; Caballero et al. 2000; Jacobson et al. 2001; Joe et al. 2003; Fan et al. 2004; Gobeil et al. 2004). Such an accumulation might interfere with TM function and lead to impaired outflow. In the TM myocilin has been shown to principally interact with optimedlin, an olfactomedin-related protein (Torrado et al. 2002), as well as binding with flotillin-1, a lipid raft protein (Joe et al. 2005).

3.3.2.2. Optineurin (OPTN)

Optineurin was originally identified in a single large British pedigree with autosomal dominant NTG and screened in 54 additional NTG families. Three sequence variants were considered disease causing, accounting for 16.7% of the cases, with the p.E50K mutation being the most common one. Another change, p.M98K, was significantly more frequent in
patients than in controls, and was suggested to confer increased susceptibility to glaucoma (Rezaie et al. 2002). However, a later study including 1048 POAG patients implicated only one of these mutations with POAG and in only one patient (Alward et al. 2003). Several other large studies found similar mutation distributions in patients and controls (Aung et al. 2003; Leung et al. 2003; Wiggs et al. 2003; Baird et al. 2004; Toda et al. 2004; Willoughby et al. 2004; Mukhopadhyay et al. 2005; Weisschuh et al. 2005; Ariani et al. 2006; Ayala-Lugo et al. 2007). Similarly to the original work, only two studies have found significant association between p.M98K and POAG (Willoughby et al. 2004; Sripriya et al. 2006), although several studies did see an increased frequency within their patient populations (Alward et al. 2003; Baird et al. 2004; Mukhopadhyay et al. 2005; Ayala-Lugo et al. 2007). It has been proposed that p.M98K may be associated with a lower IOP at the time of diagnosis, and may even modify MYOC glaucoma (Melki et al. 2003). The clinical importance of the other OPTN variants remains controversial and when all studies are considered, OPTN mutations do not appear to be a common cause of POAG.

Optineurin is a 577 amino acid protein that appears to be secreted. The protein has a bZIP motif and alternative splicing at the 5’-UTR generates at least three different isoforms, but all have the same reading frame (Rezaie et al. 2002). It is localized throughout the eye, including the TM, Schlemm’s canal, ciliary epithelium, retina, and optic nerve (Rezaie et al. 2002; Sarfarazi and Rezaie 2003). The endogenous protein is located intracellularly to the Golgi apparatus and was detected in samples of aqueous humor from human and several other species.

Optineurin appears to interact with proteins that regulate apoptosis and may be a component of the tumour necrosis factor-α (TNF-α) signalling pathway (Chen et al. 1998). In addition, the protein may interact with huntingtin, Ras-associated protein RAB8, transcription factor IIIA and two unknown kinases (del Toro et al. 2009). Although few studies have directly tested the function of OPTN, its expression in neuronal and glial cells of the retina and optic nerve indicates that it could directly affect retinal ganglion cell survival, playing a neuroprotective role in the eye and optic nerve (Rezaie et al. 2002; Sarfarazi and Rezaie 2003).

3.3.2.3. WD40-Repeat 36 (WDR36)

The third glaucoma gene, WDR36, was identified at the GLCIG locus on 5q22.1 and sequenced in 130 unrelated POAG patients. Four sequence variants were classified as disease-causing mutations (Monemi et al. 2005). However, subsequent replication studies in larger cohorts have failed to confirm a major role of WDR36 as a glaucoma-causing gene. A large
family linked to GLC1G did not present any mutations in WDR36 (Kramer et al. 2006). The authors mentioned that the family could possibly carry a mutation in the promoter, or alternatively, that another gene mapping to GLC1G causes glaucoma in this family. A two-stage study with over 400 POAG patients and over 400 age-matched controls failed to confirm the original findings (Fingert et al. 2007). The most frequent disease-associated variant in the original study, p.D658G, was found in similar frequencies in patients and controls. Two other variants were found in patients and not in controls, but the authors pointed out that this finding is not statistically significant. In addition, WDR36 has been reported to play a minor role in German (Weisschuh et al. 2007; Pasutto et al. 2008), Japanese (Miyazawa et al. 2007), and US American (Hauser et al. 2006) glaucoma patients.

The WDR36 protein has 951 amino acids, and contains at least four predicted structural motifs, with multiple G-beta WD40 repeats. In the eye, WDR36 is expressed in the lens, iris, sclera, ciliary muscles, ciliary body, TM, retina and optic nerve (Monemi et al. 2005). However, the exact physiological function of the protein remains unclear and extensive functional studies are needed to clarify the role of WDR36 variants in the glaucoma pathogenesis.

3.3.3. Glaucoma candidate genes

Sequence variants in at least 17 genes have been associated with POAG (Table 3.2), but most of these genes have been reported in one single study, and for those investigated in several studies, there is controversy as to whether they really show association or not to POAG. Therefore, the role of these genes in the aetiology of POAG has not yet been clearly established.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Chromosomal Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACP1</td>
<td>Acid phosphatase-1</td>
<td>2p25</td>
<td>(Abecia et al. 1996)</td>
</tr>
<tr>
<td>AGTR2</td>
<td>Angiotensin II receptor, type 2</td>
<td>Xq22-q23</td>
<td>(Hashizume et al. 2005)</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
<td>19q13.2</td>
<td>(Copin et al. 2002)</td>
</tr>
<tr>
<td>CDH1</td>
<td>E-cadherin</td>
<td>16q22.1</td>
<td>(Lin et al. 2006)</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1A</td>
<td>6p21.2</td>
<td>(Tsai et al. 2004)</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>Cytochrome P450, subfamily 1, polypeptide 1</td>
<td>2p22-p21</td>
<td>(Vincent et al. 2002)</td>
</tr>
<tr>
<td>EDNRA</td>
<td>Endothelin receptor, type A</td>
<td>4q31.2</td>
<td>(Ishikawa et al. 2005)</td>
</tr>
<tr>
<td>GSTM1</td>
<td>Glutathione S-transferase, mu-1</td>
<td>1p13.3</td>
<td>(Juronen et al. 2000)</td>
</tr>
<tr>
<td>IGF2</td>
<td>Insulin-like growth factor II</td>
<td>11p15.5</td>
<td>(Tsai et al. 2003)</td>
</tr>
<tr>
<td>IL1A</td>
<td>Interleukin 1-alpha</td>
<td>2q14</td>
<td>(Wang et al. 2006)</td>
</tr>
<tr>
<td>IL1B</td>
<td>Interleukin 1-beta</td>
<td>2q14</td>
<td>(Lin et al. 2003)</td>
</tr>
<tr>
<td>MTHFR</td>
<td>5,10-methylenetetrahydrofolate reductase</td>
<td>1p36.3</td>
<td>(Junemann et al. 2005)</td>
</tr>
<tr>
<td>NOS3</td>
<td>Nitric oxide synthase 3</td>
<td>7q36</td>
<td>(Tunny et al. 1998)</td>
</tr>
<tr>
<td>NPPA</td>
<td>Natriuretic peptide precursor A</td>
<td>1p36.2</td>
<td>(Tunny et al. 1996)</td>
</tr>
<tr>
<td>OCLM</td>
<td>Oculomedin1</td>
<td>1q31.1</td>
<td>(Fujiwara et al. 2003)</td>
</tr>
<tr>
<td>OPA1</td>
<td>Optic atrophy 1</td>
<td>3q28-q29</td>
<td>(Aung et al. 2002)</td>
</tr>
<tr>
<td>TAP1/2</td>
<td>Transporter, ATP-binding cassette</td>
<td>6p21.3</td>
<td>(Lin et al. 2004)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
<td>6p21.3</td>
<td>(Lin et al. 2003)</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor protein 53</td>
<td>17p13.1</td>
<td>(Lin et al. 2002)</td>
</tr>
</tbody>
</table>

Table 3.2. Genes harbouring variants with reported association with POAG.

3.4. Loci investigated

In 2000, the first genome linkage screen for POAG was completed, using an initial pedigree set of 113 affected sibpairs from 41 families (Wiggs et al. 2000). Twenty-five regions were identified, with seven regions producing a lod score ≥ 2.0 using either model-dependent (parametric) or –independent (no parametric) methods. When a second set of 69 affected
sibpairs was included in the analysis, five regions (chromosomes 2, 14, 17p, 17q, and 19) continued to produce lod scores > 2.0. Sibpair multipoint analysis also showed interesting results for the regions on chromosomes 2, 14, 17, and 19, as represented in Figure 1.4.

![Multipoint lod scores](image)

**Figure 1.4. Multipoint lod scores.** The graphs for the initial pedigree set (113 sibpairs) are shown as continuous lines. The graphs for the combined pedigree set (182 sibpairs) are shown as dashed lines (*modified from Wiggs et al. 2000*).

The Barbados family study, another genome-wide scan comprising 1327 individuals and 146 families, gave also some evidence for linkage to chromosomes 1, 2, 9, 11, and 14 (Nemesure et al. 2003).

In both studies, chromosome 14q11 was linked to POAG. Therefore, we focused this thesis on searching for candidate genes within this region. For this purpose, genetic and biological aspects of the following ten candidate genes were analyzed:

### 3.4.1. ADCY4 (adenylate cyclase type IV)

ADCY4 maps to 14q11.2 and encodes a membrane-associated enzyme member of the adenylate/guanylate cyclases family. The protein contains 1,077 residues and presents 2 guanylate cyclase domains (Ludwig and Seuwen 2002). Guanylate cyclases catalyze the
formation of cyclic GMP (cGMP) from GTP. cGMP acts as an intracellular messenger, activating cGMP-dependent kinases and regulating cGMP-sensitive ion channels. In addition to its well established role in phototransduction (Baylor 1987), cGMP is involved in many other physiological mechanisms in the retina. One important aspect of cGMP in the retina is its stimulating role in the absorption of subretinal fluid by activating the retinal pigment epithelium (RPE) pump (Marmor and Negi 1986). In the retina, cGMP-gated channels were found in photoreceptors, ganglion cells, bipolar cells and Müller cells (Nawy and Jahr 1991; Ahmad et al. 1994; Kusaka et al. 1996).

3.4.2. BCL2L2 (B-cell/lymphoma 2-like 2)

BCL2L2 maps to 14q11.2-q12 and encodes a 193-amino acid polypeptide member of the BCL-2 protein family. The proteins of this family form hetero- or homodimers and act as anti- or pro-apoptotic regulators (Chao and Korsmeyer 1998). Expression of BCL2L2 in cells has been shown to contribute to reduced cell death under cytotoxic conditions, by blocking dexamethasone-induced apoptosis (Gibson et al. 1996). The protein is expressed in a wide range of tissues, with highest levels in brain (O'Reilly et al. 2001).

3.4.3. DAD1 (defender against cell death 1)

DAD1 maps to 14q11.2-q12 and encodes a component of the oligosaccharyl transferase (OST) complex (Yulug et al. 1995). Members of this family are thought to be integral membrane proteins. DAD1 was initially identified as a negative regulator of programmed cell death in the temperature sensitive tsBN7 cell line (Nakashima et al. 1993). The DAD1 protein disappeared in temperature-sensitive cells following a shift to the nonpermissive temperature, suggesting that loss of the DAD1 protein triggered apoptosis. The protein is highly expressed in brain, and also present in retina (Wistow et al. 2002).

3.4.4. ISGF3G (interferon-stimulated transcription factor 3 gamma)

ISGF3G maps on chromosome 14, at 14q11.2 according to Entrez Gene. The 48-kDa protein encoded by this gene contains a ring finger, a motif present in a variety of functionally distinct proteins and known to be involved in protein-DNA and protein-protein interactions (Suhara et al. 1996). IFN-alpha stimulates transcription by converting ISGF3 from a latent to an active form. This conversion occurs in the cytoplasm, and only the activated factor is transported to the nucleus (Fu et al. 1990). In its latent state, ISGF3 appears to exist as two independent components, ISGF3-alpha, and ISGF3-gamma. ISGF3-alpha and ISGF3-gamma associate only following exposure of cells to IFN-alpha. ISGF3-gamma is a DNA-binding
protein that serves as the ISRE recognition component (Fu et al. 1992). Microarray and Northern blot results indicated that ISGF3G is preferentially expressed in young retina, but qRT-PCR analysis has suggested preferential expression in the elderly retina (Yoshida et al. 2002).

3.4.5. MMP14 (matrix metalloproteinase 14)
Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction (Ueda et al. 2002), and tissue remodelling, as well as in disease processes, such as arthritis and metastasis (Holmbeck et al. 1999). Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. However, the protein encoded by this gene is a member of the membrane-type MMP (MT-MMP) subfamily; each member of this subfamily contains a potential transmembrane domain suggesting that these proteins are expressed at the cell surface rather than secreted (Sato et al. 1994). This protein activates MMP2 protein, and this activity may be involved in tumor invasion (Nagase and Woessner 1999).

3.4.6. NRL (neural retina leucine zipper)
The protein NRL is a member of the v-maf family of transcription factors (Swaroop et al. 1992). The protein is 237 amino acid residues in size and is encoded by a gene with 3 exons (Farjo et al. 1997). It has been suggested that NRL forms a homodimer mediated by its leucine zipper motif and that this homodimer may form the structure recognized by CRX, another transcription factor (Mitton et al. 2000). Both of these DNA-binding proteins recognize sequences in the promoters of photoreceptor-specific genes such as those encoding rhodopsin and interphotoreceptor retinoid binding protein (Kumar et al. 1996; Chen et al. 1997). NRL is expressed in brain and retina, preferentially in rod photoreceptors (Liu et al. 1996; Swain et al. 2001). The protein regulates retinal development and/or differentiation by acting as a “molecular switch” that signals the cells to develop into rods rather than cones (Swaroop et al. 1992). Mutations in this gene have been associated with retinitis pigmentosa and retinal degenerative diseases (Bessant et al. 1999; Nishiguchi et al. 2004).

3.4.7. OXA1L (oxidase cytochrome c assembly 1-like)
The OXA1L gene (5 kb), mapping to the 14q11.2 region (Molina-Gomes et al. 1995), is composed of 10 exons and 9 introns and contains a 24 N-terminal amino-acid stretch (Rotig et al. 1997). The 42kDa OXA1L protein is required for the insertion of integral membrane
proteins into the mitochondrial inner membrane and is also essential for the activity and assembly of cytochrome-c oxidase, a complex of 13 proteins involved in cellular energy metabolism (Coenen et al. 2005; Stiburek et al. 2007). The gene is expressed in several tissues, including retina (Hillier et al. 1996).

3.4.8. SALL2 (salivary protein-like 2)
The SALL2 gene encodes a member of the spalt proteins family. In humans, mutations in some of these genes are associated with several congenital disorders (Sweetman and Munsterberg 2006). SALL2 has been proposed as a tumor suppressor gene as its mouse orthologue binds to the oncogenic polyoma large T antigen and has been shown to upregulate p21 expression (Li et al. 2001; Li et al. 2004). The protein present several zinc-finger domains, usually present in regulatory proteins, and other proteins that interact with DNA. The SALL2 gene is expressed in a subset of human tissues, with highest expression in brain, probably in distinct sets of neurons (Kohlhase et al. 1996).

3.4.9. ZNF219 (zinc finger protein 219)
The ZNF219 gene is a member of the Kruppel-like zinc finger gene family. The isolated cDNA contains an open reading frame of 2169 nucleotides encoding 723 amino acids (Sakai et al. 2000). The ZNF219 protein contains 9 zinc finger structures and may be related to the regulation of transcription and developmental regulation (Sakai et al. 2003). The gene is mainly expressed in brain, but also in lens, eye anterior segment, optic nerve, retina, retinal pigment epithelial (RPE) and choroid (Bonaldo et al. 1996).

3.4.10. RPGRIP1 (retinitis pigmentosa GTPase regulator-interacting protein 1)
The gene consists of 25 exons, encoding a 1259 amino acid protein with a predicted molecular weight of 144 kDa (Dryja et al. 2001). As represented in Figure 3.5., RPGRIP1 encodes three structurally different regions: two predicted coiled coil domains and two leucine zipper motifs at the N-terminal half of the protein, which possibly mediate homotypic and/or heterotypic interactions; two protein kinase C conserved region 2 motifs (C2) in the central domain of the protein, probably implicated in Ca^{2+}-dependent membrane docking of proteins and/or in mediating protein-protein interactions; and a bipartite nuclear localisation signal and an RPGR-interacting domain (RID) at the C-terminal region (Roepman et al. 2000).
Multiple splice variants of RPGRIP1 exist and have been found to distribute to subcellular specific sites (Lu and Ferreira 2005). RPGRIP1 localizes to the photoreceptor outer segments in humans and is also expressed in the inner retina, namely in the amacrine cells (Mavlyutov et al. 2002). RPGRIP1 is also found in centrosomes during the cell cycle and in basal bodies, which are equivalent to centroles in post-mitotic differentiated cells like photoreceptors (Shu et al. 2005). RPGRIP1 has been found to interact with RPGR (Boylan and Wright 2000), RanBP2 (Castagnet et al. 2003) and NPHP4 (Roepman et al. 2005). Mutations in RPGRIP1 most commonly cause Leber congenital amaurosis (Dryja et al. 2001), although a homozygous missense mutation in one family with late onset cone-rod dystrophy has also been reported (Dryja et al. 2001; Ferreira 2005). Recently, altered RPGRIP1 levels were identified in a mouse model for Fabry disease, in which patients have a vascular dysregulation (Arts et al. 2008).

3.5. The aims of this thesis

Primary open-angle glaucoma is a complex disease, influenced by multiple genetic and environmental factors. Several candidate genes have been investigated in this study, in order to elucidate their role in the aetiology of this complex trait, with special emphasis on the following aspects:

- Screening of MYOC and CYP1B1 in the patients and control collectives.
- Candidate genes selection and systematic mutational screening by direct sequencing.
- Systematic linkage disequilibrium analysis of RPGRIP1.
- Functional analysis of the variants found in RPGRIP1 through yeast two-hybrid-based assays and immuno technics.
4. Materials and methods

4.1. Subjects

The study protocols were approved by the ethics review board of the Medical Faculty of the University Hospital of Erlangen-Nuremberg, in accordance with the tenets of the Declaration of Helsinki, and prior to inclusion all the individuals were each informed about the intentions of these studies and gave their consent to be included.

4.1.1. Patients

4.1.1.1. Recruitment

The patients included in the initial study were recruited at the Ophthalmologic Department of the University Hospital of Erlangen-Nuremberg, Erlangen. The group of patients consisted of 399 subjects of German (Caucasian) origin: 270 with primary open-angle glaucoma (high-pressure POAG), 47 with juvenile open-angle glaucoma (JOAG), and 82 with normal-tension open-angle glaucoma (NTG).

The replication group comprised 383 unrelated patients: 304 with NTG and 79 presenting POAG. The patients of this group were clinically investigated at the University Eye Hospital in Würzburg and Tübingen following the same clinical procedure as in Erlangen.

4.1.1.2. Diagnosis

All individuals underwent standardized clinical examinations for glaucoma comprising slitlamp biomicroscopy, gonioscopy, automated visual field testing (Octopus G1; Interzeag, Schlieren, Switzerland), fundus photography (Carl Zeiss Meditec, Oberkochen, Germany), optional laser scanning tomography (HRT I and II; Heidelberg Engineering, Heidelberg, Germany) of the disc and a 24-hour Goldmann-applanation intraocular pressure (IOP) tonometry profile with five measurements. Manifest high-tension POAG was defined as the presence of glaucomatous optic disc damage (in at least one eye), visual field defects in at least one eye, and intraocular pressure higher than 21 mm Hg in one eye without therapy. Causes of secondary glaucoma, such as primary melanin dispersion and pseudoexfoliation, were excluded. Glaucomatous optic nerve damage was defined as focal loss of neuroretinal rim or nerve fiber layer associated with a specific visual field defect. According to Jonas, stage 0 optic disc was defined as normal, stage I with vertical elongation of the cup and neuroretinal rim loss at the 12 and 6 o’clock positions, stage II with focal rim loss, stage III and IV with advanced rim loss, and stage V, as absolute optic disc atrophy. Disc area was measured with HRT or estimated with a Goldmann lens and slitlamp (Haag-Streit, Köniz,
A pathologic visual field was defined by a pathologic Bebie curve, three adjacent test points with more than 5 dB sensitivity loss or at least one point with a more than 15-dB loss. Patients showing glaucomatous changes of the optic disc and visual field but no IOP elevation over 21 mm Hg after a 24-hour IOP-measurement (sitting and supine body position) without therapy, received a diagnosis of NTG. Patients were classified as having JOAG when age at onset in the index case was below 40 years and no other ocular reason for open-angle glaucoma was visible.

4.1.2. Controls
To aid in the detection of new disease-associated variants, 376 unrelated control subjects of German origin were recruited at the Ophthalmologic Department of the University Hospital of Erlangen-Nuremberg, Erlangen, and at the Ophthalmologic Department of the University Hospital of Würzburg. At the time of examination and inclusion in this study, the age ranged from 51 to 92 years (mean, 73.9 ± 6.4). These age- and sex-matched control subjects underwent ophthalmic examinations: they presented IOP below 20 mm Hg, no glaucomatous disc damage, visual acuity was at least 0.8, and the media were clear for examination. They had neither any family history of glaucoma.

Control DNA samples for the replication study were obtained from 104 unrelated subjects of German descent selected at the University Eye Hospital in Würzburg and Tübingen with the same criteria described above.

4.2. DNA standard methods

4.2.1. Genomic DNA isolation

4.2.1.1. Automated DNA isolation
Genomic DNA samples were extracted from peripheral blood leukocytes by automated techniques (AutoGenFlex 3000) using Flexigene chemistry as indicated by the manufacturers.

4.2.1.2. DNA isolation from COS-1 cells
To isolate genomic DNA from COS-1 cells, the Genomic DNA from Tissues and Cells kit, in which the DNA passes through a column resin, and proteins, detergents, and low molecular weight compounds are retained, was used according to manufacturer’s instructions.
4.2.2. Quantification of dsDNA

Using the formula 1 Unit Absorbance (260nm) = 50µg dsDNA/ml, concentration of DNA samples was measured in a photometer.

4.3. PCR (polymerase chain reaction) and sequencing

4.3.1. Polymerase chain reaction (PCR)

Kary Mullis was awarded the Nobel Prize in Chemistry in 1993 (shared with Michael Smith) for his development of the basic method for performing PCR, a technique that he invented during a night time car ride in 1983 and is now often indispensable in medical and biological research labs to produce copies from specific DNA fragments by means of two oligonucleotides (primers) that are complementary to DNA sequences that flank the desired region. These primers define the position where the DNA polymerase (usually the Taq-DNA-Polymerase from *Thermus aquaticus*) should start polymerization, because they provide the 3´-OH end, on which the DNA-Polymerase is dependent. The term “chain reaction” is used because the method is comprised of a certain number of cycles in which the number of molecules increases exponentially. These cycles are achieved with a thermocycler and include 3 temperatures steps: denaturing (high temperature to denature the double strand helix), annealing (calculated temperature for primer hybridization), and elongation (optimal polymerization temperature of the polymerase).

Usually, 10-20 ng of DNA template were used, plus 100 µM each of deoxyribonucleotide (dATP, dCTP, dGTP, dTTP), 10 pmol of each primer, 0.5 Units of Taq-DNA polymerase, 10% 5M betaine, DMSO at 5% final concentration, and PCR-Buffer, in a total reaction volume of 25 µl. A “touchdown” cycler program was used, which consists of 5 min. at 94°C (initial denaturation), and 10 cycles of: 20 sec. denaturation at 94°C, 1 min. annealing at 65°C (descending 1°C in each of the following 9 cycles) and 1 min. elongation at 68°C, followed by 30 cycles: 20 sec. at 94°C, 1 min. at 55°C and 1 min. at 68°C. Finally, a 10 min. elongation step at 68°C. Different DNA-polymerases were used: the recombinant WinTaq-polymerase, produced at the own Institute according to Engelke (Engelke et al. 1990), Platinum Taq DNA polymerase or Ampli Taq Gold polymerase.

4.3.2. Agarose gel electrophoresis

In order to separate DNA molecules (PCR products) by size, agarose gel electrophoresis was used. Negatively charged nucleic acid molecules move through an agarose matrix with an
electric field (usually 120V). Shorter molecules move faster and migrate further than longer ones. Increasing the agarose concentration of the gel reduces the migration speed and enables separation of smaller DNA molecules. The agent ethidium bromide is incorporated in the gel and intercalates in the DNA, allowing the visualization of reddish-orange bands of DNA when the gel is exposed to ultraviolet light. These DNA bands can also be cut out of the gel, and then be dissolved to retrieve the purified DNA. Agarose concentration of the gel oscillates between 1 and 2% for normal size PCR products (< 7 kb). Between 3 and 10 µl of the PCR product were mixed with bromophenol blue before loading on the gel buffered with TBE.

4.3.3. Gel extraction of PCR products
The QIAquick Gel extraction kit was used for cleanup of DNA fragments from agarose gels according to manufacturer’s instructions. It is based on the dilution of the agarose and binding of the DNA to a column, with subsequent washing and elution of the pure DNA fragment.

4.3.4. Purification of PCR products

4.3.4.1. Purification of PCR-products with magnetic beads
For high throughput purification of PCR products, the AMPure system was used, as it provides an efficient removal of unincorporated dNTPs, primers and salts used during PCR amplification, which can interfere with downstream applications. The strategy is based on the binding of the PCR amplification products to magnetic beads, allowing their separation from the rest of the reaction mixture and contaminants. Finally, the PCR amplicons are separated from the beads and transferred in a new plate. The whole process is performed automatically with the use of the pipetting station Beckman Coulter Biomek NX.

4.3.4.2. Purification of PCR-products with Millipore Cleanup Kit
Another automated PCR purification method using Millipore’s Montage PCR96 Cleanup Kit was achieved, according to the manufacturer’s instructions. This protocol includes one filtration step followed by resuspension and recovery of the sample in a final volume of 100 µl. The whole process was set up automatically on a Tecan Miniprep 75-2 station with two vacuum manifolds on the deck.

4.3.4.3. Purification of PCR-products with QIAquick PCR Purification Kit
For fast purification of few PCR products, the QIAquick PCR Purification Kit was used in a microcentrifuge, according to the manufacturer’s instructions. This system combines a spin-column technology with the selective binding properties of a silica-gel membrane: DNA
adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities are washed away and the pure DNA is eluted with USF water. Another kit (Plasmid DNA Purification kit) from Macherey-Nagel was also used, following the same principles.

4.3.4.4. Enzymatic purification of PCR-products
For fast purification of few PCR products, a combination of two enzymes, exonuclease I and antarctic phosphatase, was also used. Exonuclease I catalyzes the removal of nucleotides from single-stranded DNA in the 3' to 5' direction, degrading excess single-stranded primer oligonucleotide from the reaction mixture containing double-stranded extension products. Antarctic Phosphatase catalyzes the removal of 5' phosphate groups from DNA, removing unincorporated dNTPs.
A 10µl mixture containing 4 units of Exonuclease I and 2 units of Antarctic Phosphatase was added directly to the PCR reaction and then incubated in a thermocycler at 37°C during 15 minutes, followed by inactivation of the enzymes at 80°C during 15 minutes.

4.3.5. Sequencing of purified PCR products with the Sanger method
Frederick Sanger was awarded the Nobel Prize (his second) in Chemistry in 1980 for the development of an enzymatic method to determine the precise sequence of nucleotides in a sample of DNA. His approach utilizes 2', 3'-dideoxynucleotide triphosphates (ddNTPs), molecules that differ from deoxynucleotides by having a hydrogen atom attached to the 3' carbon rather than an OH group. These molecules terminate DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide.
Briefly, 5 µl mixture containing 0.2 µl BigDye Terminator v3.1 (DNA polymerase, dNTPs and 4 ddNTPs, each labelled with a different fluorophore), 2 µl of 5x Sequencing Buffer, 0,3 µl of sequencing primer (10 µM) and water are added to 5 µl of purified PCR product and subjected to the standard sequencing reaction program: 25 cycles of 10 sec. at 96°C, 10 sec. at 55°C and 2 min. at 60°C. The samples were then analyzed on an ABI Genetic Analyser 3730.

4.3.6. Purification of sequencing products with magnetic beads
The Agencourt CleanSEQ system was used for the removal of unincorporated dye-terminators in the sequencing reaction. Similarly to the PCR purification process (see 2.3.4.), it is based on the binding of the sequencing products to magnetic beads, allowing their separation from the rest of the reaction mixture. Beads are washed with 85% ethanol. Finally, the products are separated from the beads and can be transferred in a new plate. The whole
process is also performed automatically with the use of the pipetting station Beckman Coulter Biomek NX.

4.3.7. RT-PCR (reverse transcription polymerase chain reaction)

Synthesis of first-strand cDNA from purified total RNA was performed with SuperScript III Reverse Transcriptase and oligo(dT) primers. This cDNA was then used as template for PCR, using specific primers for the gene of interest. First, the RNA was denatured by heating to 65°C during 5 minutes a 13 µl mixture containing 1 µl oligo(dT) primer (500 µg/ml), 1 µl of dNTP mix (10 mM each), total RNA (between 1 and 2 µg) and water. Then, 4 µl 5x First Strand buffer, 1 µl DTT (0.1 M), 40 units RNase OUT, and 200 units Superscript III were added, and the cDNA synthesis took place in a thermocycler by heating 60 min. at 50°C and 5 min. at 85°C (enzyme inactivation). In all cases, quality of the cDNA was controlled by co-amplification of the housekeeping gene GAPDH.

4.3.8. Microsatellite analysis

Microsatellites are polymorphic loci present in nuclear and organellar DNA that consist of repeating units of 1-6 base pairs in length. They are typically neutral, co-dominant and were used as molecular markers.

Microsatellite markers were amplified in singleplex reactions in a final reaction volume of 15 µl containing 10 mM Tris, 1.5 mM MgCl₂, 100 µM each dNTP, 0.35 U DNA polymerase, 7.0 pmol of each primer, and 20 ng of genomic DNA. One of the primers was end-labelled with a fluorescent dye (FAM, TET, or TEX). For amplification, a touchdown PCR program was used with an annealing temperature decreasing from 61°C to 55°C over 6 cycles, followed by 31 cycles with an annealing temperature of 55°C. Products were usually pooled according to product size and fluorescent label and analyzed on an ABI Genetic Analyzer 3100.

4.4. Cloning and plasmid procedures in bacteria

4.4.1. Cloning of plasmids and PCR products in a cloning vector

The TOPO TA cloning vectors pCR2.1-TOPO and pCR4-TOPO were used for different purposes. Competent E. coli (One Shot TOP10, XL-1 Blue, or DH5α) were transformed with 0,2-0,5 µl of the desired plasmid by incubation on ice followed by heat-shock of the cells for 30 seconds (45 seconds in the case of XL-1 Blue) at 42°C. After 1 hour incubation at 37°C and 220 rpm, the cells were spread in prewarmed selective plates and incubated overnight at 37°C. In order to clone PCR fragments, a previous ligation reaction had to be performed by
mixing 3 µl of PCR product, 1 µl of vector and 1 µl salt solution. Then, 2 µl of the ligation reaction were transformed as described above. The presence of the desired cloned product in the vector was checked by PCR of single colonies the next day.

4.4.2. Miniprep plasmid preparation
Purification of DNA plasmids from competent *E. coli* (One Shot TOP10, XL-1 Blue, or DH5α) was performed with the QIAprep Miniprep Kit in a microcentrifuge, according to manufacture’s instructions. The method is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica columns in the presence of high salt. Finally, the pure plasmid DNA is washed and eluted from the columns. All the plasmids were checked by means of sequencing prior to using them in further applications.

4.4.3. Midiprep plasmid preparation
The isolation of large amounts of plasmid DNA of high purity was performed with the QIAGEN Plasmid Midi Kit according to the manufacturer’s instructions. Briefly, one *E. coli* colony harboring the plasmid of interest was inoculated in 3ml of LB medium and incubated at 37°C with shaking for approx. 8 hours. The 3ml pre-culture was then poured in 200 ml of LB medium and incubated with shaking overnight. The bacteria in this culture were then precipitated through centrifugation (4°C, 15min, 6000rpm). The plasmid purification protocol is based on a modified alkaline lysis procedure, followed by binding of plasmid DNA to QIAGEN anion-exchange resin under low-salt and pH conditions. RNA, proteins, dyes, and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. All the plasmids were checked by means of sequencing prior to using them in further applications.

4.4.4. Site-directed mutagenesis
Michael Smith was awarded the Nobel Prize in Chemistry in 1993 (shared with Kary Mullis) for developing this technique in which a mutation is created at a defined site in a DNA molecule, usually a plasmid.

Site-directed mutagenesis was performed using the QuickChange II Site-Directed Mutagenesis Kit according to the instructions of the manufacturer. The kit is based on the replication of both plasmid strands with PfuTurbo DNA polymerase with two primers containing the desired mutation, and subsequent digestion of the parental DNA template through DpnI endonuclease treatment. Most of the remaining plasmids should then carry the mutation and were used to transform XL1-Blue Supercompetent cells with a heat shock at
42°C. Colony selection was performed by means of PCR and sequencing of the mutagenised site using the ABI Prism Big Dye terminator cycle sequencing kit and analyzed on an ABI Genetic Analizer 3730 to ensure that the constructs were correct.

4.4.5. Gateway cloning

The Gateway cloning system recreates the lambda phage recombination reactions in vitro through a cocktail of recombination proteins and a set of vectors containing the att sequences they recognize. Two main reactions are involved (Figure 4.1.). In one, the LR reaction, a cDNA or other DNA sequence flanked by attL sites is transferred by recombination from an Entry Clone into a Destination Vector, which contains attR sites. In this process, the Destination Vector conveys some functionally useful elements, such as a promoter, fusion tag, new replicon, or selection marker, to the final recombination product. The resulting molecule, called an Expression Clone, is a subclone of the starting DNA sequence, correctly positioned (same orientation and reading frame) in a new vector backbone. The second Gateway pathway is the BP reaction, in essence the reverse of the LR reaction. The BP reaction transfers a DNA insert, flanked by 25 bp attB sites, from an Expression clone, into a vector donated by a plasmid containing attP sites. The final product is an Entry Clone containing the transferred DNA sequence.

Figure 4.1. The Gateway reactions. The scheme shows the four types of plasmids and the two enzymatic reactions involved in Gateway cloning reactions. Red arrows represent the fragment of interest. Taken from www.invitrogen.com.

In this study, expression clones carrying cDNA corresponding to the C2 domains of RPGRIP1 were mutagenized and linearized prior to be subcloned into the Donor Vector pDONR201 via the BP reaction to create the entry clones. Positive clones were selected from kanamycin plates and verified by restriction enzyme digestion. The cDNA inserts of the entry clones were subcloned into the destination vectors p3xFLAG/DEST or pcDNA3-HA/DEST via an LR reaction, thus maintaining the correct reading frame with the fusion tags. Positive
clones were selected from ampicillin plates and verified by restriction enzyme digestion and nucleotide sequencing.

4.5. Yeast two-hybrid experiments

The yeast two-hybrid system is a molecular genetic test for protein-protein interaction. The system utilizes the product of the yeast gene \textit{GAL4}, a protein with two functional domains that activates transcription of genes involved in galactose metabolism. The DNA binding domain (BD) of the \textit{GAL4} protein interacts with DNA sequences within the promoter region of \textit{GAL1} and the transcriptional activating domain (AD) of the \textit{GAL4} protein stimulates transcription (Fields and Song 1989). In this thesis, wild type and mutated RPGRIP1\textsubscript{C2-N+C2-C} proteins (fused in frame to \textit{GAL4}-AD) were assessed for interaction with NPHP4, fused to the \textit{GAL4}-BD domain.

The yeast reporter host strain used was \textit{Saccharomyces cerevisiae} PJ69-4a, with the following genotype: \textit{MATa}, trp1-901, leu2-3,112, ura3-52, his3-200, gal4\Delta, gal80\Delta, GAL2-ADE2, LYS2::GAL1-HIS3 met2::GAL7-lacZ (James et al. 1996). This strain contains three separate reporter genes (\textit{HIS3}, \textit{ADE2} and \textit{lacZ}) each under the independent control of three different \textit{GAL4} promoters (\textit{GAL1}, \textit{GAL2} and \textit{GAL7}, respectively) that provide a high level of sensitivity with respect to detecting weak interactions, coupled with a low background of false positives. PJ69-4a also contains an endogenous \textit{MEL1} gene, which can serve as a fourth reporter or be used as an alternative to GAL-7/lacZ. For selection of yeast clones that have been cotransformed with the AD and BD plasmids, it carries the auxotrophic markers leucine (\textit{leu2}, to select for the AD plasmid) and tryptophan (\textit{trp1}, to select for the BD plasmid).

4.5.1. Yeast cotransformation

To test for interaction, the corresponding RPGRIP1 prey and NPHP4 bait plasmids were cotransformed into PJ69-4a following the next procedures:

4.5.1.1. Lithium acetate (LiAc)-mediated cotransformation of fresh growing cells

In order to introduce the desired plasmids in the cells, yeast competent cells were prepared and suspended in a LiAc solution with the plasmids DNA to be cotransformed (400ng each), along with excess of carrier DNA (5 µl Herring Testes carrier DNA denatured). Polyethylene glycol (PEG) with the appropriate amount of LiAc was then added and the mixture of DNA and yeast was incubated at 30°C for 30 min. After the incubations, DMSO was added and the cells were heat shocked, which allows the DNA to enter the cells. The cells, resuspended in 200 µl NaCl 0.9%, were then plated on the appropriate medium to select for cotransformants.
containing the introduced plasmids. Because the selection was nutritional, an appropriate -WL synthetic dropout (SD) medium was used.

4.5.1.2. Transformation of frozen competent cells
Although the highest transformation efficiencies are obtained with freshly grown cultures, the moderately efficient transformation of frozen cells was also used to save time.

4.5.1.2.1. Preparation of frozen competent cells
Cells were grown in YPAD until their density represented approx. 0.6-1x10^7 cell/ml (OD between 0.6-1.0), washed in 0.5 vol of 1.0 sorbitol, 10mM bicine-NaOH (ph 8.35), 3% ethylene glycol, 5% DMSO, and resuspended in 0.2 vol of the same solution. 0.1ml aliquots were slowly frozen (to improve their viability) using a Nalgene Cryo 1°C freezing container (Cat. No. 5100-0001) and store at -70°C until needed.

4.5.1.2.2. Transformation of frozen competent cells
5 µl carrier DNA and 400 ng of each plasmid DNA were quickly added on top of the frozen cells. Once melting was completed, 700 µl of a 40% PEG1000, 0.2M bicine-NaOH (pH 8.35) solution was added and incubated at 30°C for 1 hour. Cells were then spinned down and the pellet resuspended in 200 µl of a 0.15M NaCl, 10nM Bicine-NaOH (pH 8.35) solution. 100 µl were then plated onto the –WL medium.

4.5.2. X-α-Galactosidase assay
X-α-Gal is a chromogenic substrate for α-galactosidase (also known as melibiase), an enzyme which enables yeast to use the disaccharide melibiose as a carbon source during growth or fermentation. In S. cerevisiae this enzyme is encoded by the MEL1 gene which is regulated by several GAL genes and it was included in the yeast two-hybrid system as a reporter gene of the cotransformation. Secretion of this enzyme in response to GAL4 activation leads to hydrolysis of X-α-Gal in the medium causing yeast colonies to turn blue. X-α-Gal was included in the medium prior to pouring plates. One ml of X-α-Gal stock solution (20 mg/ml in DMF) was added per 1 litre medium. Plates were incubated at 30°C until blue colonies form.

4.5.3. β-Galactosidase assays
The gene encoding β-galactosidase (lacZ), a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides, of E. coli has been used as a reporter of the interaction of RPGRIP1 and NPHP4 proteins. When the yeast are cotransformed with the
expression vectors containing the interacting proteins and production of LacZ is disrupted the cells exhibit no β-galactosidase activity, meaning that the interaction is disrupted.

4.5.3.1. β-Galactosidase liquid assay
In order to quantify the β-galactosidase activity in solution directly from colonies growing on solid medium, the Pierce Yeast β-Galactosidase Assay Kit was used according to the manufacturer’s instructions. Briefly, the protein was extracted, and after incubation the solution turned yellow from the hydrolysis of ONPG to ONP and galactose in a mildly alkaline solution. The β-galactosidase activity was then calculated in average using the equation: β-galactosidase=1000*A_{420}/t*V*OD_{660}. The average is taken from four readings and four clones that contain the same bait and prey plasmids.

4.5.3.2. β-Galactosidase colony-lift filter assay
Although this assay provides only qualitative results, it was a relatively sensitive method to screen colonies for production of β-galactosidase by the activation of the lacZ reporter gene. In this assay, the colorless X-Gal is used as a substrate by β-galactosidase, with turns it into a blue product. To transfer the transformants to a piece of filter paper, this paper was placed over the surface of the plate with the growing colonies. Air bubbles were carefully removed and the filter paper was pressed firmly onto the surface of the plate to ensure contact of all clones with the filter for 3 min. The filter paper was then carefully lifted, using forceps, and submerged colony side up in liquid nitrogen for 10 seconds. After the filter has frozen completely, it was removed from the liquid nitrogen, thawed at room temperature and then incubated with another filter presoaked in 5ml of Z buffer containing 2-mercaptoethanol (2.7ml/liter) and X-Gal stock solution. The cotransformed colonies turned blue.

4.6. Assays in mammalian cells
The cell lines used in the experiments were COS-7 and COS-1, established from CV-1 (Cercopithecus aethiops), which were transformed by an origin-defective mutant of SV-40 virus. They are fibroblast-like cells that grow as adherent monolayers.

4.6.1. Culture conditions
Cells were routinely grown in DMEM supplemented with L-glutamine, 10% foetal calf serum, penicillin (10 U/ml) and streptomycin (10µg/ml) in T75 flasks at 37°C and 5% CO₂. When they reached 90% confluence, they were splitted 1:10 by trypsinization with 1µl
Trypsine-EDTA 0.5%, a pancreatic enzyme that breaks the extracellular matrix which allows the cells to adhere to the container.

4.6.2. Stock preparation
In order to store the cells, they were frozen at -80°C using 10% DMSO as cryoprotectant in order to preserve them active after thawing.

4.6.3. Cotransfection methods
Different procedures were followed in order to introduce foreigner DNA into the cells:

4.6.3.1. Nucleofection
This method is based on the physical procedure of electroporation, using a combination of optimized electrical parameters with cell-type specific reagents to transfer plasmid DNA directly into the cell nucleus and the cytoplasm. COS-7 cells were cotransfected with plasmid DNA by nucleofection with Nucleofector kit V (Amaxa) and program A-24, according to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were washed with PBS, lysed in 250 µl ice-cold IP lysis buffer and scraped.

4.6.3.2. Cationic lipid transfection using Lipofectamine and PLUS reagents
Lipofectamine™ 2000 was used to introduce the different expression vectors into COS-1 cells. The positively-charged components of this reagent form a complex with the negatively-charged genetic material, and then "escort" it through the membranes of cells. The PLUS™ reagent was used for pre-complexing DNA so as it enhances the transfection efficacy. Briefly, 2.5 µg of each plasmid DNA and 20 µl PLUS reagent were diluted in 200 µl OptiMEM, incubated at room temperature for 15 min, then mixed with 200 µl of OptiMEM containing 10 µl Lipofectamine, and incubated at room temperature for 20 min to form DNA-PLUS-Lipofectamine complexes. The DNA-PLUS-Lipofectamine mixture was then added to the cells and incubated for 5 hours at 37°C, 5% (vol/vol) CO₂. Then, the cell media was replaced with normal media, and incubation was continued at 37°C, 5% (vol/vol) CO₂. Twenty-four hours after transfection, the cells were washed with PBS, lysed in 250 µl ice-cold IP lysis buffer and scraped.

4.6.4. Coimmunoprecipitation
Coimmunoprecipitation relies on the ability of an antibody to stably and specifically bind complexes containing a bait protein and its interacting partner. The antibody provides a means of immobilizing these complexes on a solid matrix.
COS-7 or COS-1 subconfluent cells were transiently cotransfected with plasmid DNA and proteins were expressed for 24h. The cells were subsequently washed in PBS and lysed in ice-cold lysis buffer. Lysates were cleared by centrifugation at 4°C for 10 min at 14000rpm. FLAG- and HA-tagged proteins were immunoprecipitated by using, respectively, ANTI-FLAG M2 affinity gel (Sigma) and anti-HA antibody (Sigma). Immunoprecipitation was performed overnight at 4°C accomplished through interaction with Protein A/G beads, so that irrelevant proteins can be washed away. Beads were then washed four times with lysis buffer and the immunocomplexes were resolved by SDS/PAGE followed by Western blot analysis with tag-specific primary antibodies.

4.6.5. Immunofluorescence

Immunofluorescence is a technique allowing the visualization of a specific protein or antigen in cells or tissue sections by binding a specific antibody chemically conjugated with a fluorescent dye. Expression of fluorescent proteins was induced here to visualize the subcellular localization of RPRIP1 and NPHP4 proteins using a fluorescence microscope. NPHP4 and RPGRIP1\textsuperscript{C2-C2-N} were cloned into the vectors pDEST-733 (C-terminal monomeric red fluorescent protein (mRFP) tag) and pDEST-501 (C-terminal enhanced cyan fluorescent protein (eCFP) tag) respectively. Mutations were introduced in the RPGRIP1\textsuperscript{C2-C2-N} construct by using the QuickChange site-directed mutagenesis kit. All constructs were verified by nucleotide sequencing. The resulting vectors (2.5 µg each) were transfected in COS-1 cells using Lipofectamine and PLUS reagents. Cells were grown overnight on glass microscope slides, fixed in 3.7% formaldehyde for 10 min, permeabilized with 0.5% Triton-X 100 in PBS for 10 min and stained directly with 1 µg/ml of Dapi for 3 min. Slides were prepared with 100 µl Mowiol and analyzed by fluorescence microscopy.

4.7. Standard protein methods

4.7.1. Western Blot

A western blot is a method to detect protein in a given extract. It uses gel electrophoresis to separate denatured protein by mass. The proteins are then transferred out of the gel and onto a membrane, where they are “probed” using antibodies specific to the protein. The name was given to the technique by W. N. Burnette in 1981 as a play on the name Southern blot, a similar technique for DNA detection developed earlier by E. Southern.
Samples for SDS-PAGE were prepared by mixing aliquots of the cell lysate with LDS-NuPAGE Sample Buffer and heated at 70°C for 10 min. Protein samples were run on NuPAGE 4–12% gradient Bis-Tris gels at 200 V for 50 minutes with MOPS SDS Running Buffer. For western blot analysis, gels were electrotransferred to a nitrocellulose membrane for 1 hour. Non-specific binding sites were blocked by incubation in TBS containing 0.5% Tween-20 and 5% non-fat dry milk powder. Proteins were detected by chemiluminescence.

4.7.2. Chemiluminiscence

Chemiluminiscence detection methods depend on incubation of the western blot with a substrate that will luminescence when exposed to the reporter on the secondary antibody. The light is then detected by a photographic film. Proteins were detected by chemiluminescence using a mouse anti-FLAG M2 monoclonal antibody and an anti-mouse secondary antibody conjugated with rabbit peroxidase.

4.8. In-situ hybridization

4.8.1. Probe preparation

Three different fragments of GenBank accession number NM_020366 were amplified and subcloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit and sequenced to confirm identity. These constructs were then linearized with SpeI, purified with the Qiaquick PCR Purification Kit and used to generate antisense and sense probes. The manufacturer’s instructions were always followed.

4.8.2. In-vitro transcription and whole-mount in-situ hybridization.

Through in-vitro transcription, the plasmid DNA was translated into RNA to be used as probe in whole retina tissue. This work was made by A. Krysta as part of her Diploma thesis. The human retina of the donor eye (70, man, no known eye-diseases) was isolated 6 hours after death and fixed for 1.5 hours.

4.9. Bioinformatic tools

4.9.1. PCR primer design

For the design of the PCR primers, Primer3 was normally used with default conditions, except reduced self complementarity. In cases where the coding sequence of a whole gene had to be
screened for mutations, the Exon Locator and Extractor for Resequencing program was used. After the input of the mRNA accession number for the gene, the program can design primers for all exons.

Primer3: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

4.9.2. Sequencing analysis

The program package DNA-Star (GATC Biotech), which allows several sequences to be analyzed at once and compared to the reference sequence, was used for analysis of sequences. First, quality of the electropherograms was controlled with Sequencing Analysis and Chromas. Those passing quality control were then analysed with SeqMan.

4.9.3. Microsatellite analysis

The Genotyper program (Applied Biosystems, Foster City, CA, USA) was used for genotyping of microsatellites.

4.9.4. Genome browsers

For annotation on genomes, the web-based UCSC Genome Browser and Ensembl were used.

UCSC Genome Browser: http://genome.ucsc.edu/cgi-bin/hgGateway
Ensembl: http://www.ensembl.org/index.html

4.9.5. Single nucleotide polymorphism (SNP) and mutation databases

Publicly available information on SNPs and mutations was retrieved from the following databases.
HapMap: http://www.hapmap.org/cgi-perl/gbrowse/hapmap_B35/
HGMD: http://www.hgmd.cf.ac.uk/ac/index.php

4.9.6. Linkage disequilibrium visualization

For measure and graphical visualization of linkage disequilibrium between SNPs, either from the HapMap data or from own sequencing or genotyping, the programs Haploview (Barrett et al. 2005) and LDmax, as implementation of the program GOLD (Abecasis and Cookson 2000) were used. According to the LD-structure, those segments in which SNPs alleles showed strong linkage disequilibrium (almost no ancestral recombination) were defined as haplotype blocks (also called LD blocks).
4.9.7. Haplotype reconstruction
A combination of alleles at different loci on the same chromosome is a haplotype. Based on an accelerated EM algorithm, the HaplovieView software (Barrett et al. 2005) estimates haplotypes and their frequencies in a whole group of DNAs. For determination of individual haplotypes, the software PHASE (Stephens et al. 2001), which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data, was applied.

4.9.8. Selection of haplotype tag SNPs (htSNPs)
Most of the haplotype structure (allele combination) in a particular chromosomal region can be captured by genotyping a smaller number of markers than all of those that constitute the haplotype. The crucial markers to type (called tagging SNPs) would be those that distinguish one haplotype from another. Selection of the htSNPs in each block in order to cover more than 90% of the haplotype diversity in a given population was performed either with HaplovieView (Barrett et al. 2005) or with SNP tagger (Ke and Cardon 2003).
SNP tagger: http://www.well.ox.ac.uk/~xiayi/haplotype/

4.9.9. Multiple sequence alignment
Evolutionary conservation was investigated with protein sequence alignment generated by ClustalW 1.8 software. The graphic representation was performed with Boxshade.
ClustalW 1.8: http://searchlauncher.bcm.tmc.edu/multi-align
Boxshade: http://www.ch.embnet.org/software

4.9.10. Promotor prediction and promoter database
Several probabilistic models have been developed to predict promotor regions in the genome. The web sites of promotor prediction programs FirstEF and ElDorado were used.
FirstEF: http://rulai.cshl.edu/tools/FirstEF/Readme/README.html
ElDorado: http://www.genomatix.de/products/Gene2Promoter

4.9.11. Open reading frame (ORF) search
In order to find all ORFs in a sequence, the web-based tools ORF Finder was used.
4.9.12. Transcription factor binding site (TFBS) prediction

Several tools have been designed for searching potential binding sites for transcription factors in any sequence. I used the web tools Transfac, Consite, TFSearch, Match, and Pupas View (Conde et al. 2005) in order to obtain as much information as possible.

Transfac: http://www.gene-regulation.com/cgi-bin/pub/databases/transfac/search.cgi
Consite: http://mordor.cgb.ki.se/cgi-bin/CONSITE/consite/
TFSearch: http://www.cbrc.jp/research/db/TFSEARCH.html
Match: http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi
Pupas View: http://pupasuite.bioinfo.cipf.es/

4.9.13. Statistics

Fisher's Exact Test: http://www.langsrud.com/fisher.htm
Odds ratio: http://www.hutchon.net/ConfidORnullhypo.htm

4.10. Nomenclature

GenBank accession NM_020366 was used as the cDNA reference sequence. The nomenclature recommendations of den Dunnen and Antonarakis (den Dunnen and Antonarakis 2001) were followed. Nucleotide +1 is the A from the ATG-translation initiation codon. For amino acid numbering the translation initiation methionine is considered +1.

4.11 Reagents and materials

4.11.1. Kits

Cell Line Nucleofector Kit V Amaxa, Cologne
DyeEx 2.0 spin kit Qiagen, Hilden
FlexiGene DNA kit Qiagen, Hilden
GATEWAY Cloning System GibcoBRL, Karlsruhe
Genomic DNA Isolation Kit for Tissue and Cells Nexttce, Leverkusen
Montage SEQ96 kit Millipore, Schwalbach
Plasmid DNA Purification kit Macherey-Nagel, Düren
QIAGEN plasmid midi kit Qiagen, Hilden
QIAprep spin miniprep kit Qiagen, Hilden
QIAquick gel extraction kit Qiagen, Hilden
QIAquick PCR Purification Kit Qiagen, Hilden
QuickChange II Site-Directed Mutagenesis kit Stratagene, Amsterdam
4.11.2. Instruments

- Autoclave: Hiclave HV85 (HMC, Engelsberg)
- Autoradiography cassettes: Siemens, Erlangen
- Bunsen burner: Fireboy plus (Integra Biosciences, Wallisellen)
- Centrifuges: Centrifuge 5415D (Eppendorf, Hamburg), Centrifuge 5415R (Eppendorf, Hamburg), Centrifuge 5810 (Eppendorf, Hamburg), Minifuge RF (Heraeus, Hanau), Varifuge 20RS (Heraeus, Hanau), Varifuge F (Heraeus, Hanau)
- Electrophoresis chambers: PerfectBlue Gelsystem (peqlab, Erlangen), XCell SureLock (Invitrogen, Karlsruhe)
- Film dryer: Mistral 2 (Jobo, Gummersbach)
- Gel documentation: BioDocAnalyze 2.0 (Biometra, Göttingen)
- Ice machine: Ziegra, Isernhagen
- Incubator: BBD6220 (Heraeus, Hanau)
- Incubator-shaker: Innova 4000 (New Brunswick Scientific, Nürtingen), Innova 4300 (New Brunswick Scientific, Nürtingen)
- Steril benches: BSB6A (Gelaire, Meckenheim), LaminAir HA2472GS (Heraeus, Hanau)
- Magnetic stirrers: Ikamag RH (Janke&Kunkel, Staufen), KMO2 (Janke&Kunkel, Staufen)
- Microscope: Labovert (Leica, Solms)
- Neubauer chamber: Brand, Wertheim
- pH-meter: Knick, Mering
- Pipettes: Pipetman (Gilson, Bad Camberg), Research Pro (Eppendorf, Hamburg), Multipipette Pro (Eppendorf, Hamburg), EasyPET (Eppendorf, Hamburg), CAPP (Dunn, Asbach)
- Plates mixer: Incutec, Mössingen
- Platform shaker: Unimax 1010 (Heidolph, Schwabach)
4.11.3. Enzymes

AmpliTaq Gold
Antarctic Phosphatase
DNase I, RNase free
Exonuclease I
Pfu Turbo DNA polymerase
PfuUltra III Fusion F DNA Polymerase
Platinum Pfx DNA polymerase
Platinum Taq DNA polymerase
Proteinase K
Pwo DNA polymerase
Restriction endonucleases:
AflIII, ApalI, DpnI, PstI, ScaI, SpeI
T4 DNA ligase
Taq DNA polymerase, recombinant
Trypsin-EDTA
WinTaq DNA polymerase

4.11.4. Consumables

Cell scrapers
Chemiluminescence film
Culture plates
CryoTube vials

Power supply
Power Pac 300 (BioRad, Munich)
EPS 3500XL (Pharmacia, Munich)
Precision balance
Sartorius MC1 (Sartorius, Göttingen)
Robotics
Tecan Miniprep 75-2 (Tecan, Crailsheim)
AutoGenFlex STAR (Genelimited, UK)
Coulter Biomek NX (Beckman, Krefeld)
Rotating Mixer
RM5 (Assistent, Sondheim)
Spectrophotometer
Ultrospec III (Biotech, Freiburg)
Tecan GENios (Tecan, Crailsheim)
Sequencer
ABI Prism 3730 (Applied Biosystems, Darmstadt)
ABI Prism 3100 (Applied Biosystems, Darmstadt)
Thermocyclers
MJ Research (Biozym, Hessisch Oldendorf)
MBS Satellite O.2G (Thermo, Ulm)
Thermomixer
Thermomixer compact (Eppendorf, Hamburg)
Vacuum
QIAvac96 manifold vacuum (Qiagen, Hilden)
Vortex
Janke&Kunkel, Staufen
Filters
Inoculation loops
Microscope slides
Microscope slide coverslips
Millipore Montage PCR Cleanup filter plates
Millipore Montage SEQ Cleanup filter plates
Nitrocellulose membranes
NuPAGE 4-12% Bis-Tris Gel
Parafilm
Pasteur pipettes
Pipette Tipps

Serological pipettes
Steril tubes
Syringes
Thermotubes
Thermowell 96-well plates
Thermowell sealing mats

4.11.5. Reagents

Adenine hemisulfate
Agar-agar
Agarose Seakem LE
Agencourt Ampure
Agencourt CleanSEQ
Ampicillin
Anti-FLAG M2 Monoclonal antibody
Anti-HA antibody
Betaine
Bicin
BigDye Terminator v1.1 Cycle Sequencing
Boric acid
Bromophenol blue
Chloroform
4',6-Diamidino-2-phenylindol Dihydrochlorid (DAPI)
Dimethylformamide (DMF)
Dimethylsulfoxid (DMSO)
Dinatrium salz (EDTA)
Dithiothreitol (DTT)

Schleicher & Schuell, Dassel
Nunc, Roskilde
Marienfeld, Lauda-Königshafen
Roth, Karlsruhe
Millipore, Schwalbach
Millipore, Schwalbach
Invitrogen, Karlsruhe
Invitrogen, Karlsruhe
Pechiney, chicago
Joseph Peske, Aindling-Arnhoffen
Eppendorf, Hamburg
Greiner, Karlsruhe
Sarstedt, Nümbrecht
Joseph Peske, Aindling-Arnhoffen
Greiner, Karlsruhe
Joseph Peske, Aindling-Arnhoffen
Discardit II (BD, Fraga)
PeqLab, Erlangen
Costar, Schwerte
Costar, Schwerte

Sigma-Aldrich, Taufkirchen
Merck, Darmstadt
Biozym, Hessisch Oldendorf
Beckman, Krefeld
Beckman, Krefeld
Roth, Karlsruhe
Sigma-Aldrich, Taufkirchen
Sigma-Aldrich, Taufkirchen
Sigma-Aldrich, Taufkirchen
Roth, Karlsruhe
Applied Biosystems, Darmstadt
Roth, Karlsruhe
Roth, Karlsruhe
Merck, Darmstadt
Roth, Karlsruhe
Sigma-Aldrich, Taufkirchen
Merck, Darmstadt
Roth, Karlsruhe
Biosynth, Staad
dNTPs

Dodecyl sodium sulphate (SDS)

Enhancing Roti-Lumin detection system

Ethanol

Ethidium bromide

Fetal calf serum

Fixer for X-ray films

Formaldehyde

D-Glucose

Glycerine

Goat anti-mouse IgG-HRP

Isopropanol

KCl

KH2PO4

LDS-NuPAGE Sample Buffer

Lipofectamine 2000

Liquid nitrogen

Lithium acetate (LiAc)

β-Mercaptoethanol

Methanol

MgCl2

MgSO4

Na2HPO4

NaCl

NaH2PO4

NaOH

Non-fat dry milk

Novex Tris-Acetate SDS running buffer

NuPAGE Antioxidant

NuPAGE transfer buffer

Penicillin/Streptomycin

Penicillin/Streptomycin/L-Glutamine

Phenol

PLUS reagent

Polyethylene glycol (PEG)

Polyvinylalkohol (Mowiol)

Ponceau S

Prestained Protein-Markers

Protease Inhibitor Cocktail Tablets

Protein A/G PLUS-Agarose IP reagent

peqLab, Erlangen

Invitrogen, Karlsruhe

Sigma-Aldrich, Taufkirchen

Roth, Karlsruhe

Roth, Karlsruhe

Invitrogen, Karlsruhe

Tetenal, Norderstedt

Roth, Karlsruhe

Sigma-Aldrich, Taufkirchen

Roth, Karlsruhe

Santa Cruz Biotechnology, New York

Roth, Karlsruhe

Roth, Karlsruhe

Merck, Darmstadt

Invitrogen, Karlsruhe

Invitrogen, Karlsruhe

Linde Healthcare, Munich

Sigma-Aldrich, Taufkirchen

Sigma-Aldrich, Taufkirchen

Roth, Karlsruhe

Invitrogen, Karlsruhe

Merck, Darmstadt

Roth, Karlsruhe

Roth, Karlsruhe

Merck, Darmstadt

Roth, Karlsruhe

Roth, Karlsruhe

Lasana, Herford

Invitrogen, Karlsruhe

Invitrogen, Karlsruhe

Biochrom, Berlin

Invitrogen, Karlsruhe

Roth, Karlsruhe

Invitrogen, Karlsruhe

Sigma-Aldrich, Taufkirchen

Roth, Karlsruhe

Roth, Karlsruhe

peqLab, Erlangen

Roche, Mannheim

Santa Cruz Biotechnology, New York
pUC Mix Marker 8  peqLab, Erlangen
Reducing Agent  Invitrogen, Karlsruhe
Roentgen developer for X-ray films  Tetenal, Norderstedt
Sequencing Buffer 5x  Applied Biosystems, Darmstadt
Sodium azide  Sigma-Aldrich, Taufkirchen
D-Sorbitol  Sigma-Aldrich, Taufkirchen
Tris  Roth, Karlsruhe
Tris-HCl  Roth, Karlsruhe
Triton X-100  Pharmacia Biotech, Uppsala
Trypton  Roth, Karlsruhe
Trypton/Pepton  Merck, Darmstadt
Tween 20  Roth, Karlsruhe
X-α-Gal  Glycosynth, Warrington
X-β-Gal  Biosynth, Staad
Xylene cyanol  Roth, Karlsruhe
Yeast extract  Merck, Darmstadt
Yeast nitrogen base w/o amino acids  Sigma-Aldrich, Taufkirchen

### 4.11.6. Media and solutions

Agar plates  20 g Agar- agar
up to 1 l LB Medium

DNA-Loading Buffer (6x)  0.25 % Bromophenol blue
0.25 % Xylene cyanol
30 % Glycerine

Dulbecco's Modified Eagle Medium (D-MEM/F-12)  Invitrogen, Karlsruhe

IP Lysis Buffer  50 mM Tris-HCl
150 mM NaCl
0.5% Triton-X-100

LB Medium  10 g NaCl
10 g Tryptone
5 g Yeast extract; pH 7.0
up to 1 l bidest. Water

Opti-MEM I Reduced-Serum Medium  Invitrogen, Karlsruhe

PBS (10x)  80 g NaCl
14.4 g Na₂HPO₄
2 g KCl
2.4 g KH₂HPO₄
up to 1 l bidest. water

SD Medium  6.7 g yeast nitrogen base w/o amino acids
182.2 g D-Sorbitol. pH 5.8
40 ml Glucose 50%
up to 1 l bidest. water

TBE (1x)  90 mM Tris
90 mM boric acid
1.25 mM EDTA; pH 8.3

**TBS-Tween**
24.2 g Tris
80 g NaCl
15 ml 32% HCl; pH 7.6
10 ml Tween-20
up to 1 l bidest. water; pH 7.6

**TE Buffer**
10 mM TrisHCl
1 mM EDTA

**YPAD Medium**
20 g Peptone
10 g Yeast extract
0.04 g Adenine 99%
40 ml Glucose 50%; pH 6.5
up to 1 l bidest. water

**Z-Buffer**
24.4 g Na2HPO4·2 H2O
5.5 g Na2HPO4· H2O
0.76 g KCl
0.246 g MgSO4; pH 7.0
up to 1 l bidest. water

### 4.11.7. Oligonucleotides (5’- 3’, for each gene in alphabetical order)
From Invitrogen (Karlsruhe) or Thermo Scientific (Ulm).

**ADCY4**

ADC1f  
GCTTTGAGCGGGTGAGAAA

ADC1r  
GACAGAAACGAGAAGCATCCAG

ADC2f  
TTGATCTTACGTTGAGACACC

ADC2r  
GCTATCAAGGCTGTTGAG

ADC3f  
CTCACCAGGCTTGAATAGC

ADC3r  
ACTTGGAGTCACAGCTCAACAA

ADC4f  
TGACACCACACTCAAATACACAC

ADC4r  
CTCACCAGGCTTGAATAGC

ADC5f  
AAGGTGATCAGTGTAGGAG

ADC5r  
GTCAGATGTCAGCATACAGCAC

ADC6f  
GGAGTCAAGTATGAGAAAGAT

ADC6r  
TGGAGATCTCCTGATGGTTTGC

ADC7f  
TCTACCCTTCTGACCTCTGCA

ADC7r  
AAAGAGCTCTGTGTTTACAGGAGGT

ADC9f  
CCTGTAACACAGGCTTCCTCTTG

ADC9r  
GAGGGACAGTAAAGGACACAGAC

ADC10f  
GATCTCTTCTGTGCAGAGAT

ADC10r  
TATCTGTCAGGAGATGGGA

ADC11f  
TTGAGTGACAGAGGAGGGAG

ADC11r  
CTCTTTGGTTCCTCAGTCTG

ADC12.2f  
AAGACCTGCTGCTTCTCCAG

ADC12.2r  
TGAATCAGCTGTACGTTGGTTG

ADC13f  
GCAAGAAGTGAGGAACCAAGAG

ADC13r  
TGATAGCCCTACCAGTTCTCA

ADC14f  
TTGAGTGACAGAGGAGGGAG

ADC14r  
GCTTTGAGCGGGTGAGAAA

ADC15f  
AGCTGCAACAGCAGCCTCTAG

ADC15r  
TCATCTACGTCCTGTCGTGTG

ADC16f  
ATAGCATCACCTCCCTCCTTC

ADC16r  
CAGATGTTAGATGTCTGGAGA

ADC17f  
AGTGCACAGAGGAGGGAG

ADC17r  
GGGCAATGACAGACACTGATAC

ADC18f  
TCACACCAGTGTATCAGTGT
ADC18r: TCTGCTGTATCCCATCTCACC
ADC19f: GGTGAGATGGGATACAGCAGA
ADC19r: GACCTCTAGAAGGGAGAGGACA
ADC20.2f: CTGCTTCCTTTCTTCCTTCCAC
ADC20.2r: AATTAGGCCCCTATGGGATCC
ADC21f: GACCTCTAGAAGGGAGAGGACA
ADC21r: TGAGAGATGTGTCAGGAAGGAGA
ADC22f: ACCCTCTCCTTCTGACACTATC
ADC22r: GTCCCACTAATAAGGCCCATCAC
ADC23r: GATGGTCGCTAAGCTGTGTTCAG
ADC24r: ATGGGAGATACAGGAGGACT
ADC25f: GGGAGAGTACAGGAGGACT
ADC25r: GGGGAAGAAGAAGAATTCCCACT

BCL2L2

BCLe2f: CCGGAGGACAGTCATTAACCAT
BCLe2r: GGTCTCCTTAGTCTATACCTTCT
BCLe3f: CCGGGTATGTCTGTGTTCATTA
BCLe3r: CAGTCCCATCCAATCTCTCTTC
BCLe4f: TCACATCAGCAACAACAGCA
BCLe4r: AGACCAGCTTTGCAGAAGGA

BM668528

BM668528f: TCACATCAGCAACAACAGCA
BM668528r: GAGCACACACTTAGGCCCACA

CYP1B1

CYP1B1_e1f: CAGTCCCTAAAAACCCGAGG
CYP1B1_e1r: CCACCCGCTACCTGTAAATACT
CYP1B1_e2p1f: ACCCAACGGCAGCTCAGTC
CYP1B1_e2p1r: CGATGGTGCCAGAAAGC
CYP1B1_e2p2f: ATAGTGGTCTGTAATGGCG
CYP1B1_e2p2r: GGAAGTACTGCAGCCAGGG
CYP1B1_e3p1f: CTATGGCATCC
CYP1B1_e3p1r: GGTGAGATGGGATACAGCAGA
CYP1B1_e3p2f: GCCTGTCACTATTCCTCATGC
CYP1B1_e3p2r: CAGCTTGCCTCTTGCTTCTTA
CYP1B1_e3p3f: TGTGAATCATGACCCAGTGAA
CYP1B1_e3p3r: TTCATTGGGCCCTTTAAGTCT

DAD1

DADe1f: CTGCACGATTAGTTGTTAGGC
DADe1r: GCAATGGCAGGCTCTTCTCATA
DADe2f: CAGCTTGCCTCTTGCTTCTTA
DADe2r: CAGTCCCATCCAATCTTCTTCT

ISGF3G

ISGe2f: GCCTTGAAAGCCAGCTTATGTG
ISGe2r: GCCTTGAAAGCCAGCTTATGTG
ISGe3f: GCCGTAAAGCCAGCTTATG
ISGe3r: ACAGAGAAAGGTCAGGCTT
ISGe4f  TGAGCCCTACAAGGTGTATCAGT
ISGe4r  CACCTCTCCCTCTGGTTACTGT
ISGe5y6f  CTGGGCAACAAGAGTGAAACT
ISGe5y6r  ACTGCCTAGGGCTATGGTAATGT
ISGe7f  GAGGGGAGGGAGTTGTTC
ISGe7r  GCCACACAGGATATGCAACA
ISGe8f  TGAGCCCTACAAGGTGTATCAGT
ISGe8r  CACCTCTCCCTCTGGTTACTGT
ISGe9f  CTGGGCAACAAGAGTGAAACT
ISGe9r  ACTGCCTAGGGCTATGGTAATGT

MMP14

MMPe1f  GAGAAGGGAGGGAGACAGAG
MMPe1r  AAAGCCCTCCTCTCCGAATA
MMPe2y3v2f  CATTGTCGGGGAGGTAGAGG
MMPe2y3v2r  CCTGCATAAGCACAATGGG
MMPe4v2f  GAATGTGCCCCCTTTTATCC
MMPe4v2r  GGGAAACCACCCCTAAAGAT
MMPe5y6v2f  GAGGCTGAGGGAGAGGAC
MMPe5y6v2r  ACCATGCCAGCCCATAG
MMPe7y8v2f  TGGGGGACTGAACCAGAGAC
MMPe7y8v2r  ACTGAGGGAATTTGGGGTG
MMPe9v2f  CACCTGTTGATTCGGTCC
MMPe9v2r  CCTGTAAGCTGTTGGAGG
MMPe10.1v2f  TAACAGAGCTTCCCTCGCTC
MMPe10.1v2r  TTTCACAGCGAGGTCTGAGG
MMPe10.2v2f  AGGGGAACCCT
MMPe10.2v2r  CTGCTTGGCTTGGCCTG

MYOC

MYOC_ex1aSf  CTCTGTTCCCTCTCCATGAAG
MYOC_ex1aSr  CTGGTCAAGGTCAAATTTGA
MYOC_ex1bSf  AGGGCAATGCAGTCAATGGG
MYOC_ex1bSr  AGCAGTGCTACTAGCCCATCT
MYOC_ex2f  ATAGTCAATCCCTGAGCATT
MYOC_ex2r  TCTGCTCCAGGGAGAAGTTAAT
MYOC_ex3aSf  CTCAGAGCTGGCCCTTTA
MYOC_ex3aSr  ATCCACAGCCAAGTCAATGGT
MYOC_ex3bSf  CTACCCTACACCAGGAGAC
MYOC_ex3bSr  TGTCAGGGTCCTTGACATAC
MYOC_ex3cSf  GACATGACATTGGCTGAGAT
MYOC_ex3cSr  GACCATGTTCATCCTCTGG

NPHP4

N4_reseq_R  GGATTTCCTCATGAGCTGGAA
N4fl501_1_RP_F  CGACCACTACGACAAGAC
N4fl501_1_RP_R  GTACAGCCGCAACCTTTTGT
N4fl501_2_RP_F  TTTCAGGGGACACAGACAG
N4fl501_2_RP_R  CAAGTTAACACAACAAATTTGCATTC

NRL

NRL_1f  CTCAGAGAGCTGGCCCTTTA
NRL_1r  CGACACCTGCTACCCCTTGAGA
NRL_2f  GAGGGGAGGGAGTTGTTC
NRL_2r  GCCACACAGGATATGCAACA
NRL_3_1f  GCTCTGGAACGAAACACAGACT
NRL_3_1r
NRL_3_2f
NRL_3_2r
NRL_3_3f
NRL_3_3r
NRL_3_4f
NRL_3_4r
NRL_Af
NRL_Ar
NRL_A1f
NRL_A1r
NRL_A2f
NRL_A2r
NRL_Bf
NRL_Br
NRL_Cf
NRL_Cr
NRL_C1f
NRL_C1r
NRL_in1.1f
NRL_in1.1r
NRL_in2.1f
NRL_in2.1r
OXA1L
OXAc1f
OXAc1r
OXAc2f
OXAc2r
OXAc3f
OXAc3r
OXAc4f
OXAc4r
OXAc5f
OXAc5r
OXAc6f
OXAc6r
OXAc7f
OXAc7r
OXAc8f
OXAc8r
OXAc9y10f
OXAc9y10r
PCK2
PCK2ex2f
PCK2ex2r
RPGRIP1
RPGRIP1_e1f
RPGRIP1_e1r
RPGRIP1_e2f
RPGRIP1_e2r
RPGRIP1_e3f
RPGRIP1_e3r
RPGRIP1_e4f
RPGRIP1_e4r

GTCA

GTCA

TCTC

CCCAGAGCTCAGCTG

TCTCTACAAGGCTCGCTGTG

CCCAGAGCTCACTCTTCAGG

TCTCTACAAGGCTCGCTGTG

CCCAGAGCTCACTCTTCAGG

GCAAATTGTCATCCCAGGAG

GCAAATTGTCATCCCAGGAG

TCTCTACAAGGCTCGCTGTG

TCTCTACAAGGCTCGCTGTG

CCCAGAGCTCACTCTTCAGG

GCAAATTGTCATCCCAGGAG

TCTCTACAAGGCTCGCTGTG

TCTCTACAAGGCTCGCTGTG

CCCAGAGCTCACTCTTCAGG

GCAAATTGTCATCCCAGGAG

TCTCTACAAGGCTCGCTGTG

GCAAATTGTCATCCCAGGAG

OXA1L

OXAc1f

CGAGGTCATGACATTCAGGT

CGAGGTCATGACATTCAGGT

GATAGCTAACCCAGCTCTTCAAC

GATAGCTAACCCAGCTCTTCAAC

CGAGGTCATGACATTCAGGT

CGAGGTCATGACATTCAGGT

GATAGCTAACCCAGCTCTTCAAC

GATAGCTAACCCAGCTCTTCAAC

CGAGGTCATGACATTCAGGT

CGAGGTCATGACATTCAGGT

GATAGCTAACCCAGCTCTTCAAC

GATAGCTAACCCAGCTCTTCAAC

CGAGGTCATGACATTCAGGT

CGAGGTCATGACATTCAGGT

OXAc4r

TCTACTTACCTCAGCTCTGAG

TCTACTTACCTCAGCTCTGAG

GACCTTAATTCTGGCGCTCTTAG

GACCTTAATTCTGGCGCTCTTAG

TCTACTTACCTCAGCTCTGAG

TCTACTTACCTCAGCTCTGAG

GACCTTAATTCTGGCGCTCTTAG

GACCTTAATTCTGGCGCTCTTAG

TCTACTTACCTCAGCTCTGAG

TCTACTTACCTCAGCTCTGAG

GACCTTAATTCTGGCGCTCTTAG

GACCTTAATTCTGGCGCTCTTAG

OXAc5f

TAGACCTAATGCTCCAAGGAGT

TAGACCTAATGCTCCAAGGAGT

OXAc5r

CTACCTTACCHCAATTCCAGT

CTACCTTACCHCAATTCCAGT

OXAc6f

AACCCTCTCATTCTCCCCTGTGA

AACCCTCTCATTCTCCCCTGTGA

OXAc6r

CTACTTACCHCAATTCCAGT

CTACTTACCHCAATTCCAGT

OXAc7f

GGGATATGGGGAAGTAAAGATGTTG

GGGATATGGGGAAGTAAAGATGTTG

OXAc7r

GAACACAGAAGAAGGACTCAGAAA

GAACACAGAAGAAGGACTCAGAAA

OXAc8f

AGTTCTGACCTCAGTGGATGAG

AGTTCTGACCTCAGTGGATGAG

OXAc8r

GCCGTGTAAGAGAAGAAGCAAGAG

GCCGTGTAAGAGAAGAAGCAAGAG

OXAc9y10f

AAGGTAAGGGCCTATCCTCCTGT

AAGGTAAGGGCCTATCCTCCTGT

OXAc9y10r

ACATCTCTGTGTGCCACAGTTC

ACATCTCTGTGTGCCACAGTTC

PCK2

PCK2ex2f

TGCA

PCK2ex2r

GGTCTGTTTACCCCA

GGTCTGTTTACCCCA

RPGRIP1

RPGRIP1_e1f

RPGRIP1_e1r

RPGRIP1_e2f

RPGRIP1_e2r

RPGRIP1_e3f

RPGRIP1_e3r

RPGRIP1_e4f

RPGRIP1_e4r

RPGRIP1_e4f
SALL2

SALe1f: TTACAATGGGAGCTGCAGAA
SALe1r: CCCTGCATCTCAACTCCTTCC

SALe2_1f  ACCCCCAACTAGCGGTTACT
SALe2_1r  TTCTGGAGGTAATGGGTGTTG
SALe2_2f  GAGGAGTCTCCAGGGGCAATT
SALe2_2r  ATGAGGCGAGGCAATCAG
SALe2_3f  GCATCCTTCTCTGCTGGAG
SALe2_3r  GTTGCCTCTGTACTGGGTGT
SALe2_4f  GTGGCAACCTCAAATGTGAC
SALe2_4r  TTCTGGAGGTAATGGGGTTG
SALe2_5f  AGGAGTCTCCAAGGCATTT
SALe2_5r  CTTGCGGCTCAATCTTCTCT
SALe2_6f  GCCTCACCCTCTGACACATC
SALe2_6r  TTCTGTGCTGCTCTCCTCA
SALe2_7f  AGAGAGCAGCAGCAGAAAGG
SALe2_7r  AAGGGTCAACCAGGGGAAAG

ZNFe2f  GGGTAGGGAGTGACTTTACTGCT
ZNFe2r  GGCAGGAGAGGAGTATACAGTT
ZNF3p1f  CTTCACCCCTTGCTCTACGC
ZNF3p1r  GCCTCCCAAATCCAGCAAC
ZNFe3p2f  GTGCTGGAGTTGGAAGAGGC
ZNFe3p2r  GGCCTTGAGAAACCAAGAC
ZNFe3p3f  AGGCCGAACCGAGATCAGT
ZNFe3p3r  CCACCTCTCTCCTCCCTCA
ZNFe3p4f  AAGAGAGGAGGTGGGAGTG
ZNFe3p4r  AAGGAAGCTACGAGGGAGTG
ZNFe4f  TTCAACCTCTAGTGTTTCGTTAG
ZNFe5p1f  AAATCAAAGTGTTGGGAGAG
ZNFe5p1r  GAAGAGCAGGCGGTGGAG
ZNFe5p2f  AACCCCTGGACCTGTCCTT
ZNFe5p2r  AAGCCCTGGACCTGTCCTT

Other regions

M13f  GTAAAACGACGCGCCAG
M13r  CAGGAACAGCTATGAC
GAPDHf  GTGGAGTCCACTGGCGTCTTC
GAPDHr  CTCCGAGCGCCTGTCCACAC
T3  ATTAACCCCTACTAAAGGGA
T7  TAATACGACTCACTATAGGG

ZNFe3p3f  AGGCCGAACCGAGATCAGT
ZNFe3p3r  CCACCTCTCTCCTCCCTCA
ZNFe3p4f  AAGAGAGGAGGTGGGAGTG
ZNFe3p4r  AAGGAAGCTACGAGGGAGTG
ZNFe4f  TTCAACCTCTAGTGTTTCGTTAG
ZNFe5p1f  AAATCAAAGTGTTGGGAGAG
ZNFe5p1r  GAAGAGCAGGCGGTGGAG
ZNFe5p2f  AACCCCTGGACCTGTCCTT
ZNFe5p2r  AAGCCCTGGACCTGTCCTT
5. Results

This thesis was aimed to the identification of new genes predisposing to POAG in German patients not carrying any known POAG-causing mutation. For this purpose, all patients were screened for mutations in MYOC, CYP1B1, OPTN, and WDR36, the known glaucoma-causing genes. In order to identify novel predisposing genes, a candidate gene approach was followed and ten selected genes were screened for mutations by direct sequencing in an exploratory collective of 46 patients and 46 controls. Based on the in silico characterization of the mutations found in the coding region of these genes, RPGRIP1 was prioritized as the best candidate. Therefore, an extended screening of 399 POAG patients and 376 controls, together with a further replication study in 383 POAG patients and 104 controls, systematic linkage disequilibrium analysis and functional studies for RPGRIP1 were performed.

5.1. Screening of MYOC and CYP1B1 in POAG patients

At the time this project was starting, only a small collective of 46 POAG patients was available for the study. I performed the mutation screening of MYOC and CYP1B1 in this collective. The rest of the cohort (399 patients) was screened for mutations in these two genes and also in OPTN and WDR36 as soon as they were recruited. In this thesis, I report the results of the initial mutation screening of MYOC and CYP1B1 in the first collective comprising 46 POAG patients.

Variation in the coding sequence of MYOC was found in 8 out of 46 cases, presenting every individual only one variant. Two patients presented the p.R76K (rs2234926) polymorphism and one more the synonymous variant p.Y347Y. Four patients carrying a p.T243P variant were identified and also one carrier of a p.Q368X mutation, accounting together for the 10.7% of the cases (5/46). The rest presented no mutation. No functional characterization of the variants found was performed, due to the lack of a validated functional assay for myocilin.

Five coding SNPs (p.R48G, p.A119S, p.V432L, p.D449D and p.N453S) were found in CYP1B1, always in heterozygous form. Three mutations (p.G61E, p.Y81N and p.E229K) were also identified, accounting for 8.7% of the cases (4/46). The rest presented no mutation. The results are summarized in Table 5.1.
### Table 5.1. Sequence variants detected in MYOC and CYP1B1 in the exploratory collective.

If corresponding to a SNP, rs number is also indicated.

The extended systematic mutation screening of the whole collective of 399 POAG patients and 376 controls led to the identification of 11 amino acid substitutions in CYP1B1, apart from known polymorphic variants (Pasutto et al. 2009). In order to study the biological effect of selected CYP1B1 mutations, a functional characterization of the enzymatic activity of the protein was performed in collaboration with Gabriela Chavarria-Soley, from the group of congenital glaucoma at the Institute of Human Genetics at the Friedrich-Alexander-Universität Erlangen-Nürnberg. Each mutation was embedded in its corresponding founder SNP background haplotype, consisting of 5 frequent coding SNPs (p.R48G, p.A119S, p.V432L, p.D449D and p.N453S) in which they occur in the normal population (Stoilov et al. 2002). A marker decrease of the relative enzymatic activity (11%) was observed for variant p.G61E which was classified as *bona fide* mutation. Variant p.Y81N showed an intermediate reduction in activity (17%), thus leading to its classification as hypomorphic alleles. On the other hand, variant p.E229K was classified as polymorphism, as its frequency was not statistically different between POAG patients (2%) and controls (3%), despite its milder effect (26%) on relative enzymatic activity. When including only the variants with impaired function, a total of 13 patients (3.6%) and 1 healthy subject (0.2%) were found. Thus

<table>
<thead>
<tr>
<th>Gene</th>
<th>Coding variants</th>
<th>Patients (46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYOC</td>
<td>p.R76K rs2234926</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>p.T243P</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>p.Y347Y</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>p.Q368X</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>8</strong></td>
</tr>
<tr>
<td>CYP1B1</td>
<td>p.R48G</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>p.G61E</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>p.Y81N</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>p.A119S rs1056827</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>p.E229K</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>p.V432L rs1056836</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>p.D449D</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>p.N453S rs1800440</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td><strong>132</strong></td>
</tr>
</tbody>
</table>
CYP1B1 mutation frequency in POAG patients is significantly higher in patients than in controls (p=0.0018, Fischer’s exact test; OR=5.4, 95%CI= 1.9 ±15.5), providing evidence of a strong association of these mutations with the disease (Pasutto et al. 2009).

5.2. Screening of candidate genes on chromosome 14q11-q12

In a genome-wide scan involving an initial pedigree set of 113 affected sib-pairs and a second pedigree set of 69 affected sib-pairs, putative loci on 2p14, 14q11, 14q21-q22, 17p13, 17q25, and 19q12-q14 were linked to POAG (Wiggs et al. 2000). In this thesis, the candidate region 14q11, covering 6.3 Mb between markers D14S261 and D14S121, was searched for genes presenting adequate pattern of expression, appropriate function and/or structural similarity with the known glaucoma-causing genes. I found a total of 10 genes out of 266 which met these criteria (Figure 3.1.).

![Figure 3.1](image.png)

Figure 3.1. View of the UCSC Genome Browser for chromosome 14q11.2, showing RefSeq Genes. Red boxes indicate the selected candidate genes.

Systematic screening of these positional and functional candidates was performed in an exploratory collective of 46 German POAG patients. Direct sequencing of 5’-3’ UTRs, exons
and flanking intronic regions led to the identification of 156 SNPs, 46 of them with a minor allele frequency (MAF) >0.15. From the 37 variants found within the coding regions, 10 were nonsynonymous changes not previously reported in any database. Each variation was found in one different patient. The results are summarized in Table 5.2.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>mRNA length</th>
<th>Identified SNPs</th>
<th>SNPs MAF&gt;0.15</th>
<th>Coding variants</th>
<th>Novel non synonymous variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZNF219</td>
<td>3040</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>p.E251-P252del p.H462Q</td>
</tr>
<tr>
<td>SALL2</td>
<td>1624</td>
<td>9</td>
<td>1</td>
<td>6</td>
<td>p.D770N</td>
</tr>
<tr>
<td>DAD1</td>
<td>683</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>MMP14</td>
<td>2335</td>
<td>18</td>
<td>9</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>BCL2L2</td>
<td>3510</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>NRL</td>
<td>2102</td>
<td>16</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>ISGF3G</td>
<td>4798</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>ADCY4</td>
<td>3415</td>
<td>26</td>
<td>12</td>
<td>3</td>
<td>p.S373R</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>156</strong></td>
<td><strong>46</strong></td>
<td><strong>37</strong></td>
<td><strong>10</strong></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2. Summary of the variations found in the initial screening of 46 German POAG patients.

The novel variants were further characterized. Three amino acid changes (ZNF219-p.E251-P252del, OXA1L-p.S482dup and ADCY4-p.S373R) were identified also in a control collective of 46 individuals. Five of the remaining variants (ZNF219-p.H462Q, SALL2-p.D770N, OXA1L-p.I292L, OXA1L-p.R436I) affected amino acid positions not conserved among different mammalian species (human, mouse, rat, cow and chimpanzee). In contrast, the three missense variants found in RPGRIP1 (p.R363T, p.R812H and p.G1240E) were both not present in controls and affecting evolutionary conserved amino acids (Figure 5.2).
Different bioinformatics tools were also used in order to investigate *in silico* if these variants altered promotor or transcription factor binding sites, but none of them seemed to affect them.

### 5.3. RPGRIP1

After the systematic characterization of the coding variants found in the screening of the exploratory collective of patients, RPGRIP1 arose as a potentially good candidate, so I focused on further characterizing this gene, both genetically and functionally.

#### 5.3.1. RPGRIP1 haplotype block structure

A total of 84 SNPs with MAF>0.05 were identified by direct sequencing of 27 amplicons covering a region spanning 34Kb in 46 patients. From these, the 14 SNPs showing MAF>0.15 were used to determine the haplotype block structure of RPGRIP1. Two blocks were identified using Haploview software (Figure 3.3.A). These data correlates well with the LD structure for this gene published by HapMap (Figure 3.3.B).
Figure 3.3. **Linkage disequilibrium (LD) structure of RPGRIP1.** Each square plots the level of linkage disequilibrium (LD) between a pair of SNPs in the region; comparison between neighbouring SNPs lies along the first line. Red colouring indicates strong LD, blue indicates intermediate or uninformative LD, and white indicates weak LD. The area limited by a black line show the LD-block. *(A)* LD plot constructed with the SNPs at MAF>0.15 identified in this study. *(B)* LD plot from the HapMap Project. The black line corresponds to the blocks from our study.

### 5.3.2. Association of RPGRIP1 with POAG in German patients

Screening for mutations was extended to 399 POAG patients originating from the same German region, leading to the identification of 14 amino acid substitutions in RPGRIP1 (Table 5.3.). Together, the missense variants accounted for 6.5% of the patient population (26/399). Five of these variants were detected in 8 out of 376 control subjects, accounting for only 2.1% of the control group (8/376). All patients were negative for *MYOC, OPTN, CYP1B1* or *WDR36* mutations. This data provided association of RPGRIP1 with POAG in this collective (*p*-value=0.003, 2-Tail Fischer’s exact test) with odds ratio (OR) =2.8 (95% CI=1.4±5.5).
Table 5.3. **RPGRIP1 sequence variants found in patients and control individuals from the first collective.** * indicates those variants located in or very near to the C2 domains of RPGRIP1 protein.

The group of patients carrying mutations comprised both late juvenile and adult onset POAG with age at diagnosis varying from 24 to 81 years (Table 5.4.). Among them, 22 had elevated maximum IOP ranging from 22 to 40 mmHg, while 6 had pressure measurements in the normal range.
Table 5.4. Clinical data from patients harbouring a mutation in RPGRIP1.

### 5.3.2.1. Replication study

Interestingly, more than half of the mutations (8/14) were located nearby the region coding for the C2 domains of the protein. To replicate the observed association data, I screened the coding region of the RPGRIP1 C2 domains in a further German cohort of 383 glaucoma patients (304 NTG and 79 POAG) and 104 control subjects. Six amino acid substitutions were identified in 9 patients (2.3%) and in 2 control subjects (1.9%) (Table 5.5.). Altogether, the
distribution of RPGRIP1 C2 domain variants found in German patients from both cohorts remained statistically significant between patients and controls ($p$-value=0.013, 2-Tail Fischer’s exact test; OR=2.5, 95% CI=1.2±5.3).

<table>
<thead>
<tr>
<th>Nucleotide alteration</th>
<th>Amino acid change</th>
<th>Patients (383)</th>
<th>Controls (104)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.1808G&gt;C</td>
<td>p.C603S</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>c.1913C&gt;T</td>
<td>p.T638I</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>c.2441G&gt;T</td>
<td>p.R814L</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>c.2521G&gt;A</td>
<td>p.A841T</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>c2555G&gt;A</td>
<td>p.R852Q</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>c.2648G&gt;A</td>
<td>p.G883D</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Subtotal</strong></td>
<td></td>
<td><strong>9</strong></td>
<td><strong>2</strong></td>
</tr>
<tr>
<td>**Total ***</td>
<td></td>
<td>(n=782)</td>
<td>(n=480)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 5.5. **Amino acid alterations identified in the C2 domains of RPGRIP1 in patients and control individuals from the replication cohort.** * refers to the total of patients and controls from the first screening and the replication study carrying variations in the C2 domains of RPGRIP1.

The group of patients from the replication group carrying mutations comprised late onset POAG with age at diagnosis varying from 44 to 88 years (Table 5.6.). Among them, only one had elevated IOP; the rest presented pressure measurements in the normal range.
<table>
<thead>
<tr>
<th>Subject ID</th>
<th>RPGRIP1 variant</th>
<th>Phenotype</th>
<th>Age at diagnosis (years)</th>
<th>MAX IOP (mmHg; R/L)</th>
<th>Optic disc (Jonas)</th>
<th>Disc area (mm2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>154</td>
<td>C603S</td>
<td>NTG</td>
<td>50</td>
<td>10/10</td>
<td>0/0</td>
<td>2.4/2.4</td>
</tr>
<tr>
<td>210</td>
<td>T638I</td>
<td>NTG</td>
<td>65</td>
<td>17/16</td>
<td>0/I</td>
<td>ND</td>
</tr>
<tr>
<td>58</td>
<td>R814L</td>
<td>POAG</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>290</td>
<td>A841T</td>
<td>NTG</td>
<td>29</td>
<td>23/23</td>
<td>I/0</td>
<td>0.7/0.7</td>
</tr>
<tr>
<td>24</td>
<td>R852Q</td>
<td>NTG</td>
<td>78</td>
<td>19/18</td>
<td>I/I</td>
<td>0.7/0.7</td>
</tr>
<tr>
<td>47</td>
<td>R852Q</td>
<td>NTG</td>
<td>67</td>
<td>18/19</td>
<td>III/0</td>
<td>2.4/2.1</td>
</tr>
<tr>
<td>148</td>
<td>R852Q</td>
<td>NTG</td>
<td>88</td>
<td>15/16</td>
<td>ND</td>
<td>1.9/1.7</td>
</tr>
<tr>
<td>149</td>
<td>R852Q</td>
<td>NTG</td>
<td>83</td>
<td>20/18</td>
<td>I/II</td>
<td>2.6/2.6</td>
</tr>
<tr>
<td>292</td>
<td>G883D</td>
<td>NTG</td>
<td>44</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 5.6. Clinical data from patients from the replication cohort harbouring a mutation in the C2 domains of RPGRIP1.

5.3.3. Segregation analysis of RPGRIP1 coding variants.

Due to the availability of some relatives, familiar segregation could be studied in 6 cases (p.439X, p.P585S, p.Q589H, p.R812H, p.A837G and p.I838V). In most of them, no helpful information could be extracted due to the lack of DNA from affected relatives or to the absence of any other affected members in the families. This was not surprising, due to both the complex character of POAG (with reduced penetrance) and the late onset of the disease (with reduced possibility of finding elder relatives to study the segregation of the mutations). The pedigree of patient 10540 illustrates well these problems: the index patient was the only available case, and although one carrier was identified (Figure 5.4., individual IV:2), this person was non affected, but still young (28) to make a certain diagnosis. This person might still develop the disease at a later age.
Figure 5.4. Pedigree from patient 10540 carrying the p.R812H variation in RPGRIP1. Index patient (10540) is marked with a red square. The sequence variant could not be found in the family members III:12 (99044), III:15 (22639), III:16 (22709), and IV:1 (99042). Individual IV:2 (99041) carries the variant. DNA from the rest of relatives was not available for the study.

5.3.4. Mutations in RPGRIP1 disrupt interaction with NPHP4 in POAG patients

Once the association of RPGRIP1 with POAG was established, I next focused on investigating the functional relevance of some of the mutations found. This work was made in collaboration with Ronald Roepman from the Nijmegen Centre for Molecular Life Sciences at the Radboud University of Nijmegen (The Netherlands), who has previously reported RPGRIP1 to interact with NPHP4 through its C2 domains (Roepman et al. 2005). Due to the availability of functional assays to verify this interaction, a subset of 8 amino acid substitutions located in or very near to the interacting domain of RPGRIP1 could be functionally characterised. To investigate the effect of these RPGRIP1 variations in the interaction with NPHP4, the mutations were introduced in the appropriate expressing vectors and analyzed using the yeast two-hybrid system. To complement these results in a cell-based assay, coimmunoprecipitation and colocalization studies were performed.

5.3.4.1. Yeast two-hybrid system

The different reporter genes from the yeast two-hybrid system previously described (see 4.5. Yeast two-hybrids experiments) were assayed in order to verify the interaction between RPGRIP1 and NPHP4.
5.3.4.1.1. Qualitative growth on SD-WLHA plates

Only yeast cells cotransformed with both pAD/RPGRIP1 and pBD/NPHP4 are able to grow on dropout plates containing SD-agar –Leu –Trp –His –Ala, as result of the activation of the HIS3 and ADE2 reporter genes. In this study, RPGRIP1 mutants p.Q589H, p.A764V, and p.R812H retained the ability to grow on a SD-WLHA selection plate. In contrast, RPGRIP1\textsuperscript{C2-N+C2-C} carrying the mutation p.R598Q was not able to grow on the dropout selection plate, indicating a severely decrease in the interaction of RPGRIP1 with NPHP4. Mutants p.A635G, p.T806I, p.A837G, and p.I838V showed a mildly low ability to grow, although not much different from the wild-type. As a negative control, a truncated RPGRIP1 construct carrying a nonsense mutation p.R890X and therefore not able to interact with NPHP4 was used.

5.3.4.1.2. α-galactosidase activity

Activation of the MEL1 reporter gene enables the yeast cells to produce α-galactosidase, causing yeast colonies to develop a blue colour. The α-galactosidase activity of RPGRIP1 mutants p.Q589H, p.A764V, and p.R812H was not different from that of the control RPGRIP1\textsuperscript{C2-N+C2-C} when interacting with NPHP4. RPGRIP1s p.A635G, p.T806I, p.A837G and p.I838V showed a reduced enzymatic activity. On the other hand, p.R598Q-RPGRIP1 and the negative control presented not detectable α-galactosidase activity.

5.3.4.1.3. β-galactosidase activity

Qualitative and quantitative assays were performed to report the β-galactosidase activity, which can be regarded as a measure of the binding affinity of the interaction between RPGRIP1 and NPHP4 proteins.

5.3.4.1.3.1. Qualitative filter lift assay

When a filter lift β-galactosidase assay is performed on a plate, clones produce a blue product as result of the lacZ reporter gene activation. As shown in Figure 5.5., p.R589Q-RPGRIP1 (nr. 2) did not activate the β-galactosidase reporter in the filter lift assay, suggesting absence of interaction between this RPGRIP1 mutant and NPHP4. As expected, the same result was obtained for the negative control RPGRIP1-p.R890X. On the other hand, RPGRIP1 variants p.A764V and p.R812H manifested a strong activation of the β-galactosidase and therefore interaction between RPGRIP1 and NPHP4. The RPGRIP1 mutants p.A635G, p.T806I, p.A837G, and p.I838V presented a reduction in the enzymatic activity in comparison with the wild-type.
5.3.4.1.3.2. Quantitative β-galactosidase enzyme activity assay

An ONPG assay was used to quantify the β-galactosidase activity of the yeast cells, as result of the lacZ reporter gene activation. As a negative control, p.R890X-RPGRIP1 was used, indicating the somewhat leaky activation of this reporter gene without selection for transactivation. Binding with NPHP4 was severely disrupted when the RPGRIP1\textsuperscript{C2-N+C2-C} fragment contained the p.R598Q mutation. Although milder, an impaired interaction between the two proteins was also revealed by RPGRIP1 variants p.A635G, p.T806I, p.A837G and p.I838V. In contrast, RPGRIP1s p.Q589H, p.A764V and p.R812H did not cause any decrease in the interaction between RPGRIP1 and NPHP4, presenting similar β-galactosidase activity to that of the wild-type protein.
Figure 5.6. **Quantification of NPHP4-RPGRIP1 interactions by a liquid β-Galactosidase assay.** The black bars indicate the average enzymatic activity (in arbitrary units). The error bars show standard deviation.

### 5.3.4.2. Coimmunoprecipitation

In order to establish if the interaction of RPGRIP1 with NPHP4 was affected by any of the mutations, epitope-tagged full-length NPHP4 (N4^{FL}) and RPGRIP1^{C2-N+C2-C} constructs were expressed in COS-1 cells and coimmunoprecipitation assays using anti-FLAG antibodies were performed (Figure 5.7.).

As expected, HA-NPHP4^{FL} clearly coimmunoprecipitated with FLAG-RPGRIP1^{C2-N+C2-C} (lane 9). The negative control, Flag-tagged leucine-rich repeat kinase-2 fragment (LRKK2^{LRR}) (40kDa) did not coimmunoprecipitate with HA-NPHP4^{FL} (lane 10) indicating that the coimmunoprecipitation of RPGRIP1^{C2-N+C2-C} was specific. The RPGRIP1 p.R598Q alteration severely disrupted the interaction with NPHP4 (lane 2), suggesting a pathologic character of this variant. Introduction of the other amino acid exchanges showed no significant effect on the RPGRIP1-NPHP4 interaction (lanes 1, 3-8).
Figure 5.7. Immunoprecipitation (IP) of wild-type and mutated FLAG-RPGRIP1\textsubscript{C2-N+C2-C} and HA-NPHP4\textsubscript{FL}. Coimmunoprecipitation is shown in Top. The middle two blots show 6% input of the COS-1 lysate protein mixtures as well as immunoprecipitation of HA-tagged NPHP4\textsubscript{FL} with anti-HA beads.

5.3.4.3. Colocalization of RPGRIP1 and NPHP4 in COS-1 cells

In COS-1 cells expressing only the full-length NPHP4 fused to mRFP, the protein was localized in specific structures around, but not in, the nucleus. In cells only transfected with RPGRIP1\textsubscript{C2-N+C2-C}-eCFP, the protein was localized in the nucleus. Coexpression of NPHP4\textsubscript{FL} with RPGRIP1\textsubscript{C2-N+C2-C} fully retained the latter to the cytoplasm, resulting in the in vivo colocalization of both proteins. Coexpression of NPHP4 with RPGRIP1 mutants p.Q589H, p.A635G, p.A764V, p.T806I, p.R812H, p.A837G, or p.I838V resulted in the colocalization of both proteins in the cytoplasm as well. Interestingly, in cells expressing both NPHP4 and p.Q589H-RPGRIP1, both proteins colocalized in the cytoplasm but a substantial nuclear signal could also be detected for the RPGRIP1 mutant, suggesting that this amino acid substitution influenced the (co)localization results (Figure 5.8.).
Figure 5.8. Colocalization of RPGRIP1 and NPHP4 upon overexpression in COS-1 cells. (A) DAPI staining of the cell nuclei (blue signal). (B) single transfection of mRFP-NPHP4 (red signal). (C) single transfection of eCFP-RPGRIP1 (green signal). (D-F) Coexpression of both RPGRIP1 and NPHP4 wild-type proteins in the same cell. The dashed line delimitates the nuclear compartment. (D, mRFP signal; E, eCFP signal; F, overlay of D-E). (G-I) Coexpression of p.R598Q-RPGRIP1 and NPHP4 in the same cell (G, mRFP signal; H, eCFP signal; I, overlay of G-H).

5.3.4.4. Classification of the RPGRIP1 variants

In view of the results of the different yeast two-hybrid experiments together with the coimmunoprecipitation and colocalization assays, I was able to determine the functional relevance and systematically classify those RPGRIP1 variants located within the C2 domains of the protein, as summarized in table 5.7.
<table>
<thead>
<tr>
<th>Nucleotide alteration</th>
<th>Amino acid change</th>
<th>Patients (399)</th>
<th>Controls (376)</th>
<th>Functional classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.1767G&gt;T</td>
<td>p.Q589H</td>
<td>4</td>
<td>2</td>
<td>polymorphism</td>
</tr>
<tr>
<td>c.1793G&gt;A</td>
<td>p.R598Q</td>
<td>3</td>
<td>0</td>
<td><em>bona fide</em> mutation</td>
</tr>
<tr>
<td>c.1904C&gt;G</td>
<td>p.A635G</td>
<td>2</td>
<td>0</td>
<td><em>bona fide</em> mutation</td>
</tr>
<tr>
<td>c.2291C&gt;T</td>
<td>p.A764V</td>
<td>1</td>
<td>0</td>
<td>polymorphism</td>
</tr>
<tr>
<td>c.2417C&gt;T</td>
<td>p.T806I</td>
<td>1</td>
<td>0</td>
<td><em>bona fide</em> mutation</td>
</tr>
<tr>
<td>c.2435G&gt;A</td>
<td>p.R812H</td>
<td>1</td>
<td>1</td>
<td>polymorphism</td>
</tr>
<tr>
<td>c.2510C&gt;G</td>
<td>p.A837G</td>
<td>2</td>
<td>0</td>
<td><em>bona fide</em> mutation</td>
</tr>
<tr>
<td>c.2512A&gt;G</td>
<td>p.I838V</td>
<td>2</td>
<td>0</td>
<td><em>bona fide</em> mutation</td>
</tr>
</tbody>
</table>

Table 5.7. Identified and functionally validated RPGRIP1 mutations and polymorphisms.

The β-galactosidase activities of the RPGRIP1 p.Q589H, p.A764V and p.R812H variants were similar to that of the wild-type protein, indicating that these variants probably do not alter the interaction between RPGRIP1 and NPHP4. The results obtained in the qualitative yeast two-hybrid and colocalization assays, suggesting interaction between both proteins, corroborated also the classification of these variants as non pathological polymorphisms. An impaired interaction with NPHP4 was, however, revealed by RPGRIP1 p.A635G, p.T806I, p.A837G and p.I838V variants, which suppressed the enzymatic activity to a similar level to that of the negative control p.R890X. In addition, variant p.R598Q resulted in an even lower enzymatic activity than p.R890X, implying a complete disruption of the RPGRIP1-NPHP4 interaction, which could be confirmed by coimmunoprecipitation and colocalization assays. This led to their classification as *bona fide* mutations.

The rest of the variants identified in the mutation screening and located far from the C2 domains of RPGRIP1 could not be characterized, as there is no functional assay available at this time. The group of our collaborator Ronald Roepman is currently working on this issue.

To summarize, the association and functional results herein reported suggest that rare heterozygous loss of function variants in RPGRIP1 are a risk factor for POAG and reaffirm the hypothesis that genetic predisposition to this disease is mainly cause by rare variants rather than common SNPs.
5.3.5. **RPGRIP1 undergoes significant alternative splicing**

Different RPGRIP1 isoforms have been reported in the literature (Mavlyutov et al. 2002; Castagnet et al. 2003; Lu and Ferreira 2005). In order to identify novel splice variants, primers spanning the whole genomic sequence of RPGRIP1 were used to perform RT-PCR on cDNA from different human eye tissues and blood. Using primers complementary to exon 12 and 14 previously reported (Lu and Ferreira 2005) five alternative splicing isoforms could be identified in sclera, retina and blood (Figure 5.9.).

![RT-PCR of RPGRIP1 comprising exons 12-14 from different human cDNA eye tissues](image)

**Figure 5.9. RT-PCR of RPGRIP1 comprising exons 12-14 from different human cDNA eye tissues.** (1) sclera. (2) choroids. (3) cornea. (4) retina. (5) blood.

The amplicons comprised 223, 256, 330, 440 and 650 bp, with the one with 256 bp being the most abundant and encoding the canonical exons 12, 13, and 14. Sequence analysis also revealed that the isoform with 223 bp had exon 13 truncated by 33 nucleotides, leading to an in-frame deletion of 11 amino acids. In isoform 650 bp, the whole intron 13 (450 bp) was translated, resulting in an in-frame insertion of 150 amino acids. Isoforms 330 and 440 contained in each case the first 117 and 246 nucleotides of intron 12, coding for in frame insertions of 39 and 82 amino acids respectively (Figure 5.10). A replication of these results with a different primer pair could not be performed due to time constraints as I concentrated on the functional validation of the variants found in the screening.
Figure 5.10. Summary of mRNAs detected by sequencing of RT-PCR products overlapping coding regions of exons 12 and 13 of RPGRIP1. RefSeq exons 12-14 of RPGRIP1 (top) and five isoforms identified (bottom) are shown.
6. Discussion

6.1. Genetics of POAG as a complex disease

Unlike single gene Mendelian diseases, complex genetic disorders (such as asthma, diabetes, schizophrenia and glaucoma) are caused by the combined effect of multiple variants in a number of genes coupled with environmental factors. Despite the recent improvements in the throughput of genetic and genomic techniques and the increased availability of gene and marker data, we remain largely ignorant of the overall genetic architecture of complex traits like POAG, including the total number of genes, the typical effect sizes for risk alleles, and the genetic interactions among them.

6.1.1. Study design

There are a number of approaches to disease gene identification and many arguments to support the merits of one strategy over another (see also 3.1.2. Methods for genetic dissection of complex diseases). Their relevance in dissecting glaucoma is discussed below.

The application of a linkage approach depends on the availability of large pedigrees or, if not possible, in combining data from a large number of affected sibpairs, permitting the identification of genes responsible for a disease without any preconception of the biological mechanisms underlying the disorder. This strategy led to the identification of the three currently known POAG causing genes (MYOC, OPTN and WDR36). Although these genes with large effect were found segregating with the disease in pedigrees, in the majority of the sporadic cases occurring in the general population the underlying genetic cause of the disease remains unknown and these three genes do not play a significant role, due to the high heterogeneity attributable to complex traits, in which each gene with phenotypic relevance is thought to make a relative small contribution to the overall disease susceptibility. In the absence of unfeasibly large sample sizes, these small effects are likely to be below the threshold of detection (Risch 2000). In order to circumvent this problem, the threshold of acceptable LOD score is typically relaxed from 3 to 2, or sometimes even lower (Pericak-Vance et al. 1998). Subsequently, we would expect to see a number of hits due to chance alone with a comprehensive genome scan at this threshold. If this first problem is solved and a statistically significant evidence of linkage is obtained, extensive candidate gene studies are still required to identify the causal gene within the region. Despite of being successful in the identification of glaucoma genes until now, I consider that conventional linkage analysis is
not the ideal strategy for glaucoma research, as it depends on the availability of large pedigrees, which are especially difficult to recruit for late onset diseases such as glaucoma, in which most of the cases appear sporadically in unrelated patients. It may explain, at least partially, why only three causative genes for POAG were identified after 16 years of research. In the last few years, association studies based on genotyping have demonstrated potential for identifying SNPs and haplotypes associated with a range of common clinical phenotypes, such as myocardial infarction, breast cancer or age-related macular degeneration. Different projects aim to identify as much as possible of the underlying genetic variation in human populations, including the International HapMap Project (www.hapmap.org) or the 1000 Genomes Project (www.1000genomes.org). However, only a small fraction of observed phenotypic variation is currently attributable to identified allelic variants, and only a few of the many risk loci reported have been confirmed and replicated. Furthermore, once the association has been identified, fine localization of the risk variants and functional characterization of their biological effect are still needed. Reported associated alleles with POAG show marginal $p$-values and subsequent studies fail to replicate the initial findings. An adequate phenotyping and good characterization of patients and controls is also crucial in any association study. The main sign of glaucoma is cupping of the optic nerve head, but this observation is somewhat subjective and difficult to diagnose early in the disease. Subsequently, young people are often not well characterised and the diagnosis can vary also between ophthalmologists. Lack of success of the association approach in finding genes responsible for POAG may be due to all these different factors playing together. The need of application of objective diagnostic criteria for glaucoma worldwide and establishment of large international collaborations to achieve the high number of patients and controls required for such association studies becomes therefore clear in order to detect the low effects supposed to this kind of trait.

An alternative option to the traditional linkage and association approaches are candidate gene resequencing studies, which directly test the association between a particular allele of a gene that may be involved in the disease (i.e., a candidate gene) and the disease itself. My search for glaucoma genes exemplifies the pros and contras of this kind of strategy. Briefly, I looked for candidate genes in a region with reported linkage to POAG in genome-wide linkage scans and performed mutation analysis in a large group of patients and controls. The first difficulty arose by the concept of candidate gene itself. The perfect candidate should play a relevant role in the disease under investigation. However, not much is known about the cellular and biochemical events that are necessary for retinal ganglion cell function/survival or regulation.
of intraocular pressure. In this thesis, genes considered as potentially good candidates for POAG are genes that modulate apoptosis, together with genes expressed in the eye and/or sharing protein domains or interacting with known glaucoma genes, although the question of how representative the pathogenesis of MYOC, OPTN or WDR36 glaucoma is for the majority of sporadic POAG cases remains still open. In addition, the selected candidate genes were also positional candidates because they map to the reported glaucoma linked locus 14q11 (Wiggs et al. 2000). The second challenge lies in discerning which sequence variants are pathogenic and which are simply polymorphisms. Amino acid replacements found in candidate genes were analyzed for the biochemical severity of the missense changes, the localization and/or context of the altered amino acid in the protein sequence and their degree of evolutionary conservation. In addition, the frequency of the variants in patients should be statistically significant higher than in controls. However, once again the complex nature of this trait complicates the picture, and innocent polymorphisms could show a statistically significant association with POAG by virtue of linkage disequilibrium if they are located within or near the causative gene. A segregation analysis in unrelated but clinically similar families should offer the most convincing evidence for causality. However, the possibility of genetic heterogeneity in the families, incomplete penetrance at the individual level, phenocopies and/or gene-gene or gene-environment interactions can not be rule out so easily. The late age of onset of the disease further complicate things, because individuals classified as healthy at 40 may develop the disease at 50 years, and even in the same family, age of onset can be variable. The last challenge is to explain the biological effect of the variants. Being POAG a complex disorder, the contribution of each variant to the development or progression of the disease is supposed to be limited. A main function of the candidate gene, RPGRIP1, is to serve as a scaffold for a large protein complex acting in signalling pathways of different retinal cell subpopulations (Oti et al. 2008). Through yeast two-hybrid screening of a retinal cDNA library, RPGRIP1 was found to specifically bind through its C2 domains to NPHP4 (Roepman et al. 2005). At this point, we started a collaboration with Ronald Roepman from the Nijmegen Centre for Molecular Life Sciences at the Radboud University of Nijmegen (The Netherlands) to functionally test the effect of our variants in this interaction.

6.1.2. Common variants versus rare variants

Two theories have been proposed to understand the genetic architecture underlying common diseases.
The most widely accepted common disease/common variant hypothesis (CD-CV) holds, as its name suggest, that disease-predisposing alleles for common diseases are common alleles with a relative high frequency (>0.01) in the population, each with a small contribution to the pathophysiology of the disease (Lander 1996). These common susceptibility alleles are potentially detectable in large-scale patient-control association studies using a huge number of frequent SNPs as markers, as it has become possible nowadays due to the availability of array genotyping platforms.

In contrast, the common disease/rare variant hypothesis (CD-RV) affirms that common diseases are due to a large number of rare variants at many different loci. Due to their low frequencies, rare variants will not be detectable by population association studies; their discovery depends on extensive resequencing of carefully selected candidate genes in relatively large numbers of carefully chosen cases, together with a thorough analysis of the functional effects of any suspected variants (Bodmer and Bonilla 2008). POAG is characterized by a high locus and allelic heterogeneity, with different rare variants in numerous genes (Allingham et al. 2009). For these reasons, I sequenced the entire RPGRIP1 coding sequence in a large cohort of POAG patients, as I expect to find association to rare variants rather than to common SNPs, in line with previous findings in other glaucoma genes such as MYOC and WDR36.

6.2. Screening of MYOC and CYP1B1 in POAG patients

Knowledge about the genetics of glaucoma is far from complete. Together with WDR36 and OPTN, MYOC is one of the genes with a recognized role in the pathophysiology of POAG, although the exact mechanism remains unknown. Different large studies have shown that only 3% to 5% from POAG patients carry mutations in the MYOC gene. More than 100 potentially disease causing mutations have been identified so far in the MYOC gene (Human Gene Mutation Database Cardiff, HGMD), most of them located in exon 3, which contains an olfactomedin-like domain. MYOC is secreted into the aqueous humour and expressed in the trabecular meshwork, which is responsible for the drainage of aqueous humour. Apparently, mutant myocilin, lacking the olfactomedin-like domain, is not correctly processed in the endoplasmic reticulum and accumulates into insoluble aggregates (Zhou and Vollrath 1999). The presence of increasing amounts of mutant protein induces a fraction of the soluble, native myocilin to move to the insoluble fraction. Interestingly, mice with a targeted disruption of the myocilin gene do not exhibit a pathological phenotype, indicating that a loss-of-function effect does not cause the glaucomatous phenotype in humans (Tamm 2002).
Heterozygous mutations in CYP1B1 are associated with primary congenital glaucoma (PCG), but their contribution to the occurrence of POAG remains controversial. CYP1B1 belongs to the P450 gene superfamily, with more than 300 members. More than 50 PCG-causing mutations have been described, from which around 50% cause a truncated protein, with loss of the heme-binding domain coded in exon 3 or loss of highly conserved amino acids with important function. CYP1B1 is assumed to participate in the normal development and function of the anterior eye chamber (Choudhary et al. 2007) and has also a role in retinoic acid synthesis (Chen et al. 2000). Some of the proposed functions of retinoic acid are the establishment of cell polarity (Sen et al. 2005) and to act as an antiapoptotic factor (Ahn et al. 2005). Therefore, CYP1B1 could play a role in retinal ganglion cell survival. If this scenario is true, it is conceivable that carrying mutations in the heterozygous state could predispose an individual to develop adult onset glaucoma.

The objective of my thesis was to identify new genes associated with POAG. For this reason, it was decided to screen first the whole collective of patients for mutations in MYOC, CYP1B1, OPTN and WDR36 in order to identify those patients carrying mutations in any of these reported glaucoma-causing genes. I performed the initial screening of MYOC and CYP1B1 in 46 patients, the initial collective that we have recruited at the time I started working on this project. The extended screening of the whole collective of 399 patients was performed with the help from technical assistants.

One patient carrying the common p.Q368X glaucoma-causing mutation in MYOC and four more patients carrying a p.T243P variant were identified in the exploratory collective of 46 POAG patients. Future studies will be required in order to elucidate if this variation represents only a SNP or a disease-causing mutation, as currently no functional test for myocilin is available.

Three mutations (p.G61E, p.Y81N and p.E229K) were found in CYP1B1 in 4 out of 46 patients of the initial collective (8.7%). Further screening of the whole POAG collective (399 patients) led to the identification of 11 amino acid substitutions in CYP1B1 in 13 patients. All these variants have been reported before both in PCG and POAG cases, and some of them also in healthy subjects (Aklillu et al. 2002; Kumar et al. 2007). The CYP1B1 mutation rate in our POAG patient cohort resulted significantly increased over expectancy (p=0.0018, Fischer’s exact test), confirming association of rare CYP1B1 variants with POAG (Pasutto et al. 2009). A functional characterization of these variants was also performed. Each mutation was embedded in its corresponding SNP haplotype and a functional analysis of their enzymatic activities was performed. As for every other enzyme, the action of CYP1B1 at the
cellular level depends on its abundance as well as its activity. The relative activity of the common CYP1B1 variants was found between 35% and 45% of the maximum activity, implying that relative activity values of 35% are still enough to prevent an individual from developing glaucoma. The relative activity was drastically reduced to less than 11% of the maximum in the p.G61E mutant, confirming its role as a bona fide mutation. The reduction in relative activity for the amino acid changes p.E229K and p.Y81N was 26% and 17%, respectively. These are intermediate relative activity values, lying between the bona fide mutations (with less than 11% of the maximum relative activity) and the common variant with the weakest activity (with 35% of the maximum). This intermediate reduction led to classify p.Y81N not as bona fide mutation but as a hypomorphic allele (Chavarria-Soley et al. 2008; Pasutto et al. 2009). A milder effect was found for p.E229K (26%). However, this variant was identified in similar frequency in patients and controls (2 and 3%, respectively) suggesting it should be reclassified as a polymorphism (Pasutto et al. 2009) in contrast to previous reports (Chavarria-Soley et al. 2008). These results supports the hypothesis that CYP1B1 has a broader significance for glaucoma pathogenesis than initially thought, ranging from a causal effect in autosomal recessive PCG and other anterior segment dysgenesis disorders, to a risk factor in POAG.

6.3. RPGRIP1 as a candidate gene for POAG

6.3.1. Selection of RPGRIP1

The genes identified so far are responsible for a very small fraction of glaucoma cases. For most patients, mutations in other loci (not identified up to now) are probably the cause of the disease. The search for these loci by linkage analysis is complicated by the fact that most families are too small for the analysis to have enough power. In a previous study, a two-step affected-sib-pair (ASP) analysis was performed, with 113 ASPs from 41 families (Wiggs et al. 2000). As a result, seven loci with a multipoint LOD score greater than 1 and five loci with a score greater than 2 were identified. The Barbados family study of Open Angle Glaucoma, comprising 1327 individuals and 146 families, could not reveal linkage to the MYOC locus, but gave some evidence for six chromosomal regions (Nemesure et al. 2003). Locus 14q11 was linked to POAG in both studies, so I decided to focus my work on finding candidate genes within this region. For this purpose, the complete exonic regions of ten carefully selected candidate genes mapping to 14q11 were sequenced in an exploratory collective of 46 POAG patients. All these genes were positional and functional candidates, as they are expressed in the eye, share protein domains or interact with other glaucoma genes and/or
affect molecular pathways that could be relevant in the pathophysiology of the disease, e.g. apoptosis. Ten coding variants were found in five of them (ZNF219, RPGRIP1, SALL2, OXAIL, and ADCY4). Familiar segregation studies were performed if relatives were available and a total of 46 healthy individuals of comparable age who had normal ophthalmologic examinations served as controls for resequencing, leading to the exclusion of three of the variants found as disease causing. The biochemical properties of the amino acidic substitutions and the evolutionary conservation of the affected amino acid positions were also studied. This led to the exclusion of five more variants. The remaining three missense variants were located in RPGRIP1. For all these reasons, RPGRIP1 was selected for further characterization as a good candidate gene for POAG.

6.3.2. Association of RPGRIP1 with POAG

At the very beginning of the project, our strategy was to identify glaucoma genes through systematic analysis of the linkage disequilibrium (LD) and case-controls association studies. When I started this work, only preliminary HapMap data was available, with no information about the RPGRIP1 locus. To solve this inconvenience, 14 SNPs with MAF>0.15 found in our first collective of 46 POAG patients were used to construct the LD structure of RPGRIP1, covering a region spanning 34 kb. A clear block pattern, with two different blocks was identified. This result correlates with the current LD structure of RPGRIP1 established by HapMap. However, lack of a big collective of patients and controls necessary to perform a case-control association study, together with increasing evidence that this was not an adequate approach to deal with the complex aetiology of POAG (as discussed above), prompted me to abandon this strategy. Instead of that, I aimed to identify glaucoma-causing genes through systematic mutation screening of ten positional and functional candidate genes, concentrating afterwards on RPGRIP1, the best candidate gene among this subset.

This systematic mutation screening in 399 patients led to the identification of 14 amino acid substitutions in RPGRIP1, accounting for 6.5% of the patients population (26/399). Due to the fact that glaucoma is a complex trait, the variants are expected to be more common in the affected patients, but they are also expected to be found in control individuals. In fact, 8 of these variants were detected in controls, representing 2.1% (8/376) of the control collective. These results demonstrate strong association of our rare variants in RPGRIP1 with POAG (p-value=0.003). As most of the patients carried a mutation located in or very near to the C2 domains of RPGRIP1 (16 patients against 3 control subjects, p-value=0.007), and to replicate the observed association data, the complete coding region of these C2 domains was screened in a further German cohort of 383 patients and 104 controls. Also in this second group,
different missense variants were detected, but at a lower frequency rate (2.3%). Pooling all the data together, the RPGRIP1 variants found in the C2 domains still showed significant association with POAG (p-value=0.013, 2-Tail Fischer’s exact test; OR=2.5, 95% CI=1.2±5.3).

The differences in frequency of the RPGRIP1 C2 domains variants between the exploratory (6.5%) and the replication (2.3%) patient cohorts might indicate differential genetic factors contributing to the development of the pathology. This fact is not surprising, as POAG is characterized by a high locus and allelic heterogeneity. One possible factor that might contribute to these differences is the intraocular pressure (IOP): the second cohort was composed mostly by patients with normal-tension glaucoma (NTG), in contrast with the discovery group, in which patients carrying RPGRIP1 mutations presented mainly high IOP. Glaucoma is frequently associated with harmful and high IOP. In fact, experimentally elevating IOP can induce glaucomatous neuropathy. IOP is affected by aqueous humor (AqH) production in the ciliary body and by its drainage through the trabecular and uveoscleral drainage pathways. Different studies suggest that high IOP can contribute to the retinal ganglion cell death through several mechanisms, such as altering the vascular perfusion or causing direct injury to the cellular soma or to the optic nerve head. Environmental factors may also contribute to this difference.

6.3.3. Expression of RPGRIP1 in retina

The RPGRIP1 gene is subjected to complex alternative splicing, encoding several different isoforms in the retina of human, bovine, rodents and dog. These distinct RPGRIP1 isoforms present differential expression in the inner retina (specifically among the amacrine cells and different ganglion cell populations), the outer segment of rods and cones, the cytoskeleton of photoreceptors and decorating microtubules, and may participate selectively in different subcellular processes, providing a rationale for the distinct phenotypes caused by genetic lesions in RPGRIP1 in human (Castagnet et al. 2003). Further characterization of the region between exons 12 and 14, which undergoes significant alternative splicing, was performed by Lu and Ferreira. They conclude that the production of several RPGRIP1 isoforms underlies the presence of isoform ratios (Lu and Ferreira 2005), which may differ among and within neuronal cell types and could have disease implications as reported elsewhere for other genes and diseases (Hong et al. 1998). Their studies also demonstrated that some RPGRIP1 isoforms undergo limited proteolytic processing, yielding a small fragment that can translocate to the nucleus, although the exact mechanism of this event is not yet clear (Lu and Ferreira 2005). Altogether, their results further strengthen the model of the selective
participation of distinct RPGRIP1 isoforms in different subcellular processes and molecular pathogenesis of RPGRIP1-allied diseases.

My RT-PCR experiments detected alternative splicing, as well as expression of RPGRIP1 in retina, sclera and blood from a healthy donor. Together with the two isoforms previously reported (Lu and Ferreira 2005), three novel isoforms were identified between exon 12 and 14, resulting in the in-frame insertion of several amino acids. Thus, our results reflect that the number of isoforms reported for RPGRIP1 could be underestimated. In addition, it is likely that mutations located anywhere in this genomic region (such as variations p.P585S and p.Q589H found in our collective of POAG patients) could lead to aberrant transcripts and play a role in the pathophysiology of the disease. However, my preliminary results could not be replicated due to time constraints, as I concentrated on the functional validation of the variants found in RPGRIP1, so the novel isoforms herein reported still need to be validated and their possible function remains to be resolved.

6.3.4. Functional characterization of RPGRIP1 mutations

Autosomal recessive mutations in RPGRIP1 most commonly cause Leber congenital amaurosis (LCA) in human, a severe systemic retinopathy (Dryja et al. 2001). However, the biological role of the RPGRIP1 protein in retinal function and pathogenesis is not completely clear. The finding of different RPGRIP1 interacting partners such as RPGR (Boylan and Wright 2000; Roepman et al. 2000), NPHP4 (Roepman et al. 2005), and RanBP2 (Castagnet et al. 2003) and its consistent involvement in a large spectrum of different retinal phenotypes suggest that the function of RPGRIP1 in the retina is to serve as a scaffold for a large protein complex acting in different signaling pathways of distinct retinal cell subpopulations (Roepman et al. 2005). Most of these interacting partners associate to the RPGRIP1 region containing the C2 domains, which are necessary for the relocation (and proteolytic cleavage) of the N-terminal domain of RPGRIP1 to the cell nucleus (Lu et al. 2005).

NPHP4 is one of the currently known RPGRIP1 interacting partners. This protein localized in the cytoplasm. Interestingly, coexpression of NPHP4 with RPGRIP1 results in the colocalization of both proteins in the cytoplasm (Roepman et al. 2005). Mutations in NPHP4 are involved in nephronophthisis type 4 and Senior-Løken syndrome (SLSN), a combination of nephronophthisis and progressive retinal degeneration (Schuermann et al. 2002). Most of the mutations found in our patients (16/28) were located in or very near to the C2 domains of RPGRIP1, the region where NPHP4 and RPGRIP1 are reported to interact. In collaboration with Ronald Roepman, from the Radboud University of Nijmegen (The Netherlands), I characterized the effect of our RPGRIP1 mutations in the interaction of both proteins through
yeast two-hybrid experiments and two cell-based assays: coimmunoprecipitation and colocalization assays with fluorescence proteins.

RPGRIP1 alteration p.R598Q severely disrupts the interaction with NPHP4. Yeast cotransfected with both constructs were able to grow on selective plates, and the enzymatic activity of the β-galactosidase was even lower than that of the negative control. Negative coimmunoprecipitation results also confirmed a complete disruption of the RPGRIP1-NPHP4 interaction. Moreover, in cells coexpressing both proteins, RPGRIP1 could still translocate to the nucleus, which indicates that the protein did not interact with NPHP4, so the latter could not retain the first to the cytoplasm. Altogether, these results suggest that this is a bona fide mutation, with a serious effect on RPGRIP1 function, impairing its interaction with NPHP4. The biological significance of this disrupted interaction in the pathology of POAG still needs to be clarified with further functional assays. The effects of RPGRIP1 mutants p.A635G, p.T806I, p.A837G, and p.I838V on RPGRIP1-NPHP4 interaction were less pronounced. An impaired interaction between NPHP4 and these RPGRIP1 variants was revealed through qualitative yeast two hybrids experiments. In addition, their β-galactosidase activities were reduced to an intermediate value lying between the wild type protein and the negative control. These mutant proteins were able to coimmunoprecipitate with NPHP4, but less than the wild type RPGRIP1, and all these RPGRIP1 constructs colocalized with NPHP4 in the cytoplasm when cotransfected to COS-1 cells. For all these reasons, I conclude that these are bona fide mutations, although their effect on protein-protein interaction is not very pronounced. This is not surprising, being POAG a complex disease; it is unquestionable that additional risk factors, both genetic and environmental, are needed to develop the disorder. In addition, POAG presents a late onset, meaning that the genetic defects can be compensated for many years before manifesting, being therefore not suitable to detection with this kind of functional assays. Moreover, RPGRIP1 presumably acts as a scaffold for recruitment of multiple partners, and one or more of these partners might compensate for loss of activity of one of the other complex members (Roepman et al. 2005). Being RPGRIP1 part of such a complex interactoma, it is plausible that these mutations could also affect its interaction with other not yet known binding partner(s) of the C2 domains, leading to the glaucomatous phenotype through different molecular and biochemical pathways. The importance of the elucidation of the molecular disease mechanisms associated with both RPGRIP1 dysfunction and POAG (patho)physiology becomes therefore clear.

On the other hand, the results obtained for RPGRIP1 variants p.Q589H, p.A764V and p.R812H in all the experiments were not significant different from those of the wild type
protein, indicating that these variants do not affect the RPGRIP1-NPHP4 interaction and may be classified as non pathological polymorphisms. In fact, RPGRIP1 variant p.Q589H was previously reported as a polymorphism (Dryja et al. 2001), as it was found in 1/57 LCA patients and 1/92 control subjects, so our functional assays validate this earlier classification.

Interestingly also, when considering only the C2 domain mutations causing impaired RPGRIP1-NPHP4 interaction, a total of 5 variants in 10 patients (2.8%) was identified in the discovery group, and none in control subjects. This represents a significant increase over expectancy (p-value = 0.001, two-tail Fischer’s exact test; OR= 7.0, 95% CI=2.2±23.1) and therefore strong association of these RPGRIP1 mutations with POAG. These data support as well my finding that RPGRIP1 might be a relevant genetic factor in the pathogenesis of POAG.

6.3.5. Summary

a) Replicated association of RPGRIP1 mutations with POAG was found in a collective of German patients.

b) Many splicing isoforms have been identified in several tissues. Further studies to characterize the biological role of these alternative transcripts have to be performed.

c) Functional characterization of RPGRIP1 amino acid changes p.R598Q, p.A635G, p.T806I, p.A837G, and p.I838V led to the classification of these variants as bona fide mutations, as they disrupt the interaction with NPHP4. None of them were identified in the control collective. A major effect was revealed for RPGRIP1 mutation p.R598Q.

d) RPGRIP1 variants p.Q589H, p.A764V, and p.R812H did not affect the interaction with NPHP4. In addition, two of them (p.Q589H and p.R812H) were also found in controls. Therefore, they seem to be non pathological polymorphisms.

6.4. Final conclusions and future perspectives

In conclusion, this study demonstrates association of RPGRIP1 with POAG and gives functional evidences for involvement of RPGRIP1 mutations in the pathogenesis of the disease, as part of an intricate interactoma. Dissection of this macromolecular complex will provide further clues to the molecular pathogenesis of the disease and may identify additional candidate genes for glaucoma.

The results herein reported also support that POAG belongs to the same category of traits under the common disease-rare variant theory such as epilepsy (Weber and Lerche 2008) and
macular degeneration (Swaroop et al. 2007), reaffirming the hypothesis that genetic predisposition to this late onset disease is mainly caused by rare variants with large effect located in numerous genes rather than by common SNPs. According to this hypothesis, the expectation for POAG is that many alleles involved in the aetiology of the disease will tend to have minor allele frequencies. This could have important consequences for designing future studies aimed at discovering new glaucoma causing genes and should encourage synergistic collaboration between several disciplines, including genetics, proteomics, system biology, disease biology and bioinformatics in order to provide a deeper understanding of the glaucoma pathogenesis and elucidate the molecular causes underlying the disease.
7. Bibliography


phenotypes in subjects from the framingham heart study." Hypertension 36(4): 477-83.


8. Abbreviations

°C  degree Celsius  
A  adenine  
AD  activating domain  
ADCY4  adenylate cyclase 4  
B.C.  before Christ  
BCL2L2  B-cell/lymphoma 2-like 2  
BD  binding domain  
bp  base pair(s)  
C  cytosine  
cDNA  complementary DNA  
cGMP  cyclic guanosine monophosphate  
CI  confidence interval  
CNVs  copy-number variations  
CYP1B1  cytochrome P450 subfamily I polypeptide 1  
ddNTPs  2', 3'-dideoxynucleotide triphosphates  
DAD1  defender against cell death 1  
DNA  deoxyribonucleic acid  
DNase  deoxyribonuclease  
dNTP  dinucleotide triphosphate  
Da  dalton  
DTT  dithiothreitol  
ds  double strand  
EDTA  ethylene diamine tetraacetic acid  
e.g.  for example (exempli gratia)  
fl  full length  
g  gram  
G  guanine  
GAPDH  glyceraldehyde-3-phosphate dehydrogenase  
gDNA  genomic DNA  
h  hour  
HW  Hardy-Weinberg  
htSNP  haplotype tagging SNP  
i.e.  that is (id est)  
IFNα  interferon alpha  
IOP  intraocular pressure  
IP  immunoprecipitation  
ISGF3G  interferon-stimulated transcription factor 3 gamma  
JOAG  juvenile open angle glaucoma  
K  kilo (10³)  
kb  kilobase pair(s)  
l  liter  
LB  Luria Bertani medium  
LCA  Leber congenital amaurosis  
LD  linkage disequilibrium  
LOD  logarithm of the odds ratio  
M  molar (mol/liter)  
m  meter  
m  milli (10⁻³)  
min.  Minute  
MMP14  matrix metalloproteinase 14  
mRNA  messenger RNA  
MYOC  myocilin
9. Publications

Articles


Abstracts


Fernández-Martínez L, Pasutto F, Chavarria-Soley G, Michels-Rautenstrauss K, Mardin C, Rautenstrauss B, Kruse F, Reis A. *Association of functional CYP1B1 variants in German patients with primary open-angle glaucoma (POAG)*. German Society of Human Genetics (GfH) Annual Meeting, Hannover, April 8-14, 2008

Fernández-Martínez L, Pasutto F, Chavarria-Soley G, Michels-Rautenstrauss K, Mardin C, Rautenstrauss B, Kruse F, Reis A. *Association of functional CYP1B1 variants in German patients with primary open-angle glaucoma (POAG)*. German Society of Human Genetics (GfH) Annual Meeting, Hannover, April 8-14, 2008

Fernández-Martínez L, Mardin C, Pasutto F, Kruse F, Reis A. *Systematic mutational screening of candidate genes in a putative glaucoma locus on chromosome 14q11 in German patients*. German Society of Human Genetics (GfH) Annual Meeting, Heidelberg, March 8-11, 2006


Fernández-Martínez L, Pasutto F, Mardin C, Kruse F, Reis A. *Determination of the linkage disequilibrium (LD) structure for a putative glaucoma locus on chromosome 14q11 in German patients*. German Society of Human Genetics (GfH) Annual Meeting, Halle, March 9-12, 2005.

Fernández-Martínez L, Pasutto F, Reis A. *Systematic linkage disequilibrium (LD) analysis for screening candidate genes in glaucoma*. Spanish Society of Biotechnology Annual Meeting, Oviedo (Spain), July 19-23, 2004
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Lore

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11. Curriculum vitae

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