PRO RETINA FOUNDATION

7th PRO RETINA
Research-Colloquium Potsdam

CONFERENCE REPORT

Retinal Degeneration

Vision and Beyond

An Interdisciplinary Dialogue

April 8th/9th, 2011

Potsdam, Seehotel am Templiner See

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FOR PREVENTION BLINDNESS

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PRO RETINA DEUTSCHLAND E. V. & THE PRO RETINA-FOUNDATION FOR PREVENTION BLINDNESS

WHO WE ARE

The patient-organisation, "Pro Retina Deutschland e.V.", was founded in 1977 as „Deutsche Retinitis Pigmentosa-Vereinigung" by patients and their relatives intended to organize help for themselves. The three objectives mentioned in the constitution are to actively support research, to give psychological and social advice for its members and to strengthen public information.
Every member can join one of the 60 regional groups, which are spread throughout Germany.
At present (2010), PRO RETINA Deutschland e.V. counts more than 5,800 members. The Board, the Counsellors, the leaders of the regional groups and all active members are working on a non-profit basis, but they are supported by a fulltime working staff at our office which is located in Aachen (www.pro-retina.de).

WHAT WE DO IN RESEARCH

The jewel of all this work is the Pro Retina-Foundation for Prevention Blindness, which was founded in 1996.
From the early beginning we have created a stable network with researchers and ophthalmologists for joined information and advice. We support research projects with direct financial funding – since the "Foundation for Prevention of Blindness" was established in 1996, more than two million Euro have been donated. We actively initiate research projects and therapy tests and contribute to their implementation.

Every year, we award two research prices and organize and support national and international seminars and conferences on relevant topics. We are financing PhD grants in order to foster research activities and networking between researchers.

We are consulted by a Scientific and Medical Advisory Board ("Wissenschaftlicher und Medizinischer Beirat", WMB) and a Working Group on Clinical Questions ("Arbeitskreis Klinische Fragen", AKF). In this Working Group scientists of different medical and other relevant disciplines are taking part.

The main objective is to secure a long-term support for research activities, e. g. by granting financial means for the development of new research projects or by financing the initial phase of relevant projects.

It is envisaged to increase the capital of the foundation to a minimum of Euro 5,000,000, which are to result in a steady source of funding for the support of research, independent from changing income of donations.
We guarantee that the benefits of the Foundation will only be dedicated to the research of retinal diseases, with the wider objective to develop applicable therapies for the patients.

PRELIMINARY PROGRAMME

Friday, April 8th 2011

13:00–13:05 **Opening remarks:** Franz Badura (PRO RETINA Foundation, research division)

13:05–13:25 **Introduction:** Eberhart Zrenner (Chairman, Scientific Medical Advisory Board) Tübingen: "*Hereditary retinal degeneration – Roadmaps of national and international research*"

13:25–13:45 **Spotlight:** Bernd Wissinger, Tübingen: *"HOPE and orphan diseases in Germany"*

13:45–14:30 **Opening lecture:** Heinz Wässle, Frankfurt: *"The eye as a window of the brain"*

14:30–15.30 Coffee break and scientific chitchat

15:30–17:30 **Session 1**
Channelrhodopsins: Applications and photochemistry
Chairman: Prof. Klaus W. Rüther

- 15:30 – 16:00 Peter Hegemann, Berlin: *"Optogenetic tools for Retinal Prosthesis"*
- 16:00 – 16:30 Nir Grossmann, London: *"Engineering Optogenetic Retinal Prosthesis"*
- 16:30 – 17:00 Botond Roska, Basel: *"Restoring visual function with optogenetic tools"*
- 17:00 – 17:30 Franz Bartl, Berlin: *"Photochemistry of Channelrhodopsins"*

17:30–18:00 **Keynote lecture:** Roderick R. McInnes, Montreal: *"Responses of the Retina in inherited photoreceptor degenerations and prospects for their treatment"*

18:00 Dinner

19:30 **Swingin' poster session**

PRELIMINARY PROGRAMME

Saturday, April 9th 2011

08:30–10:30 **Session 2**
Organelles and retinal degeneration
Chairman: Prof. Olaf Strauß

- 08:30 – 09:00 Jürgen Kopitz, Heidelberg: *"Lysosomal dysfunction of retinal Pigment Epithelium contributes to the pathogenesis of age-related Macular Degeneration"*
- 09:00 – 09:30 Patrick Dolph, New Haven: *"Endocytosis-mediated cell death in Drosophila"*
- 09:30 – 10:00 Mike Cheetham, London: *"The X-linked retinitis pigmentosa protein RP2 in vesicle traffic and cilia function"*
- 10:00 – 10:30 Miguel Seabra, London: *"Rab GTPases, membrane traffic and retinal degeneration"*

10:30–11:30 Coffee break

11:30–12:55 **Session 3**
Selected poster presentation and Poster Award 2011
Chairman: Prof. Bernhard H. F. Weber

12:55–13:00 Concluding remarks

13:00 Lunch and end of meeting

PRO RETINA
POTSDAM-MEETING

SPEAKER ABSTRACTS

Hereditary retinal degenerations – Roadmaps of national and international research

Eberhart Zrenner

Department for Ophthalmology, University of Tuebingen

Congenital retinal degenerations are the most frequent cause of blindness in young adults. Research into causes is now well under way and has yielded new approaches to diagnosis and therapy, several of which are being clinically tested already. These include:

High throughput mutation screening, allowing cost effective fast assessment of the genetic origin
Proteomics, unraveling networks of proteins involved in the pathophysiological mechanism
Molecular imaging, visualizing cells and molecules involved in the degeneration process
Multimodal mapping for improved differentiation of disease development
Gene Therapy: A good number of animal models with retinal degenerations have been treated successfully. Three gene therapy studies of Leber's congenital amaurosis demonstrate the safety and the efficacy of subretinal transvection based on viral vectors. Further gene therapy studies on Stargardt's disease, Usher-syndrome, retinoschisis and achromatopsia are at the preparatory stage.
Neurotrophic factors: Encapsulated cell factories that produce ciliary neurotrophic factors (CNTF) are being transferred into the vitreous body; the study is now already in phase 3 and it has produced interesting rescue results.
Electrostimulation: Ocuvision GmbH has developed a transcorneal method of electrical stimulation, shown in a controlled clinical study, to enlarge the visual field and to increase the amplitude of rod b-waves; animal experiments have give reason to believe that endogenous growth-stimulating factors are released by electrostimulation.

"Visual cycle modifiers" are being used by Acucela Inc. to alleviate metabolic disorders arsing from enzyme defects in the rhodopsin cycle.
Antioxidative treatment: Animal models have demonstrated that antioxidative "cocktails" slow down the rate of degeneration; in Spain a corresponding clinical study is under way.
Stem-Cell Transplants have shown new possibilities of replacing cells; however, the control mechanisms have not yet been understood well enough to warrant a clinical trial.
Optogenetic approaches bring light sensitive ion channels into the cytoplasmic membrane of retinal neurons. At present, too much light is still necessary, and the temporal resolution is yet too limited, even though in mouse models proof of concept has been achieved.
Electronic subretinal implants have been able to restore a certain degree of vision in a couple of patients participating in current clinical studies. For totally blind patients this method may be a helpful approach.

Summary: It can be expected that not only therapies tailored to individual mutations, but also generally neuroprotective substances are going to emerge from pre-clinical and clinical studies; thus strong efforts should be made to bring these new therapeutic approaches into clinical application. This needs to be complemented by newly emerging techniques of molecular imaging and electrophysiological function testing that allow highly sensitive monitoring of safety and efficacy of such therapeutic approaches. In parallel further efforts are necessary to unveil the causes of retinal degeneration on molecular and cellular level to understand the exact mechanisms of the degeneration process to further discover effective therapeutic strategies for the various forms of retinal degeneration.

The Eye as a Window of the Brain

Heinz Wässle

Department of Neuroanatomy, Max-Planck-Institute for Brain Research, Deutschordenstrasse 46, D-60528 Frankfurt/Main, Germany

Our eyes send different 'images' of the outside world to the brain – an image of contours (line drawing), a colour image (watercolour painting) or an image of moving objects (movie). This is commonly referred to as parallel processing, and starts as early as the first synapse of the retina, the cone pedicle. Here, the molecular composition of the transmitter receptors of the postsynaptic neurons defines which images are transferred to the inner retina. Within the second synaptic layer – the inner plexiform layer – circuits that involve complex inhibitory and excitatory interactions represent filters that select 'what the eye tells the brain'.

In the first part of the talk, the different morphological types of retinal ganglion cells will be introduced and their physiological roles will be discussed. There are at least 15 types of ganglion cells, which all cover the retina with their dendritic fields.
They send 15 images of the outside world to the visual centres of the brain.
In the second part it will be shown that the 12 different types of bipolar cells represent parallel channels that transfer the visual signals from the outer to the inner plexiform layer. The pathway selecting the chromatic signals of L-, M- and S-cones will be discussed in more detail. Finally the molecular composition of the glutamate receptors at the cone output synapse will be presented.

New Channelrhodopsin Variants for Application in Visual Prosthesis

Peter Hegemann, Matthias Prigge, and Franziska Schneider

Biology, Experimental Biophysics, Humboldt-Universität zu Berlin

Purpose: The death of photoreceptor cells caused by retinal degenerative diseases often results in a complete loss of retinal responses to light. Bi et al. 2006 and Lagari et al. 2008 were the first converting inner retinal neurons to photosensitive cells by expressing microbial rhodopsins as Channelrhodopsin (ChR) and Halorhodopsin in these cells as a possible strategy for imparting light sensitivity to retinas lacking rods and cones. One limiting step for ChR application is the low light sensitivity of ChR-expressing cells and the high blue-light intensities needed to produce a usable visual signal. This is caused by the low unitary conductance of wild type ChRs and the limited number of molecules that can be expressed in secondary retinal cells.

Methods: Our research group is studying light-gated ion channels (Channelrhodopsins, ChR) from green algae and constructed hybrids by exchanging transmembrane helices between different ChR-species. Based on computer modeling we identify amino acid residues that might be critical for the ChR-function replace these by other residues by side directed mutagenesis. The new proteins are tested respective electrical properties in Xenopusoocytes and HEK293 cells. Spectral properties are tested on purified recombinant ChRs that are produced in COS-cells or the yeast Pichiapastoris. In case the ChR-variants gained properties that are of potential interest for retinal prosthesis they are tested in blind mice in the group of Zhuo Pan (Detroit).

Results: We will present engineered ChRs-variant with shorter or extended open state lifetime (Berndt et al. 2009, Gunaydin et al. 2010), higher conductance, and reduced degree of inactivation in continuous light. We recently combined helices of ChlamydomonasChR1 and Volvox VChR1 and have generated the ChR-hybrid C1V1 with red-shifted absorption spectrum (λ_{max}= 540 nm) and excellent expression and membrane targeting thus allowing depolarization of host cells with 600 nm light of moderate intensities. This hybrid exhibits an open state lifetime of 90 ms, which is considered as ideal for retinal prosthesis in order to get maximal photocurrents with retained visual motion. Finally we will present approaches for the modification of the ion selectivity of the various ChR wild type and hybrids.

Conclusion: Engineering of Channelrhodopsin allows the production of directly light-activated ion channels with properties that are superior over the existing ChRs for restoration of photosensitivity to animal models of retinitis pigmentosa and possibly to human patients.

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Engineering ChR2-based Retinal Prosthesis

N. Grossman, K Nikolic, P Degenaar

Background: During the last decades there have been immense efforts worldwide to develop retinal prosthesis solution for patients who suffer from retinal dystrophies such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP). Hitherto efforts have been based on implanted arrays of electrodes. Despite some recent progress, it is becoming apparent that this approach is unlikely to restore sufficient level of vision due to fundamental engineering limitations.

Purpose: Herein, a new type of retinal prosthesis that is based on genetic expression of a microbial light sensitive ion channel, Chanelrhodopsin-2 (ChR2), and a remote light stimulation is developed. In this case the optoelectronic circuitry is fully external and hence would not suffer from the problems of electrical retinal implants.

Methods: An optical hardware was developed by processing gallium nitride semiconductor wafers. Proof-of-concept tests were done on retina slices of blind *rd1* mice in which the retinal ganglion cells (RGCs) were transfected with ChR2-YFP using electroproration.
A model that represents the response of ChR2-expressing neurons to light stimuli was developed and implemented in Simulink *(Mathworks Inc)*. The induction of ChR2's conductivity with light was modelled with a branched four-state model. The effect of the induced conductance on the neuron was determined with a cable model which contains active Hodgkin-Huxley type elements.

Results: We developed an optical hardware that has both the light intensity and the parallelism to evolve into a realistic optoelectronic visual prosthesis. The optical module is capable of generating >4000 pixel stimulation images on the ChR2-sensetiized retina with a single cell resolution. We demonstrated its functionality *ex-vivo* on blind mice retinas with RGCs expressing ChR2.
We investigated the dynamics of the light-to-spike stimulation process via ChR2. We found that in pulsed mode illuminations, the stimulation efficiency is limited by ChR2 adaptation and incomplete recovery of the rest potential between spikes. We showed that a better spiking yield can be achieved by using short and intense pulses. We also found through simulation that while increasing the lifetime and shuttering speed of ChR2 will provide only limited improvement in the spiking efficiency, reducing the threshold irradiance by increasing the conductance will circumvent the ChR2 adaptation and allow constant dynamic range.

Conclusions: This work examined the engineering challenges in developing a ChR2-based retinal prosthesis and presented initial results from pre-clinical testing.

Botond Roska

Friedrich Miescher Institute for Biomedical Research, Neural Circuit Laboratories, 4058 Basel, Switzerland

Retinitis pigmentosa (RP) refers to a diverse group of progressive, hereditary diseases of photoreceptors that lead to incurable blindness and affect two million people worldwide. Artificial photoreceptors constructed by gene delivery of light activated channels or pumps ("optogenetic tools") to surviving cell types in the remaining retinal circuit has been shown to restore photosensitivity in animal models of RP at the level of the retina, cortex as well as behaviorally. The translational potential of this optogenetic approach has been hinted by in vitro studies using post mortem human retinas. I will discuss recent developments in this expanding field and the potentials/limitations of the future use of optogenetic-engineering in RP patients.

Photochemistry of Channelrhodopsins

Eglof Ritter[1], Patrick Piwowarsiki[1], Katja Stehfest[2], Peter Hegemann[2] and Franz Bartl[1]

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Phobic responses and phototaxis in green algae are mediated by channelrhodosins (ChRs), light gated ion channels that serve as sensory photoreceptors [1-3]. In neurosciences they have been recently used to depolarize specific cells with light. Despite such promising applications, molecular details of their light-induced photoreaction including formation of the conducting state, the isomerization state of the chromophore and the role of specific amino acids for the formation of the different intermediates are largely unknown. We present a model of the photocycle of slow cycling mutants C128T and C128S with 200 and 2000 fold extended life time of the conducting state as compared to wildtype [4], respectively. For these mutants under prolonged illumination conditions the photocycle exhibits a branch-off in which blue shifted species accumulate. The recovery of the ground state from these intermediates is slow and occurs within a time scale of 100 – 1000 s.
Based on UV/ Vis and FTIR spectroscopic data [5, 6] and on HPLC retinal extraction we investigated the isomerization state of the chromophore and it connection to the various intermediates of the photocycle.

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Inherited photoreceptor degenerations: molecular mechanisms of cell death and survival, and opportunities for treatment.

Roderick R. McInnes, Lady Davis Institute, McGill University, Canada

Several fundamental questions are posed by inherited photoreceptor (PR) degenerations. First, why do the mutant PRs die? And second, how can the mutant PRs live and function normally for years to decades, despite expressing the mutation from their birth, yet ultimately die because of the mutation? Third, what molecular and cellular responses are elicited in the mutant PRs by the presence of the mutation, during the years to decades (or months, in a mouse) prior to their death? Finally, can we interfere with these responses to prolong the life of the mutant PRs? Great insight into these difficult questions has been obtained over the last decade. I will give an overview of what is known, and what isn't. I will also report our recent findings on the roles of the cell signalling molecules Endothelin 2 and Stat3 in resisting or mediating PR death. Finally, I will briefly discuss recent exciting work indicating that cell replacement and gene therapy hold much promise for the treatment of this important group of genetic diseases.

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Lysosomal Dysfunction of Retinal Pigment Epithelium Contributes to the Pathogenesis of Age-related Macular Degeneration

J. Kopitz[1], T.U. Krohne[2], F.G. Holz[2]

[1]University Hospital Heidelberg, [2]University Eye Hospital Bonn

Purpose: Several lines of evidence suggest that age-related damage to the retinal pigment epithelium (RPE) plays a key role in the pathogenesis of ARMD. Lipid peroxidation, a fundamentally deteriorative aging reaction, might represent an initial trigger of RPE dysfunction and damage. The retina is exposed to light in an extremely oxygen-rich environment, and unsaturated fatty acids are present in high concentrations in the photoreceptor membranes of the retina, thereby creating optimal conditions for lipid peroxidation processes. The resulting highly reactive aldehydes are capable of easily forming protein adducts, thereby damaging protein structure and function. If such damage occurs in the photoreceptors of the retina, lipid peroxidation products and damaged proteins will be cleared away by continous shedding of photoreceptor outer segments (POS). Shedded POS are disposed of by the retinal pigment epithelium (RPE) via phagocytosis. Thus postmitotic RPE cells in their lysosomal compartment have to deal with an enormous load of toxic lipid peroxidation products and damaged proteins.

Methods: Effects of lipid peroxidation on phagocytosis, transcytosis and degradation of POS was assayed in cultured human RPE cells with radiolabelled POS. Autophagy was assayed in radiometric pulse-chase experiments. Lipofuscinogenesis was evaluated by FACS analysis and fluorescence microscopy. Michael adduct formation between lipid peroxidation products and cathepsins in isolated RPE lysosomes was analyzed by mass spectrometry.

Results: *In vitro* experiments with cultured human RPE cells indicate that diminished susceptibility to lysosomal proteolysis after protein modification by lipid peroxidation products is a causative factor in the formation of intracellular deposits (lipofuscin), and basolateral waste deposition in polarized RPE cultures. In addition to their altered susceptibility to lysosomal proteolysis carbonylated proteins may also be active as competitive inhibitors of general proteolytic pathways. Finally increased levels of reactive substances like MDA and HNE, either taken up by phagocytosis or generated by phototoxic lipofuscin, will have further adverse effects on lysosomal function, including further substrate stabilization by alkylation and inactivation of lysosomal cysteine proteinases (cathepsins B, L and H) by forming Michael adducts with active site cysteine residues. Altogether these pathogenic mechanisms severly impair general cellular functions, as examplified by strikingly reduced phagocytic and autophagic activities.

Conclusion: Lysosomal dysfunction of the RPE caused by lipid peroxidation products and resulting carbonylated proteins may considerably contribute to the induction of general RPE dysfunction and degeneration which preceedes photoreceptor loss in ARMD patients.

Endocytosis-mediated Cell Death in Drosophila

Yashodhan Chinchore, Ron Kinser, and Patrick Dolph

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Purpose: In Drosophila, as in humans, many mutations that disrupt the phototransduction signaling cascade trigger cell death. We have found that mutations that block visual transduction, result in stable complexes between rhodopsin and its regulatory molecule arrestin. These complexes trigger photoreceptor cell death. The long-term goal of our laboratory is to elucidate the cell death signaling pathway that results from rhodopsin/arrestin complexes.

Methods: Retinal degeneration was ascertained by fixing Drosophila eyes and embedding them in plastic resin. Fixed and embedded tissue was sectioned and viewed by light microscopy. Immunofluorescence was performed on dissected retinas with antibodies specific to Drosophila retinal proteins. Protein levels were determined by western and slot blot analysis. RNA levels were determined by quantitative RT-PCR.

Results: Stable complexes between rhodopsin and arrestin result in light-dependent photoreceptor cell degeneration. Early in the degenerative process there is massive internalization of rhodopsin and arrestin via receptor-mediated endocytosis. The internalized rhodopsin is unable to traffic properly through the endosomal system and instead becomes trapped in late endosomal structures, where the rhodopsin aggregates and becomes insoluble. The misfolded rhodopsin triggers the translocation of cathepsins from the endosomal compartment to the cell body where cell death is induced. Cell death is not triggered by classic apoptotic molecules; caspases, IAP's, and Bcl-like molecules play a minimal role in this pathway. Instead, the aggregated rhodopsin triggers cell death by inducing the innate immunity pathway. Mutations in essential components of the Drosophila immunity cascade block cell death in a genetic background where rhodopsin complexes are induced. This includes mutations in the Drosophila NF-κB homologue relish. Interestingly, expression of an activated form of NF-κB in many different tissues is capable of triggering cell death.

Conclusion: We have uncovered a novel cell death pathway in Drosophila photoreceptors where the stable complexes between rhodopsin and arrestin trigger the internalization and aggregation of rhodopsin in the endosomal system. Cathepsin translocation eventually activates the innate immunity pathway and NF-κB function results in cell death. NF-κB is not regarded as a pro-apoptotic molecule and typically has anti-apoptotic functions. Therefore, this defines a novel role for NF-κB in a subset of tissues and cell types.

The X-linked retinitis pigmentosa protein RP2 in vesicle traffic and cilia function

Nele Schwarz[1], Tatiana Novoselova[1], R. Jane Evans[1], Kerstin-Nagel Wolfrum[2], Uwe Wolfrum[2], Alison J. Hardcastle[1] and Michael E Cheetham[1]

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[2] Johannes Gutenberg University of Mainz, Muellerweg 6, 55099 Mainz, Germany

Purpose: Photoreceptors are complex ciliated sensory neurons. The basal body and periciliary ridge of photoreceptors function in association with the Golgi complex to regulate the export of proteins from the inner segment to the outer segment sensory axoneme. Here, we investigated the potential role of the X-linked retinitis pigmentosa protein RP2 in periciliary traffic.

Methods: RP2 ultrastructural localization was investigated by immunofluorescence and immunoelectron microscopy. RP2 function was probed in ARPE19 and SK-N-SH cells by siRNA and expression of mutant proteins. Immunoprecipitation, yeast-two hybrid and proteomic approaches were used to investigate RP2 interacting proteins.

Results: The retinitis pigmentosa protein RP2, which is a GTPase activating protein (GAP) for Arl3, localised to the ciliary apparatus, namely the basal body and the associated centriole at the base of the photoreceptor cilium. Targeting to the ciliary base was dependent on N-terminal myristoylation. RP2 also localised to the Golgi and periciliary ridge of photoreceptors. Depletion of RP2 and dysregulation of Arl3 resulted in dispersal of vesicles cycling cargo from the Golgi complex to the cilium, including the intraflagellar transport protein IFT20. Furthermore, RP2 bound specifically to a phototransduction protein, thereby facilitating its membrane association and traffic.

Discussion: Collectively, the data suggest that RP2 may cause retinal degeneration through defects in the traffic of proteins to the outer segment.

Rab GTPases, membrane traffic and retinal degeneration

Miguel C Seabra[1,2]

[1] *NHLI, Imperial College London, UK*
[2] *CEDOC; Faculdade de Ciências Médicas, Universidade Nova de Lisboa, Portugal*

Purpose: Several inherited human disorders have been associated with defects in Rab GTPase activity, either directly or indirectly. Our studies focus on Choroideremia (CHM), an X-linked late-onset retinal degeneration characterised by progressive dystrophy of photoreceptors, retinal pigment epithelium (RPE) and the choroid. CHM is due to a defect in Rab Escort Protein 1 (REP1), a cofactor required for the prenylation of Rab GTPases. CHM is therefore a disease of intracellular membrane traffic but the events that trigger degeneration remain to be elucidated.

Methods: We have generated mouse models for Choroideremia using conditional gene knock-out (KO), cre-lox technology and studied them using a combination of techniques including histopathology, in vitro assays and functional analysis.

Results: We have shown that RPE disease accelerates photoreceptor degeneration, highlighting the central role of RPE in pathogenesis. Furthermore, in a RPE-restricted Rep1 KO, the RPE ages prematurely, accumulating pathological changes at 5-6 months of age, which are more exuberant than those observed in 2-year old controls. Extracellularly, we observed thickening of Bruchs membrane and accumulation of basal laminar deposits (BLamDs) and intracellularly, disorganisation of basal infoldings and accumulation of lipofuscin, the age pigment. This is accompanied by defects in intracellular membrane traffic pathways, including melanosome movement and phagosome processing.

Conclusions: These phenotypes suggest that multiple chronic defects in membrane traffic pathways accelerate the ageing process in the RPE. Furthermore, the striking similarities between the present observations and those reported in age-related macular degeneration (AMD), a leading cause of blindness in the developed world, suggest that membrane traffic defects may contribute to the pathogenesis of AMD and could represent a new focus of therapeutic strategies.

POSTER ABSTRACTS

Activity and expression of PARP and calpain in P23H-1 and S334ter-3 mutant rhodopsin transgenic rats

B. Arango-Gonzalez, F. Paquet-Durand, S. Mencl, A. Sahaboglu, E. Zrenner, and J. Kaur.

Centre for Ophthalmology, Institute for Ophthalmic Research, Division of Experimental Ophthalmology, Tübingen, Germany

Purpose: The aim of this project was to determine processes involved in retinal degeneration in rhodopsin transgenic rats. To this end, we used P23H-1 and S334ter-3 rhodopsin transgenic rats and examined the expression and activity of poly [ADP-ribose] polymerase (PARP) and Calpain during photoreceptor cell death.

Methods: Retinas of P23H-1, S334ter-3 and wild-type Sprague Dawley (SD/CD) rats at postnatal (PN) days 0-30 were examined by conventional histological techniques, TUNEL assay, immunohistochemistry and immunoblotting using antibodies against PARP, PAR, calpain, and calpastatin. PARP and calpain activity at the cellular level was investigated with specifically adapted enzymatic *in situ* assays (Paquet-Durand et al., 2006; 2007).

Results: P23H retina demonstrated high levels of TUNEL-positive photoreceptors when compared to CD, showing a peak of cell death at PN15. In S334ter animals, the highest percentage of TUNEL-positive cells was found at PN12. We observed PARP activity in a subset of photoreceptors in P23H and S334ter retinas, but only very rarely in CD retinas. As an indirect measure for PARP activity, we performed immunostaining for poly (ADP-ribosyl)ated (PAR) proteins. This staining showed numerous positive photoreceptors in both transgenic rats, nevertheless, their amount was higher in the S334ter. No positive cells were detected in CD retina. Western blot analysis of PARP and PAR also showed an up-regulation in mutants. Immunostaining for an 85kDa, caspase-cleaved PARP fragment identified only a subset of cells in transgenic retinas, but not in CD. Calpain activity was considerably increased in photoreceptors in mutant rats, with only very few cells positive in CD retinas. Increased calpain-3 ONL immunolabelling was observed in both transgenic rats, being more intense in S334ter. Immunostaining against calpastatin, the endogenous inhibitor of calpain, was less intense in both rhodopsin transgenic rats when compared to wild-type, in particular at the level of the inner segments. Immunoblot confirmed decreased calpastatin expression in both mutant models.

Conclusions: Our results indicate that activation of PARP and calpain are important hallmarks in the general mechanism causing photoreceptor cell death. These results are in line with previous studies using other animal models for retinitis pigmentosa, which highlights the possibility of using calpain and/or PARP inhibitors as therapeutic agents to prevent or delay photoreceptor degeneration.

Allelic imbalance in retinal expressed disease genes: A common phenomenon?

Sukirthini Balendran, Bernd Wissinger, Simone Schimpf-Linzenbold

Molecular Genetics Laboratory, Institute of Ophthalmic Research, Tuebingen, Germany

Purpose: In retinal dystrophies, reduced penetrance and variability in disease expression with respect to onset, course, and severity is a well-documented feature. This makes reliable genotype/phenotype correlations as well as individual disease prognosis difficult. Although the basis of this variability is largely unknown, it is commonly accepted that secondary genetic factors (modifier-genes) are key factors for the determination of the development, severity, and course of a disease. A good example is the *Prpf31* gene. Mutations in *Prpf31* cause retinitis pigmentosa (RP) with reduced penetrance. Two independent studies showed that the transcript level of the non-mutant allele is highly correlated with the risk of disease expression and that higher expression of the non-mutant transcript protects asymptomatic carriers from RP. Based on such known examples, we hypothesize that *cis*-acting variants governing gene expression levels play a crucial role in phenotypic variation and disease penetrance in hereditary retinal disorders. The aim of this project is the identification of such *cis*-acting gene variants and the determination of their impact on disease expression.

Methods: To demonstrate if and how common allelic imbalance (AI) in retinal expressed genes are and due to the absence of human donor eyes, experiments were done in cross-breeds of five different inbred mouse strains as a proof-of-principle experiment.
Up to now, more than 20 different retinal genes were screened for heterozygous cSNPs applying PCR and sequencing. We applied Pyrosequencing assays on RT-PCR amplified cDNAs generated from retinal RNA to determine allelic expression differences based on the identified cSNPs. Results were calibrated for equimolar ratios by used genomic DNA as a control.

Results: Using the Pyrosequencing technology, we identified an AI in 7 retinal disease genes. In two of those genes we can see the AI already on genomic level suggesting a copy number variation. Screening of the *Pde6c* gene revealed a 116-bp insertion on cDNA level that results in a premature termination codon leading, due to the nonsense mediated mRNA decay, to a downregulation of the mutant transcript. For the remaining genes the cause of the AI has to be verified.

Conclusions: Our results show that allele-specific differences in gene expression are common in retinal expressed genes. But until now, only in few cases *cis*-variants which cause an AI could be identified.

Store-operated calcium entry sustains increased calcium level in photoreceptor cells and may affect vision in mouse

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Purpose: The store-operated mechanism in vertebrate photoreceptors consists of store-operated Ca-permeable (SOC) channels in the plasma membrane and STIM1 (stromal interaction molecule 1), a sensor which transmits information about store-depletion from the endoplasmic reticulum to SOC channels. The resulting store-operated calcium entry (SOCE) regulates baseline $[Ca^{2+}]i$ in light-adapted cells. We characterized the functional role of SOCE in mammalian vision by selectively eliminating STIM1 from rod and cone photoreceptors.

Methods: STIM1 rod- and cone-specific conditional knockout (cKO) lines were developed using the conventional cre-loxP excision. STIM1 and Cre recombinase expression was determined with RT-PCR, Western blot and immunofluorescence. Fura-2 calcium imaging was performed in dissociated cells from control and cKO mouse retinas. Visual function was tested using ERG and the optomotor tracking response.

Results: IHC revealed photoreceptor-specific expression of Cre and deletion of the STIM1. Significant changes in visual acuity were observed for both rod and cone STIM1 cKO mouse lines. Rod cKOs exhibited lower scotopic acuity (0.239±0.013 cycles/deg) compared to wild type (0.256±0.004 c/deg) and strain control animals (0.254±0.007 cycles/deg) with no difference in photopic acuity. Cone cKOs were characterized by a decreased photopic acuity (0.358±0.007 cycles/deg vs 0.384±0.007 cycles/deg in controls) and no change in scotopic acuity. Baseline $[Ca^{2+}]i$ decreased in rods from 97±6 nM in wild type to 64±11 nM in rod cKO mice whereas no difference was observed in Müller cells. SOCE responses in isolated cKO photoreceptors were significantly decreased with respect to controls in both response amplitude and ratio of cells expressing this phenomenon.

Conclusions: Loss of STIM1 from rods slightly compromises rod vision whereas selective elimination of STIM1 from cones causes photopic vision deficits. The observed visual phenotypes suggest that STIM 1-stimulated store-operated calcium entry in photoreceptors significantly contributes to the regulation of steady-state $[Ca^{2+}]i$ and the flow of visual information from photoreceptors to higher order visual centers.

Vesicular traffic associated protein Magi2 interacts with the Ush1g protein sans

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Purpose: The human Usher syndrome (USH) is the most common cause of combined deaf-blindness. The encoded molecules are integrated into protein networks by scaffolds including the USH1G protein SANS (scaffold protein containing ankyrin repeats and SAM domain). Previous data indicated that SANS is involved in photoreceptor transport processes related to cilia. To decipher the cellular role of SANS, we screened with a yeast-2-hybrid (Y2H) for new interacting partners in the retina and validated putative interactions.
Methods: Results obtained from Y2H screens of a retinal cDNA library were validated by independent assays: GST-pull downs, co-transfection and co-immunoprecipitations. Correlative immunofluorescence and immunoelectron microscopy were used to study subcellular distribution of confirmed interactors. Functional assays elucidated the role of SANS associated complexes more precisely.

Results: Y2H revealed three PDZ-domain containing proteins as putative interactors. Here we identified Magi2 (membrane associated guanylate kinase inverted-2) as a novel component of the USH interactome and confirmed its direct interaction to SANS. Immunofluorescenceshowed partial co-localization of both interactors in mouse retina especially at the periciliary-ciliary region in apical inner segment. In addition, electron microscopy revealed an association of the Magi2-SANS complex with transport vesicles in this region. Further analysis of the complex indicated an association with the endocytosis/exocytosis machinery in retinal cells.

Conclusions: Direct binding of SANS to vesicle-associated Magi2 and subcellular distribution of both interaction partners in photoreceptor cells further support a role of SANS-organized protein complexes in periciliary-ciliary transport processes. In addition, our results provide further evidence for a role of SANS-Magi2 assembly in endo-/exocytotic processes associated with cilia function.

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Expressionsmuster der ether à-go-go related gene K^+ Kanäle (erg; Kv11) in der Mausretina

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Zielsetzung: Durch einfallendes Licht wird das Membranpotential der meisten retinalen Neurone nur graduell geändert, ohne dass Aktionspotentiale ausgelöst werden. Für das Fine-Tuning dieser Änderungen benötigen die Zellen K^+-Kanäle mit Aktivierungsschwellen um -40 mV. Die Mitglieder der ether-à-go-go related gene K^+-Kanäle (erg; Kv11) mit Aktivierungsschwellen zwischen -30 und -60 mV sind also hervorragend dafür geeignet. Alle drei Mitglieder dieser Familie wurden bei ihrer Klonierung in der Retina nachgewiesen. Wir wollten in der vorliegenden Untersuchung die Expressionsmuster der erg-Kanäle in der Retina aufklären, um erste Rückschlüsse auf ihre Aufgaben in der Retina ziehen zu können.

Methoden: Mit Hilfe von RT PCR-Experimenten haben wir die Expression der erg-Kanäle in der humanen und Mäuseretina nachgewiesen. Die Expressionsmuster der erg Kanal-Untereinheiten wurde mit Hilfe von immunhistologischen Färbungen von Kryoschnitten der Mäuseretina mit spezifischen Antikörpern gegen die einzelnen Untereinheiten und durch geeignete Doppelfärbungen untersucht.

Ergebnisse: Die drei erg Kanal-Untereinheiten haben in der Retina sehr unterschiedliche Expressionsmuster. M-erg1 zeigt die stärkste Immunreaktivität in den Innensegmenten der Photorezeptoren, dem äußeren Teil der inneren plexiformen Schicht, und der inneren nukleären Schicht. Doppelfärbungen mit Anti-PKCα-Antikörpern zeigt, dass m-erg1 in der inneren nukleären Schicht ausschließlich in Stäbchen-Bipolarzellen vorkommt. Immunreaktivität für m-erg2 konnte in der inneren und äußeren plexiformen Schicht nachgewiesen werden. Die Kolokalisation mit vGluT1 zeigt, dass m-erg2 in präsynaptischen Endigungen von Photorezeptoren und Bipolarzellen exprimiert ist. M-erg3 schließlich konnte gar nicht in der neuronalen Retina nachgewiesen werden, dafür aber sehr prominent in der apikalen Membran vom retinalen Pigmentepithel.

Schlussfolgerung: Wegen ihrer unterschiedlichen Expressionsmuster erfüllen die drei erg-Kanal-Untereinheiten vermutlich sehr unterschiedliche Aufgaben in der Retina. Die Expression von erg1-Kanälen im Zellkörper und in Dendriten von Bipolarzellen und in den Innenseg-

menten von Photorezeptoren deutet auf eine Beteiligung an der Signalverarbeitung innerhalb von Photorezeptoren und Bipolarzellen hin. M-erg2 hingegen wurde vornehmlich präsynaptisch in Axonterminalen von Photorezeptoren und Bipolarzellen nachgewiesen, wo sie an der Kontrolle der Glutamatausschüttung beteiligt sein können. M-erg3 schließlich scheint ausschließlich in der apikalen Membran des retinalen Pigmentepithels exprimiert zu sein, wo diese Kanäle einen Beitrag zur K^+-Homöostase und/oder der Osmoregulation leisten können.

Retinal progenitor cells acquire tri-potentiality following in vitro expansion

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Purpose: One of the treatment strategies proposed for retinal degenerations is to replace lost neurons with cells expanded in vitro, primed to specific neuronal fates that after transplantation would integrate into the retina and restore its visual function. Here we investigated the differentiation potential of in vitro expanded retinal stem cells (RSCs).

Methods: RSCs cultures were generated by isolating retinal cells from eyes of postnatal day 0 (PN0) actin:GFP or actin:dsRed mice and expanded in medium containing N2 and growth factors (EGF, FGF2). For in vitro oligodendrocyte differentiation primary retinal cells as well as expanded RSCs from early and late passages were subjected to a protocol involving stepwise replacement of growth factors by forskolin, PDGFα, ascorbic acid and triiodothyonine. Expanded as well as oligodendrocyte-differentiated RSCs were characterized by immunocytochemistry and RT-PCR. Additionally, intravitreal transplantation experiments using expanded and primed RSCs into the eyes of adult wild-type mice were performed. Dissected retinae were either subjected to transmission electron microscopy or flat mounted and analyzed by fluorescence/confocal microscopy following immunohistochemistry.

Results: Retinal cells can be expanded in vitro and maintain the potential to generate glial (GFAP-positive) and neuronal (β-III-tubulin-positive) cell types. However, RT-PCR analysis shows that expanded RSCs loose expression of some genes that are characteristic for retinal progenitor cells like Rax and Chx10. In contrast to primary retinal progenitor cells, RSCs are able to respond to oligodendrocyte priming treatment with expression of oligodendrocyte markers and following transplantation develop myelin basic protein-positive and ultra-structurally normal myelin sheaths around RGC axons.

Conclusion: RSC cultures can be propagated as undifferentiated cells over several passages and maintain the ability to differentiate along the glial and neuronal lineage. However, expanded RSCs do not differentiate into true retinal neurons, e.g. photoreceptors and loose expression of genes characteristic for retinal progenitor cells. They acquire the capacity to express proteins implicated in oligodendrocyte differentiation and after transplantation form myelinating oligodendrocytes. Thus, we conclude that culture expansion conditions based on high concentrations of FGF-2 and EGF lead to a loss of regional identity and conversion of RSCs into tri-potential neural stem cells.

Rhodopsin and transducin in vertebrate rods show transient interactions independent of light conditions – implications for retinal diseases

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Purpose: The early steps in vertebrate vision require fast interactions between the visual pigment rhodopsin (Rho) and the conjugate G protein transducin (G_t). The interaction is classically described by a collisional coupling mechanism driven by the free diffusion of monomeric proteins on the disc membranes of rod and cone cells. However, recent findings point to an extremely low Rho mobility and rather support the organization of the receptor into paracrystalline rafts of varying size. Moreover, increasing evidence seems in favor of interactions between Rho and G_t even prior to light stimuli, which is also difficult to reconcile with the traditional view.

Methods: We investigated the kinetics of interaction between native Rho and G_t in different conditions by biosensor technology and analysed the results in a general physiological context by employing a holistic systems modelling approach. Our analysis focused mostly on the interactions possibly occurring in the dark, and in those after receptor bleaching, a process leading to the formation of Opsin, a constitutively active form of the receptor which was found to be related to Leber congenital amaurosis.

Results: Our results point to a dynamic scaffolding mechanism, in which Rho and G_t interact already in the dark by forming transient complexes (~25% of G_t precoupled to Rho). We show that such a dynamic precoupling does not slow down the phototransduction cascade but rather is compatible with the observed photoresponses on a broad scale of light stimuli. The possible implications for pathological conditions can now be explored in the context of the whole network of interactions.

Conclusion: We conclude that an isolated Rho molecule or a Rho-G_t complex involving 25% of the available transducin in the dark can both absorb a photon and normally trigger the cascade. The general finding that Rho and G_t can interact any time during - and even prior to - the light activation opens new scenarios with novel possibilities to investigate the molecular basis of rod-related diseases.

Localization and functional role of Pannexin1 in the mouse retina

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The vertebrate protein family of pannexins is composed of three members (Panx1, Panx2 and Panx3) and the functional and physiological role of pannexin proteins is still ambiguous. Due to structural similarities of pannexins with gap junction proteins, pannexins have been hypothesized to form gap junction-like membrane channels. So far several studies have demonstrated that Panx1 fails to form gap junctions. In addition, Panx1 shows different channel properties compared to connexins. Recently, it has been postulated that Panx1 forms single membrane channels, which are thought to be associated with cellular transmembrane transport, e.g. with ATP-dependent processes via purine receptors. However, the functional role of Panx1 is just emerging.
Panx1 is expressed in various neuronal tissues including the retina (Dvoriantchikova et al., 2006). Here, we investigate the expression and the functional role of Panx1 in the mouse retina.
We used RT-PCR to identify Panx1 mRNA expression in the mouse retina. To detect Panx1 protein in the mouse retina and in dissociated retinal neurons, we performed immunohistochemical and ultrastructural analyses using anti-Panx1 antibodies. Electroretinograms (ERGs) of Panx1 wild-type and Panx1 knock-out mice were recorded to examine the functional role of Panx1.
Consistent with previous studies, we identified Panx1 mRNA expression in cells of the inner nuclear layer in the mouse retina. Panx1 proteins were located in type 3a OFF bipolar cells and in horizontal cells. In type 3a OFF bipolar, Panx1 is expressed in the entire plasma membrane without preference for a specific compartment. In horizontal cells, Panx1 was located on dendritic tips, which invaginate the photoreceptor terminals. The dark-adapted ERG of Panx1 knock-out mice revealed changes in the light response properties. In comparison with Panx1 wild-type mice, the a- and the b-wave of Panx1 knock-out mice were significantly increased, whereas implicit times (IT) of a- and b-wave were not different between both mouse genotypes. Representing the timing of an ERG component peak, the IT allows to infer on the strength of the synaptic transmission. Therefore, an unchanged IT for the b-wave indicates that synaptic transmission from photoreceptors to bipolar cells was not altered.
In summary, we suggest Panx1 forms single membrane channels in type 3a bipolar cells and in horizontal cells, which play an important role in the signal transduction of the rod pathway.

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Photoreceptor precursor cell transplantation: The outer segment in focus

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Purpose: Retinitis pigmentosa is characterized by a significant progressive loss of photoreceptor cells with no effective treatment available up to date. Current studies are focussing on a cell transplantational approach to replace degenerated photoreceptors. The key feature of every photoreceptor, to have a functional outer segment (OS) with properly aligned disc membrane staples, has not been studied in detail following transplantation so far. In our study we show an ultra-structural analysis of OS integrity on the background of cell transplantational approaches.

Methods: As reporter animals we used knock-in human rhodopsin-GFP fusion construct mice (rhoGFP) and double transgenic mice composed of the rhoGFP mouse plus a chicken beta-actin promoter driven DsRed transgene. Transplanted cells were gained from postnatal day 4 reporter mice retinas. Young photoreceptors were enriched prior to transplantation using CD73 antibody and magnetic associated cell sorting. Finally, donor cells were transplanted into the sub-retinal space of adult wild-type mice. Further analysis was done by immunohistochemical staining of 30µm vibratome sections and ultra-thin cryosections. For electron microscopy analysis a protein A gold staining was applied on labelled cryosections.

Results: GFP-positive OSs were visible in transmission electron microscopy. We were able to rediscover transplanted rhoGFP cells. The transplanted cells which integrated into the outer nuclear layer (ONL) frequently start to develop an OS with well aligned membrane disc staples. Surprisingly, a lot of cells remaining in the subretinal space are also successful in forming OSs with intact and organized disc membrane staples.

Conclusions: We could show that our method is very suitable for detailed examination of OSs of transplanted photoreceptor cells. Well integrated cells develop OSs with native aligned disc staples. Surprisingly, cells which stay in the subretinal space develop OS, too. This leads to the conclusion that OS formation of transplanted photoreceptor precursor cells is independent of an integration of these cells into the ONL.

Statement on proprietary interest: The authors hereby state that there are no commercial of proprietary interest of any kind of any drug, device, or equipment mentioned in this study. Neither author has any financial interest of this study. The poster was prepared solely by the authors listed.

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High-throughput genomic technologies and Autosomal-Recessive Retinitis Pigmentosa diagnosis

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Purpose: Retinitis pigmentosa (RP) refers to a group of hereditary retinal degenerative diseases with high genetic heterogeneity. It affects around 1:4000 individuals worldwide. To date, more than 50 genes have been reported to cause RP in all modes of inheritance, 33 of which are responsible for autosomal-recessive RP (arRP). There are no major disease-causing gene, no recurrent pathogenic mutations and 40% of the cases have yet to be assigned. In our group, we aim to elucidate the molecular basis of arRP by applying state of the art genomic technologies.

Methods: In our collective of 40 Spanish pedigrees, we first perform haplotype analysis using a previously developed high-throughput single nucleotide polymorphism (SNP)-based genotyping cosegregation chip for 116 SNPs of 27 arRP genes. These families also undergo a screening for 594 reported arRP mutations through a commercial chip (Asper-Ophtalmics). When no disease-causing gene is identified, next-generation DNA sequencing is being applied in order to identify new arRP genes.

Results: To date, we identified homozygous mutations in *CERKL*, *RPE65*, *PDE6A* and *MERTK* cosegregating with the disease. In addition, we could rule out 27 arRP genes as disease-causing in 14 pedigrees. Targeted resequencing of exomes and a custom sequence capture array covering all reported arRP genes together with some novel candidates is being currently performed on a SOLiD platform (Applied Biosytems).

Conclusion: The first step before undertaking the search for new arRP causative genes is to rule out all the already reported disease-causing genes. With this aim, we developed a strategy for arRP genetic diagnosis, which merged co-segregation analysis and high-throughput SNP genotyping. This allowed us to identify the genes underlying the disease in 4 pedigrees. We could rule out 27 candidate genes in 14 families too. The cosegregation analysis of the remaining families is ongoing.
Nowadays next-generation sequencing has arisen as a powerful tool to identify rare variants in inherited diseases and the potential of targeted sequence capture and high-throughput DNA sequencing to improve genetic diagnosis has been established. Our ongoing study will assess if next-generation DNA sequencing is also effective as a diagnostic tool for heterogeneous genetic disorders, in order to be incorporated into routine clinical care.

Classical candidate association study identifies a new RPE/retina-specific AMD susceptibility gene

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Background: Age-related macular degeneration (AMD) is a complex disorder of the retina/choroid and the most common cause of legal blindness in industrialized countries. Two major susceptibility loci have been identified including *CFH* on 1q32 and *ARMS2/HTRA1* on 10q26. Furthermore, three additional complement gene loci (*C2/CFB, C3* and *CFI*) have also been shown to play a role in AMD pathogenesis. These gene loci are detectable even in genome-wide association studies (GWAS) with moderate sample sizes. Other susceptibility genes with moderate to minor contributions to AMD risk are likely but difficult to detect in GWAS. However, a candidate gene approach which typically analyses a restricted number of SNPs within a preselected candidate locus in a large case-control sample can provide reasonable statistical power to detect relatively rare and weak risk effects.

Methods: 25 AMD candidate genes were selected based on functional implications in phenotypically related retinopathies, known AMD pathomechanisms and/or an RPE/retina-specific gene expression. These genes were analysed following a haplotype-tagging SNP approach (N = 109) in a German discovery sample consisting of 794 AMD patients and 612 controls. Replication of positive hits was done in a second independent German sample of 867 AMD patients and 517 controls which could be extended by two additional independent Caucasian replication studies from the US and the UK resulting in a pooled replication sample of 1,961 AMD patients and 1,067 controls.

Results: After quality control and analysis of the 109 SNPs we detected three relatively weak association signals in three independent gene loci ($0.014 \leq P \leq 0.032$). One of these signals remained statistically significant in the first replication sample ($P = 9.7 \times 10^{-4}$) strongly suggesting the corresponding RPE/retina-specific gene to be involved in AMD predisposition. Follow-up of this SNP in the US and the UK sample revealed consistent association signals ($P = 0.017$ and $P = 0.033$; respectively) and resulted in a highly significant association in the pooled replication sample ($P = 1.0 \times 10^{-6}$). The observed differences in effect allele frequency in all four studies were similar (13.3–14.3 % in the AMD cases; 10.3–10.9 % in the controls) revealing odds ratios from 1.35 to 1.57. Further fine-mapping of the gene locus narrowed the candidate region to approximately 150 kb that exclusively encompassed the candidate gene in question and so far indicated 15 correlated variants whose functional consequences remain to be elucidated.

Conclusions: The current candidate gene approach successfully indicated a novel AMD susceptibility gene with an RPE/retinal-specific expression revealing a consistent association with AMD in four independent Caucasian case-control-studies. The observed effect allele frequencies and odds ratios classified the locus to be a moderate AMD risk modifier. Ongoing functional analysis of the new AMD susceptibility gene might uncover a functional variant. Further knowledge of the function of the novel gene may point to a new mechanism underlying AMD pathology.

Modulation of ion channels in RPE cells by sublytic complement activation

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Question: The retinal pigment epithelium (RPE) is part of the blood-retinal barrier. Insufficient suppression of the complement system at the RPE is associated with age-related macular degeneration. Sublytic complement activation at the cell surface of RPE cells leads to VEGF secretion which is thought to be controlled partly by L-type calcium channel activation. Since complement activation results in calcium influx, we investigated the ion channel-dependent cellular response to complement exposure.

Methods: Electrophysiological recordings: human ARPE-19 cells, Ba^{2+} as a charge carrier to dissect L-type calcium currents.

Results: Complement activation by treatment with NHS for 10 minutes produced a shift in the voltage dependence of L-type calcium channels towards more positive values (heat-inactivated control: -14.77 mV, NHS: -10.77 mV; p=0.028). Current clamp experiments (physiological conditions) revealed an expeditious hyperpolarization in contrast to control from -38.31 ± 2.74 mV (mean ± SEM) to -62.95 ± 1.45 mV after 70.74 ± 5.13 s of onset of treatment, followed by a depolarization to -3.15 ± 0.64 mV (after 10 min). Voltage clamp experiments showed a linear membrane conductance during the hyperpolarizing phase; hyperpolarizing currents were carried by potassium ions, insensitive to paxilline (50 nM) and iberiotoxin (100 nM); followed by an outwardly rectifying membrane conductance at the late depolarization.

Conclusion: Our experiments demonstrate that the application of NHS leads to complex modulations of membrane conductance in human RPE: activation of non-rectifying ion channels, initial hyperpolarization and final depolarization. Additionally, L-type calcium channels show a strong shift in voltage-dependence.

Progression of age-related geographic atrophy: Role of the fellow-eye

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Purpose: To investigate if the stage of age-related macular degeneration (AMD) of the fellow eye is associated with atrophy progression over time in patients with geographic atrophy (GA) secondary to AMD.

Methods: A total of 300 GA eyes of 193 patients, recruited from the prospective FAM (Fundus Autofluorescence in age-related macular degeneration)-Study, were classified in 3 groups according to the AMD stage of the fellow-eye at baseline: (1) bilateral GA, (2) early AMD and (3) exudative AMD. GA areas of study eyes were quantified on fundus autofluorescence images using semi-automated image analysis and progression rates calculated using a 2-level linear mixed-effects model.

Results: At baseline, 148 patients belonged to group 1, 16 to group 2 and 29 to group 3, respectively. Univariate analysis showed an average population-specific progression rate of 1.64 mm^2/year (95% CI [1.478;1.803]) for group 1, 0.74 mm^2/year [0.146;1.342] for group 2 and 1.36 mm^2/year [0.937;1.787] for group 3. Although there was a statistical significant influence of the size of baseline atrophy on GA progression rate (conditional F-test: p=0.001), adjustment for this parameter still revealed a statistical significant relationship between the disease status of the fellow eye and atrophy enlargement over time (conditional F-test: p=0.033).

Conclusions: The AMD disease stage of the fellow eye determined at baseline examination is associated with atrophy enlargement and thus the extension of corresponding absolute scotoma over time. This may indicate manifestation-dependent disease activity. The identification of prognostic determinants on atrophy progression may not only help to add to our understanding of underlying pathophysiological mechanisms, but also for the design of future interventional trials in GA patients.

Towards a Genetic Risk Model for Age-Related Macular Degeneration (AMD)

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Purpose: Age-related macular degeneration (AMD) is the leading cause of blindness in the Western World and affects an estimated 50 million people worldwide. AMD is a multifactorial trait involving both genetic and environmental effects although the precise aetiology still remains elusive. Age, smoking, and to a lesser extent diet and sunlight exposure are among the most commonly reported risk factors for disease onset. A genetic contribution to AMD is well established by familial aggregation analyses and twin studies. Recently, variations in the complement pathway as well as other loci have been found to be associated with AMD. Testing single susceptibility variants is of limited value for prediction of complex diseases. Genotyping and evaluating several disease associated variations (genetic profiling) however, could be the key to accurately predict disease risk.

Methods: Our study included 979 AMD patients and 793 matched controls recruited from the Lower Frankonian area at the University Eye Clinic of Würzburg (Dr. Claudia N. Keilhauer). Genotyping of patients was performed by various methods including i-plexing, Taqman assays, and direct sequencing. Conditional and unconditional logistic regression was carried out with R, a scripting language used in statistics and epidemiology.

Results: We genotyped previously reported variations that were found to be associated with AMD in European samples and found the most parsimonious set of variations by conditional logistic regression analysis. Ten SNPs showed significant and independent association with late stage AMD. These SNPs were used to build a multivariate logistic regression model. With the log-odds ratios obtained from this model, a genetic risk score for each individual study member was calculated and a hypothetical population assuming different prevalences was modeled. By adjusting the prevalence in this population, we can allow our population to age and thus can predict disease risk at any given age.

Conclusion: Here we suggest a model to predict disease risk for late stage AMD from a well characterized case control study by modeling a hypothetical population and linking the prevalence in this population with age strata obtained from previous publications. This enables us to predict a person's risk to develop late stage AMD assuming the genotype at the ten SNPs used in the model is known.

Retinal structural and functional changes in retinitis pigmentosa patients with confirmed rhodopsin mutations

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Purpose: Commonly central visual acuity is preserved for long time in patients with retinitis pigmentosa. The aim of this study is to examine their retinal structure by means of OCT and to investigate the relationship between structural and functional changes in retinitis pigmentosa patients with confirmed rhodopsin mutations.

Methods: Eighteen eyes in nine retinitis pigmentosa patients (1 (11,1%) female, 8 males (88,9%)) with confirmed rhodopsin mutations were included into the prospective observational study. The mean age was 48±15 years. Spectral domain optical coherence tomography (SD-OCT) with 25 volume *b-scans* was used to acquire high resolution retinal images for every individual. The mean of retinal thickness at 0 degree, 5 degree and 10 degree retinal eccentricity as well as the mean thickness of IS/OS+RPE and RNFL+GLC were assessed by central macula *b-scan*. Retinal cysts were evaluated using novel multimodal mapping software. The vertical diameter of cystical changes was calculated (µm) and averaged in central macula *b-scan* for each individual. Correlation with functional parameters, in particularly visual acuity (logMAR) and static visual field 30 degree, i.e. threshold (LUE), and fixation was performed.

Results: The mean retinal thickness was 268±26 µm, 276±58 µm and 215±19 µm at 0, 5 and 10 degree retinal eccentricity, respectively ($p<0,05$). The mean thickness of IS/OS and RNFL was 36±8 µm and 62±16 µm, respectively. The mean vertical diameter of cystical changes was 67±40 µm in central macula *b-scan*. Cystical changes were observed in 7 eyes (36,84%), while in 2 eyes (28,6%) were located in ONL, 4 (57,1%) in INL and 1 (14,3%) in RNFL. Significant correlation was observed between retinal thickness (at 5 and 10 degree eccentricity) and best corrected visual acuity logMAR) ($r=-0,62$; $p<0,05$) as well as between static visual field treshold and retinal thickness (in 5 degree eccentricity) ($r=0,74$; $p<0,01$). Cystical changes were significantly correlated with fixation in static visual field ($r=-0,762$; $p<0,05$).

Conclusions: Changes in retinal structure are significantly associated with functional alterations in retinitis pigmentosa patients with confirmed rhodopsin mutations. Imaging of retinal structure by means of OCT is one mean to support diagnosis of retinitis pigmentosa as a typical configuration of the retina can be detected, and thus could be applied as sensitive tool in treatment trials.

A new conditional photoreceptor degeneration and regeneration model in the adult zebrafish retina

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Purpose: Since photoreceptor cell loss during adulthood is one of the major causes for blindness in humans, it is of huge interest to investigate retinopathy studies in regenerating model organisms. Unlike mammals, zebrafish gain a tremendous ability to regenerate parts of their central nervous system (CNS) and retina. Furthermore, the wealth of tools available and the cone-dominance of the zebrafish retina make it a promising model to investigate human retinopathies.

Method: We developed in our lab a new conditional genetic lesion model in the Fgf pathway with the aim to analyze photoreceptor degeneration and disorganization in the adult retinal tissue. We characterized our model using BrdU birthdating experiments, apoptosis assays and immunohistochemistry. Furthermore our model allows us to do lineage tracing experiments of different cell populations in the adult retina.

Results: Using conditional transgene expression we specifically ablated photoreceptor cells, while other cells are largely unaffected. Our experiments revealed that photoreceptor cells were gradually dying and almost completely lost within one week after onset of transgene expression. More importantly, we could also show that these processes are completely reversible in the adult zebrafish and regeneration can be traced from dividing progenitors into newly differentiated photoreceptors. Thus, a very fast and profound regeneration response is able to restore the layered structure by integrating new photoreceptor cells into the existing adult retina.

Conclusion: Here we introduce a new transgenic and inducible genetic lesion model to study adult photoreceptor degeneration and regeneration. This model will be used to investigate photoreceptor maintenance and the mechanisms that allow regeneration to occur in the adult vertebrate retina.

Identification of Rx1 (RAX) and Rx-L (RAXL1) target genes in vertebrate development

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Members of the Rax (Rx)-family of homeobox-containing transcription factors are known to play an important role during neural development, in particular the development of the visual system. In humans, mutations of the RAX gene, often within the DNA-binding domain, led to clinical anophthalmia or microphthalmia. However, mutations in a related gene, RAXL1, were associated with age related macular degeneration and cone-rod dystrophy-11. In *Xenopus, Rx1(Rax)* specifies the eye field in concert with other EFTFs (eye field transcription factors), like *ET, Six3, Pax6* and *Lhx2*. During later development *Rx1* keeps retinal progenitor cells in a proliferative state. More recently, we identified a second member of this family from *Xenopus* and we were able to show that *Rx-L (RaxL1)* is important to program retinal progenitor cells into photoreceptor cells at the expense of amacrine and biopolar cells. Little is known about the target genes or potential interacting partners of *Rx-L* and *Rx1*. All vertebrate Rx-family members share a highly conserved homeodomain and within its C-terminus a so-called Rx- and OAR-domain of yet unknown functions. We aim to understand the role of both genes and more specific the role of the putative functional domains outside the homeodomain. Thus, we employ microarray experiments to identify possible target genes of *Rx1* and *Rx-L* using neuralized embryonic stem cells and employ domain swap constructs in *in vivo* lipofection experiments of frog embryos to define putative functions of certain Rx-specific domains.

Development of a humanized Mouse-Model for X-linked Retinitis Pigmentosa caused by a point mutation in the *RPGR* gene

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Introduction: Mutations in the gene encoding the retinitis pigmentosa GTPase regulator (RPGR) are the most frequent causes for X-linked RP in humans. Most of the responsible mutations can be found in a specific repetitive region of the ORF15, which is therefore called the "mutation hot spot" of RPGR. Point mutations in ORF15 cause a frame shift, leading to a modified C-terminal amino acid chain and potentially causing a toxic gain of function of the mutated protein. The purpose of this study is to develop a mouse model that contains a 1 base pair deletion, which provokes a change of the amino acids at the C-terminal end similar to the mutated human proteins.

Methods: In addition to the pathologic mutation 2793delA and two silent mutations (2650subT-C, 3071subA-T), the targeting vector contained positive and negative selection markers (neo, DTA), and a restriction site for the homing endonuclease ISceI for later applications in gene therapy. Genotyping of resulting offspring was performed by PCR, RT-PCR, restriction digestion and sequencing. For histological analysis, whole eyes were fixed in Bouin's solution and embedded in paraffin. Slices of 7µm were stained with hematoxylin/eosin and images were obtained using a digital microscope (Keyence B8000).

Results: The targeting vector was introduced into C57/BL6-129sv hybrid ES cells and positive clones were implanted into surrogate mother mice. Chimeric animals were genotyped and back-crossed into BL/6 background. Histological analysis of the retina reveals a progressive reduction of the size of the retina as well as delocalized nuclei among the IS (inner segments) and OS (outer segments) of photoreceptors as early as 12 weeks post natal.

Discussion: The newly generated mouse model displays a degenerative phenotype, suggesting the activation of a similar or at least related pathologic pathway compared to human patients. Therefore, this model may help to gain further insight into the pathological mechanisms involved in retinal degeneration, the expression pattern of mutated RPGR-ORF15 forms, the influence of point mutations in the ORF15 repetitive region on expression and splicing of the mRNA, and the biochemical reason for the toxicity of such proteins.

Towards a stem cell-based intraocular delivery system

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Objective: The application of neuroprotective factors that attenuate photoreceptor degeneration is among the strategies to develop mutation-independent treatments for hereditary retinal degenerations and other pathologies involving photoreceptor death. A continuous and long-lasting intraocular delivery of neuroprotective factors might be achieved by transplantations of stem cells engineered to express these factors. To establish such a stem cell-based intraocular delivery system, we generated novel lentiviral vectors to express neurotrophic factors in murine neural stem (NS) cells and the neural precursor cell line C17.2. Transplantation experiments into the *rd1* mutant mouse were performed to evaluate the therapeutic potential of this stem cell-based delivery system.

Methods: To express neurotrophic factors in murine NS cells, we have generated bicistronic lentiviral vectors encoding a CMV early enhancer/chicken ß-actin (CAG) hybrid promoter, a "gene of interest" (GDNF or CNTF), and a fluorescence/resistance fusion gene. For co-expression of transgenes, cells were transduced with several vectors each encoding a different combination of fluorescence and resistance genes. Engineered stem cell cultures and clonal stem cell lines were analyzed for expression of transgenes using immunocytochemistry and immunoblot analysis, and grafted into *rd1*, *rd10* or wild-type mice to study their fate *in vivo* and to analyze the therapeutic potential of this stem cell-based delivery system.

Results: Lentiviral vectors allowed rapid derivation of engineered stem cell cultures and clonal stem cell lines. Immunocytochemistry and immunoblot analysis confirmed expression of GDNF and CNTF in both cell populations, and their secretion into the culture supernatant. Co-expression of transgenes was also successfully achieved, as indicated by the co-expression of two or more reporter genes. Intraocular transplantation experiments revealed that grafted cells survived and expressed the transgenes for at least one month. Importantly, intravitreal injections of clonally derived C17.2 cell lines with a forced expression of CNTF significantly attenuated photoreceptor degeneration in the *rd1* mouse mutant.

Conclusions: We have developed a stem cell-based intraocular delivery system that enables us to target functionally relevant quantities of neuroprotective factors to the retina. This stem cell-based delivery system will be of use to evaluate the therapeutic potential of other factors with neuroprotective activity on photoreceptors, as well as combinations thereof, in mouse models of retinal dystrophies.

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Towards identification of the genetic defect underlying North Carolina Macular Dystrophy (NCMD)

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Purpose: NCMD is an autosomal dominant macular dystrophy with congenital or infantile onset and a remarkable variation of clinical phenotypes. The NCMD gene locus has been mapped to chromosome 6q16. Genotyping of three German NCMD families identified an ancestral core haplotype that defines a 1.85 Mbp candidate interval. Direct sequencing of all annotated genes including the abundant retinal PRDM13 gene in these NCMD patients failed to identify the disease causing mutation. Here, we performed genetic mapping in novel NCMD families to screen for additional NCMD-associated haplotypes and furthermore assessed the role of chromosomal rearrangements in NCMD.

Methods: Individuals from 14 families with clinical symptoms of NCMD were genotyped with microsatellite markers located within the critical NCMD interval. Southern blot analysis was used to screen for larger chromosomal abnormalities around the PRDM13 locus.

Results: Additional five NCMD families and one simplex case were identified to carry the ancestral haplotype. One recombination event further refined the minimal candidate region to a 1.64 Mbp interval. Moreover, additional and distinct haplotypes were found in two German families. So far, Southern blot analysis of DNA from genetically-mapped NCMD patients with five out of nine probes covering the entire PRDM13 region showed no chromosomal rearrangement.

Conclusions: The failure to detect the NCMD-associated founder mutation by direct sequencing of the coding sequence of all annotated genes in the NCMD candidate region raises the possibility of genomic rearrangements that possibly affect regulatory regions as the cause of NCMD. Thus, extensive analysis by Southern Blotting will be performed to screen for chromosomal abnormalities. The identification of NCMD families with haplotypes different from the founder haplotype suggests independent mutations underlying NCMD. Mutational screening of these patients is currently ongoing.

PU.1 and NFkB control *Activated Macrophage/Microglia WAP domain protein (AMWAP)* expression during microglial activation

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Purpose: Microglial activation is a common phenomenon in retinal dystrophies. To identify genes involved in this process, we used DNA-microarray analysis of microglial cells from wild-type and retinoschisin-deficient mice, a prototypic model for inherited retinal degeneration. We identified strong expression of a novel microglia-restricted gene that was termed *Activated Macrophage/Microglia WAP domain protein (AMWAP)* (Karlstetter et al., J Immunol, 2010). AMWAP acts as a negative regulator of microglial activation and shows broad serine protease-inhibition and anti-bacterial potential. We hypothesize that AMWAP secretion from alerted microglia is triggered by *disease associated molecular patterns (DAMPs)* present in the degenerating retina. The aim of this study was to characterize the transcriptional regulation of AMWAP during different inflammatory conditions.

Methods: Cell culture experiments using mouse microglia and macrophages were used to investigate induction of AMWAP gene expression by TLR agonists and Interferon-gamma (IFNγ) in time-kinetics and dose-response experiments. qRT-PCR was performed to determine AMWAP transcript levels. *In vivo* binding of the transcription factors PU.1 and NfkB to the AMWAP promoter was analyzed by chromatin immunoprecipitation followed by qPCR.

Results: Microglia-specific AMWAP expression critically depends on the myeloid-specific transcription factor PU.1, which binds to the proximal promoter region of AMWAP. AMWAP transcript levels are also sensitive to IFNγ and several Toll-like receptor agonists mimicking disease associated molecular patterns (DAMPs) in the degenerating retina. Up-regulation of AMWAP by TLR signaling was mainly mediated by NfkB. Blockade of new protein synthesis by cycloheximide reduced this effect, indicating an influence of further transcription factors that are newly synthesized upon TLR stimulation.

Conclusion: AMWAP transcription is mainly regulated by PU.1 and depends on NfkB signaling during pro-inflammatory activation of microglial cells. Other transcription factors like *Signal Tranducers and Activators of Transcription (STATs)* could be involved in AMWAP expression under specific inflammatory conditions.

Immunhistochemical characterization of the outer plexiforme layer in animal models of retinal degeneration and gene transfer

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Introduction: The transient detachment of the neuroretina from the RPE through a subretinal injection of saline causes reactive gliosis in several animal models. The question of whether the subretinal injection of an AAV vector suspension causes permanent alterations within the synaptic circuits of the outer plexiform layer (OPL) remains unknown. The aim of this study was to develop immunhistochemical tools for the characterization of the OPL in two different animal models for retinal degeneration and gene transfer, the wistar rat and the Swedish Briard dog with mutations in the RPE65 gene.

Methods: Retinas have been obtained from a total of 6 untreated dogs, 4 affected and 2 unaffected, as well as from 6 rats that underwent AAV mediated gene transfer. The tissues were fixed in 4% PFA, cryoprotected in sucrose suspension and snap frozen in liquid nitrogen prior to sectioning using a cryostat. Antibodies that have been used: CtBP2 (synaptic ribbons), PKCα (rod bipolar cells), calbindin (horizontal cells), dhp (L-type calcium channel in photoreceptor terminals), and PSD95 (photoreceptor axon terminals).

Results: In rats, all tested antibodies allowed a strong and specific detection of the respective epitopes. Pre- and postsynaptic structures are macroscopically not altered after subretinal injection and absolute numbers of synaptic ribbon structures do not differ significantly between injected and control retina. In dogs, only CtBP2, PKCα, and calbindin could be used for characterizing the OPL. In young animals (less than 3 years), the OPL structure does not differ between affected and unaffected animals. At later time points, pathologic changes can be observed.

Discussion: We identified a battery of antibodies that can be used to analyze the morphological structures within the OPL in rats and dogs. The neuronal circuits in the OPL of healthy rat retinas undergoing AAV mediated gene transfer are not altered following the subretinal injection, by the presence of viral particles or the expression of GFP as transgene. In dogs, the effects of AAV mediated gene therapy following subretinal injection can be analyzed using the above tested antibodies, which may reveal new information about the difference in treatment efficiency between dogs and man.

Genes and mutations in autosomal recessive achromatopsia

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Purpose: Achromatopsia (syn.: rod monochromatism, total colorblindness) is a rare autosomal recessive inherited retinal disorder characterized by the absence of color discrimination, low visual acuity, photophobia and congenital nystagmus. Here we provide valid and reliable data on the genetic basis of achromatopsia in the Caucasian population including the prevalence of the known disease genes *CNGA3, CNGB3, GNAT2* and *PDE6C*.

Methods: Genetic testing comprised subsequent and continuous analysis of the coding exons and flanking intron sequences of *CNGA3, CNGB3, GNAT2* and *PDE6C* in over 600 independent patients with a clinical diagnosis of autosomal recessive achromatopsia by means of PCR and direct Sanger sequencing. Segregation analysis was carried out where DNA samples from family members were available.

Results: We were able to identify mutations in 579 patient from 397 individual families with a clinical diagnosis of autosomal recessive achromatopsia: 216 patients (141 families) carried mutations in *CNGA3*, 336 patients (237 families) in *CNGB3*, 16 patients (12 families) carried mutations in *GNAT2*, and 11 patients (7 families) in *PDE6C*. Single heterozygous mutations were observed in 6 cases in *CNGA3* and in 27 patients in *CNGB3*. The complete mutation spectra comprised <100 different mutations in *CNGA3*, 58 mutations in *CNGB3*, 14 mutations in *GNAT2* and 11 mutations in *PDE6C*.

Conclusions: *CNGB3/ACHM3* remains the major locus for achromatopsia accounting for ~50% of all affected patients, while mutations in *CNGA3/ACHM2* are responsible for ~30% of achromats in our patient sample. In contrast, *GNAT2/ACHM4* and *PDE6C/ACHM5* only play a minor role with 2.5% and 1.5% of patients carrying mutations in these genes, respectively. Our data and patient sample will be valuable to support clinical and therapeutic studies on achromatopsia, which is currently being considered as a major target for retinal gene therapy.

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The Secretion of ARMS2 Suggests a Novel Protein Export Mechanism

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Purpose: Over one-third of all proteins synthesized in eukaryotic cells enter the secretory pathway. In the best characterized cases, the first translated, N-terminal, signal peptide and its receptor directs the ribosome to the endoplasmic reticulum (ER). Besides, a subgroup of proteins is fully synthesized in the cytosol, and reaches the ER only post-translationally. Yet another group of proteins is secreted without traversing the ER-Golgi system.
ARMS2 is one of the three candidate genes strongly associated with age-related macular degeneration (AMD) and subjected to unconventional secretion. Here we set out to determine the overall trafficking patterns of this exceptional protein.

Methods: A series of mutant ARMS2 proteins were expressed in cultured cells and tested for intracellular trafficking. Protein secretion was followed by fluorescent microscopy (ApoTome, Zeiss) and Western blotting of extra- and intracellular fractions. Proteins involved in the export of ARMS-2 were identified by co-immunoprecipitation using anti-ARMS2 antibody and identified by mass spectrometric analysis. The structure of ARMS2 was predicted by using the I-TASSER server and visualized by PyMOL.

Results: We found that the di-isoleucine motif within the last eight amino acids (-SIIHTAAR*) are indispensable for proper targeting. Besides, we identified two further regions each spanning ~20 amino acids, which are also involved in this process. The modeling of ARMS2 suggests that the di-isoleucine motif forms a highly hydrophobic interface between these two stretches. Several further proteins were found in the human genome possessing a C-terminal di-Ile motif, and a few of them have been cloned, mutated and tested in an analogous way to ARMS2. Nevertheless, no further proteins were found up to now, where such a di-Ile motif would play a similar crucial role in targeting. Co-immunoprecipitation results indicate the involvement of the poorly characterized vesicular SNARE protein SEC22B in the transport process that is ER-dependent but Golgi-independent.

Conclusions: The secretion of ARMS2 suggests an alternative secretory pathway, probably depending on the 3D structure of the protein. The vSNARE protein SEC22B may play a pivotal role in hauling ARMS2. Because drusen deposition matches that of ARMS2 on the other side of the Bruch´s membrane, this secretory mechanism might play an important role in the pathogenesis of AMD.

Small molecule-reprogrammed human induced pluripotent stem cells differentiate into functional RPE and rescue retinal degeneration in vivo

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Purpose: The recent development of technologies capable of producing induced pluripotent stem (iPS) cells from adult somatic tissues may permit their use to generate autologous retinal pigment epithelium (RPE) grafts for the treatment of retinal diseases such as age-related macular degeneration and Stargardt disease. Current reprogramming techniques require retroviral transduction of four transcription factors which in part possess oncogenic potential. Given the associated risk of tumor formation, the use of iPS cells reprogrammed with a reduced number of these factors would be advantageous. We evaluated human iPS cells that were reprogrammed using small molecules and only two or one exogenous transcription factors for their capacity to differentiate into functional RPE grafts.

Methods/Results: iPS cells were generated from primary human epidermal keratinocytes by lentiviral transduction of two factors (Oct4, Klf4) or one factor (Oct4) and additional treatment with small molecules. iPS cells reprogrammed by standard four factor transduction (Oct4, Sox2, Nanog, Lin28) served as controls. RPE cells could be differentiated from four factor, two factor and one factor-derived iPS cells. Cells expressed RPE-specific markers (bestrophin, CRALBP, RPE65), formed epithelial monolayers with intracellular tight junctions, and exhibited homogenous polygonal morphology and pronounced pigmentation. Cells performed RPE-specific functions such as vectorial apical-to-basolateral fluid transport and photoreceptor outer segment phagocytosis. Subretinally transplantated cells reduced retinal degeneration in the Royal College of Surgeons (RCS) rat, a model of RPE-mediated retinal degeneration.

Conclusion: We demonstrate the capability of two and one factor-derived human iPS cells to differentiate into cells with RPE-specific morphology and function and to rescue retinal degeneration in vivo. Further optimization of reprogramming and differentiation efficiency is crucial for future therapeutic application of iPS cell-derived RPE cells as autologous grafts in retinal diseases.

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ARMS2 expression is influenced by risk variants at the 10q26 AMD locus but not causative for increased AMD susceptibility.

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Backround: AMD is a multifactorial disease of the central retina and a leading cause of irreversible vision loss in developed countries. Genetic variants in chromosomal region 10q26 have been strongly associated with the disease and are located within a 23.3 kb region of strong linkage disequilibrium. These polymorphisms center over two nearby genes, *ARMS2* (age-related maculopathy susceptibility 2) and *HTRA1* (HtrA serine peptidase 1). With the exception of a single non-synonymous variant (rs10490924), the remaining associated polymorphisms appear not to have any obvious functional consequences but may influence expression levels of either *ARMS2* or *HTRA1* (1, 2). The aim of our study was to analyze the effect of risk and non risk associated polymorphisms on the expression of *ARMS2* and to detect putative functional consequences of altered *ARMS2* mRNA levels.

Methods: Cos-7 cells were transfected with genomic, risk and non risk associated *ARMS2* variants, and *ARMS2* expression was compared by qRT-PCR and immunocytochemistry. The influence of an indel variant (c.* 372_815del443ins54) in the 3'-region of *ARMS2* on its expression was tested by generating chimeric constructs of risk and non risk associated haplotypes. The expression of different *ARMS2* isoforms was also analyzed in human post-mortem retina/RPE samples heterozygous for different 10q26 haplotypes via semi-quantitative sequencing. Finally, the effect of *ARMS2* knockdown and overexpression on the transcriptome of BeWo cells was analyzed using the GeneChip Human Exon 1.0 ST Array (Affymetrix).

Results: In in vitro assays, *ARMS2* mRNA levels transcribed from the risk haplotype were significantly reduced compared with non-risk mRNA isoforms. Analyzing chimeric *ARMS2* constructs, this effect could specifically be assigned to the insertion/deletion polymorphism (c.(*)372_815del443ins54) in the 3'-untranslated region of *ARMS2*. Analysis of human retina/RPE samples heterozygous for the risk haplotype confirmed the *in vitro* results demonstrating that the risk haplotype decreased *ARMS2* transcript levels. However, we provide *in vivo* evidence that a common non-risk-associated variant (rs2736911) also leads to decreased *ARMS2* transcript levels. In addition, an up- or downregulation of *ARMS2* expression in BeWo cells had no influence on the BeWo transcriptome.

Conclusion: Our data suggest that pathogenic effects due to *ARMS2* mRNA or protein deficiency are unlikely to account for AMD pathology.

Literature: 1) Fritsche et al., 2008, Nat. Genet., 40:892-896; 2) Dewan et al., 2006, Science, 314: 989-992

Microglial Activation and Transcriptomic Changes in the Blue Light Exposed Mouse Retina

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Microglia are important components of the ocular immune system and may contribute to age related macular degeneration. Exposure to blue light induces oxidative protein modifications similar to those observed in drusen and elicits retinal immune responses. To study the underlying cellular events, we analyzed microglial activation and monitored transcriptomic changes in blue light-induced retinal lesions. MacGreen mice with EGFP-tagged retinal microglia were exposed to blue light. At different time intervals, eyes were prepared for immunofluorescence microscopy, microarray analysis and qRT-PCR. Retinal whole mounts and cross sections showed that EGFP labeled microglia rapidly migrated towards the retinal lesion. Prominent transcriptomic changes occurred after 12h, peaked at 24h and declined at 72h. We identified more than 100 differentially expressed genes, including transcripts related to microglial activation, apoptosis and regenerative signaling. A comparison of our results with published datasets from white light damage indicates overlapping but also distinct molecular mechanisms. This study extends our knowledge of transcriptomic changes in light induced models of retinal degeneration.

Imbalance of complement regulatory proteins CFHR1, CFHR3 and factor H influences risk for Age Related Macular Degeneration (AMD)

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**contribute equally*

Purpose: Age related macular degeneration (AMD) is a degenerative eye disease in older individuals. Deletion of the factor H related genes *CFHR1* and *CFHR3* (Δ*CFHR1*/ *CFHR3*) is protective from AMD, but the underlying mechanism of this effect is still unclear. The deletion is discussed to be linked to one of the two protective polymorphisms of the *factor H* (CFH) gene, SNP (rs1061170) and SNP (rs2274700).

Results: Here we show in a German cohort of 530 AMD patients that Δ*CFHR1*/*CFHR3* is protective to AMD and that this effect is independent of the two polymorphisms in CFH. These findings suggested involvement of *CFHR1* and *CFHR3* protein functions in AMD. We characterized *CFHR3* functions and identified a novel human complement regulator that has cofactor activity for the serine protease factor I for cleavage of C3b. In addition *CFHR3* has anti-inflammatory effects and blocks C5a generation and C5a mediated chemoattraction of neutrophils. *CFHR3* and also *CFHR1* and factor H plasma concentrations are relevant for complement regulation, as all three proteins compete for binding to the central complement component C3b. Δ*CFHR1*/*CFHR3* enhances local factor H binding and regulation, which can explain the protective effect of Δ*CFHR1*/*CFHR3* from AMD.

Conclusions: The findings underline the central role of complement regulation in AMD and define a critical balance of the complement regulators *CFHR1*, *CFHR3* and Factor H in human plasma.

The new chimeric alternative complement inhibitor COMP_CFH15-20 protects surfaces from complement mediated damage

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Purpose: The human complement cascade regulates tissue homeostasis, controls inflammation and modulates the adaptive immunity. However, defective regulation of this self amplifying cascade results in tissue damage and is a major risk factor for the development of age-related macular degeneration (AMD). Therefore intervention in the human complement system is both relevant and attractive for treatment of complement mediated diseases such as AMD. One major aspect of therapeutic complement control is whether inhibition is achieved systemically or locally. Given the central role of the alternative regulator Factor H and its surface attachment for the protection of retinal surfaces, we utilized the surface attachment region of Factor H, as a central sensor of cellular damage, to direct a complement inhibitor to retinal surfaces and to sites of immune stress.

Methods / Results: To this end we generated a recombinant fusion protein, that combines the surface attachment region of Factor H (i.e. domains SCR15-20) with the established complement inhibitor compstatin. The dual activities of this novel COMP_CFH15-20 protein were assayed regarding C3/C3b binding, and by comparing the complement inhibitory activities in fluid phase and on the surfaces of nucleated cells in the presence of regulator-reduced human plasma. COMP_CFH15-20 efficiently blocked complement amplification on activator and non-activator surfaces of erythrocytes, on living as well as on degenerated nucleated cells. Bound to epithelial cells COMP_CFH15-20 protected these surfaces from complement mediated damage in a dose-dependent manner, and the tagged compstatin was about 30 % more efficient than the native one.

Conclusion: COMP_CFH15-20 is a promising new complement regulator. It allows potent local complement control, specifically at surfaces damaged by immune stress and additionally abolishes -some- adverse effects, that occur upon systemic complement inhibition.

Store operated calcium entry in short-time culture of pig retinal pigment epithelium

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Retinal pigment epithelium (RPE) interacts with photoreceptors and supports visual function. Many of these functions such as visual cycle or secretion are regulated by increases in intracellular Ca^{2+} as second-messenger.
Store operated calcium entry (SOCE) is an important mechanism in calcium signaling to regulate many cellular processes. In native RPE cells little is known about I_{CRAC} channels activation. We aim to show activation of SOCE through I_{CRAC} channel pathway in primary pig RPE cells close to *in vivo* conditions.

Methods: RT-PCR and FURA 2AM Ca^{2+} imaging in pig RPE cells 48 h after preparation.

Result: RT-PCR revealed expression of I_{CRAC} channel proteins Orai 1-3 and in addition Stim 1and 2 proteins. Activation of SOCE was induced by depletion of Ca^{2+} store (ER) in the presence or absence of thapsigargin (1 µM) under extracellular Ca^{2+} free conditions. Re-adding of extracellular Ca^{2+} increased intracellular Ca^{2+} by 15 fold of basal calcium (n = 9). This was blocked with 2-APB (75 µM) but not with SKF (50 µM). Due to the large increase of Ca^{2+} trigged by thapsigargin, 2-APB (5 µM) had no effect but when the depletion was only with extracellular Ca^{2+} free, 2-APB (5 µM) increased intracellular Ca^{2+} from 123 nM to 151 nM.

Conclusions: Short-time RPE cell culture which is close to *in vivo* conditions show functional presence of Orai probably regulated by Stim activation. Our findings bring a new pathway of calcium signaling in the RPE that give insight on function of endogenously expressed proteins in this tissue which are involved in Ca^{2+} signaling.

Molecular in vivo imaging of VEGF using fluorescence-labeled Bevacizumab in an animal model

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Purpose: The aim of this study is to develop a fluorescent molecular probe linked to a humanized monoclonal vascular endothelial growth factor (VEGF)- antibody against VEGF for *in vivo* imaging of free VEGF in the retina.

Methods: Bevacizumab was covalently attached to a near-infrared indocyanine dye yielding a soluble conjugate. *In vivo* reflectance (exc. 488 nm and 830 nm) and fluorescence (exc. 488 nm and 790 nm, em. 500–700 nm and >800 nm) imaging was performed in eleven Dark Agouti rats using confocal scanning laser ophthalmoscopy (cSLO). Multiple retinal lesions around the optic nerve head were placed by argon laser photocoagulation on day one. On day seven the fluorescent bevacizumab conjugate was intraperitoneally injected and its retinal uptake recorded *in vivo*. Animals were imaged at pre-defined time intervals for up to 35 days.

Results: *In vivo* imaging before dye injection showed ill-defined retinal lesions on day seven. Directly following injection (on day seven) no fluorescence was visible in all animals, while a strong signal was present on day eight in the retinal vasculature and within roundish laser lesions. Furthermore, multiple fluorescent spots were visible. Over time, a continuous decrease of the fluorescence intensity was observed within retinal blood vessels and laser lesions for up to 23 days after injection. Fluorescent spots were visualized up to 35 days following injection.

Conclusion: This study demonstrates that the fluorescent bevacizumab conjugate can be visualized in the retinal vasculature using *in vivo* imaging. Its spatio-temporal relationship at the site of VEGF-hyperexpression can be determined. Following further expanded investigations in animal models, molecular imaging of VEGF may be applicable in patients for earlier diagnosis and more refined individualized anti-VEGF therapies to optimize functional outcomes.

Expression of recombinant ARMS2 in *Pichia pastoris*

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Purpose: Age-related macular degeneration (AMD) is the most frequent cause of blindness in people older than fifty years. The hallmark of AMD is the accumulation of deposits (drusen) at the macula, which leads to the degeneration of pigment epithelial cells in the retina. AMD is complement associated as complement genes such as *factor H, CFHR1, CFHR3, C2, C3, factor B* determine susceptibility to the disease. Nucleotide polymorphisms in two further genes (*ARMS2* and *HTRA1*) with advanced AMD susceptibility were identified in a number of independent genetic studies. However, the precise physiological role of the encoded gene products are still unclear and also reports about expression and cellular localization are inconsistent. Thus the function of *ARMS2/HTRA1* remain to be fully evaluated. To determine the physiological role of *ARMS2*, recombinant expression and efficient purification of the protein is of high interest. Here we show expression of the *ARMS2* protein in the *Pichia pastoris* expression system and its purification to homogeneity.

Methods: Condon optimized cDNA of *ARMS2* was cloned and protein was expressed in Pichia pastoris. The recombinant protein was purified by nickel-affinity chromatography. The identity of *ARMS2* was confirmed by MALDI Tof analysis. The recombinant protein was separated by SDS PAGE and stained with silver or detected by immune staining. Different antibodies were compared for detection.

Results: The *ARMS2* protein was recombinantly expressed in *Pichia pastoris* system followed by chromatocraphical purification steps that revealed a highly purified protein. The high yield of protein expression will be used to immunisation of rabbits to generate specific antiserum. The purification of *ARMS2* under physiological conditions allows functional assays such as binding of *ARMS2* to human cells. The recombinant protein together with the antiserum we will focus on the identification of the *ARMS2* function and how the polymorphisms in the *ARMS2* gene influence protein functions. These findings will help to understand the pathomechanism of AMD.

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Disease-associated missense mutations in bestrophin-1 affect cellular trafficking and anion conductance

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Purpose: Bestrophin-1 is an integral membrane protein encoded by the BEST1 gene and localized predominantly to the basolateral membrane of the retinal pigment epithelium (RPE). Mutations in the BEST1 gene were first associated with Best vitelliforme macular dystrophy (BMD), central retinopathy with autosomal dominant inheritance and variable penetrance. To date, over 120 disease-causing mutations are known. While initially thought to impair Cl^- channel function, the molecular pathology of BEST1 mutations is still controversial. To further study the cellular effects of BMD mutations, we have now analyzed the subcellular localization and anion conductance of 13 disease-associated BEST1 mutations.

Methods: Wild-type bestrophin-1 and 13 missense mutations located in four mutational hot spots of bestrophin-1 were generated by site-directed mutagenesis and stably expressed in polarized MDCK II cells, an established model of apical to basolateral protein sorting. Cells were subsequently co-stained with anti bestrophin-1 antibody and antibody markers for subcellular compartments. Pearson's correlation coefficient (PCC) was employed to quantitatively evaluate co-localization. To investigate the impact of missense mutations on the ability of bestrophin-1 to increase anion permeability of the cell membrane, iodide fluxes were measured by halide sensitive YFP^{I152L} fluorescence quench.

Results: Wild-type bestrophin-1 was targeted to the basolateral membrane and showed strong co-localization with the plasma membrane marker monocarboxylic acid transporter 1 (MCT1). In contrast, 9 out of 13 bestrophin-1 mutants revealed no co-localization with the MCT1 membrane protein and mainly accumulate in the Golgi complex and the endoplasmic reticulum. Functional analysis of bestrophin-1 mutants by Iodide fluxes in HEK-293 cells showed that all mutants exhibited significant reduction in anion conductance.

Conclusions: Together, our data indicate that a defective intracellular trafficking could be a common cause of BMD accompanied by an impaired anion conductance representing a loss of anion channel function likely due to mistargeting of mutant proteins.

Cleavage of Fas Ligand Regulates Vessel Leakage in Laser Induced CNV

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Purpose: In age-related macular degeneration (AMD) choroidal neovascularization (CNV) is the main cause of severe visual loss, in part due to vessel leakage that causes retinal edema. Fas ligand (FasL) has been shown to be an important inhibitor of neovascularization by inducing apoptosis in Fas+ endothelial cells. However, FasL is a membrane-bound protein (mFasL) that can be cleaved by metalloproteases to release a soluble form (sFasL). Membrane FasL is pro-apoptotic and pro-inflammatory, while sFasL is anti-apoptotic and anti-inflammatory. FasL is constitutively expressed in immune privileged sites and within the eye is found in the retina and RPE cells. Preventing FasL cleavage by treating with doxycyline to inhibit MMPs has been shown to reduce CNV. We extended these studies by examining the effect of FasL cleavage in vessel leakage using a membrane-only FasL mouse (ΔCS.1) in which the FasL metalloproteinase cleavage sites in exon 2 was mutated. ΔCS.1 mice only express mFasL and no sFasL.

Methods: A mouse model of laser-induced CNV was used in which four laser spots were placed in each quadrant using a red krypton laser. Groups of WT B6 and ΔCS.1 mice received laser treatment and at days 5 and 14 post-treatment high-resolution SD-OCT and fluorescein angiography (FA) were performed. Choroidal flat-mounts and retinal sections were analyzed histologically.

Results: Laser-induced damage led to significantly increased vessel leakage in WT, but not in mFasL- only mice, as observed by FA 5 and 14 days post treatment. Reduced vessel leakage was not due to a smaller retinal lesion in mFasL- only mice, since SD-OCT analysis revealed equal size lesions in WT and mFasL- only mice.

Conclusions: Laser treatment of ΔCS.1 mice that express only mFasL resulted in significantly less vessel leakage. These data indicate that cleavage of FasL and production of sFasL is pathogenic and contributes to vessel leakage and retinal edema.

Retinal degeneration and microglial activation in mouse models of neuronal ceroid lipofuscinoses

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Purpose: Neuronal ceroid lipofuscinoses (NCL) are early onset lysosomal storage disorders characterized by vision loss, mental and motor deficits, and spontaneous seizures. Patients remain in a vegetative state for several years before premature death occurs. Neuropathological analyses of human autopsy material and brain from NCL animal models revealed neuroimmune processes closely associated with neuronal degeneration. It is currently unclear whether this phenomenon is confined to the brain or also occurs in the retina. Therefore, the aim of our study was to characterize the relation between retinal degeneration and microglial activation in different mouse models of NCL.

Methods: Retinal degeneration of the NCL mutant mouse strains $Cln3^{\Delta ex7/8}$ KI and $Cln6^{nclf}$ was characterized by detailed structural analyses at different ages. Microglial morphology and migration was analyzed by immunohistochemistry. Visual acuity was determined by measuring the optokinetic response in an Optomotry system.

Results: Our data show that there is a migration of microglia from the plexiform layers to the nuclear layers in $CLN6^{nclf}$ and $Cln3^{\Delta ex7/8}$ KI retinas, which is consistent with an alerted state of microglia. Moreover, the cell shape of these cells changed from a ramified form to an amoeboid form. Histological analyses revealed that this microglial activity was accompanied by a prominent retinal degeneration. Optomotry tests showed that the $CLN6^{nclf}$ and $Cln3^{\Delta ex7/8}$ KI mice had a progressive decline in visual acuity as they aged.

Conclusion: Our results identified a coincidence of microglial activation, retinal degeneration, and vision loss in $CLN6^{nclf}$ and $Cln3^{\Delta ex7/8}$ KI mice. We therefore hypothesize that therapies aimed a modulating retinal microglia activation could be helpful to preserve vision in NCL patients.

L-type Ca^{2+} channels, Ca^{2+}-activated K^+ channels and their key role in the phagocytic function of the retinal pigment epithelium

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Objective: In the retinal pigment epithelium (RPE) the activation of L-type Ca^{2+} channels regulates different physiologically important cell functions such as phagocytosis of shed photoreceptor outer segments. Recently a direct coupling of large-conductance voltage- and Ca^{2+}-activated K^+ channel (BK channel) activity to L-type Ca^{2+} channel activation via a negative feedback control has been described (Wimmers et al. 2008, Molecular Vision). Aim of this study was to investigate the relevance of these interactions for phagocytosis.

Methods: To analyze the role of BK channels and L-type Ca^{2+} channels Cav1.3 in phagocytosis mice with deficiencies for both channel proteins were used (BK-/-, Cav1.3-/-). For in vivo measurement of circadian rhythm of retinal phagocytosis we labeled the outer segment protein rhodopsin in retinal cross-sections of mice and quantified rhodopsin-positiv phagosoms in the RPE at two time points during the day: 30 minutes and 8 hours after onset of light in the morning. An in vitro approach for RPE cell phagocytosis of photoreceptor outer segment fragments was established.

Results: In the morning, phagocytic activity showed in wild-type mice a peak of 11±4 phagosomes/ 100 µm retina which decreased to a base level of 4±2 phagosomes/ 100 µm. In the BK-/- mice we observed an increase of the peak activity to 15±5 phagosomes / 100 µm and a return to lower base level of 2.5±2 phagosomes/ 100 µm. Both, the peak phagocytic activity in the morning and during the afternoon were significantly different ($P < 0.001$). In the Cav1.3-/- mice we observed a significantly increased phagocytic activity at the second time point, with the number of 5±1 phagosomes / 100 µm increased compared with the wild-type control. Phagocytosis of RPE cells in vitro was increased by iberiotoxin, a specific BK channel blocker. Additionally we observed in 16 months old BK-/- mice a decrease in photoreceptor outer segment length, measured as ratio outer segment length/ inner segment length of 1.83±0.6 compared with 2.23±0.8 in wildtype mice.

Conclusion: In conclusion, the absence of BK channels and Cav1.3 channels lead to altered circadian regulation of phagocytic function of the RPE in vivo. Inhibition of both channels to modulate phagocytic activity in vitro. These data indicate for the first time a role of ion channels in the regulation of phagocytosis by the RPE.

Gene Repair by Zinc Finger Nucleases mediated Homologous Recombination of USH1C

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Purpose: Human Usher syndrome (USH) is the most frequent cause of inherited combined deaf-blindness. It is clinically and genetically heterogeneous, assigned to three clinical USH types of which the most severe type is USH1. No effective treatment for the ophthalmic component of USH exists. Gene based strategies are attractive for the treatment of hereditary retinal degenerations. Here we establish a strategy for gene repair of the *USH1C* gene by homologous recombination (HR). We replace a mutated segment of *USH1C* by an exogenous rescue plasmid encoding for the healthy gene. To increase the efficiency of HR, double-strand breaks are introduced at the mutated chromosomal region using zinc finger nucleases (ZFN).

Methods: We screened the mutated *Ush1c* sequence for suitable ZFN binding sites and generated ZFN by modular assembly. We demonstrated the ZFN cleavage capability *in vitro* by semi-quantitative duplex PCR. Gene repair by ZFN in cells was analyzed by TseI digestion, PCR sequencing, indirect immunofluorescence and Western blot.

Results: ZFN were successfully generated, expressed in cell culture and the nuclear localization was verified by indirect immunofluorescence. PCR demonstrated cleavage of the target sequence *in vitro* by ZFN. Successful gene repair mediated by ZFN induced HR was shown on genomic level by TseI digestion and sequencing. Expression of the repaired *Ush1c* gene product harmonin demonstrated ZFN activated gene repair on protein level, analyzed by indirect immunofluorescence and Western blot.

Conclusions: Gene repair by ZFN induced HR represents a powerful technique to correct genetic defects. This ensures sustained and tissue-specific expression of the gene product. Restoration of some USH1C protein might stop or slow down progression of retinal degeneration and greatly improve the life quality of USH1 patients.

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Identification of risk genes for age-related macular degeneration and drusen formation in rhesus macaques as animal model

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Aim: For age-related macular degeneration (AMD) in humans, the genes *ARMS2* and *HTRA1* in the region 10q26 are both promising candidates for being involved in pathogenesis. However, the associated variants are located in a region of strong linkage disequilibrium (LD) and, thus far, in humans the determination of the causative gene was not possible. Rhesus monkeys suffer from drusen, a major hallmark of AMD, and the drusen-phenotype shares susceptibility factors with AMD. Moreover, the existence of genetically homogenous cohorts offers an excellent opportunity to determine drusen-specific risk factors. The aim of this study is to analyze the LD structure of the 10q26 homologous genomic region in rhesus monkeys and to investigate whether variants in *ARMS2* or *HTRA1* are associated with the drusen-phenotype as well.

Methods: We studied a genetically homogeneous cohort of 91 rhesus monkeys descending from the island Cayo Santiago that are currently housed in the German Primate Centre in Göttingen. Within this group, ophthalmoscopic examinations revealed a naturally high drusen prevalence of about 55% in monkeys >5 years. By sequencing fragments in a 20kb-region around *ARMS2* and *HTRA1* we detected 56 polymorphisms. As one deviates from Hardy-Weinberg-Equilibrium, 55 variants could be used for creating an LD-Plot as well as testing for association.

Results: We observed strong LD between these markers and were able to define two haplotype blocks. One of these blocks spanned the whole *ARMS2* and the 5' part of *HTRA1* – almost perfectly resembling the situation found in humans. Tests for association revealed a variant in the promoter region of *HTRA1* and two variants in 5'-UTR of *ARMS2* to be associated with drusen.

Conclusion: The strong LD – conserved in comparison with the human LD – indicates a functional connection between the two candidate genes. This matches with current findings in humans showing that a polymorphism in 3'-UTR of *ARMS2* influences the *HTRA1*-expression. To further evaluate a functional connection in our rhesus monkey cohort, reporter assays involving the three identified variants are needed. The results might help to resolve whether *ARMS2*, *HTRA1* or an interaction of both genes is responsible for the observed effect in this region.

Expression ofrod cyclic–nucleotide gated (CNG) channelis critical for *rd1* mouse retinal degeneration

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Purpose: Retinitis pigmentosa (RP)is a severe and often blinding human retinal degeneration. One of the most commonly studied animal models for RPis the *rd1* mouse which is characterized by abnormally high levels of rod photoreceptor cGMP. Despite several decades of research, the mechanisms causing photoreceptor degeneration have remained poorly understood. Here, we set out to study the role of cyclic-nucleotide-gated (CNG) channels in the degenerative process, by crossing CNGb1$^{-/-}$ animals with *rd1* mutant mice.

Methods: Wild-type, *rd1*, CNGb1$^{-/-}$ single, and *rd1* x CNGb1$^{-/-}$ double mutant animals were analysed *in vivo* using optic coherence tomography and scanning laser ophthalmoscopy imaging techniques, combined with electroretinographic functional testing. *Ex vivo* analysis included assessment of general retinal morphology, rod and cone photoreceptor markers, degenerative markers (calpain activity, TUNEL), cGMP contents, and electron microscopicultrastructural analysis of rod outer segments.

Results: In *rd1* x CNGb1$^{-/-}$ double mutants devoid of regular CNG channels, cGMP levels werestrongly elevated, but rod photoreceptor viability and outer segment morphology were greatly improved. Activity of calcium (Ca^{2+})-activated calpain-type proteases was strongly reduced in double mutants when compared with *rd1* retina. Importantly, cone photoreceptors, the basis for high-resolution daylight and colour vision, survived and remained functional for extended periods of time.

Conclusions: We demonstrated the major importance ofCNG channels for rod photoreceptor loss supporting the hypothesis of deleterious Ca^{2+}-influx as a cause of rapid rod cell death. Presence or absence of CNG channels was directly correlated with calpain activity, suggesting that CNG channel-dependent Ca^{2+}-influx caused excessive calpain-mediated proteolysis.The survival and functionality of cone photoreceptors in double mutant animals illustrates how therapeutic strategies targeting rods may preserve useful human vision for extended periods of time. Specifically, our results suggest that targeting rod CNG channels, rather than general Ca^{2+}-channel blockade, is a promising symptomatic approach to treat otherwise incurable forms of cGMP-related RP.

Localization of the Human P-Protein to a Subset of the Microtubuli Network Indicates Involvement in Intracellular Transport of L-DOPA Carrying Vesicles

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Purpose: Mutations in the human *OCA2* gene underlie autosomal recessive ocular and oculocutaneous albinism. *OCA2* encodes the P-protein a transmembrane protein of the melanosomal membrane. No function could be associated with the P-protein by now. Predictions based on the domain structure of P-Protein indicate a function in transmembrane transport of small molecules or ions. To approach the impact of mutations in *OCA2* we investigated the localization of intrinsic P-protein expression in ARPE19 cells.

Methods: ARPE19 cells (ATCC No. CRL-2302) were seeded on slides with flexiperm silicon frames at passage 3-20. After growth to sub-confluency the cells were probed with a P-protein specific antibody (Abnova, *OCA2*, H00004948-M02, 1:100) to show the localization of the intrinsic expression of *OCA2* in ARPE19 cells. Counter staining was performed using an α-tubulin specific antibody (Santa Cruz SC-53030, 1:100) and a dopamine directed antibody (Abnova, PAB14696, 1:100). Immunofluoerscence stining was recoded using an epifluorescence microscope and a confocal laser-scanning microscope. Experiments were performed in triplicate.

Results: anti-P-ab labeled structures within ARPE19 cells indicated microtubule association. α-tubulin-ab labeled intracellular structures comparable to those labeled by anti-P-ab. Restricted co-localization of P-protein immunoreactivity (IR) and α-tubulin IR was detected. Dopamine IR co-localized with anti-P-ab IR but showed a different intracellular distribution.

Conclusion: Co-localization of P-protein from intrinsic *OCA2* expression and α-tubulin in ARPE19 cells indicate a function in the distribution of P-protein carrying structures along a subset of the microtubuli network. L-DOPA is a common intermediate of melanin and dopamine synthesis and has been considered the neuro-active substance involved in neuronal guidance of the optic nerve. Dopamine IR did not label structures showing P-protein IR. Therefore, involvement of P-protein in dopamine transport seems unlikely.

Light Induced Structural Changes of Channelrhodopsin-2 monitored by Vibrational Spectroscopy

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Channelrhodopsins (ChRs) are light-gated cation channels – sensory photoreceptors that mediate phototaxis in green algae. They have become widely used as tools for light-dependent cell depolarization in the field of neuroscience. Similar to other rhodopsins, they bear the chromophore retinylidene covalently linked to a lysine of the opsin apoprotein. Although several photocycle models and reaction schemes have been proposed on the basis of electrophysiological and spectroscopical data, details of the photoreactions and the light-induced conformational changes of the protein still remain obscure.

Here we present FTIR (fourier-transform-infrared) and UV-Vis spectroscopic investigations and retinal extraction data of wild type ChR and mutants in which the lifetime of the conducting state is altered. These mutants can therefore be used to study several photocycle intermediates that cannot be stabilized in case of the wild type.

Based on the data we propose a model of the photocycle that connects the chromophore isomerization state and structural alterations of the protein. Furthermore, we show that extended illumination causes a branching which leads into a sideway that contains new blue-shifted intermediates. This should be considered when ChR is used as neuroscience tool, especially under conditions of high light intensities or when long-term illumination is applied.

Elucidation of physiological and pathophysiological aspects of Bestrophin-1, the gene mutated in Best vitelliforme macular dystrophy

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Background: Bestrophin-1 (Best1) encodes an integral membrane protein of the retinal pigment epithelium and is mutated in Best vitelliforme macular dystrophy (BVMD), an autosomal dominant form of macular degeneration with highly variable expressivity and reduced penetrance. The function of Bestrophin-1 and the pathophysiology of the disease are not well understood. We have previously targeted the murine Bestrophin-1 gene and have generated a knock-out mouse deficient in murine Best1 ($Best1^{-/-}$) and a knock-in mouse carrying the BVMD-causing mutation Y227N ($Best1^{N/N}$). Extensive phenotyping and molecular as well as biochemical analyses showed that Bestrophin-1 manipulation in the murine retina is well tolerated. Here, we have extended our analyses to the testis, a tissue with high expression of Bestrophin-1 in the mouse.

Methods: RT-PCR was used to investigate Bestrophin-1 expression in several tissues across species. Temporal and spatial expression profiles of Bestrophin-1 was established in murine testis with sperm marker Slxl-1, Leydig cell marker 17HsdßIII and Sertoli cell marker Ptgds. Bestrophin-1 protein was detected by immunhistochemistry in epididymal tissue sections with polyclonal antibodies against murine Bestrophin-1. Isolated epididymal spermatozoa of adult male mice were labeled by immunofluorescence. Initial characterization of fertility was addressed by subjecting isolated spermatozoa to *in vitro* capacitation and Ca^{2+} ionophore triggered acrosome reaction. Sperm number and various parameters of spermatozoa motility were assessed using a computer-assisted semen analysis system.

Results: Crosspecies expression of Best1 revealed a dominant expression in human RPE when compared to testis. In contrast, rabbit, rat and particularly mouse revealed a more prominent expression in testis. Testing developmental stages of murine testis, Bestrophin-1 expression was found to greatly overlap with Slxl-1. This suggests that the Best1 expression correlates with post-meiotic rebuilding of germ cell architecture and polarization. Labelings of epididymal tissue sections demonstrated a specific Bestrophin-1 staining within the lumen while protein staining of isolated spermatozoa refined Bestrophin-1 localization to the sperm head and the acrosomal region. Bestrophin-1 staining was still observed in capacitated and acrosome-reacted sperms. Breeding experiments of $Best1^{-/-}$ and $Best1^{N/N}$ males so far give no indication of impaired fertility.

Conclusion: Both RPE cells and spermatozoa are highly polarized cells. High expression and a defined localization makes the murine sperm a promising tool for further elucidating functional aspects of normal and mutated Bestrophin-1.

Investigating the interaction between CACNA1F and retinoschisin, the protein mutated in X-linked juvenile retinoschisis

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Background: Mutations in the *RS1* gene are known to cause X-linked juvenile retinoschisis (XLRS), a common form of juvenile macular degeneration in males. The protein encoded by *RS1*, termed retinoschisin, is specifically expressed in the retina from where it is secreted to bind to the photoreceptor inner segment membranes. Studies with retinoschisin-deficient mice show that the protein is essential for maintaining the structural integrity of the retina (Weber et al., 2002). Its exact function in the retina is still unknown. In 2009, Shi et al. described an interaction between retinoschisin and the L-type voltage-gated calcium channel (L-VGCC) in chicken, more specifically with the CACNA1D subunit that is mainly expressed in cones. In the human and murine retina, CACNA1F is the member of the L-VGCC family which is predominantly expressed in photoreceptors and was associated with congenital stationary night blindness (Lodha et al., 2010). The aim of this study was to verify the putative interaction between retinoschisin and CACNA1F in a murine model of *Cacna1F* deficiency (*Cacna1F^{nob2}*).

Methods: Expression of L-VGCC family members in human and murine retina and other tissues was analyzed by RT-PCR. The interaction of human retinoschisin and *CACNA1F* was tested by *in vitro* assays analyzing the binding of recombinant retinoschisin to HEK293 cells transfected with human *CACNA1F*. In addition, the influence of *Cacna1F* deficiency on *RS1* expression was determined in the *Cacna1F^{nob2}* mouse by quantitative qRT-PCR, RT-PCR and semi-quantitative Western blot analysis. Binding of retinoschisin to retinal membranes was compared between wildtype and *Cacna1F* deficient retinae.

Results: Expression analysis of the various *CACNA1* subtypes confirms *CACNA1F* as the most prominent *CACNA1* subtype in the human retina, whereas the murine retina expresses different *Cacna1* subtypes at comparable intensities. In vitro binding assays with HEK293 cells fail to show an interaction of human retinoschisin with full-length *CACNA1F*. *Cacna1F^{nob2}* mice reveal no influence on retinoschisin expression both on the transcript and protein level. In addition, the retinal membranes of *Cacna1F^{nob2}* mice exhibit no difference in their binding capacity to retinoschisin.

Conclusion: Taken together, our initial data do not support a predominant role of *CACNA1F* in anchoring retinoschisin to retinal cell surfaces.

Literature:

Weber et al. (2002): Inactivation of the murine X-linked juvenile retinoschisis gene, Rs1h, suggests a role of retinoschisin in retinal cell layer organization and synaptic structure. PNAS 99 (9). P. 6222-6227.

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Clinical Trial with Subretinal Implants and the Current Results

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Purpose: In hereditary retinal disorders the amount of photoreceptors progressively decrease leading mostly to blindness with no available therapy. We developed subretinal implants which can replace the function of the photoreceptors in the end-stage of these diseases.

Methods: In the current clinical trial patients with an end-stage hereditary retinal disease received a new permanent subretinal Alpha IMS chip (Retina Implant AG) with 1500 pixels, in the worse eye. The implant is powered wirelessly with a subdermal coil in the retroauricular region and can be used for the first time outdoors as well as at the patients' homes.
Visual functions to determine the spatial resolution and visual acuity were measured as well as skills related to activities of daily living both in standardized conditions. Additionally all subjects used the visual implant outdoors as well as in their homes and described their experiences and perceptions.

Results: In most patients the standardized tests showed an increase in visual functions compared to the performance prior to implantation as well as to switched-off implant condition. The learning curve shows a fast progress in the using of the implant during the first week. The evaluation of activities of daily living showed significant improvements in discrimination, localization and identification of objects. Spatial resolution up to 0.33 – 0.44 cpd could be obtained by means of the visual implant. The eye-hand coordination was possible.
While moving freely outdoors or at their homes the patients could distinguish tableware in restaurants and parts of dishes, facial characteristics of persons (e.g. mouth shape, glasses etc.). Some of them were able to read big letters, recognize objects on the horizon or characteristics of clothes, differentiate shades of grey. Motion perception was reported by seeing a hand or a pencil moving against a contrasting background, or a swimming white goose in the nature.

Conclusions: Subretinal electronic implants are able to mediate useful visual functions in blind patients with hereditary retinal diseases.

Identification of chemical compounds suitable to improve surface expression of trafficking deficient mutant CNGA3 channels using a calcium imaging based bioassay

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Purpose: *CNGA3* and *CNGB3* encode for the A3 and B3 subunit of the cone cyclic nucleotide-gated (CNG) channel, which is a crucial component of the phototransduction cascade. CNG channels enable the cGMP-dependent influx of sodium and calcium into the cone photoreceptor outer segment. Mutations in *CNGA3* and *CNGB3* are the major cause for achromatopsia, an autosomal recessively inherited retinal disorder characterized by a strongly impaired or in many cases complete loss of cone function. Several *CNGA3* mutations have been shown to affect protein folding and/or trafficking thus lowering the channel density in the plasma membrane in heterologous expression systems. Following the establishment of a bioassay, we are performing a medium-throughput screening for chemical and pharmacological chaperones, which may help to overcome the folding/ trafficking deficits of mutant CNG channels.

Methods: HEK293 cells were co-transfected with plasmids encoding for the wild type or mutant (E228K) *CNGA3* channel and pCAeq encoding for apoaequorin. Apoaequorin is the protein component of aequorin, a calcium sensitive photoprotein. After reconstitution of aequorin with coelenterazine, calcium binding leads to the oxidation of coelenterazine to coelenteramide, resulting in the emission of light. Following transfection, cells were treated for 24 hours with chaperones and the emitted light was recorded before and after the activation of the CNG channels with 8-Br-cGMP.

Results: Cells expressing mutant CNG channels reveal a significant reduction in the luminescence signal compared to cells expressing wild type CNG channels. In a first screening, mutant channels were treated with 17 different compounds. We were able to detect an increase in the luminescence signal in the presence of glycerol a known chemical chaperone. Nifedipine, a blocker of L-type calcium channels was also found to have an effect on protein folding/trafficking indicating its function as pharmacological chaperone.

Conclusion: The established assay has shown to be sensitive and effective and thus is suitable for the screening of substances which may improve the trafficking/protein folding of mutant CNG channels. Trafficking deficient channels carrying the mutation E228K were used as an indicator to show that glycerol and nifedipine in fact improve protein folding/trafficking.

Multi-modal mapping for photoreceptors imaging in retinitis pigmentosa patients with confirmed rhodopsin mutations

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Purpose: Morphological alterations affecting the photoreceptor layer are commonly observed in retinitis pigmentosa patients. Recently available high resolution retinal imaging modalities allow to precisely observe disease severity, to overlap several imaging modalities, and to highlight areas with photoreceptor preservation in vivo. The aim of this study is to apply a multi-modal mapping approach for analyzing structural information of the outer retina acquired by optical coherence tomography (OCT) and autofluorescence imaging in retinitis pigmentosa patients with confirmed rhodopsin mutations.

Methods: Nine patients (8 males; 1 female) with confirmed rhodopsin mutations were prospectively included into the study. The mean age was 47.66±15.5 years. High-resolution spectral domain OCT (24-48 B-scans per OCT scan) was used for retina cross-sectional structural imaging. Custom mapping software (MultiModalMapper) with novel mapping modes was used for the segmentation and registration two retinal image modalities. The area of the preserved photoreceptor layer was segmented for each B-scan by an experienced mapper. A two-dimensional thickness profile showing the area with preserved photoreceptors was reconstructed. The area was only reconstructed between two neighboring B-scans at a specific horizontal position if both B-scans showed preservation at this horizontal position. The reconstructed area was mapped on the segmented hypofluorescence ring area. The mean area of the overlapping portion of the hypofluorescence ring and the preserved photoreceptors was calculated for the whole cohort and individually.

Results: The mean area sizes were 3,081±3,229mm^2 and 2,176±2,275mm^2 for the hypofluorescence ring and the preserved photoreceptor layer, respectively ($p>0.05$). The mean overlapping portion of both areas was 0.74±0.17, whereas the hypofluorescence area was totally (100%) covered by the preserved photoreceptor area in each case. The actual overlapping portion can be expected to be larger, since the reconstructed area with preserved photoreceptors is clipped at its borders, especially when the overall number of B-scans is small and the distance between neighbouring B-scans is large.

Conclusion: Multi-modal mapping provides useful and significant information about the localization of areas with preserved photoreceptors in retinitis pigmentosa patients with confirmed rhodopsin mutations. The novel multi-modal analysis tool provides precise retinal structure analysis for inherited retinal degenerations and has the potential to be applied in clinical trials as additional quantitative and qualitative tool.

Interaction of Rab27A and the calcium channel subunit $Ca_v1.3$ in the RPE

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Rab27A is a small GTPase in the retinal pigment epithelium (RPE), regulated by REP1. REP1 mutations cause choroideremia, a rare X-chromosomal recessive disease leading to blindness. Observations in a conditional knockout mouse model indicate that the disease starts with a loss of function of the RPE, whose secretion is important for the normal structure of the choroid. Rab27A is known to play important roles in secretion processes. As the pore-forming subunit of the voltage-dependent calcium channel of the RPE, $Ca_v1.3$, has an essential function in secretion, too, we were interested, whether there could be an interaction between both proteins. Therefore, we performed immunoprecipitation and immunocytochemistry experiments in HEK293- and CHO-K1- cells as expression system for Ca^{2+}-channel subunits and Rab27A. Co-precipitation of $Ca_v1.3$ and Rab27A indicates possible direct interaction of these two proteins. There is no co-precipitation between the auxiliary calcium-channel subunit $ß_3$ and Rab27A. That $Ca_v1.3$, $ß_3$-subunit and Rab27A can form complexes was shown by indirect co-immunoprecipitation of $ß_3$ and Rab27A. These results were supported by immunocytochemical staining, where co-localization of $Ca_v1.3$, $ß_3$ and Rab27A in the plasma membrane could be shown. To identify a binding domain between $Ca_v1.3$ and Rab27A, we analyzed sequence homologies within $Ca_v1.3$, and could detect a domain in the intracellular loop between homologous repeats II and III of $Ca_v1.3$, homologous to MyRIP (myosin VIIa/ Rab27A interacting protein). We examined the interaction between Rab27A and the $Ca_v1.3$-MyRIP-mutant, which lacks that Rab27A binding domain. Here we found that there is no binding of Rab27A to the $Ca_v1.3$-mutant. Since there is a normal binding of the $Ca_v1.3$-MyRIP-mutant with the calcium-channel-subunit $ß_3$, a specific loss of function effect is confirmed. Immunocytochemical staining of the mutant showed that there is still a good co-localization of the $Ca_v1.3$-MyRIP-mutant and $ß_3$, whereas there is almost no co-localization with Rab27A anymore. The mutant does not go to the membrane as good as the wild type $Ca_v1.3$. In conclusion we showed in heterologous expression system that Rab27A and $Ca_v1.3$ bind to each other. We could detect the binding region on $Ca_v1.3$, which is homologous to the Rab27A-binding domain of MyRIP. As there is a physical binding between these two proteins, it is likely that Rab27A influences the activity of $Ca_v1.3$ and therefore the secretory activity of the RPE.

CRX, NRL, and FIZ1 control retina-specific expression of *Fam161a*, the mouse ortholog of the human RP28 gene

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Purpose: Retinitis pigmentosa (RP) describes a group of inherited retinal diseases leading to progressive vision loss. Homozygosity mapping in a consanguineous Indian family assigned the *RP28* locus to chromosome 2p11-15 (Gu *et al.* J Med Genet 1999). CRX chromatin-immunoprecipitation coupled to parallel sequencing (ChIP-Seq) (Corbo et al. Genome Research 2010) and targeted resequencing of the locus identified nonsense mutations in the *FAM161A* gene (Langmann *et al.* American Journal of Human Genetics 2010). *Fam161a* is an uncharacterized gene and here we studied its transcriptional regulation in the mouse retina.

Methods: CRX-, NRL-, FIZ1, and RNA-Pol-II-directed ChIP from adult mouse retinas were performed to demonstrate *in vivo* binding of these proteins to the *Fam161a* locus. Transcription factor bound *cis*-regulatory regions were fused to dsRed reporter vectors and *in vitro* electroporations of explanted living mouse retinas were carried out. After eight days in culture, flat mounts and retinal sections were analyzed by fluorescence microscopy.

Results: Independent large-scale datasets from ChIP-Seq, ChIP-Chip, and ChIP-PCR revealed *in vivo* binding of CRX, NRL, and FIZ1 to the promoter and first intron of *Fam161a* in adult mouse retinas. Co-occupancy of these genomic regions with RNA-Polymerase II demonstrates a permissive chromatin conformation required for high level transcription. *In vitro* electroporations of mouse retinas with dsRed reporter plasmids detected a phylogenetically conserved CRX-bound region in intron 1 that acts as a strong retina-specific enhancer in explanted retinas.

Conclusion: The retina-specific expression of *Fam161a* depends on a CRX-bound region in the first intron, whereas its spatiotemporal expression may be controlled by interactions of CRX, NRL, and FIZ1 at the promoter region.

Establishing and characterizing a light lesion paradigm to study retina regeneration in adult zebrafish

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Purpose: Loss of photoreceptor cells is a common symptom in hereditary and age related human eye diseases. In contrast, Zebrafish is capable of regenerating the whole retina after injury and therefore serves as an important model organism to understand the principles of regeneration. Light lesions cause extensive cell death in the photoreceptor cell layer and trigger a special photoreceptor regeneration response that we want to study in further detail. We characterized and compared two different lesion paradigms, the previously described method by Bernardos and colleagues and our own approach.

Methods: The light lesion method previously described by Bernardos et. al. was slightly modified and will be referred to as direct illumination. Our own light lesion paradigm employs a microscope to direct the light on one eye of an anaesthetized fish leaving the other eye as a control and will be called indirect illumination. All fish have been dark adapted for 5 days and were exposed to intense white light for 30 minutes.

Results: The exposure of zebrafish retinas to intense focused light using indirect illumination causes a small lesion in the central part of the retina, anterior to the optic nerve head. Transgenic fish expressing GFP in rods or UV cones as well as immunohistochemistry show that photoreceptors die in a distinct area. Furthermore, we show that cones are more sensitive to intense light treatment than rods. Cell death initiates within 12 hours post lesion (hpl) and proceeds until 24 hpl. Proliferation of cells in all layers begins within the first 24 hpl and peaks at 3 days post lesion. Regeneration after indirect illumination completes within 28 days. These observations are confirmed in the direct illumination approach.

Conclusions: The light lesion paradigms induce cell death selectively in the photoreceptors of the zebrafish retina. The new approach using indirect illumination is as reliable as the direct illumination approach and has additional advantages. We will use this model to understand the role of Fgf signalling in the regenerating retina by interfering with the Fgf pathway after inducing a lesion. Furthermore we hope to elucidate the molecular mechanisms of regeneration in more detail by the help of transgenic lines.

High-throughput mutation screening in patients with retinal dystrophies and optic nerve degenerations

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Objective: Retinal dystrophies (RDs) and optic nerve degenerations (ONDs) are the most severe hereditary eye diseases in terms of visual function loss and lack of treatment. They are characterized by a high degree of genetic heterogeneity: currently more than 180 genes are implicated in RDs and ONDs. The genetic heterogeneity in these diseases remains a great challenge for DNA diagnostics. Conventional Sanger sequencing is both laborious and expensive and more efficient approaches in microarray formats are only available for a subset of genes/mutations (i.e. Asper chips). Moreover, only 50% of cases are explained by mutations in those genes that are known to date.
Our aim is to improve mutation screening in RDs and ONDs by developing a fast and cost-efficient targeted resequencing approach to screen for all known RD- and OND-associated genes simultaneously.

Methods: Enrichment of genomic DNA is performed for the coding and flanking intronic/UTR sequences of 190 genes involved in RDs and ONDs (SureSelect in Solution by Agilent). Sequencing is performed using barcoded libraries on the SOLiD 4 platform (Life Technologies). A diagnostic pipeline was developed that enables identification of (i) regions with sufficient coverage (>20), (ii) potentially pathogenic single nucleotide variants (SNVs), (iii) small indels and (iv) large indels including whole exon deletions and duplications. All variants and indels are verified by Sanger sequencing or quantitative PCR, respectively. Both gene list and enrichment design are updated, extended and balanced with each round of ordering of the Agilent SureSelect in solution kit.

Results: Up to know we have processed 48 samples by this method. The relative coverage of targeted regions was sufficient to perform SNV and indel calling in all cases. Different types of mutations including SNVs as well as copy number variations were detected in so far unsolved cases.

Conclusion: We have successfully established a high-throughput sequencing platform for in-depth parallel mutation screening of known RD and OND genes. We have developed a data analysis pipeline that not only identifies single nucleotide variants but also small indel mutations as well as larger copy number variants based on relative coverage ratios.

Read-through of a nonsense mutation as a treatment option for Usher type 1C

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Purpose: The Usher syndrome (USH) is the most frequent cause of inherited combined deaf-blindness. It is clinically and genetically heterogeneous, assigned to three clinical USH types of which the most severe type is USH1. The *USH1C* gene encodes the PDZ domains containing scaffold protein harmonin which is expressed in form of numerous alternatively spliced variants. Hamonin binds directly to all USH1 und USH2 proteins and is a key organizer the USH protein networks. So far no effective treatment for the ophthalmic component of USH exists. Aminoglycosides are known to facilitate read-through of nonsense mutations, but their clinical use is limited due to their toxicity. Synthetic redesign of a clinical approved aminoglycoside to reduce toxic side-effects resulted in NB30 and NB54. In addition, PTC124 is a new promising compound for translational read-through of nonsense mutations leading to a premature termination stop. It is currently gauged in clinical phase II for nonsense mutations in non-ocular diseases. Here we compared the potential of PTC124, NB30 and NB54 in comparison as a treatment option for patients carrying a nonsense mutation in the *USH1C* gene (p.R31X).

Methods: Read-through was validated in cell culture, in retinal explants and *in vivo*. Restoration of scaffold function of the USH1C protein harmonin was tested in GST-pull downs and by co-staining. Biocompatibility was determined in murine and human retinal explants by TUNEL-assays.

Results: We demonstrated translational read-through of the p.R31X mutation in *USH1C* not only in cell culture and in retinal explants, but also in mice *in vivo*. Our assays concerning the molecular function of the restored protein showed that the recovered harmonin expression restored harmonin's scaffolding function and F-actin bundling activity in the cell. Furthermore, we compared the biocompatibility of PTC124, NB30 and NB54 with the clinically approved read-through inducing aminoglycoside gentamicin. In this evaluation, PTC124, NB30 and NB54 showed a much better biocompatibility in murine retina cultures and human retinal explants.

Conclusions: High retinal compatibility of PTC124, NB30 and NB54 combined with their transcriptional read-through efficacy emphasize the high potential of these molecules as therapeutic agents for the p.R31X nonsense mutation in USH1 as well as in other retinal genetic conditions.

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SFD-associated mutations affect the abundance and protease-sensitivity of extracellular matrix-bound TIMP3

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Purpose: Mutations in the gene encoding tissue inhibitor of metalloproteinases-3 (TIMP3) cause Sorsby fundus dystrophy (SFD), a late-onset macular degeneration characterized by choroidal neovascularisation and death of retinal pigment epithelial (RPE) and photoreceptor cells. The objective of this study was to gain further insight into the mechanism by which mutant TIMP3 affects extracellular matrix (ECM) degradation and remodeling causing the thickening of Bruch's membrane in early stage disease.

Methods: Primary lung fibroblast cells were derived from wild-type (wt), *Timp3*$^{S156C/+}$ and *Timp3*$^{S156C/S156C}$ mice. *De novo* ECM production was determined by L-[^{3}H]-proline incorporation. Human cDNAs of wt-TIMP3 and TIMP3 carrying several SFD-associated as well as SFD-unrelated mutations were cloned into pCEP4 vector and overexpressed in Hek293 cells. Some of these constructs were also expressed with a C-terminal 5xHis-tag. ECMs of fibroblasts and transfected Hek293 cells were subjected to proteolytic digestion and the digests were analyzed by ELISA and Western blotting.

Results: Mutant S156C-TIMP3 fibroblasts showed a significantly increased ECM production and TIMP3 accumulation relative to wt-TIMP3 cells. ELISA and densitometric analysis of Western blots of proteolytic digests revealed that S156C-TIMP3 from fibroblast ECM and most of the recombinant ECM-bound mutant TIMP3 molecules showed a significantly increased sensitivity to trypsin digestion whereas S156M-TIMP3 and H158R-TIMP3 exhibited a moderate difference in trypsin sensitivity when compared to wt-TIMP3. No difference in elastase, heparinase III and chondroitinase ABC sensitivity was observed between ECM-bound mutant and wt-TIMP3.

Conclusions: The accumulation of TIMP3 and extracellular material in cultured cells expressing mutant TIMP3 resembles *in vivo* observations in SFD eyes. Increased trypsin sensitivity of ECM-bound mutant TIMP3 suggests a different conformation of these molecules that may prevent or induce interaction of TIMP3 with putative partners in the ECM. Alternatively, altered ECM composition may affect trypsin sensitivity of TIMP3.

Cone-rod dystrophy associated with amelogenesis imperfecta in a child with neurofibromatosis type 1

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Purpose: To report a case of a 9-year-old child with neurofibromatosis type 1 (NF1) and Jalili syndrome, the latter denoting a rare combination of cone-rod dystrophy and amelogenesis imperfecta.

Methods: Detailed ophthalmological and electrophysiological examinations were carried out and blood samples were taken from the patient and her father for molecular genetic analysis by direct DNA sequencing of the NF1 and the ancient conserved domain protein 4 (CNNM4) gene.

Results: The diagnosis of neurofibromatosis type 1 (NF1) could be confirmed clinically and genetically. Furthermore, cone-rod dystrophy and amelogenesis imperfecta could be observed as typical features of a rare condition, acknowledged as Jalili syndrome. The diagnosis was assured on the basis of clinical examinations and molecular genetic analysis of the CNNM4 gene, which was previously shown to cause Jalili syndrome.

Conclusion: Our case shows a unique combination of NF1 and Jalili syndrome. The random association of two diseases is unusual and deserves attention. This case highlights the importance not only of detailed clinical examination, but also of molecular genetic analysis, which together provide a precise diagnosis.

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