ON THE ROLE OF CCDC66 GENE PRODUCTS IN RETINA AND EXTRA-RETINAL TISSUES OF THE CCDC66-DEFICIENT MOUSE MODEL FOR RETINAL DEGENERATION

by

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A thesis submitted in partial fulfilment of the requirements for the degree of

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from the International Graduate School of Neuroscience

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This research was conducted at the Department of Human Genetics within the Faculty of Medicine at the Ruhr University under the supervision of Prof. Dr. J. T. Epplen

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Statement
I certify herewith that the dissertation included here was completed and written independently by me and without outside assistance. References to the work and theories of others have been cited and acknowledged completely and correctly. The “Guidelines for Good Scientific Practice” according to § 9, Sec. 3 of the PhD regulations of the International Graduate School of Neuroscience were adhered to. This work has never been submitted in this, or a similar form, at this or any other domestic or foreign institution of higher learning as a dissertation.
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<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AAA</td>
<td>acetic acid anhydride</td>
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<td>Abi-1</td>
<td>abelson interactor protein 1</td>
</tr>
<tr>
<td>abs</td>
<td>absolute</td>
</tr>
<tr>
<td>AC</td>
<td>anterior commissure</td>
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<td>AD</td>
<td>activator domain</td>
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<td>amp</td>
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<td>binding domain</td>
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<td>BLAT</td>
<td>Basic Local Alignment Tool</td>
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<td>bp</td>
<td>basepair</td>
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<td>°C</td>
<td>degree celsius</td>
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<td>DIG</td>
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<td>DITABIS</td>
<td>Digital Biomedical Imaging System</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
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<td>dimethylformamide</td>
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<td>enhanced green fluorescence protein</td>
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<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>EM</td>
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<td>inner limiting membrane</td>
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Abstract

IV. Abstract

A new mouse model with a gene trap mediated interruption of the Ccdc66 gene exhibits Retinitis pigmentosa (RP)-like slow retinal degeneration. Ccdc66 expression is localized to retinal photoreceptors (Gerding et al. 2011). Initial evidence indicates expression also outside of the retina, but the role of Ccdc66 expression and the underlying mechanisms of degeneration are unknown. This thesis aimed at characterizing the localization and function of the Ccdc66 gene products in order to study Ccdc66-mediated processes in retina, brain and beyond.

Ccdc66 expression and localization studies were performed by in situ hybridization for RNA and by X-gal staining for reporter gene expression. The protein expression was investigated by Western blotting and immunofluorescence, and the morphological evaluation of selected regions of interest was conducted by electron microscopy. A yeast two-hybrid screen with a retinal cDNA library was performed in order to identify potential interaction partners of the protein CCDC66.

The results confirmed Ccdc66 expression in the retinal photoreceptors and revealed that it is present in additional retinal layers (inner nuclear layer, ganglion cell layer, inner and outer plexiform layer) during postnatal developmental stages P4, P10, P17, 1 month, 1.5 and 10 months. Time-related changes in expression appeared with highest intensity at P17 and declined until adulthood in the photoreceptor inner segments and increased from birth to P17 in the plexiform layers. These expression differences were accompanied by cytoskeleton-associated processes like maturation of the photoreceptor and outgrowth/maturation of synaptic contacts in the plexiform layers. The Ccdc66 expression study was further extended to the brain at P4, P10, P17, 1 month, 1.5 and 10 months and additional tissues in adult mice. Strongest Ccdc66 expression was found in the brain ventricle ependyma, the rostral migratory stream, olfactory bulb, the adjacent olfactory epithelium and hippocampal formation, while weaker expression was demonstrated in the cerebellum by X-gal staining. Likewise, Ccdc66 could be detected in epithelia of the fallopian tube and lung, in kidney tubules, in maturing sperm cells and female germ cells.

Amongst the structures of the central nervous system, a developmental time course of Ccdc66 expression pattern was shown at the external plexiform and glomerular layers of the olfactory bulb, the stratum radiatum and molecular layer of the...
hippocampal formation. These developmental changes paralleled processes such as neurite outgrowth/maturation as shown for the retina. Moreover, analysis of brain regions with strongest Ccdc66 expression revealed neurodegeneration within olfactory sensory nerve terminals within the glomeruli of the olfactory bulb, whereas hippocampal formation and ependyma did not reveal changes in 11 month-old mice. Thus Ccdc66 expression does obviously not only result in retinal degeneration.

In order to further identify the general function of the protein CCDC66, its cellular localization could be predominantly verified for the nuclear and cytoskeleton compartment in retinal and brain extracts at >P17. This is in line with protein CCDC66 detected in soma, processes and in (or in close proximity) to the nuclei of neurons, stem cells, astrocytes and mainly oligodendrocytes in a neural cell culture derived from embryonic day 12.5 neurospheres after 7 days of differentiation.

Furthermore, the identification of Epidermal growth factor receptor kinase substrate 8 and Multiple PDZ domain protein 1 as possible interaction partners of CCDC66 reflects widespread function of CCDC66 with association to the cytoskeleton compartments. Both proteins are involved in cytoskeleton-related processes, regulating cell shape by giving structural stability and enabling dynamics in the retina, brain and partly in other tissues.

In conclusion, these findings point to protein CCDC66 as a structural/cytoskeleton associated cellular component in specialized structures such as retinal photoreceptors. Ccdc66 gene expression also plays a pivotal role in the nervous system and furthermore it could give insights into a brain-specific phenotype in future studies. The presented findings might promote to categorize the Ccdc66-deficient mouse rather as a model for a syndromic than for an isolated type of retinal degeneration. This could bring further insights into gene function and pathology in human disease and possible future therapies.
Chapter 1 - General introduction

The human eye, especially the retina, represents the border of physical and chemical signal transduction of light. Together with subsequent brain structures the retina provides the possibility of representing our environment visually and enables us to interact and orientate in a spatial and social setting. Such a highly specified system like the human retina is prone to disturbances in numerous ways. The spectrum of diseases affecting this delicate piece of tissue is broad and heterogeneous, regarding affected structures of the retina and resulting phenotypes. The disease course and severity ranges from visual impairment to irreversible vision loss (Kanski 2007). Patients experience severe problems and enormous changes in all situations of life. So far, they have to cope and accept these circumstances, for the reason that there is no complete cure available at the moment. A general difficulty in the treatment of retinal degeneration is the heterogeneity, because there is not a single target or pathway for the development of therapeutic approaches. Thus, disease mechanism independent approaches, like retinal implants or general stabilization of the cone pathway that might rescue vision also in primarily rod affecting disease types are eagerly awaited (Maghami et al. 2014; Dalkara et al. 2015).

In order to explore the different underlying mechanisms for effective therapies animal models are generated and characterized. Based on models, therapy approaches encompassing retinal electrode implants, stem cell application as well as gene replacement strategies are developed, with the initial successes by prolonging or improving vision in several animal models such as mouse, rat and dog and even in humans (Acland et al. 2001; Acland et al. 2005; Bainbridge et al. 2008; Bennicelli et al. 2008; Hauswirth et al. 2008; Jacobson et al. 2006; Lund et al. 2006; Maguire et al. 2008; Margalit et al. 2002; Menn et al. 2006; Tan et al. 2009). A new retinal degeneration mouse model, the Ccdc66-deficient (Ccdc66−/−) mouse exhibits one yet unknown mechanism of retinal degeneration, and it is the main focus of this work. The characterization and understanding of this gene and its products might bring insights into the present degeneration mechanism and of the option for future therapies. In order to introduce mechanisms in retinal degeneration, the structure of the mammalian retina is described in the following.
1.1 The mammalian retina

The mammalian retina is a multi-layered assembly of cells involved in the transmission of electric stimuli driven by a light impulse (figure 1; Swaroop et al. 2010). The light passes the lens and all retinal layers until it hits the photoreceptor cells that are located in the outer nuclear layer (ONL), where the stimulus is converted into a chemical signal. This information is then transferred by interneurons to the ganglion cells (GC) that finally transmit the signal to subsequent brain structures (for an overview see Wässle and Boycott 1991; Wässle 2004). Disturbances at any point of this pathway may result in vision impairments and even complete vision loss.

![Figure 1: Structure of the mammalian retina](image)

**Figure 1:** Structure of the mammalian retina Scheme of retinal cells and their connections; C - cones, R - rods, B – bipolar cell, H – horizontal cell, M – Müller cell, G – Ganglion cell; modified from Swaroop et al. 2010.
1.1.1 The outer nuclear layer and photoreceptor structure

The photoreceptor cells include rods that are mainly active in the achromatic vision and cones that are predominantly active during chromatic vision. The photoreceptor somata form the outer nuclear layer (ONL). Photosensitive molecules each consisting of an opsin protein component and the chromophore 11-cis retinal reside in the disc membranes of the receptor outer segments (OS) (Steinberg et al. 1980) that in turn are embedded in the retinal pigment epithelium (RPE). The pigmented cells of the RPE recycle the disk membranes and 11-cis retinal of the photoreceptors. Furthermore, the RPE provides vitamin A and glucose and is involved in $K^+$ and $Cl^-$ metabolism of the photoreceptors. It prevents the reflection of light and thereby provides enhanced visual acuity (Strauss 2005). The photoreceptor OS are connected to the inner segments (IS) by a connecting cilium, a microtubule based structural element (consisting of the so-called axoneme and basal body) which is anchored to the ciliary rootlet (reviewed in Wheway et al. 2014). The IS contain accumulations of mitochondria and the endoplasmic reticulum (ER) (Liu et al. 2007). The nuclei of the photoreceptors are located in the ONL, their synaptic terminals in the outer plexiform layer (OPL).

1.1.2 Subsequent layers and signal transmission

Beside the photoreceptor cells four major types of neurons are present in the retina encompassing bipolar, amacrine, horizontal and ganglion cells as well as the retinal principal glia, the Müller cells (figure 1 (Swaroop et al. 2010); Bhattacharjee and Sanyal 1975). The somata of these cells are located in the inner nuclear layer (INL), except for the GCs whose somata that built a separate layer (ganglion cell layer - GCL). Some displayed cells of the nuclear layers are located in circumjacent plexiform layers (Pérez De Sevilla Müller et al. 2007). The bipolar cells interconnect the photoreceptor and ganglion cells. This vertical conduction pathway is modulated by horizontal cells on the photoreceptor bipolar synapses in the outer plexiform layer (OPL) and by amacrine cells on bipolar ganglion cell synapses in the inner plexiform layer (IPL) (Ghosh et al. 2004). Signal transmission is based on graduate potentials until the ganglion cells generate and forward the information by action potentials along the optic nerve that is built by their axons in the area of the optic disc. Projections of the retinal ganglion cells encompass several cortical or sub-cortical
brain areas including the retino-geniculate-cortical, retino-tectale and pretectale, hypothalamic and the accessory optic system (Cooper et al. 1989). As mentioned, despite the neuronal portion the Müller cells represent the retinal glia cells and they constitute the limiting membranes preventing the retina from structural damage through mechanical strength (Omri et al., 2010; Pedler, 1961) by forming enormous branches that sprout in the ONL enclosing photoreceptor cell bodies, dendrites and also pass the nerve fiber layer. They are further involved in widespread functions from homeostasis over processing of visual information to vascularization and angiogenesis (Bringmann et al. 2006; Eichler et al. 2000; Eichler et al. 2004; Liu et al. 2007; Newman and Reichenbach 1996; Pierce et al. 1995).

1.2 Inherited retinal degeneration

Given the complexity in the transmission of visual information, the cells of the visual pathway from retina to brain, are sensitive to damage that may lead to severe implications for an individual's visual abilities. Beside retinal degeneration caused by trauma/mechanical damage, drugs, toxics or vitamin deficiency there are numerous groups of inherited retinal degenerations (IRD) (Hayes 1974; SanGiovanni et al. 2007; Snodderly 1995; Seddon 1994; Won et al. 2011). Within the last decade more than 260 loci including about 220 genes associated with retinal degeneration were identified (Retinal Information Network (https://sph.uth.edu/retnet/)). Nevertheless, a considerable number of mutations causing retinal degeneration still remain to be identified. For example, only 50% of the mutations for autosomal recessively inherited Retinitis pigmentosa (RP) have been identified and for Leber congenital amaurosis (LCA) 30% are unknown (figure 2). The IRDs display broad variations regarding their mode of inheritance, underlying genetic defects, age of onset and phenotypic severity (Sahel et al. 2014). Different parameters can be used to classify them like the mode of inheritance, affected genes (groups/pathways) or the predominant affected type of photoreceptor (cone-rod or rod-cone dystrophy like RP; reviewed in Hamel 2007; Phelan and Bok 2000).
### Figure 2: Prevalence of mutations in genes causing genetically heterogeneous retinal dystrophies in man. Modified from den Hollander et al. 2010. arRP – autosomal recessive Retinitis pigmentosa; LCA – Leber congenital amaurosis.

#### 1.2.1 Non-syndromic inherited retinal degeneration

The phenotype or pathological mechanism of retinal degeneration is primarily retina exclusive if the affected genes are directly involved in the visual cycle or are exclusively expressed in the retina. Partial expression in different structures or layers results in various derangements and phenotypes.

LCA is one example of such a predominant non-syndromic, autosomal recessively inherited retinal degeneration that occurs with an estimated incidence of 3/100000 (Koenekoop 2004). LCA is characterized by vision loss already at birth (Kaplan 2008), reflected by no recordable electroretinogram (ERG) developing in infancy and is the main cause of blindness of children (Cremers et al. 2002). This symptom can be accompanied by nystagmus, cataract, the lack of a pupillary response to light and others (Chung & Traboulsi 2009). At least 14 genes have been linked to LCA, encoding proteins covering a variety of retinal functions. The constellation of most frequently associated genes also depends on how strictly the diagnostic criteria for LCA are considered (den Hollander et al. 2008; Khan et al. 2014). Some LCA genes are intersecting with their causative mutation also being responsible for other
diseases like the Joubert syndrome, RP etc. (den Hollander et al. 2006; Shen et al. 2015).

RP is the group of the most common retinopathies, affecting approximately 1 in 3500 people (Berger et al. 2010). The inheritance is heterogeneous with several modes of inheritance (autosomal recessive (50-60%), autosomal dominant (30-40%), X-linked (5-15%); Anasagasti et al. 2012; Rozet et al. 2002; Stone et al. 2011; Sullivan et al. 2006). The manifestation varies from infancy to adulthood with most patients diagnosed in their middle age (Tsujikawa et al. 2008). The initial symptom is night blindness followed by tunnel vision and loss of visual acuity, which decreases during the disease course and often resulting in blindness (Eballe et al. 2010; Hamel 2006; Phelan & Bok 2000; Hartong et al. 2006; Ferrari et al. 2011). Ophthalmoscopic findings include released and accumulated pigments from the RPE in the fundus. The ERG is measurable, but shows pre-ophthalmoscopic and perimetric reduced amplitudes and an increased response latency of stimuli-response. These findings can vary depending on the underlying mechanisms of the defects (Berson 1976; Berson 1981; Berson 1993). The characteristics of the classic type of RP are caused by the malformation or progressive degeneration of the photoreceptor cells, or the adjacent retinal pigment epithelium. More than 40 genes are associated with RP (see Retinal Information Network (https://sph.uth.edu/retnet/) for references). They encode proteins of the photo transduction cascade, the visual cycle, the provision and recycling of the vitamin A derivative retinal or transcription factors (for an overview see Ferrari et al. 2011).

1.2.1 Syndromic inherited retinal degeneration

As mentioned, RP can be limited to the retina, but in up to 20-30% it also appears as a part of a multi-systemic disease, for instance in Bardet-Biedel or Usher syndrome (Chang et al. 2011; Ferrari et al. 2011; Mockel et al. 2011; Yan & Liu 2010). Usher syndrome (USH) is predominantly a multi-systemic disorder encompassing retinal, hearing and sometimes vestibular dysfunctions, although mutations in the Usher genes have also been reported to be associated with non-syndromic hearing loss depending on the type of mutation (Ahmed et al. 2002, 2003; Bork et al. 2001; Chen et al. 2014; Hilgert et al. 2009; Liu et al. 1997; Oonk et al. 2014; Ouyang et al. 2002; Sobrier et al. 2005). The number of disease causing mutations is permanently
increasing and actually has recently reached 10 (Reddy et al. 2014; Gao et al. 2014). According to disease course, severity and the addition of vestibular symptoms USH1, 2 and 3 are distinguished with descending severity from 1 to 3 (Pan and Zhang 2012). The retinal affection follows a typical RP like course with rod-cone degeneration.

Bardet-Biedl syndrome (BBS) is a more diversified example for a multisystem, pleiotropic disease with a typical RP rod-cone dystrophy accompanied by major features like obesity, renal conspicuousities, polydactyly and genital abnormalities. The diagnosis is verified, if four of the above mentioned main features apply to the patient (Beales et al. 1999; Forsythe & Beales 2013). Phenotypically is Alström syndrome as widespread as BBS and characterized by progressive rod-cone dystrophy, obesity, hearing loss and diabetes mellitus with several other possible features. The genetic basis of this very rare (estimated prevalence of 1:10000 to 1:100000 (Marshall et al. 2011; Minton et al. 2006)) monogenic syndrome has been localized to the $ALMS1$ gene that presumably encodes an intracellular transport protein that is involved in cilia assembly (Andersen et al. 2003; Hearn et al. 2002; Hearn et al. 2005). Numerous parallels can be established to the BBS, but the sensorineural deafness is more severe and Diabetes mellitus occurs early, while other features like mental retardation and cognitive impairments or polydactyly are not present in Alström syndrome patients (Badano et al. 2006; Adams et al. 2007; Beales et al. 1999). In general, pleiotropy and other crucial diagnostic criteria such as missed secondary features due to mild or late manifestation for example complicate the strict assessment of present IRD. It might sometimes remain unclear, whether it is a retina exclusive disease or bears components that shift the phenotype to a syndromic disorder. The detail of phenotypic characterization and anamnesis modulate the diagnoses and misdiagnoses that are common in a variety of IRD types. Animal models are thus helpful tools to identify subtle phenotypes that might help to identify and classify respective phenotypes and disease subtypes, in human diseases.

1.3 Animal models for retinal degeneration

Disease causing mutations detected in animal models are often of pioneering relevance in mutation identification and determination of biochemical and respective
pathological mechanisms in humans and can also point to phenotype characteristics in humans. A broad variety of animal models for retinal degenerations is available, reflecting the heterogeneity in progression and severity like in human RD (Chang et al. 2005; Chang et al. 2013; Dalke and Graw 2005; Won et al. 2011). Hence, some models exhibit a fast decline of retinal function, while others have almost normal retinas for some weeks, thus representing a human RP-like slowly progressive retinal degeneration (Hart et al. 2005). For example, the retinal degeneration slow mouse with a mutation in the rds gene encoding peripherin, a neuronal intermediate filament, shows slow degeneration of the retina in the first year of development (Schalken et al. 1990). The nob2(no-b-wave2) mouse in contrast shows a non-progressive type of retinal degeneration, but a steady state of visual impairment (Chang et al. 2006). Due to mutations in the Cacna1f gene that encodes a calcium channel, the nob2 mouse exhibits disorganized synaptic connections in the outer plexiform layer as well as incorrect neurite outgrowth of bipolar and horizontal cells (Chang et al. 2006). A newly identified and trans-species model for retinal degeneration is the naturally occurring Ccdc66 (coiled coil domain containing protein 66)-deficient Schapendoes dog and, based on this mutation, a recently generated mouse model.

1.4 Ccdc66 in retinal degeneration
The insertion of 1-basepair (bp) in exon 6 of the canine Ccdc66 gene, leading to a premature stop codon (c.521_522InsA), has been identified as the underlying cause for the naturally occurring autosomal recessively inherited generalized progressive retinal atrophy (gPRA) in the Schapendoes dogs (Dekomien et al. 2010). Similarly, a gene-trap mediated interruption of the gene in the mouse leads to an RP-like retinal degeneration (Gerding et al. 2011). The gene and respective protein have been barely described up to now and all available data dealing directly with this topic originates from the department of Human Genetics of the RUB. This section summarizes the current available information and findings regarding the gene, the protein and the Ccdc66-deficient mouse model.
1.4.1 *Ccde66* gene and products in the mouse

1.4.1.1 *Ccde66* RNA

According to database entries and previous research, the *Ccde66* gene is conserved in vertebrate species (Dekomien et al. 2010). The *Ccde66* RNA splice pattern appears to be highly complex with a variety of putative mRNAs, whose functional relevance is yet unclear (for an overview see NCBI-AceView database).

1.4.1.2 CCDC66 protein

Two isoforms of ~100 - 140 kDa (long isoform) and ~37 kDa size (short isoform) were initially characterized in the postnatal mouse retina and brain suggesting a crucial role for the protein CCDC66 in photoreceptors as supported by the retinal degenerating phenotype (described in 1.4.2), hinting to a potential role in the brain (Gerding et al. 2011; Schreiber 2010). Interestingly, the putative short isoform is still detectable in the *Ccde66* transgenic mouse and might be the product of an alternative spliced *Ccde66* mRNA that skipped the gene trap. Accessible information from database entries displays a 107 kDa and two experimentally unconfirmed 103 and 90 kDa isoforms, whereas no small isoform is reported for the mouse (UniProtKB/Swiss-Prot accession no. QSNS45). In contrast, in humans and dogs long isoforms (human: ~109 kDa, ~105 kDa; dogs: ~106 kDa) as well as short experimentally yet unconfirmed short isoforms appear in the databases (humans: ~32 kDa, ~21 kDa; dogs: ~10 kDa; UniProtKB/EMBL, accession no. D0R7H6 and D0R7H3; UniProtKB/Swiss-Prot database, accession no. A2RUB6). In general the amino acid (aa) sequence of a protein holds information about present motifs that could predict also the function of the protein. Emanating from the full length CCDC66 protein, it harbours three coiled coil domains and thereby joins the long list of coiled coil protein family members (numbering up to 180 in UniProt database for the mouse; [http://www.uniprot.org/uniprot/]). Coiled coil domains are characterized by alpha helices that are wound to superhelical structures. These domains were first described as structural elements of fibrous proteins like keratin (Pauling & Corey 1953). However, it is known now that these domains do not allow conclusions about a coiled coil protein functions, since they act in a wide range of tasks (Glover & Harrison 1995; Lupas 1996). According to the Psort server ([http://www.psort.org/](http://www.psort.org/)) an
additional leucine zipper is reported to be present at the C-terminal of CCDC66. Leucine zippers are common protein domains in transcription factors, but might also play a general role in protein-protein interactions (Huang et al. 2004; Landschulz et al. 1988; Surks et al. 1999; Wemhöner et al. 2012). Furthermore, CCDC66 is reported to have a phosphorylation site (Q6NS45, modified residue: 114). Detailed, experimentally confirmed information about the structure of protein CCDC66 is not available. Reported isoforms and domains based on the UniProt database (http://www.uniprot.org/) are presented in figure 3.

![Figure 3: CCDC66 isoforms in the mouse.](image)

Figure 3: CCDC66 isoforms in the mouse. The UniProt database reports three experimentally unconfirmed CCDC66 isoforms (isoforms 1-3; 107, 103 and 90 kDa). All isoforms include three coiled-coil domains (light grey boxes) and a leucine zipper motif (dark grey boxes) that is present according to the Psort server. Exon 4 harbors a phosphothreonine (P) that is lacking in isoform 2 (UniProt). White boxes indicate encoding exons in the respective isoforms. aa – amino acid, Met – methionine.

1.4.1.3 Localization

CCDC66 protein was detected by immunohistochemistry (IHC) in the photoreceptors of the dog and human retina and the OS of the mouse photoreceptor cells paralleling their differentiation during early postnatal development (Dekomien et al. 2010; Gerding et al. 2011). The localization in the outer segments was supported by immunogold electron microscopy and revealed further reactivity in the inner segments, connecting cilium and basal body (Gerding et al. 2011). In addition, Western blot studies also hint to extra-retinal expression in the brain and to a lesser extent in non-neural tissues. However, the exact expression sites are unknown.
1.4.2 The $Cccdc66$-deficient mouse – a retinal degeneration model

To elucidate the function of CCDC66 in normal retinal function in further detail, a new mouse model has been developed with a 5` gene-trap mediated disrupted $Cccdc66$ gene (prior to mouse exon 4, accession no. NM_177111; figure 4). The gene-trap comprises a new splice acceptor site that avoids the normal splicing process of exon 3 and 4, a beta-geo ($\beta$-galactosidase and neomycin resistance) cassette. In addition it contains a polyadenylation site that results in an early transcription stop and finally generates a shortened fusion transcript of the first three exons of the $Cccdc66$ gene and the $lacZ$ reporter gene (Gerding et al. 2011).

![Figure 4: 5´gene-trap-mediated interruption of the mouse $Cccdc66$ gene. 5´ to the mouse exon four a gene-trap was introduced including a new splice acceptor site, a $\beta$-geo fusion gene ($lacZ$ gene and neomycin resistance) and a polyadenylation site. Activity of the endogenous promoter results in transcription of the fusion mRNA of exon 1 to 3 and the gene trap (Gerding et al. 2011).](image)

The homozygous $Cccdc66$-deficient mouse lacks $Cccdc66$ RNA as well as expression of the long protein isoform (Gerding et al. 2011). A putative short isoform was still detectable in the $Cccdc66^{-/-}$ mouse by Western blot analyses, implying that the respective RNA has not been eliminated by the gene trap.

However, the gene trap mediated destruction of the full length CCDC66 protein leads to retinal degeneration in the mouse, starting around postnatal day 13 (P13) with distorted photoreceptors and slowly degenerating until up to 3 months obvious by increasingly thinning of the outer nuclear layer (figure 5; modified from (Gerding et al. 2011)).
At 7 months of age photoreceptors are severely shrunken and displaced (figure 5). Years in human approximate comparable months in mouse life. This model, therefore, illustrates a slow degeneration type. ERG revealed reduced scotopic a-wave amplitudes declining over time (monitored up to 7 months of age) as well as an early reduction of the photopic b-wave (1 month of age) with the latter improving slightly during the time course (Gerding et al. 2011).

These results imply that the lack of protein CCDC66 in the Ccdc66−/− mouse causes early onset (P13), slow progressive RP like retinal degeneration (over several months) in the Ccdc66 mutant mouse model. Thus it represents a solid basis for the extensive characterization of protein CCDC66 and this type of retinal degeneration with unclear mechanism.

**Figure 5:** Progressive degeneration of the Ccdc66-deficient mouse retina. Initial degeneration signs appear thirteen days postnatal (P13) in the Ccdc66−/− mouse showing disorganized photoreceptor outer segments. Appropriate outer segments do not develop. After a slow degeneration about several months (3mo and 7mo) the retina is severely compromised in comparison to age-matched Ccdc66+/+ controls (white arrows). P – postnatal day, mo – months, OS – outer segments, IS – inner segments, ONL – outer nuclear layer, INL – inner nuclear layer, IPL – inner plexiform layer, GCL – ganglion cell layer. Toluidine-blue stained, vertical semithin sections (0.75 µm), scale bar: 50 µm. Modified from Gerding et al. 2011.

### 1.5 CCDC66 mutations in humans

In order to address the question as to whether CCDC66 deficiency causes retinal disease also in humans, thorough sequence analyses of the CCDC66 have been performed in 80 RP and 20 LCA patients (Gerding et al. 2011). Several sequence
variations were identified, but none of them was deleterious. An additional sequence variation in exon 16, resulting in an amino acid exchange (c.2042G>A p.Cys681Tyr) was detected in a RP patient in heterozygous state, but not in 172 healthy controls. There is no evidence for a causal \textit{CCDC66} mutation for RP in humans so far (Gerding et al. 2011). Nevertheless, due to the heterogenic nature of retinal diseases, it is not unlikely that a \textit{CCDC66} retinal disease causing mutation exists in humans. The lack of an intact \textit{Ccdc66} gene and functional protein results in retinal degeneration in two species (dog and mouse) and thereby provides a prime candidate gene for an RD gene in humans.

\subsection*{1.6 Objectives}
Previous investigations in the Department of Human Genetics (RUB) showed the relevance of the \textit{Ccdc66} gene and its products in mouse retinal maturation and function (Dekomien et al. 2010; Gerding et al. 2011). Moreover, \textit{Ccdc66} expression in extra-retinal tissues for example in the brain has initially been reported. The precise role of the \textit{Ccdc66} gene and its products in a functional context or cellular pathway in the retina or other organs are unknown. The functional characterization of \textit{Ccdc66} expression/protein CCDC66 shall elucidate the underlying degeneration process and contribute to the understanding of retinal degeneration mechanisms that in turn might build the basis for therapeutic approaches in the future. By characterization of \textit{Ccdc66} gene products in the mouse, two central questions should be answered:

- What is the role of \textit{Ccdc66} expression in the pathology of slow retinal degeneration?
- Does \textit{Ccdc66} expression in extra-retinal organs disclose additional roles in protein function?

In order to address these questions, the following topics are explored within the following four chapters of the presented thesis.

- The functional investigation of \textit{Ccdc66} gene products in the mouse retina is addressed in chapter 2. Therefore \textit{Ccdc66} expression sites in the retina are explored (chapter 2.1). The retinal expression of protein CCDC66 was previously analyzed by
immunohistochemistry (Gerding et al. 2011). In order to confirm and especially delineate retinal Ccdc66 expression sites, different detection methods are used. These encompass the detection of Ccdc66 RNA by in situ hybridization (ISH) as well as the visualization of Ccdc66 reporter gene expression by X-gal staining in the Ccdc66-deficient mouse. In order to capture potential dynamics in Ccdc66 expression during postnatal development, several developmental stages shall be investigated. The pathway of CCDC66 is yet unknown, but apparently leads to retinal degeneration if interrupted. The underlying process should be identified by the characterization of interaction partners of protein CCDC66 (chapter 2.2) in order to gain insight into the proteins relevancies in proper retinal function. Furthermore, these findings could provide new candidate genes for retinal degeneration.

- The functional investigation of Ccdc66 expression in the brain and consequences of Ccdc66 deficiency are outlined in chapter 3. An initial screen revealed CCDC66 expression in the brain as indicated by Western blot experiments (Schreiber 2010). The determination of Ccdc66 expression sites in the brain (chapter 3.1) should give additional hints regarding the general role of Ccdc66 expression/protein CCDC66 that might help to understand the retinal degeneration process. The functional relevance of Ccdc66 expression in the brain, or rather the consequences of impaired Ccdc66 expression, are still unclear; since the Ccdc66+/− mouse does neither constitute an obvious additional or deviating phenotype nor apparently display conspicuous behavior compared to the Ccdc66+/+ mouse. Therefore, identified sites expressing the Ccdc66 reporter gene are assessed in the Ccdc66+/− mouse on the ultra-structural level by electron microscopy (chapter 3.2). These results shall reveal mild changes due to Ccdc66 deficiency.

- The subcellular localization of protein CCDC66 in mouse retina and brain could add to the functional role of CCDC66 and might contribute to information gained about potential interaction partners. Are the interaction partners and CCDC66 located in the same compartment? A fractionation of the compartments with subsequent detection of protein CCDC66 by Western blot is performed (chapter 4). This approach shall be complemented by the double-immune detection of protein CCDC66 with neural markers in a mixed neural cell culture (chapter 4).

- Extra-retinal Ccdc66 expression in further tissues is presented in chapter 5. Non-neural tissues exhibited distinctly lower levels of CCDC66 protein expression than
retinal and cerebral tissues in an initial screen for CCDC66 positive organs by Western blot analyses. No detailed expression site was systematically investigated. In order to obtain additional information regarding the role of \textit{Ccdc66} expression a wide screen in a variety of tissues will be performed by assessment of \textit{Ccdc66} reporter gene expression. Altogether these approaches shall contribute to the understanding of the role of protein CCDC66 in proper retinal functioning.
Chapter 2 - Functional investigation of Ccdc66 gene products in the mouse retina

2.1  Ccdc66 RNA and reporter gene expression

2.1.1  Introduction

The phenotype of the new Ccdc66−/− retinal degeneration mouse model for RP implies that Ccdc66 gene expression plays a crucial role in retinal development and regular retinal function (introduction 1.4). The localization of Ccdc66 expression sites during the postnatal dynamics of the developing and differentiating retina is important in order to deduce the function of the respective protein CCDC66 and is subject of this chapter. Previous investigations detected protein CCDC66 in the mouse retina by Western blot analyses, and it could be localized by immunohistochemistry and immunogold electron microscopy to the outer segments of the postnatal developing mouse photoreceptors, partially the cilium and basal body (Gerding et al. 2011). The used antibody did not work continuously, and other commercially (#A303-339A, Bethyl Laboratories; #NBP1-91761, Novus Biologicals) or custom ordered poly-(metabion) and monoclonal antibodies (Abmart) company did not show a consistent and/or specific CCDC66 signal in, both, IHC and Western blots (data not shown). Thus, in order to verify the previous results and to reveal further and characterize other Ccdc66 expression sites explored so far, the retina is investigated by two different methodologies - the detection of Ccdc66 RNA by ISH with DIG(digoxigenin)-labeled probes and the detection of reporter gene lacZ mediated β-galactosidase expression. Thereby, the following questions should be answered and discussed:

1) Can the previous results be confirmed?
2) Are additional Ccdc66 expression sites discovered and if so where?
3) Does Ccdc66 gene expression parallelize retinal development processes which would allow drawing conclusions on the function of the respective CCDC66 protein?
2.1.2 Materials and methods

2.1.2.1 Animals

Mice of the C57BL6/J strain were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), bred at the Ruhr University of Bochum (RUB) and kept in a light/dark cycle of 12h/12h. The mice had unlimited access to commercial food and water. Ccdc66 gene trap mice were generated and genotyped as described before (Gerding et al. 2011) and housed like the C57BL6/J mice. For all analyses, tissues were taken from mice at least back crossed to generation F6. All experimental procedures complied with the animal care and utilization committees of the RUB and were performed in accordance with the German guidelines.

2.1.2.2 Tissue preparation

Mice were deeply anesthetized by CO₂ and decapitated. Tissues were dissected and immediately frozen on dry ice (for RNA isolation) or in Isopentane on dry ice (for ISH and X-Gal staining). Tissues were stored at -80°C until further use. For RNA isolation retinas (and brains, chapter 2) were taken at least from postnatal day (P)17 Ccdc66⁺/⁻ or C57BL6/J mice. For ISH and X-gal staining three mice of each genotype (Ccdc66⁺/⁺ and Ccdc66⁻/⁻) were investigated. ISH was performed on P17 mouse retina (and age-matched brain, chapter 2) and X-Gal staining performed on the P4, P10, P17, 1 month, 1.5 months and over 10-months old retina. The day of birth was defined as P0.

2.1.2.3 Preparation of cryosections

Tissues were embedded in freezing medium (#14020108926, Jung Tissue Freezing Medium, Leica), fixed in the cryostat (CM3050 S, Leica) and serial, coronal or sagittal sections were prepared (12 µm for ISH and 25 µm (retina and other tissues) or 30 µm (brain) for X-gal staining). Groups of five slices were mounted consecutively on SuperFrost® Plus microscope slides (Thermo Scientific™) in order a different staining or hybridization of adjacent sections, dried for 2 h at RT and stored at -80°C.
2.1.2.4 *In situ* hybridization

In order to localize *Ccdc66* RNA in mouse tissue, *In situ* hybridization (ISH) with a *Ccdc66* specific DIG-labeled antisense riboprobe was performed. The detection of *Rhodopsin* transcripts served as positive control, the application of respective sense probes as negative control.

Riboprobe synthesis

A previously cloned part of the *Ccdc66* gene (N579-1925 of ENSMUST00000050480) that was introduced between *EcoRI* and *SalI* interfaces and flanked by SP6 and T7 promoter sequences in the pSPT18 Vector (Roche) served as template for the synthesis of the *Ccdc66* probes. *Rhodopsin* probes were generated by PCR on cDNA.

RNA was isolated with PeqGold TriFast reagent (#30-2010, Peqlab) and purified by the use of the RNeasy mini-kit (#74104, Qiagen) according to the manufacturer’s protocol. RNA concentration was assessed and purity ensured by spectrometric absorbance measurements (ND-1000, NanoDrop Technologies). The cDNA was generated from isolated RNA by reverse transcription using the Sensiscript RT Kit (#205211, Qiagen) according to the company’s instructions and the use of an oligo(dT) primer (reverse (rev) 5’-TTTTTTTTTTTTTTTTTTTTTTTT-3’; Metabion). PCR on cDNA was performed *Rhodopsin* forward (fwd) primer for the generation of the sense probe was flanked by a SP6 promoter sequence (fwd 5’-GATTAGGTGACACTATAG ACCCAGGCCTTCAGGCCTGTGC-3’), the rev primer for the generation of the antisense probe by the T7 promoter sequence (rev 5’-TAATACGACTCAGTGAGG CACCCTCCGACCCCAAGTGTA-3’; included region: N1985-2611 ENSMUST00000032471). PCR was performed with the following sample components and cycling conditions (table 1; Biometra T professional gradient Thermocycler #3100-813-01, Biometra). Amplicons were separated on agarose gels (1.5% in 1 x TBE) containing SERVA DNA Stain G (#39803.01; SERVA Electrophoresis), controlled for size (GeneRuler 1 kb DNA Ladder, ready-to-use (#SM0314, Fermentas)) and purity and documented by the aid of the FUSION-SL documentation system and respective FUSION-CAPT software (Peqlab).
Table 1: Sample components and standard cycling conditions for PCR based template generation for *in situ* hybridization probes

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<td>rev Primer</td>
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<td>dNTPs</td>
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<td>HotStarTaq DNA Polymerase, #203203 Qiagen</td>
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PCR samples were purified with the HighPrep™ PCR kit (#AC-60050, Magbio). Based on the PCR templates riboprobes were generated by the use of the DIG RNA Labeling Kit (SP6/T7) (#11175025910, Roche) according to the manufacturer protocol with an additional precipitation step as described before (Braissant, Oliver and Wahli 1998). In short, duplicate amounts (40 µl) of the probes were generated and subsequently precipitated by the addition of 40 µl 3 M NH₄-acetate and 200 µl EtOH and incubation at -20°C for 30 min. After centrifugation (30 min at 4°C, 13000 rpm), the supernatant was discarded and probes were suspended in 40 µl RNase-free H₂O (RNase-Free Water, #129112, Qiagen). Labeling efficacy was evaluated by a dot blot in conjunction with the pre-labeled reference contained in the labeling kit.

**Hybridization**

Tissue slices were thawed, dried at RT and subsequently fixed in 4% PFA for 20 min and in acidic EtOH for 10 min at -20°C. Slices were washed in PBS (2 x 5 min, RT) and equilibrated in proteinase K digestion buffer before digestion with proteinase K for 20 min at 37°C. Sections were post fixed in 4% PFA for 5 and washed in DEPC water (2 x 5 min, RT). Tissue slices were equilibrated in TEA buffer (5 min, RT), incubated in TEA/AAA (10 min, RT) and again washed in 2x DEPC water (2 x 5 min,
RT). Sections were dehydrated in ascending EtOH concentrations (70%, 80%, 90%, 96%, 2 x 100%) and briefly dried at RT. Pre-hybridization was performed with hybridization mix for 1 h at 58°C, followed by application of hybridization mix containing the probe (Rhodopsin antisense and sense 300 ng/ml, Ccdc66 antisense and sense: 1200 ng/ml) and incubated overnight at 58°C. Slices were washed 3 x 15 min and 5 min in 2x SSC at RT, equilibrated in RNaseA digestion buffer for 5 min at 37°C and single stranded probe was removed by incubation in RNaseA solution (20 µg RNaseA/ml RNase digestion buffer) for 45 min and subsequent washing steps (3 x in 2x SSC for 15 min at 58°C, 0.5x SSC for 15 min and 0.1x SSC for 5 min at RT). Sections were equilibrated in PBS for 5 min, blocked in 5% NGS in PBS-T for 45 min and incubated with anti-DIG antibody (Roche) 1: 500 in 2% NGS in PBS-T for 1 h. Slices were washed in PBS (4 x 10 min), equilibrated in AP substrate buffer (2 min) and then incubated for 2-5 hours (optical control) in AP substrate buffer with NBT and BCIP (purple precipitation). Slices were washed with DEPC water dehydrated in an ascending EtOH concentration (see above) and cover-slipped with mounting medium (HICO-Mic, REF: 64 00 09, HIRTZ & Co. KG). Staining was documented with the Axio Imager Z.2, and the Metafer and Vslide software (MetaSystems). Utilized buffers and solutions are listed in the appendix (appendix - chapter 2.1).

2.1.2.5 X-gal staining (enzyme histochemistry)

The Ccdc66/- mouse was engineered to express a CCDC66/ β-galactosidase fusion protein (partly encoded by the Ccdc66 gene (exon 1 to 3) and the lacZ reporter gene) under control of the endogenous Ccdc66 promoter, as described in 1.4.2. By the addition of the substrate X-gal, reporter gene expression positive cells were marked by the blue precipitate released during the hydrolysis reaction through the lacZ encoded β-galactosidase. Tissue slices were dried at RT, briefly fixed in EtOH (-20°C, 7 min), transferred in PBS (5 min, RT), covered with X-gal staining solution and incubated overnight at 37°C in a humidity chamber. Alternating slices were counter-stained with neutral red solution for 3 min and rinsed with H2O and then all sections washed in PBS for 5 min. Specimens were dehydrated by immersion in ascending EtOH concentrations and cover-slipped (see 2.1.2.4, last section,
hybridization). Utilized buffers and solutions are listed in the appendix (appendix - chapter 2.1)

2.1.3 Results

2.1.3.1 Ccdc66 RNA expression in the postnatal Ccdc66+/+ and Ccdc66−/− mouse retina

In order to localize Ccdc66 expression in the postnatal mouse retina and to potentially discover additional expression sites, P17 Ccdc66+/+ and Ccdc66−/− retinas were compared by Ccdc66 ISH. Ccdc66 RNA in the Ccdc66+/+ mouse retina can be detected in the photoreceptors (figure 6 A, black arrowhead), where Ccdc66 expression has been shown earlier by IHC (Gerding et al. 2011). In addition, Ccdc66 RNA is also associated with the INL as revealed by a gradient staining strongest adjacent to the plexiform layers (figure 6 A, white arrowheads), moreover in the GCL (figure 6 A, black arrow) and in the IPL, in form of single puncta (figure 6, white arrow). In the Ccdc66−/− mouse, the staining in the IS and the INL is lacking (figure 6 B, black and white arrowheads), while the staining in the GCL and IPL is present in both, Ccdc66+/+ and Ccdc66−/− (figure 6 A and B, black and white arrows). Probe hybridization to Rhodopsin RNA serves as positive control, highlighting the photoreceptor IS where the RNA of Rhodopsin is translated and to less extent the ONL (Wolfrum and Schmitt 2000; figure 6, C and D grey arrowheads). The application of the respective sense probes (Rhodopsin and Ccdc66) serves as negative control revealing no signal in the Ccdc66+/+ or Ccdc66−/− retina (figure 6, G - H). These results confirm Ccdc66 expression in the photoreceptors and moreover disclose Ccdc66 RNA expression with varying degrees in additional retinal layers. A staining in the GCL is present in retinas of both genotypes (Ccdc66+/+ and Ccdc66−/−).
Figure 6: Expression of *Ccdc66* RNA in the *Ccdc66*+/+ and *Ccdc66*−/− mouse retina detected by *in situ* hybridization (postnatal day 17). *Ccdc66* RNA is localized in the photoreceptor IS of the *Ccdc66*+/+ mouse (A, black arrowhead). Additional staining appears in the INL at the border to plexiform layers (A, white arrowhead), in the IPL (white arrow) in the GCL (black arrow). The signal in the IS and INL is absent in the *Ccdc66*−/− retina (B, black and white arrowheads), whereas the staining in the IPL and GCL is still present to certain extent (B, black and white arrow). ISH with the *Rhodopsin* antisense probe served as positive control (C and D, grey arrowheads), the application of *Ccdc66* and *Rhodopsin* sense probes as negative controls (E - H). ISH – *in situ* hybridization, RPE - retinal pigment epithelium, OS - outer segments, IS - inner segments, ONL - outer nuclear layer, OPL - outer plexiform layer, INL - inner nuclear layer, IPL - inner plexiform layer, GCL - ganglion cell layer. 12 µm vertical cryosections, scale bar – 50 µm.

### 2.1.3.2 *Ccdc66* reporter gene expression in the postnatal *Ccdc66*−/− mouse retina and comparison to *Ccdc66* RNA expression (2.1.3.1)

In the P17 *Ccdc66*−/− mouse retina expression of the trapped *Ccdc66* gene was further visualized by X-gal staining (figure 7). Intense labeling is present in the photoreceptor IS (figure 7 A, black arrowhead), presumably the OPL and GCL (figure 7 A, grey arrowhead, black arrow) and distinct but less intense in the INL/plexiform layer.
borders (figure 7 A, white arrowheads). In addition Ccdc66 reporter gene expression is present in terms of single puncta in the IPL (figure 7 A, white arrow). The parallel staining of the Ccdc66\(-/-\) retina serves as negative control and revealed no staining (figure 7 B).

Figure 7: Ccdc66 reporter gene expression in the retina of the Ccdc66\(-/-\) mouse (postnatal day 17). In the Ccdc66\(-/-\) retina intense labeling by X-gal staining is displayed in the photoreceptor IS (black arrowhead) and OPL (A, grey arrowhead) and in the IPL (A, white arrow). The borders of the INL (A, white arrowheads) and the GCL (A, black arrow) is distinctly highlighted by X-gal staining. The parallel treatment of Ccdc66\(+/-\) slices revealed no blue color precipitation (B). Mice ages: postnatal day 17. RPE - retinal pigment epithelium, OS - outer segments, IS - inner segments, ONL - outer nuclear layer, OPL - outer plexiform layer, INL - inner nuclear layer, IPL - inner plexiform layer, GCL - ganglion cell layer. 25 µm vertical cryosections, scale bar – 50 µm.

Compared to the detection of Ccdc66 RNA in the Ccdc66\(+/-\) retina, the X-gal staining in the Ccdc66\(-/-\) retina is represented at the same sites (IS, INL boundaries, IPL and GCL; figure 8 A and B, black and white arrowheads, black and white arrow). Additional reporter gene expression is observed in the area of the OPL (figure 8 A, grey arrowhead).
Figure 8:  

*Ccdc66* expression in the *Ccdc66*+/− mouse retina by *in situ* hybridization and by reporter gene expression in the *Ccdc66*−/− mouse retina (postnatal day 17). *Ccdc66* promotor activity was detected in several layers in the *Ccdc66*+/+ and *Ccdc66*−/− mouse retina. Overlapping signals were revealed in the IS of the photoreceptors, the INL, IPL, GCL and in both. X-gal staining in of the *Ccdc66*+/− mouse retina revealed additional intense labeling of the OPL. Retinae were aged-matched (P17). RPE - retinal pigment epithelium, OS - outer segments, IS - inner segments, ONL - outer nuclear layer, OPL - outer plexiform layer, INL - inner nuclear layer, IPL - inner plexiform layer, GCL - ganglion cell layer. 25 µm (A) and 12 (B) vertical cryosections, scale bar – 50 µm.

These results further support previous findings regarding the *Ccdc66* expression in the photoreceptors and moreover display, like ISH *Ccdc66*, promotor activity in several retinal layers. Because the comparison of the two methods yields comparable results, X-gal staining was used for the investigation of *Ccdc66* expression during the postnatal development of the mouse retina.

2.1.3.3 *Ccdc66* reporter gene expression during postnatal retinal development of the *Ccdc66*−/− mouse retina

In order to explore whether *Ccdc66* gene expression parallels the retinal developmental processes that allow to hypothesize on the function of the *Ccdc66* gene products, *Ccdc66* reporter gene expression was monitored at developmental stages P4, P10, P17, 1 month, 1.5 months and > 10 months of the *Ccdc66*−/− mouse retina (figure 9 A - C, G - I) in conjunction with the *Ccdc66*+/+ as negative control that are intensely counterstained with neutral red for morphological orientation (figure 9 D
In the P4 retina (figure 9 A), prior to the completion of retinal layers organization, a faint labeling is present in the forming saccule, a protrusion that later gives rise to the photoreceptor OS (figure 9 A, black arrowhead), the GCL (figure 9 A, black arrow) and in the originating plexiform layers (OPL grey arrowhead, IPL white arrow), respectively whereas staining in the OPL is more intensive than in the IPL/GCL. The age-matched \textit{Ccdc66}^{+/+} retina does not show any marking (figure 9 D), which is also the case for all further stages (P10, P17, 1 month, 1.5 months and >10 months, figure 9 E and F, J - L) and thus it is not mentioned separately for all stages. The retina of the P10 \textit{Ccdc66}^{-/-} mouse (figure 9 B), after completion of the retinal layer formation and prior to initial degeneration signs of the photoreceptors, exhibits a comparable staining pattern to the P4 retina. Slightly increased staining intensity is present in the IS and OPL (figure 9 B, black and grey arrowhead) and signal intensities comparable to P4 in the IPL and GCL (figure 9 B, white and black arrows). Additional signals are now more distinctly observed at the INL border to the OPL (figure 9 B, white arrowheads). At this time point the \textit{Ccdc66}^{-/-} retina does not yet show obvious degeneration as reported earlier and as it is also illustrated by the comparison to the age-matched \textit{Ccdc66}^{+/+} retina (figure 9 E). In the P17 \textit{Ccdc66}^{-/-} mouse, after eye opening and initial degeneration signs in the photoreceptors present on the ultra-structural level (Gerding et al. 2011), strong labeling occurs the IS (figure 9 C, black arrowhead), now distinguishable from the OS, as well as in the OPL (figure 9 C, grey arrowhead) and INL (figure 9 C, white arrowheads). Comparable to P4 and P10 the GCL and scattered spots in the IPL are more distinctly marked by X-gal staining (figure 9 C, black and white arrows). In the retina of the 1 month-old \textit{Ccdc66}^{-/-} mouse, after the majority of synaptic contacts are established and the degeneration in the \textit{Ccdc66}^{-/-} mouse has further progressed, intense X-gal staining is limited to the IS of the photoreceptors (figure 9 D, black arrowhead). The majority of above reported markings (in the INL at the borders to the plexiform layers, OPL and IPL) are still present, but less intense than in the former stages (figure 9 D, grey and white arrowheads, white arrow). The GCL is highlighted in comparable intensity than in the younger stages (figure 9 D, black arrow). The 1.5 months-old \textit{Ccdc66}^{-/-} mouse exhibits the same distribution of labelling than in the 1 month-old retina (figure 9 E, black, grey and white arrowheads, black and white arrows), but with clear signs of degeneration present on the microscopic scale with
apparent thinning of the outer retina compared to the age-matched $Ccdc66^{+/+}$ control (figure 9 K). Likewise, in the over 10 months-old $Ccdc66^{-/-}$ retina, the distribution of the X-gal staining is suggested to be unchanged in comparison to the 1 and 1.5 months old retina (figure 9 F, black and grey arrowhead, black arrow). Yet this is difficult to make an objective assessment, because of the massive degeneration process that hardly allows to clearly distinguish the retinal layers (especially X-gal puncta within the IPL and GCL), as evidenced by comparing to the $Ccdc66^{+/+}$ control (figure 9 L).

These results show that $Ccdc66$ expression is present throughout the whole postnatal development in several retinal layers, as monitored by $Ccdc66$ reporter gene expression studies in the $Ccdc66^{-/-}$ mouse and initially confirmed by ISH in the P17 retina (2.1.3.1). The IS of the differentiating and mature photoreceptor cells are constantly and distinctly highlighted by X-gal staining with maximum signal intensity between developmental stages P17 and 1.5 months (slightly in P4, stronger in P10, P17, P28, 1.5 and slightly in >10 months-old retina). An additional strong staining appears in the OPL in the early investigated stages (increasing from P4, P10 to P17) and then decreasing with age (faint already in the P28 retina). In the IPL puncta of X-gal staining are detectable predominantly between P4 and P17. A relatively constant (partly faint) staining over time is observed in the INL borders to the plexiform layers and GCL at all investigated time points.
Figure 9: *Ccdc66* reporter gene expression in the *Ccdc66*-deficient mouse during postnatal development. *Ccdc66* reporter gene during the postnatal development of the mouse retina.
was surveyed by X-gal staining and is detected during the whole postnatal development in the IS of the photoreceptors with maximum signal intensity between P17 and 1.5 months and present in all investigated ages (slightly in P4, strong in P10, P17, 1 month, 1.5 and slightly in >10 months, black arrowheads in A – C, G - I). Additional labeling appears in the OPL increasing up to P17 and declining afterwards (grey arrowheads in A – C and G - I). X-gal marking is present in the INL in the majority (white arrowheads in B and C, G and H, in P4 and >10 months hard to assess) and in the GCL at all investigated stages (A – C and G – I, black arrows). *Ccdc66*/+/+ slices of age-matched retinae served as negative control (D – F, J – L, counterstained with neutral red for morphological reference).

**2.1.4 Discussion**

In previous IHC investigations protein CCDC66 was localized to the outer segments of mouse retinal photoreceptor cells (Gerding et al. 2011). Remarkably, the detection of *Ccdc66* expression on the RNA level by ISH presented here (2.1.3.1) revealed expression in several retinal layers beside the photoreceptors during postnatal development. The indirect evidence of *Ccdc66* presence by the *Ccdc66* reporter gene expression could be extended to several retinal layers (OPL, INL, IPL and GCL) during postnatal development (2.1.3.3). In conclusion, the expression in the photoreceptors was confirmed by other methodological approaches, and it is likely that protein CCDC66 does not only play a role in photoreceptors cells, but also in other retinal cell types or structures.

**2.1.4.1 Methodological aspects of *Ccdc66* RNA, reporter gene expression and protein CCDC66 in the mouse retina**

On the RNA level as well as by the X-gal staining as indirect proof of reporter gene expression, there are more *Ccdc66* expression sites identified than by the antibody staining against protein CCDC66 (Gerding et al. 2011). In general, previous experiments have shown that direct immunogenic detection of protein CCDC66 seems to be difficult because of no suitable antibody applicable for several methods and/or the loss of antigen specificity over time. An example taken from the literature for a missed expression site detection by antibodies that was finally identified by a reporter construct experimentally uncovered the involvement of integrin proteins in
axonal migration (Baum & Garriga 1997). Furthermore, if a protein is unstable, the immunohistochemistry might not be able to depict the target. Replacement or modification of the target by the fusion to a stable protein (for instance β-galactosidase) might result in a more stable protein level. In addition, one has to consider that the replacement or the modification of the investigated target protein can also impact on the transcription level and localization of the trapped protein product (see below, next section). Nevertheless, reporter expression enables to infer where the investigated protein is expressed and thereby disclosing the entirety of in this case Ccdc66 expression that in turn might support the derivation of the function of CCDC66.

Deviations in Ccdc66 RNA and reporter gene expression patterns – are they reconcilable?

In the P17 mouse retina, Ccdc66 expression experiments reveal consistent results in the photoreceptor IS and ganglion cells with methods ISH and X-gal staining. Regarding the INL, Ccdc66 RNA is detected in the whole width of this layer, with predominant labeling at the borders to the IPL and OPL. X-gal staining is also present at these borders to certain extent, but also appears to be strongly present in the OPL (peaked expression at P17). X-gal staining would in theory reflect the visualized Ccdc66 expression on protein level, since the translation of the trapped gene is required for the visualization. Of course, RNA and protein representations do not necessarily spatially overlap in all cases. In general, one would expect the RNA predominantly close to the nuclei at sites of translation on the ribosomes, whereas a protein product might be located at different sites. Rhodopsin RNA for example is translated in the IS of the photoreceptors and the protein functionally active in the OS (Wolfrum & Schmitt 2000). The spatially separated localization of RNA and final destination of translational product is also a well-known fact from many other proteins that are transported along protrusions of polarized cells as in axons and dendrites of neurons (Petersen et al. 2014).

Moreover the localization of RNA and protein can differ at the quantitative level, due to posttranscriptional, translational or posttranslational events. These modifications crucially impact on creating an independent, non-correlating ratio of the amount of
RNA to protein, emphasizing the benefits considering several methodologies when initially assessing expression (Tian et al. 2004).

Furthermore the signal intensities may generally differ due to the detection method and their adjustments, like the probe concentration in ISH and background staining. Transferring these conditions to the present finding regarding Ccdc66 expression, the localization of the X-gal staining differs from the detection sites of Ccdc66 RNA by ISH. Thus it is possible that the fusion protein is transported and thus does not spatially overlap with RNA localization. But this does not necessarily reflect the translocation of the wild type protein CCDC66 regarding time and distribution, if transport signals or necessary domains are lacking in the fusion protein. Only exons 1-3 of CCDC66 are present, the true transport might be incomplete. For example, a transgenic mouse with a EGFP (enhanced green fluorescence protein) knock-in under the control of the Rhodopsin promotor displays expression of the reporter with four days delay compared to normal Rhodopsin expression (Ichsan et al. 2005).

However, this comparison is easy to make for extensively characterized promoters, genes and proteins as Rhodopsin, but this it is not as easy regarding the Ccdc66 gene or its products. The identity of the wild type CCDC66 is unclear, since no targeting signals can be predicted (see chapter 4, CCDC66 localization). The finding that the signal of the X-gal staining is presented in the IS of the photoreceptors of the Ccdc66/- mouse, while protein CCDC66 is localized to the outer segments in the Ccdc66+/+ mouse retina by IHC, could also be due to the fact that in the Ccdc66/- correct OS are not formed at all (Gerding et al. 2011).

Nevertheless, in conclusion Ccdc66 seems to be expressed in the somata of cells in the ONL, INL and GCL as evidenced by RNA staining (ISH) and the CCDC66/β-galactosidase translation product (partly) transported in neurites in the plexiform layers. Since the signal of the X-gal staining is stronger in the OPL than in the INL and not present to a comparable level in both layers, one can expect that this is not only due to diffusion. Diffusion would imply an equal distribution within the whole cell (soma in nuclear layer and processes in plexiform layer), as it was assumed for above mentioned EGFP knock-in model (Ichsan et al. 2005; Chalfie et al. 1994). Furthermore, β-galactosidase is larger in size and might not diffuse as easily as the small EGFP (according to UniProt EGFP ~26 kDa, β-galactosidase ~110 kDa).
**Ccde66 RNA and reporter gene expression in Ccde66−/− retinal ganglion cell layer**

With regard to the remaining RNA detection in the GCL, one has to consider that RNA detection could only be reflected partly by the respective translational product in the Ccde66−/− mouse. This could be the case if additional RNA splice variants are detected by ISH that are possibly not affected by the trap and thus not reflected by β-galactosidase expression. For the GCL this might be an explanation for remaining labeling in the Ccde66−/− retina. This aspect will be additionally discussed in conjunction with the interpretation of the RNA analysis in the Ccde66−/− brain (3.1.4.1).

**Retinal cells and structures of Ccde66 RNA/ reporter gene expression**

Beside the assessment of photoreceptors and ganglion cells expressing the trapped Ccde66 gene, the determination of expression in the INL and plexiform layer is not as obviously assignable to certain cell types. Ccde66 RNA spreads across the entire INL, where horizontal, bipolar and amacrine and Müller cell somata are located. Ccde66 hybridization signal shows gradients with stronger labeling at the borders to the plexiform layers, where the majority of amacrine and horizontal cells are located. However, the general broad distribution of Ccde66 RNA signal indicates that it seems not to be limited to a single cell type in the INL with respect to their frequencies (amacrine cells ~40%, horizontal cells ~3%, bipolar cells ~40%, Müller cells ~16%; Jeon, Strettoi, and Masland 1998). If Ccde66 expression would be exclusively present in the horizontal cells for example, the RNA signal would likely not cover the whole width of the INL. Nevertheless, the presence of the X-gal signal at the borders of the INL supports a contribution to Ccde66 expression by horizontal and amacrine cells. In order to additionally assign Ccde66 expressing cells and those whose processes are labeled in the OPL, double labeling of retina specific cell markers should be performed in combination with an anti-β-galactosidase antibody. Another possibility to avoid indirect detection of Ccde66 expression would be the separation and sorting of INL retinal cells by flow cytometry with cell specific antibodies (for example Portillo et al. 2014) and subsequent Western blot and immune detection of protein CCDC66.
2.1.4.2 Retinal processes in parallel with temporal and spatial Ccdc66 reporter gene expression changes – functional association of Ccdc66 expression

The time-dependent changes in reporter gene expression yield important conclusions regarding the function of protein CCDC66. Housekeeping genes tend to exhibit constant RNAs and steady-state proteins, while development-related gene expression is depending on dynamic processes like cell cycle events, cell division or differentiation (Tian et al. 2004). Reporter gene expression in the developing postnatal Ccdc66−/− mouse retina would thus partly reflect a development-related expression, since it shows varying intensities in the IS of the photoreceptors and plexiform layers over time. In the IS, Ccdc66 reporter gene expression throughout the entire investigated stages of the postnatal development further suggests not only the contribution of protein CCDC66 in IS/OS formation, but also maintenance in the healthy Ccdc66+/+ retina. The Ccdc66 reporter gene expression sites in the IPL and the OPL likewise show age dependent alterations, with a slight accentuation of the IPL and stronger in the OPL at the early stages P4, P10 and P17, which is clearly reduced in older retinas. The IHC investigations of the rodent retinal IPL revealed immune reactivity of various excitatory and inhibitory synaptic markers expression mainly at P0 and latest around P5 (Guo et al. 2009; Johnson et al. 2003; Fletcher & Kalloniatis 1997; Hack et al. 2002; Kim et al. 2002; Koulen 1999; Sassoè-Pognetto & Wässle 1997; Witkovsky et al. 2005). Their expression increases to an adult comparable level around P14 – P21. The synaptic circuit generation in the OPL is established by the pedicles of differentiating photoreceptors and lateral branching horizontal cells that enter into synaptic contact from around P5 on (Blanks et al. 1974; Olney 1968; Hoon et al. 2014). Later, around P6 and P7 the processes of the bipolar cells that were mainly differentiated postnatal around P3 contribute to the synaptic network of the OPL (Hoon et al. 2014). For example, immunolabeling for VGlut1 (vesicular glutamate transporter 1) in the OPL as a marker for the excitatory synapses is detectable around P3 (Johnson et al. 2003) whereas expression of the inhibitory synapse marker VIAAT (vesicular inhibitory amino acid transporter) in the developing mouse OPL occurs around P5 to P7 increasing up to P14 (Guo et al. 2009). However, there is exhaustive literature on the formation of synapses and the description of retinal developmental marker expression.
The general conclusions on these findings is that the synaptic contacts in the plexiform layers are sequentially formed (first in the IPL, then OPL) and are generally established before sensory input by eye opening. Moreover, the expression of synaptic proteins reaches a relatively constant level around the time point of eye opening (Hoon et al. 2014). Referring to the results in the presented study, \textit{Ccdc66} reporter gene expression is detected early in both, the IPL and OPL (P4), with the signal increasing until P17 briefly after eye opening and then being lower in the other stages.

These results hint to an association of \textit{Ccdc66} expression, or protein CCDC66 and the formation of synaptic terminals and or contacts, rather than the maintenance or regular function in signaling, because signals of \textit{Ccdc66} reporter gene expression decreases. Since the establishment of the synapses in the IPL is initiated prior to the stage of the here investigated early postnatal stage P4, younger retinas should be screened for \textit{Ccdc66} expression in future.

Another postnatal developmental process in the plexiform layers is the development of the retinal vasculature that is based on the processes of angiogenesis and vasculogenesis (mouse: Fruttiger 2002; rat: Henkind and de Oliveira 1967). However, the primary vascular network is established in the form of a plexus covering the inner surface of the retina by about one week postnatal (Fruttiger 2002). Around this time vascular branches spread in the plexiform layers of the retina (Gerhardt et al. 2003; Fruttiger 2002; Stahl et al. 2010) first in the outer plexiform layer (Stahl et al. 2010; Blanks & Johnson 1986) with a vascular profile in earlier studies reported around P8 - P10 in the outer plexiform layer (Connolly et al. 1988; Blanks & Johnson 1986). In the adult, blood vessels are restricted to the nerve fiber layer and to the inner and outer borders of the INL (Fruttiger et al. 1996). Thus, even though there is partially spatial overlap of expression of \textit{Ccdc66} and forming vasculature of the retina, \textit{Ccdc66} expression is present in both, IPL and OPL already at P4, which precedes the occurrence of the vessels.

In conclusion, regarding postnatal processes in the developing retina, especially the OPL, an association of \textit{Ccdc66} expression with synaptic formation/terminal formation would be more likely than vascularization. Thereby strong \textit{Ccdc66} expression would rather relate to the establishment phase than to maintenance phase of the synaptic circuit.
2.2 Interaction partners of protein CCDC66 in the mouse retina

2.2.1 Introduction

The function of protein CCDC66 is unclear, except for the fact, that its presence is essential for proper retinal function. The identification of interaction partners shall answer the following questions:

1) Does protein CCDC66 belong to a new biochemical cascade, or act in a known pathway?

2) What might the role of CCDC66 be within the respective process?

A promising approach to screen for protein interaction partners is the yeast two-hybrid (YTH) system. Potential binding partners of protein CCDC66 (library of retinal cDNA) are fused to the activation domain (AD) of the GAL4 transcription factor (prey). In turn, CCDC66 is fused to the DNA binding domain (BD) of the GAL4 transcription factor (bait) by cloning Ccdc66 cDNA into the respective vector. Both fusion constructs (CCDC66 and library protein, each fused to one subunit of the transcription factor) will be transformed into yeast cells and co-cultivated. The spatial proximity of the two subunits of GAL4, due to respective protein-protein-interaction, leads to the transcription of reporter genes, enabling the selection of positive clones that will be cultivated and characterized by DNA sequencing of respective interacting proteins.

2.2.2 Materials and methods

In order to screen for novel interaction partners a YTH screen with defined parts of protein CCDC66 was performed using a mouse retinal cDNA library. The utilized yeast strain for the bait construct transformation (AH109; Clontech Laboratories, Inc.) was kindly provided by Dr. habil. A. Gießl (University of Erlangen-Nuremberg Department of Biology; Animal Physiology) as were the mouse retinal cDNA library in the Y187 host strain (Make Your Own “Mate & Plate™” Library for Yeast Two-Hybrid Screening, Clontech Laboratories, Inc.) and several plasmids (pGADT7, pGBK7-53 and pGADT7-T). An independent, non-auto active bait construct (PH-domain of inositol polyphosphate 5-phosphatase OCRL-1 (OCRL1 in the following) in pGBT9) as well as the “empty” pGBT9 plasmid was kindly provided by Dr. habil. K. S. Erdmann, AG biochemistry of polarized cells, RUB (at that time).
2.2.2.1 Yeast strains

AH109 served as host strain that was transformed with the CCDC66 bait constructs. It contains several reporter genes under the control of three distinct GAL4-responsive promoters (\textit{HIS3} (promotor GAL1), \textit{ADE2} (promotor GAL2), \textit{lacZ} and \textit{MEL1} (promotor MEL1)).

\textit{Y187} is the prey library host strain and contains the \textit{lacZ} (promotor G1) and \textit{MEL1} (promotor M1) GAL4-inducible reporter genes.

2.2.2.2 Generation of CCDC66 bait constructs

Five fragments of \textit{Ccdc66} cDNA constructs were chosen not to interrupt reported protein domain coding sequences according to the UniProt database and the Psort-server as well as to be approximately equal in bp length (figure 10).

![Figure 10: Ccdc66 constructs for the yeast two-hybrid screen](image)

Respective \textit{Ccdc66} sequences were amplified by PCR from cDNA, derived from mouse retinal RNA (pooled from 6 P17 mouse retinas (3 animals)) by reverse transcription as described in chapter 2, 2.1.2.4.. Primers were flanked by \textit{SalI} and \textit{XmaI} recognition sites (table 2) for subsequent cloning of the fragments into the pGBT9 plasmid resulting in fusion constructs (CCDC66-1 – CCDC66-5).
Table 2: Primer sequences for the amplification of yeast two-hybrid constructs CCDC66-1 – CCDC66-5. *Xma* and *Sai* recognition sites are underlined

<table>
<thead>
<tr>
<th>Construct</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCDC66-1</td>
<td>F TATGACCCCGGGGATGAACCTGGGAGATGGTTTA</td>
</tr>
<tr>
<td></td>
<td>R TATGACGGCTCGACTTAACACTGCTGAAATGCCTCTTG</td>
</tr>
<tr>
<td></td>
<td>Fragment length: 693 bp</td>
</tr>
<tr>
<td>CCDC66-2</td>
<td>F TATGACCGGAGGCAAGAGGCATTTTCAGCAGTGT</td>
</tr>
<tr>
<td></td>
<td>R TATGACGTGGACTTAACACTGCTGAAATGCCTCTTG</td>
</tr>
<tr>
<td></td>
<td>Fragment length: 615 bp</td>
</tr>
<tr>
<td>CCDC66-3</td>
<td>F TATGACCGGAGGCTTTTCACACCTCCACCTG</td>
</tr>
<tr>
<td></td>
<td>R TATGACGTGGACTTAACACTGCTGAAATGCCTCTTG</td>
</tr>
<tr>
<td></td>
<td>Fragment length: 612 bp</td>
</tr>
<tr>
<td>CCDC66-4</td>
<td>F TATGACCGGAGGCAAGACTCCCTCCCCAAGAAGGAC</td>
</tr>
<tr>
<td></td>
<td>R TATGACGTGGACTTAACACTGCTGAAATGCCTCTCTC</td>
</tr>
<tr>
<td></td>
<td>Fragment length: 681 bp</td>
</tr>
<tr>
<td>CCDC66-5</td>
<td>F TATGACCGGAGGAATGACTATGAAAAGGAGACTCTG</td>
</tr>
<tr>
<td></td>
<td>R TATGACGTGGACTTAACACTGCTGAAATGCCTCTTG</td>
</tr>
<tr>
<td></td>
<td>Fragment length: 381 bp</td>
</tr>
</tbody>
</table>

Amplicons were assessed and purified as described in chapter 2.1.2.4 and subsequently, in parallel to the pGBT9 plasmid, double digested with *Sai* and *Xma* at 37°C overnight. Samples were purified with Invisorb® Fragment Clean Up columns (#1020300200, Strattec biomedical) according to the manufacturer protocol. The vector was dephosphorylated by the Antarctic phosphatase, purified again and the *Ccdc66* inserts ligated (T4 Ligase) between respective interfaces of the pGBT9 plasmid at 4°C overnight (plasmid:insert ratio 1:3). All enzymes in this section were obtained from New England Biolabs GmbH. The constructs were transformed into One Shot® TOP10 Electrocomp™ *E. coli* according to the manufacturer protocol (#C4040-50, Life technologies, Inc.; Gene Pulser (BioRad Laboratories, Inc.), settings: voltage 1.7 kV, resistance 200Ω, capacitance 25 μF)) and positive clones selected by incubation on LB-amp plates (50 μg/ml amp) overnight at 37°C. Single ampicillin resistant colonies were transferred into 1.5 ml of fresh LB-amp and grown overnight. Cells were harvested by centrifugation (13000 rpm, 30 sec) and
suspended in 250 µl lysis buffer. After 5 min incubation on ice 25 µl lysozyme were added, mixed and incubated at 95°C for 1 min. Samples were kept on ice for 15 min and subsequently centrifuged (13000 rpm, 15 min) to pellet cell debris and genomic DNA. Supernatants were transferred into a fresh centrifuge tube, and plasmid DNA was precipitated by the addition of 500 µl of ice cold EtOH abs. and centrifugation (13000 rpm, 10 min). Plasmids were washed with 70% EtOH and centrifuged again (as above). Supernatants were discarded, pellets air dried and suspended in 50 µl H$_2$O. DNA concentration was determined photometrical (ND-1000, NanoDrop Technologies). The integrity of the constructs was confirmed by DNA sequencing. Amplified products of the pGBT9 plasmid inserts (PCR (see chapter 2.1.2.4); primer: fwd 5´-AAAAGTTTGCCGCTTTGCTA-3`; rev 5´-CCGGTAGAGGTG TGGTCAAT-3`) were cleaned by the HighPrep™ PCR kit (Magbio) and sequence reaction performed with BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Inc.) according to the manufacturer instructions and the use of the Microlab STARlet liquid handler (Hamilton). Sanger sequencing was performed on a capillary sequencer (Applied Biosystems 3500xL Genetic Analyzer, Life Technologies, Inc.).

2.2.2.3 Chemical transformation of yeast cells

50 µl of an AH109 yeast glycerol stock were transferred in 10 ml YPDA medium and incubated overnight at 30°C and 180 rpm until the OD$_{600}$ reaches 0.15 - 0.3. The culture was diluted in YPDA medium (1:10) and grown until the OD$_{600}$ reaches 0.4 - 0.5. Cells were centrifuged for 4 min at 1500 rpm and suspended in 20 ml H$_2$O. After another centrifugation step (see above), the supernatant was discarded and the cell pellet suspended in 1 ml 1x TE/1x LiAc. After 10 min at RT the following approach was prepared (table 3) for each co-transformation of plasmids sets and the co-transformation of control plasmids with known interaction. After incubation at 30°C for 30 min and inversion every 7 min, 70 µl DMSO were added and incubated at 42°C for 7 min. The samples were put on ice for 3 min and subsequently centrifuged for 5 min at 6000 rpm. The supernatant was discarded, transformed yeast cells solved in 100 µl TE and selected on SD minimal medium deficient in Tryptophan (-Trp) and/or Leucine (-Leu). The yeast grew for 3 to 5 days at 30°C.
### Table 3: Sample composition for the transformation of yeast

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>yeast</td>
<td>100 µl</td>
</tr>
<tr>
<td>carrier DNA (2 mg/ml salmon sperm DNA)</td>
<td>10 µl</td>
</tr>
<tr>
<td>1xTE/1x LiAc/PEG</td>
<td>600 µl</td>
</tr>
<tr>
<td>plasmid DNA</td>
<td>1 µg of each plasmid per transformation</td>
</tr>
</tbody>
</table>

#### 2.2.2.4 Auto activity test of baits and prey

Sets of bait and prey constructs were co-transformed into the yeast strain AH109. In order to test auto activity of the bait constructs, they were co-transformed with the empty prey vector (pGADT7). In order to exclude auto activity of the interaction partner candidates (prey) that emerged from the screen (see chapter 2.2.2.5), they were co-transformed with the empty bait vector as well as non-auto active independent bait (OCRL1). The potential interaction of the prey and the utilized bait in the screen shall be independently reproducible by co-transformation of the bait with its respective prey.

Co-transformations were selected on SD-Leu/-Trp and at least ten clones transferred in 100 µl PBS and thereof 5 µl on further SD selection media with expectations listed below (table 4). Note 3-AT (3-AT- 3-Amino-1,2,4-triazole) inhibits HIS3 reporter gene products, since the HIS3 reporter can be leaky that in turn could result in transcription without a specific interaction. Co-transformation of pGBK7-53 + pGADT7-T served as positive control.
Table 4: Expectations of growth of transformation sets on different selection media (+ = should grow; - = should not grow). SD – synthetic defined medium, Leu – leucine, Trp – tryptophan, His – histidine, Ade – adenine, 3-AT- 3-Amino-1,2,4-triazole

<table>
<thead>
<tr>
<th>Transformation sets</th>
<th>SD-Leu/-Trp</th>
<th>SD-Leu/-Trp/-His</th>
<th>SD-Leu/-Trp/-His/3-AT</th>
<th>SD-Leu/-Trp/-His/-Ade</th>
<th>SD-Leu/-Trp/-His/-Ade/+X-α-gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCDC66 bait + prey (if interacting)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+ turn blue</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGBK17-53 + pGADT7-T</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+ turn blue</td>
</tr>
<tr>
<td>Negative controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bait: CCDC66 construct + empty prey plasmid</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Prey: Prey + empty bait plasmid</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Prey + non-auto active independent bait</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2.2.2.5 Yeast two-hybrid screen

Per screen 50 ml of the non-auto active bait construct housing yeast (one CCDC66-1 to CCDC66-5) were incubated overnight at 30°C (150 rpm) in SD-Trp medium until the OD reached ~0.5. The culture was diluted up to 100 ml with SD-Trp and let grown for 3 - 4 hours until the OD reached ~1. Cells were centrifuged (4 min, 1500 rpm), suspended in 20 ml H2O, pelleted again (4 min, 1500 rpm) and incorporated in 5 ml SD-Trp. 600 µl of the library housing yeast was added and 45 ml YPDA medium. Mating of the cells was allowed overnight while slowly shaking (100 rpm) at 30°C. An OD of ~ 2 as well as the appearance of characteristic zygoites indicated the time point to plate the yeast on SD-Leu/-Trp/-His/-Ade plates (300 µl
per Ø 14 cm Petri dish). Positive colonies were transferred on SD -Leu/-Trp/-His/-Ade plates containing X-α-gal to prove expression of α-galactosidase by blue-white selection. The screens with CCDC66-1 and -5 as well as CCDC66-2 and -4 were performed in parallel.

2.2.2.6 Confirmation of positive interaction

Yeast colony PCR was performed with the Kappa2G robust PCR kit (Peqlab) according to the manual, by the use of buffer KappaB and the following primers: fwd: 5′-GTAAAACGACGGCCAGTCTATCTATTCGATGATGAAG-3′, rev: 5′-CAGGAAACAGCTATGACCACGATGCACAGTTGAAGTGAA-3′. Amplicons were sequenced and identified by alignment (UCSC Genome Browser, BLAT). In case the represented candidate sequence is in frame with the ATG of the pGADT7 vector, plasmids are isolated from the yeast, segregated by transformation into 

E.coli

and retransformed in order to exclude auto activity of the prey and to reproduce the interaction by co-transformation of bait and prey (see 2.2.2.4).

In order to isolate plasmid DNA from yeast, a single colony was inoculated in 1.5 ml selection medium (SD -Leu/-Trp/-His/-Ade) and grown for two days at 30°C (150 rpm). Cells were collected by centrifugation (1 min, 13000 rpm), the pellets suspended in 200 µl yeast lysis buffer and 200 µl phenol/chloroform/isoamyl alcohol and 10 glass balls (Ø 0.5 mm) were added. The suspension was shaken for two minutes and subsequently centrifuged again (5 min, 15000 rpm). The supernatant was transferred into a fresh centrifuge tube and the plasmid DNA precipitated by the addition of NaOAc (1x volume, 3 M, pH 5.2) and EtOH (2.5x volume) and centrifugation (10 min, 13000 rpm). The pellet was washed in 70% EtOH and centrifugation repeated. Plasmid DNA was suspended in 1x TE buffer and, in order to separate the plasmids, subsequently transformed into 

E.coli

and isolated again. The presence of the pGADT7 library plasmid was controlled by PCR (2.2.2.2). Required buffers and solutions are listed in the appendix (chapter 2.2).
2.2.3 Results

2.2.3.1 Auto activity tests of the baits

In order to screen for interaction partners, fragments of CCDC66 were expressed in fusion to the GAL4 DNA binding domain. A prerequisite for using constructs in the YTH screen, they were tested for auto activity in order to rule out that the transcription of the reporter genes is not activated in absence of a specific interaction partner. Five different CCDC66 bait constructs were generated (CCDC66-1 to -5). CCDC66-1 and -5 encompass N- and C-terminus of the protein. CCDC66-2 includes two coiled coil domains, CCDC66-3 the third one. Construct CCDC66-4 contains no reported domain. All of the constructs were tested for auto activity. The results for the auto activity test of CCDC66-3 are exemplarily shown in figure 11. Co-transformation of CCDC66-3 and the empty prey vector results in growth on SD -Leu/-Trp as mediated by the reporter gene activity of two plasmids. In contrast, these clones were unable to grow without supplemental His (SD -His; SD -His + 3-AT) and no colony appears on SD -Leu/-Trp/-His/-Ade + X-α-gal. Co-transformation of the control plasmids (pGADT7-T and pGBK7-53) served as positive control and enables the yeast to grow on all selection plates as expected and to turn blue on the SD -Leu/-Trp/-His/-Ade + X-α-gal. All CCDC66 constructs do not display auto-active growth and are suitable for the screen against the retinal library.
**Figure 11:** Auto activity test of CCDC66-3. Co-transformation of the bait construct with the empty prey plasmid (pGADT7) in AH109 results in growth on SD-Leu/-Trp plates, whereas no colonies appear on SD-Leu/-Trp/-His, SD-Leu/-Trp/-His + 3-AT and SD-Leu/-Trp/-His/-Ade + X-α-gal selection media. Yeast that were co-transformed with the positive control plasmids (pGADT7-T and pGBKT7-53 with reported interaction of translated products) are able to grow on all plates. SD — synthetically defined, Leu — leucine, Trp — tryptophan, His — histidine, Ade — adenine, 3-AT — 3-Amino-1,2,4-triazole.

2.2.3.2 Interaction partner candidates

The yeast two-hybrid screen with the five CCDC66 constructs (CCDC66-1 to 5) against the retinal cDNA library as prey revealed several putative interaction partners of CCDC66 in terms of positive clones on SD-Leu/-Trp/-His/-Ade selection plates. After colony transfer on SD-Leu/-Trp/-His/-Ade + X-α-gal plates following blue-white selection, persistent positive blue clones were identified by PCR, sequencing and alignment of the sequences (UCSC BLAT). The YTH screen with CCDC66-1, the construct of the N-terminal part of CCDC66, reveals 85 colonies in total, of which 81 were positive as shown by X-α-gal expression. Thereby, two candidates contribute to the total number of colonies for CCDC66-1, Protein C21orf2 homologue (abbreviated here C21orf2; 25 colonies, 4 x white, 21 x blue) and Zinc finger HIT domain-containing protein 3 (ZnHit3; 49 colonies, all blue). Construct CCDC66-2 with the two potential protein interacting coiled coil domains revealed 10 positive colonies in the screen, including three persistent positive (blue) colonies. The Pancreatic progenitor...
cell differentiation and proliferation factor (Ppdpf) is the potential interaction partner that could be identified, while the other two colonies harbor an intronic sequence or undefined sequence (several hits in UCSC BLAT). The possible interaction candidates of CCDC66-3 encompass the Epidermal growth factor receptor kinase substrate 8 (Eps8; found 3 x), Multiple PDZ domain protein 1 (Mpdz; found 1 x), Mediator of RNA polymerase II transcription subunit 4 (Med4; found 1 x) and again ZnHit3 (found 1 x). Another positive blue colony contained non-coding DNA sequences, while two others failed to be sequenced/identified. In addition, 20 white colonies were present. No potential interaction partner could be identified by the YTH screen with CCDC66-4, a construct without any reported domain. By CCDC66-5, the C-terminal construct, the same potential candidates as with CCDC66-1 are presented, C21orf2 (3 x white, 8 x in blue colonies) and ZnHit3 (1 x white, 5 x blue). Interaction partner candidates are summarized in the table 5.
**Table 5:** Positive clones on SD –Leu/-Trp/-His/-Ade (SD –Leu/-Trp/-His/-Ade + X-α-gal) and the identities of the prey

<table>
<thead>
<tr>
<th>Construct</th>
<th>Total colonies</th>
<th>Positive colony sequence identities (UCSC BLAT, sequence lengths between ~400-600 bases)</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>white</td>
<td>blue</td>
<td></td>
</tr>
<tr>
<td>CCDC66-1</td>
<td>4</td>
<td>81</td>
<td>25 x C21orf2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>49 x ZnHit3</td>
</tr>
<tr>
<td>CCDC66-2</td>
<td>7</td>
<td>3</td>
<td>intronic sequence (signal-induced proliferation-associated 1 like 2 (Sipa1l2), mRNA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>intronic sequence undefined</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pdpf</td>
</tr>
<tr>
<td>CCDC66-3</td>
<td>20</td>
<td>9</td>
<td>3x Eps8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mpdz</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Med4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ZnHit</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>sequencing failed</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>non-coding DNA</td>
</tr>
<tr>
<td>CCDC66-4</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CCDC66-5</td>
<td>9</td>
<td>18</td>
<td>11 x C21orf2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6 x ZnHit3</td>
</tr>
</tbody>
</table>

2.2.3.3 Verification of the candidates open reading frame (ORF)

The sequence referring to a potential interaction partner was controlled to be in frame with the ATG for *in vitro* translation of pGADT7 in order to ensure that the inserted sequence corresponds to the respective candidate’s protein in fusion to the GAL4 AD when translated. Interaction partner candidates and the range of their represented amino acids in respective prey are presented in figure 12. The candidates that are shown to be in an ORF with the *in vitro* ATG (GAL4 AD) encompass C21orf2, ZnHit3, Mpdz and Eps8. The latter are further analyzed and validated by the auto activity test and the validation of the positive interaction. Remaining CCDC66 interaction partner candidates (Pdpdf and Med4) showed no ORF in frame to the pGADT7 ATG (not shown) and were not further characterized for the present.
Figure 12: Amino acid sequences of the prey proteins that showed positive interaction in the yeast two-hybrid screen with CCDC66 constructs. Interaction partner candidates including open reading frames in frame with the ATG for \textit{in vitro} translation of pGADT7 are C21orf2, ZnHit3, Eps8, Mpdz, while Ppdpf and Med4 are not in frame with the DNA-binding domain (not shown). The last presented cytosine (C) in the plasmid sequence is replaced by guanine (G) in the construct sequences (marked by arrows). Start and last (ensured by sequencing) represented aa of the interaction partner candidate in the respective prey is highlighted in red. Additional aa, not candidate or pGADT7-specific, are marked in grey. aa – amino acid, Mpdz (UniProt accession number: B2RQR2; ensemble: ENSMUST00000102830, ZnHit3 (UniProt accession number: Q9CQK1; ensemble: ENSMUST00000103195; C21orf2 (UniProt accession number Q8C6G1; ensemble 1810043G02Rik-001 ENSMUST00000105397), Eps8 (UniProt accession number Q08509-1, Eps8-001 ENSMUST0000058210). The plasmid reference sequence of pGADT7 is available at Clontech (https://www.clontech.com/).
2.2.3.4 Auto activity test of the prey and validation of their positive interaction with CCDC66 constructs

The validation of the candidates is presented in the following from the N- to the C-terminus of respective CCDC66 bait constructs. The auto activity test of the CCDC66 interaction partner candidates C21orf2 and ZnHit3 (figure 13) shows that none of them is auto active as shown by co-transformation with OCRL1, reflected by no growth on respective selection media that require reporter gene expression. But neither the interaction with CCDC66-1 nor -5 and _vice versa_ could be reproduced by co-transformation. Growth of all yeast colonies on SD-Leu/-Trp/-His likely reflects the above mentioned non-specificity of the _HIS3_ reporter gene (2.2.2.4). The re-transformation of construct CCDC66-3 and ZnHit3 still needs to be further investigated. The retransformation of the interaction partner candidates Mpdz and Eps8 only revealed growth on the selection media when co-transformed with CCDC66-3, but neither with the empty bait plasmid (pGBT9) nor with the independent prey OCRL1 (figure 14). Hence, this interaction could be confirmed by growth of the yeast that is depending on the specific combination of co-transformed CCDC66 bait construct and prey candidates.
Figure 13: Auto activity test of prey C21orf2 and ZnHit3 and retransformation with CCDC66-1 and -5. Transformation of the prey C21orf2 or ZnHit3 resulted in no growth neither when co-transformation with the independent bait OCRL1 nor with the CCDC66 bait constructs CCDC66-1 or -5. Yeast co-transformed pGADT7-T and pGBK7-53 served as positive control and grows on all plates and turned blue (dark grey) in the presence of X-α-gal. SD – synthetically defined, Leu - leucine, Trp – tryptophan, His – histidine, Ade – adenine, 3-AT - 3-Amino-1,2,4-triazole.
Figure 14: Auto activity test of prey Mpdz and Eps8 and proof of positive interaction with CCDC66-3. Co-transformation of the bait CCDC66-3 in combination with the prey Mpdz and Eps8 resulted in growth on SD -Leu/-Trp/-His, SD -Leu/-Trp/-His + 3-AT and blue colonies on SD -Leu/-Trp/-His/-Ade + X-α-gal selection media. Co-transformation of Mpdz and Eps8 with the empty bait plasmid pGBT9 and the independent bait OCRL1 served as control to exclude auto activity of the prey and resulted in no growth. Co-transformation of the positive control plasmids (pGADT7-T and pGBKT7-53) revealed colonies on all plates and turned blue in the presence of X-α-gal. SD – synthetically defined, Leu – leucine, Trp – tryptophan, His – histidine, Ade – adenine, 3-AT - 3-Amino-1,2,4-triazole.

2.2.4 Discussion

The function of an unknown protein might be indirectly explored by its association with other proteins of known function. The identification of these interaction partners, if they are familiar, allows hypothesizing about the function of the unknown target protein, by integrating its potential role in the framework of information its interaction partners have offered. A YTH screen with defined parts of protein CCDC66 as bait revealed several putative interaction partners encompassing C21orf2, Zinc finger HIT domain-containing protein 3 (ZnHit3), Pancreatic progenitor cell differentiation and proliferation factor (Pdpdf), mediator complex subunit 4 (Med4), Multiple PDZ domain protein 1 (Mpdz) and Epidermal growth factor receptor kinase substrate 8 (Eps8) that will be discussed separately with the prime candidates (Mpdz and Eps8) first and the others following.
2.2.4.1 Mpdz and Eps8 – prime candidates to interact with CCDC66

Mpdz and Eps8 were identified as positive interaction partners of the same bait construct CCDC66-3. This part of CCDC66 contains two of the three coiled coil domains and thus likely represent a segment that enters into protein-protein interactions (Lupas 1996). Mpdz is a large scaffold protein that contains 13 PDZ domains but no catalytic motif. The identified sequence of Mpdz in the YTH screen encompasses the PDZ domain 9 (according to the UniProt database). Mpdz was initially identified by its interaction with the C-terminus of the serotonin receptor 5HT$_{2C}$ (Ullmer et al. 1998). With respect to this interaction Mpdz is supposed to be associated with the assembly and targeting of signaling complexes (Becamel et al. 2001). Besides the 5HT$_{2C}$ receptor, Mpdz is associated with the modulation of several other receptor pathways by stabilization, signaling and targeting (Balasubramanian et al. 2007; Rama et al. 2008; Krapivinsky et al. 2004; Sindic et al. 2009). Furthermore, Mpdz has several interactions with proteins and protein complexes for example at neuronal gap junctions with Afadin and Connexin-36 (Becamel et al. 2001), a variety of adhesion proteins and claudin proteins at autotypic tight junctions in Schwann cells (Poliak et al. 2002) and in polarized epithelial cell tight junctions (Liew et al. 2009; Jeansonne et al. 2003; Hamazaki et al. 2002). By its interactions with Afadin via NG2 for example, Mpdz is associated with the RhoGTPase signaling cascades (Binamé et al. 2013; Kuriyama et al. 1996; Boettner et al. 2000). The RhoGTPases are important molecular switches in the regulation and up setting of polarity and mediate morphogenesis, migration, cell division and morphological neuroplasticity by the generation of protrusions (Zegers & Friedl 2014; Auer et al. 2011; Collins & Tzima 2014).

In general Mpdz is a multivalent scaffold protein that is present in diverse brain regions, including hippocampus (pyramidal neurons, granule layer of the dentate gyrus), choroid plexus, olfactory bulb (olfactory sensory neurons (Dooley et al. 2009) and many more (Becamel et al. 2001; Sitek et al. 2003). Furthermore it is expressed extra-neural in kidney (Sindic et al. 2009) and spermatozoa (Heydecke et al. 2006).

In the human and mouse retina Mpdz is associated with the formation and regulation of tight junctions and localizes to the sub apical region, the basal part of the IS (van de Pavert et al. 2004; Hamazaki et al. 2002) in a complex with adherence and trans-membrane molecules Crb1, Pals1 and Mpp4 (van de Pavert et al. 2004; Roh &
Margolis 2003; Roh et al. 2002). It was associated with retinal degeneration and dysplasia in the chicken and cases of human RP and LCA (Ali et al. 2011). The presence of Mpdz in the region of the IS of the photoreceptors overlaps with the detection of Ccdc66 reporter gene expression (2.1.3) and an interaction would spatially be possible.

The role of this potential interaction is not clear, but the hypothesis of the contribution of protein CCDC66 to outer segment formation and maintenance as well as the careful speculation of being involved in dendrite or synapse formation (with respect to the plexiform layers 2.1.3.3) a scaffolding support of CCDC66 by Mpdz as well as a signal cascade initiation via Mpdz that leads to differentiation could be possible. Beside the fact that the interaction has to be additionally confirmed, further experiments are required to investigate the possibilities of mechanism in CCDC66 and Mpdz interaction (see below).

Eps8 was identified in the YTH screen with presented aa at least representing half of the PH (pleckstrin homology; Lemmon 2007) domain of Eps8 (PH domain aa: 69 - 129, representation in the screen: at least aa 97 – 285). The PH domain was initially defined by its capability to bind phosphoinositides even though ~90% of these domains do not (Lemmon 2007). Eps8, as the name implies, is involved in the down-stream pathway of the epidermal growth factor receptor (EGFR; Fazioli et al. 1993). The EGFR is auto phosphorylated in consequence of ligand binding and subsequently activates signal transduction pathways that are involved in regulating cellular proliferation, differentiation and survival (EGFR function reviewed in Herbst 2004). Eps8 is reported to regulate the internalization of EGFR (Lanzetti et al. 2000) and affects the down-stream pathway of this receptor by activating the Rac pathway together with the eps8 SH3 domain binding protein 1 (E3b1 = abelson interactor (Abi-1)) Eps8 (Scita et al. 1999; Scita et al. 2001). Rac is one of the three best characterized RhoGTPases that, as mentioned above, control mediations of a variety of signals in response to the activation of receptors (Etienne-Manneville & Hall 2002). E3b1 is located at sites of actin cytoskeleton remodeling dynamics at epithelial cell-cell junctions and dendritic spines (Biesova et al. 1997; Grove et al. 2004). It is involved of the Rac-based actin-polymerization machinery (Scita et al. 2001). Beside this association of Eps8 with this actin remodeling pathways, Eps8 is proposed to be directly involved in actin dynamics with bundling and capping
properties, a characteristic that in general promotes the hypothesis of regulating dynamics and architectures of different cellular protrusions. For example in dendritic spines Eps8 impairs spine enlargement by its capping activities that in turn is regulated by brain-derived neurotrophic factor (BDNF) that inhibits Eps8 actin capping and leads to spine formation (Hertzog et al. 2010; Disanza et al. 2004; Disanza et al. 2006; Menna et al. 2009). Eps8 is involved in stereo cilia growth and hair bundle length (rather than maintenance) of the inner and outer hair cells of the acoustic system and is localized to the tips of the hair cells in a complex with myosin XVa and whirlin (Olt et al. 2014; Manor et al. 2011; Zampini et al. 2011). An Eps8L2-deficient (Eps8 like protein) mouse model has been associated with progressive hearing loss (Furness et al. 2013) and a biallelic nonsense mutation c.88C > T (p.Gln30*) of Eps8 was shown to be the pathological mutation responsible for autosomal recessive profound deafness in two siblings born to consanguineous Algerian parents (Behlouli et al. 2014). In the course of that work, Eps8 was localized within the macaque retina to a small extent in the IS of rod photoreceptors, the inner and outer plexiform layer co-localized with the presynaptic protein synaptophysin. In case these results concerning the retinal expression are transferable to the mouse (IHC on the mouse retina should be investigated in the future), an overlap of expression with CCDC66 is likely feasible. The proposed interaction of Eps8 further supports the participation of CCDC66 in dynamical processes (OS built and preservation, process formation etc.), but comparable to Mpdz, additional experiments are required to validate at first the interaction of Eps8 and to determine the mechanism. Therefore, immunoprecipitation experiments should be performed. The potential interaction partner constructs are linked to a hemagglutinin (HA)-tag which allows the identification of a true physical protein interaction also without any nominal antibody against protein CCDC66.

In conclusion, both interaction partners are involved in cytoskeleton dynamics or scaffolding processes and act in a broad variety of functions. They share the association with predominantly polarized cells and structures of challenging cytoskeleton demands (tip links of the hair cells or tight junctions). Furthermore, they are linked to RhoGTPases pathways that are crucial for cell migration, differentiation and protrusion outgrowth (Auer et al. 2011). By the identification of these potential CCDC66 interaction partners as well as by the apparent role of protein CCDC66 in
the formation and probably maintenance of constantly renewed OS it is tempting to speculate on a role of protein CCDC66 in structural dynamics. This hypothesis is supported by the parallel Ccdc66 reporter gene expression to formation of synapses/protrusions/dendrites in the plexiform layers of the retina.

2.2.4.2 ZnHit3, C21orf2, Ppdpf and Med4 – false positive interaction partners?

Construct CCDC66-1, the N-terminus of CCDC66 that, according to the databases entries, harbors no functional domains (2.2.2.2), revealed the candidates ZnHit3 and C21orf2 as interaction partners in the screen. Both were further identified with CCDC66-5 as bait whereby the screen was performed in parallel with the screen with CCDC66-1. ZnHit3 was additionally obtained by the screen with the CCDC66-3 bait construct that contains two of the three reported coiled-coil domains.

ZnHit3 (Thyroid hormone receptor interactor 3) is suggested by sequence similarities to specifically interact with the thyroid receptor and requires the presence of the thyroid hormone for its interaction (according to UniProt database entries). The receptor in turn acts as transcription factor (Forrest & Vennström 2000) in a variety of neural tissues and is involved in migration, proliferation and differentiation (Thompson & Potter 2000; Forrest et al. 1990; Forrest et al. 1991). Within the retina thyroid hormone receptors are associated with guidance of retinal progenitors, proliferation, ganglion cell genesis and photoreceptor maturation (Ng et al. 2001; Ma et al. 2014; Roberts et al. 2006; Sevilla-Romero et al. 2002). In the chicken retina three thyroid hormone receptors were identified that vary in their distribution during development (Sjöberg et al. 1992). While TRα is described to be expressed in all retinal layers during neurogenesis and TRβ2 is restricted to the ONL and promotes photoreceptor development, TRβ0 is present in the inner and nuclear layer (Sjöberg et al. 1992). A general overlap of CCDC66 and ZnHit3 is thus possible, but has to be investigated in detail, if the outstanding re-transformation of CCDC66-3 and ZnHit3 reveals positive results (see below).

C21orf2 is supposed to be involved in cytoskeleton organization in the context of cilia formation and preservation and plays a role in the regulation of cell shape and morphology (Bai et al. 2011). It was identified as a candidate gene in the pathogenesis in two individuals with a simplex case of cone-rod dystrophy (Abu-Safieh et al. 2013), whereas its distribution in the retina is currently not documented.
in the literature. Nevertheless, C21orf2 as well as ZnHit3 were identified as potential interaction partner candidates in the present screen and proven false by the validation experiment. Their co-transformation with the CCDC66-bait constructs, they were identified with, did not reflect a confirmed interaction. This is the case for CCDC66-1 and CCDC66-5, but still has to be performed for CCDC66-3. However, that ZnHit3 already appeared as a false positive interaction partner of two CCDC66 constructs, curtails the expectation of ZnHit3 being a positive interaction partner for the third. In addition, what already diminished the probability of the positive interaction of these candidates prior to the control experiment was that these candidates were also isolated from white colonies (after the blue white selection), suggesting that their presence is not automatically associated with a positive interaction. The high number of colonies harboring these two candidates might be due to an enrichment of these cDNAs within the library.

Another possibility for the preliminarily positive interaction evaluation by blue staining and later failed growth is that the yeast identified as positive in the screen yield more than one plasmid, so that the real interacting protein that is responsible for the detected positive interaction might have been missed. But in this case, because in total 36 times C21orf2 and 55 times ZnHit3 were identified by sequencing and it is rather unlikely that over 80 true interaction partners “hide” behind always the same two actually identified clones.

Ppdpf is involved in development and differentiation (UniProt) and was revealed as a potential interaction partner of construct CCDC66-2. There is hardly any literature available on the proteins function. A dissertation appeared on the characterization of CEP164 und Ppdpf in the mitotic context (Anderhub 2013). Ppdpf is involved in correct segregation of chromosomes during mitosis, localizing to mitotic spindles. Furthermore it was demonstrated to interact with acetylated tubulin and kinesin Eg5. Apart from these findings, the association has been reported of the zebrafish homologue protein Exdpf in proliferation and differentiation of exocrine precursors (Jiang et al. 2008). The EMBL-EBI expression atlas reports expression in a variety of tissues including colon, heart, kidney, liver, lung, skeletal muscle, spleen, testis, thymus and brain. ISH labeling of the mouse brain are available at the Allen Institute for Brain Science and state expression in the isocortex, olfactory areas, cortical subplate and the cerebellum. Ppdpf expression in the mouse retina is present as
evidenced by array results deposited at the Gene Expression Omnibus (GEO) database. The prey Ppdpf was not treated as a prime candidate for true positive interaction with its respective bait CCDC66-2, because the open reading frame reflecting its amino acid sequence has not been in frame with the DNA binding domain of the GAL4 transcription factor. Nevertheless, it should be also tested in the future, since the possibility of translational frameshift remains (Ketteler 2012) that sometimes allow correct translation even in the presence of frameshift mutations. In case re-transformation would show a reproduced positive interaction of Ppdpf and CCDC66-2, the respective Ppdpf sequence should be cloned in frame and prove the interaction again.

The same is the case for Med4 that is part of the mediator complex that links gene specific-transcription factors and the polymerase II system (Napoli et al. 2012). Med4 has recently been reported as modifier in patients with retinoblastoma resulting in low penetrance (Dehainault et al. 2014), but it has not been reported otherwise in the retinal context. In general, it should be considered that the possibility of false positives is higher among candidate proteins that directly impact on DNA binding (Ppdpf) or transcription (Med4).

The fact that the construct CCDC66-4 revealed no interaction partner candidates might be due to the fact that there is no interaction domain within this construct, or a non-reported domain that might be disrupted by the freely selected borders of the CCDC66 constructs. CCDC66-4 could for example be rearranged by integrating more sequence regions up- and/or downstream.
Chapter 3 - Functional investigation of Ccdc66 gene products in the mouse brain

3.1 Ccdc66 RNA and reporter gene expression in the mouse brain

3.1.1 Introduction

Extra retinal Ccdc66 expression, with increased incidence in several brain areas in comparison to extra-cerebral organs (see chapter 5), was initially demonstrated by Western blot analyses (Schreiber 2010). IHC was performed on mouse brain slices with the antibody initially used for the retinal investigation (anti-CCDC66, #G14 sc-102418, Santa Cruz Biotechnology Inc. (Gerding et al. 2011)), but too much background prohibited the distinction between specific and non-specific signals, also in comparison to the Ccdc66−/− mouse.

In order to specifically identify Ccdc66 expression sites in the brain Ccdc66 RNA detection by ISH and the assessment of β-galactosidase activity in the Ccdc66−/− mouse was performed on Ccdc66+/− and Ccdc66−/− mouse brain slices. Furthermore, in order to capture possible development-related dynamics of Ccdc66 expression, β-galactosidase activity was monitored during the postnatal development of the Ccdc66−/− mouse brain. Thereby the following questions shall be answered and discussed in this chapter:

1) Where are the precise localizations of Ccdc66 expression within the brain?
2) Does the detection of Ccdc66 expression in turn hint at the general function of the respective protein CCDC66?
3) Does Ccdc66 reporter gene expression in the brain parallelize with postnatal development processes that allow drawing conclusions on the function of protein CCDC66?

3.1.2 Materials and methods

3.1.2.1 Animals, tissues and preparation of cryosections

The mouse brains for the ISH and Ccdc66 reporter gene expression analysis were dissected from the animals for the retinal analyses (three mice of each genotype and age) and treated in the same manner (see 2.1.2.1 - 2.1.2.3).
3.1.2.2 *In situ* hybridization

ISH was performed as described in 2.1.2.4 with the probe for *Ccdc66* RNA detection in the retina. Positive antisense control probes for the different brain regions (olfactory bulb, hippocampus and cerebellum) were selected from the Allen Institute for Brain Science gene expression (ISH) atlas (available at http://www.brain-map.org/) with the prerequisite that respective genes are solely expressed in a defined region in the area of interest. Primers for the generation of antisense and sense probes are listed below (table 6). All probes were used with a final concentration of 300 ng/ml hybridization mix.

**Table 6: Positive control probes for *in situ* hybridization on brain slices**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Primer sequence (5'-3'); <strong>SP6</strong> (fwd) / <strong>T7</strong> (rev) promoter</th>
<th>Probe length [bases]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Thyrotropin releasing hormone (Trh)</strong></td>
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<td></td>
</tr>
<tr>
<td>Fwd primer</td>
<td>GATTTAGGTGACACTATAGAAGACCTCCAGCGTGTC</td>
<td>996</td>
</tr>
<tr>
<td>Rev Primer</td>
<td>TAATACGACTCATATAGGGTTGGCCATATGGGGCAGAT</td>
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</tr>
<tr>
<td><strong>Protein kinase C, delta (Prkcd)</strong></td>
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<tr>
<td>Fwd primer</td>
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<tr>
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</tr>
<tr>
<td><strong>Glutamate receptor, ionotropic, delta 2 (grid2) interacting protein (Grid2ip)</strong></td>
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<td></td>
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<tr>
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<tr>
<td>Rev Primer</td>
<td>TAATACGACTCATATAGGGAGGAAGTTGATCTTAAGGGCCAG</td>
<td></td>
</tr>
</tbody>
</table>

3.1.2.3 X-gal staining

X-gal staining was performed as described in 2.1.2.5.

3.1.3 Results

3.1.3.1 *Ccdc66* RNA expression in the postnatal *Ccdc66*+/+ and *Ccdc66*−/− mouse brain

In order to localize *Ccdc66* RNA expression sites in the mouse brain, ISH to *Ccdc66* RNA was performed on P17 *Ccdc66*+/+ and *Ccdc66*−/− mouse brains. Thereby the focus was directed to the broadly categorized regions olfactory bulb, hippocampus
and cerebellum that ought to present Ccdc66 expression sites, as indicated on the protein level by Western blot analyses (Schreiber 2010).

**Olfactory bulb**

In the Ccdc66+/+ mouse olfactory bulb Ccdc66 RNA is presumably localized to the granule, mitral and glomerular layer as well as inner and external plexiform layers as displayed by prominent dark spots (figure 15 A, white arrowheads). Furthermore, scattered, faint staining is present in the olfactory nerve fiber layer (figure 15 A, white arrowhead). In the age-matched Ccdc66−/− mouse olfactory bulb these regions appear as well (figure 15 B, white arrowheads). No obvious difference between both genotypes was observed. In general, a high level of Ccdc66 antisense probe hybridization is visible on slices of both genotypes, a fact that impedes a clear evaluation of Ccdc66 positive cells and in addition an explicit difference between Ccdc66+/+ and Ccdc66−/− sections. Application of the Thyrotropin releasing hormone (Trh) antisense probe served as positive control and highlights distinctly, in both Ccdc66+/+ and Ccdc66−/−, the glomerular layer (figure 15 C, D, black arrowheads) as reported from the Allen Institute (experiment 71016631, Trh-RP_050725_03_A12, specimen 05-2304) and slightly the mitral and granule layer as well. Respective sense probe hybridization (Ccdc66 and Trh) serves as negative control and displays no or faint staining on slices of both genotypes (figure 15 E - H).
Figure 15: Expression of *Ccdc66* in the olfactory bulb of *Ccdc66*+/+ and *Ccdc66*−/− mice by *in situ* hybridization (P17). In the *Ccdc66*+/+ mouse olfactory bulb *Ccdc66* RNA was localized to the GrL, ML, GL, IPL, EPL and OlfNL as evidenced by purple color precipitates (A, white arrowheads). In the *Ccdc66*−/− mouse the staining pattern is comparable (B, white arrowheads). Detection of *Trh* RNA in the GL of *Ccdc66*+/+ and *Ccdc66*−/− olfactory bulb sections serves as positive control (C and D, black arrowheads). *Ccdc66* and *Trh* sense probes were applied as negative controls and reveal no or faint staining (E - H). GrL - granule layer, ML - mitral layer, GL - glomerular layer, IPL – inner plexiform layer, EPL – external plexiform layer, OlfNL olfactory nerve layer, *Trh* - *Thyrotropin releasing* hormone. P – postnatal day, 12 µm frontal cryosections, scale bar - 100 µm.

**Hippocampus**

In the hippocampus of the *Ccdc66*+/+ and *Ccdc66*−/− mouse, *Ccdc66* RNA expression is apparently present in a broad variety of structures (figure 16 A and I). These encompass the pyramidal layer of area CA1 to CA3 (figure 16 B and C, J and K), the granule cell layer of the dentate gyrus (figure 16 D and L) as well as dispersed puncta in the molecular layer neighboring the CA regions (*stratum radiatum*) (figure 16 B and C, J and K) and the dentate gyrus (inner molecular and polymorph layer; figure 16 D and L).
Figure 16: *Ccdc66* in situ hybridization on hippocampus sections of the *Ccdc66*+/+ and *Ccdc66*-/- mouse (P17). In the *Ccdc66*+/+ and *Ccdc66*-/- mouse hippocampus *Ccdc66* expression sites detected by ISH (purple staining) include the PyL of CA1-3 (A – C, I - K) and the GrCL of the DG (A, D, I, L). Scattered labeled sites further appear in the stratum radiatum (SR) of CA1-3 (A – C, I - K), the
DG PL and IML (A, D, I, L). Adjacent CTX (E, M) and TH (F, N) exhibit Ccdc66 RNA expression as well. Prkcd transcript detection in the TH and CA3 conducted as positive control (H and P). The Ccdc66 sense probe ISH serves as negative control and results in no labeling of Ccdc66+/+ or Ccdc66−/− sections (G and O). PyL - pyramidal layer, GrCL - granule cell layer, DG - dentate gyrus, PL – polymorph layer, IML - inner molecular layer, CTX – cortex, TH – thalamus, Prkcd - Protein kinase C delta, P - postnatal day. 12 µm frontal cryosections, scale bar in A, I, G, H, O, P - 500 µm, B, C, D, J, K, L - 100 µm, E, F, M, N - 50 µm.

Moreover, adjacent thalamic and cortical areas show Ccdc66 antisense probe hybridization on hippocampus slices of both genotypes (figure 16 E and F, M and N), whereas in the cortex of the Ccdc66−/− mouse the staining appears even stronger than in the Ccdc66+/+ mouse. Except of this, no obvious difference is detected between results of the hybridization to Ccdc66 RNA when comparing Ccdc66+/+ and Ccdc66−/− mouse hippocampus. Detection of protein kinase C delta (Prkcd) transcripts conducted as positive control and is specifically detected in the thalamus and CA3 (see Allen Institute for reference: experiment 70301274, Prkcd - RP_050309_02_B09, specimen 05-1525). The generation of the sense probe against Prkcd could not be optimized, and the data are not shown. However, hybridization with the Ccdc66 sense probe reveals no staining on Ccdc66+/+ or Ccdc66−/− sections and serves as an adequate negative control (figure 16 G and O).

Cerebellum

ISH to Ccdc66 RNA on slices of the cerebellum of the P17 Ccdc66+/+ mouse exposes a strong hybridization signal by distinct precipitates in the granular, pyramidal but less in the molecular layer (figure 17 A, black arrowheads). In the age-matched Ccdc66−/− mouse, Ccdc66 antisense probe hybridization reveals a less intense staining in the granular and Purkinje layer and a dispersed faint marking in the molecular layer (figure 17 B, black arrowheads). As positive control for the ISH on cerebellum slices, the detection of the glutamate receptor, ionotropic, delta 2, interacting protein 1 (Grid2ip) expression in the pyramidal layer was monitored (figure 17 C and D; for reference see Allen Institute, experiment 71670479, RP_050503_01_C04, Specimen 05-2603) in conjunction with the sense probes of investigated targets as negative controls (Ccdc66 and Grid2ip, figure 17 G and O).
Figure 17: *Ccdd66* expression in the *Ccdd66*+/+ and *Ccdd66*−/− mouse cerebellum assessed by *in situ* hybridization (P17). In the *Ccdd66*+/+ mouse cerebellar GraL and PuL and single sparse areas in the MoL exhibit intense *Ccdd66* antisense hybridization signals presented as contrasted dark staining. On slices of the *Ccdd66*−/− mouse cerebellum likewise a staining is revealed in the GraL and PuL, but less intense. Observation of *glutamate receptor, ionotropic, delta 2 interacting protein 1* (*Grid2ip*) expression in the PuL on sections of the *Ccdd66*+/+ and *Ccdd66*−/− cerebellum serves as positive control, the application of *Ccdd66* and *Grid2ip* sense probes as negative control. GraL - granular layer, PuL - Purkinje layer (dashed lines), MoL - molecular layer, P - postnatal day 17. 12 µm frontal cryosections, scale bar – 100 µm.

These results show that *Ccdd66* RNA expression seems to be present in all investigated brain regions (olfactory bulb, hippocampus and cerebellum) and within those in a broad variety of structures. These findings further confirm that *Ccdd66* expression is not limited to the retina. Moreover, in case the *Ccdd66* ISH specifically reflects *Ccdd66* RNA expression sites this would indicate a considerable level of *Ccdd66* RNA expression in the *Ccdd66*−/− mouse brain (see discussion 3.1.4).
3.1.3.2 Ccdc66 reporter gene expression in the postnatal Ccdc66\(^{-/-}\) mouse brain and comparison to Ccdc66 RNA expression

In order to prove the identified Ccdc66 expression sites by ISH in the mouse brain, respective regions of the age-matched (P17) Ccdc66\(^{-/-}\) mouse brain were monitored for the gene trap enabled β-galactosidase expression via X-gal staining, and the results were compared to Ccdc66 RNA expression (3.1.3.1).

Olfactory bulb

Reporter gene expression in the Ccdc66\(^{-/-}\) mouse reveals β-galactosidase activity in the mitral, glomerular and granule layer as well as blurred labeling of the inner and external plexiform layer (figure 18 A, black arrowheads). In addition, a strong reporter gene expression is presented in the olfactory nerve layer. The parallel treatment of the Ccdc66\(^{+/+}\) mouse with the X-gal staining solution serves as negative control and shows no labeling (figure 18 B).

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**Figure 18:** Ccdc66 reporter gene expression in the olfactory bulb of the Ccdc66\(^{+/+}\) mouse (P17). In the Ccdc66\(^{+/+}\) olfactory bulb X-gal staining results in labeling of the GL and ML and reveals, in addition to the Ccdc66 ISH, strong signals in the IPL and EPL and OlfNL (A, black arrowheads). The parallel treatment of Ccdc66\(^{+/+}\) slices revealed no blue color precipitation by X-gal staining (B, counterstained with neutral red). GL - glomerular layer, ML - mitral layer, IPL - inner and EPL - external plexiform layer, OlfNL - olfactory nerve layer, P - postnatal day. 30 µm frontal cryosections, scale bar – 100 µm.
With both, detection of *Cc*dc66 reporter gene expression by X-Gal staining in the *Cc*dc66\(^{-/-}\) (figure 19 A, black arrowheads) and *Cc*dc66 RNA labeling by ISH in the *Cc*dc66\(^{+/+}\) mouse (figure 19 B, black arrowheads), the same layers of the olfactory bulb are highlighted (granule layer, inner and external plexiform layer, mitral and glomerular layer (figure 19 A and B, black arrowheads). In addition, in the olfactory nerve layer of the *Cc*dc66-deficient mouse, a strong \(\beta\)-galactosidase activity is perceived without correspondingly vigorous mark in the ISH. In general, the intensities of the two staining procedures do not reflect RNA/reporter gene abundance, but patterns resemble each other closely.

**Figure 19:** Comparison of *Cc*dc66 reporter gene and *Cc*dc66 RNA expression in the mouse olfactory bulb (P17). Both X-gal staining on *Cc*dc66\(^{-/-}\) (A) and ISH on *Cc*dc66\(^{+/+}\) (B) label a variety of layers of the olfactory bulb (A, B, black arrowheads) yet not with equal intensity. GrL – granule layer, IPL – inner plexiform layer, ML – mitral layer, EPL – external plexiform layer, GL – glomerular layer, OlfNL – olfactory nerve layer, P – postnatal day, ISH – *in situ* hybridization. 30 µm (A) and 12 (B) frontal cryosections, scale bar - 100 µm.

**Hippocampus**

In the *Cc*dc66\(^{-/-}\) mouse hippocampus a broad variety of structures are highlighted by visualized *Cc*dc66 reporter gene expression (figure 20 A - G). A massive staining occurs in the dentate gyrus inner molecular layer (figure 20 D) and the ventricle (figure 20 E). The pyramidal layer of the regions CA1 to 3 and the dentate gyrus granule cell layer are distinctly marked (figure 20 A - D). Further staining is present in the stratum radiatum (predominantly CA1, less in CA3, figure 20 B and C) and the
polymorph layer of the dentate gyrus (figure 20 D). The hippocampus surrounding cortical and thalamic areas also disclose distinct X-gal staining (figure 20 F and G). X-gal staining treatment of the Ccdc66<sup>+/−</sup> mouse hippocampus reveals no labeling of any mentioned layer and serves as negative control (figure 20 H).

Figure 20:  

**Ccde66 reporter gene expression in the hippocampus of the Ccdc66<sup>−/−</sup> mouse (P17).** Visualization of Ccc66 reporter gene expression on hippocampal sections of the Ccdc66<sup>−/−</sup> mouse (A-G) highlights the PyL and the SR (region CA1 in B, CA3 in C), the GrCL of the DG (D) and mostly the IML of the DG (D) as well as the lateral V (E). Adjacent CTX and TH also display distinct X-gal staining reaction products (F and G). The Ccdc66<sup>−/−</sup> mouse served as negative control and no marking in any layer is detected by similar treatment to the Ccdc66<sup>−/−</sup> slices (H, counterstained with neutral red for morphological reference). PyL pyramidal layers, SR - *stratum radiatum*, DG - dentate gyrus, GrCL - granule cell layer, IML - inner molecular layer, CTX - cortex, TH - thalamus, V - ventricle, P - postnatal day. 30 µm frontal cryosections, scale bar in A, H - 500 µm, B, C, E, F, G - 100 µm, D - 200 µm.
The distribution of the reporter gene expression pattern in the Ccdc66+/− hippocampus (figure 21 A) is directly comparable to the pattern of the Ccdc66 RNA localization by ISH in the Ccdc66+/+ (figure 21 B) hippocampus - with some differences regarding the intensities. In the Ccdc66+/− dentate gyrus, a prominent difference towards the Ccdc66+/+ is the additional strong accentuation of the inner molecular layer (figure 21 A, black asterisk). A comparable signal is present in the lateral ventricle (figure 21 A, red asterisk), both are only weakly labeled by ISH in the Ccdc66+/+ (figure 21 B, black and red asterisks). Ccdc66 RNA labeling in the Ccdc66+/+ mouse hippocampus in turn, showed more reactivity in the polymorph layer of the dentate gyrus (figure 21, B, white arrow) and adjacent cortical and thalamic areas (figure 21 B, black arrows) compared to the reporter gene expression in the Ccdc66−/− hippocampus (figure 21 A, white and black arrows).

Figure 21: Comparison of Ccdc66 reporter gene and RNA expression visualized by X-gal staining and in situ hybridization in the mouse hippocampus (P17). Layers that are labeled by X-gal staining in the Ccdc66−/− (A) or via ISH in the Ccdc66+/+ (B) encompass the pyramidal layer of area CA1 (to CA3) (A and B, white arrowheads), the granule cell layer of the DG (A and B, white asterisks), and the SR of CA1 (A and B, black arrowheads) and the polymorph layer (PL; A and B, white arrows) of the DG. In contrast to the Ccdc66+/+, in the Ccdc66−/− hippocampus an intense proof of β-galactosidase activity labels the IML of the DG (A and B, white asterisk) and lateral V (A and B, red asterisks). DG – dentate gyrus, SR - stratum radiatum, V – ventricle, IML - inner molecular layer, P - postnatal day, ISH – in situ hybridization. 30 µm (A) and 12 (B) frontal cryosections, scale bar - 500 µm.
Cerebellum

In the $Ccdc66^{-/-}$ mouse cerebellum X-gal staining marks β-galactosidase activity in the Purkinje, granular and molecular layers (figure 22 A, black arrowheads). The $Ccdc66^{+/+}$ cerebellum section was treated similar and bears no staining of these layers thus representing the adequate negative control (figure 22 B).

Figure 22: $Ccdc66$ reporter gene expression in the $Ccdc66^{-/-}$ mouse cerebellum visualized by X-gal staining (P17). In the $Ccdc66^{-/-}$ cerebellum $Ccdc66$ reporter gene expression is present in the PuL, GraL and dispersed in the MoL as indicated by β-galactosidase mediated blue precipitates (black arrowheads). In the $Ccdc66^{+/+}$ mouse, no labeling was detected after same staining treatment (B, counterstained with neutral red for morphological reference). PuL - Purkinje layer (dashed lines), GraL - granular layer, MoL - molecular layer, P - postnatal day. 30 µm frontal cryosections, scale bar - 100 µm.

In comparison, the $Ccdc66$ RNA detection in the cerebellum of the $Ccdc66^{+/+}$ and the reporter gene expression on $Ccdc66^{-/-}$ cerebellar slices cover identical structures (figure 23 A and B, black arrowheads).
These results demonstrate that previously identified Ccdc66 expression sites by ISH within Ccdc66+/+ (and Ccdc66-/-) olfactory bulb, hippocampus and the cerebellum are supported by the detection of Ccdc66 reporter gene expression in respective regions of the Ccdc66-/- mouse by X-gal staining. Due to the presentation of comparable marking patterns of Ccdc66 expressing regions and in order to simplify matters of the visualization, the X-gal staining was used for the indirect investigation of Ccdc66 expression during the postnatal development of the Ccdc66-/- mouse brain (3.1.3.3). In the here presented chapter, the Ccdc66-/- P17 mouse brain exposes most remarkable X-gal staining in the olfactory nerve layer of the olfactory bulb, the inner molecular layer of the dentate gyrus as well as in the ventricle coating. Hence, these structures were further investigated in detail during postnatal development, whereas the cerebellum was less prioritized and not further explored in the framework of this study. In turn, the results of the initial assessment of Ccdc66 reporter gene expression in the P17 mouse Ccdc66-/- indirectly suggests the more detailed investigation of an additional structure - the olfactory epithelium, whose axons form the strongly labelled olfactory nerve layer in the olfactory bulb.
3.1.3.3 *Ccdc66* reporter gene expression in the postnatal developing mouse brain

In order to explore potential dynamics in *Ccdc66* expression during postnatal development, X-gal staining was performed on brains which were in addition age-matched to investigated retinal developmental stages. Here I focussed on the regions defined above (on the former page), namely olfactory epithelium and olfactory bulb, hippocampus and brain ventricle lining ependyma.

**Olfactory epithelium and olfactory bulb**

The olfactory epithelium was investigated for *Ccdc66* reporter gene expression during postnatal development of the *Ccdc66*" mouse. In order to obtain a detailed view of the olfactory epithelium it is exemplarily presented of a 10 months-old *Ccdc66" mouse (figure 24 A) in conjunction with a schematic drawing (figure 24 B). Strong labeling by X-gal staining is present in olfactory sensory neurons (figure 24 white asterisks) including their apical dendrites and terminal knobs that likewise displayed blue precipitates (figure 24 A, black and white arrows), whereas supporting cells of the epithelium showed no or only faint blue coloration (figure 24 A, black asterisks).
Figure 24: **Ccdd66 reporter gene expression in the olfactory epithelium of a ten months-old Ccdd66⁻/⁻ mouse.** A) Strong Ccdd66 reporter gene expression is present in OSN (white asterisks) of the olfactory epithelium, including their dendrites (black arrows) and TK (white arrows) that are strongly marked by X-gal staining in contrast to unlabelled supporting cells (SC). B) A scheme of the olfactory epithelium is shown (modified from Monell Center Advancing Discovery in Taste and Smell; http://www.monell.org/). C) The identically treated Ccdd66⁺/+ serves as negative control, showing no labeling by X-gal staining. Counterstaining with neutral red marks OSN and SC. OSN - olfactory sensory neurons, D - dendrite, TK - terminal knobs, Ci - cilia, SC - supporting cell. 25 µm cryosections, scale bar in A and C - 20 µm.

At all investigated developmental stages of the Ccdd66⁻/⁻ mouse (P4, P10, P17, 1 month, 1.5 and >10 months) the olfactory sensory neurons of the olfactory epithelium show Ccdd66 reporter gene expression and are distinctly labeled by X-gal staining (figure 25 A - F, white asterisks). The supporting cells (figure 25 A - F, black asterisks) are not labeled at any time point as indicated by the recess of staining in the apical area surrounding the apical dendrites of the olfactory sensory neurons. The staining appears noticeably less intense at P4 and probably slightly decreased at 1.5 and >10 months of age. X-gal staining of respective age-matched Ccdd66⁺/+
olfactory epithelia reveals no labeling at any time point (figure 25 G - L). Thus sections were counterstained with neutral red in order to visualize the cells.

Figure 25: Ccdc66 reporter gene expression in the olfactory epithelium during postnatal development of the Ccdc66⁻⁻ mouse. At all investigated time points of the postnatal development of the Ccdc66-deficient mouse (P4, P10, P17, 1 mo, 1.5 mo, >10 mo), the olfactory epithelium displays Ccdc66 reporter gene expression in the OSN (A - F, white asterisks). An attenuation of the signal intensity is present in the P4 and presumably slightly in the 1.5 and >10 months-old mice when compared to X-gal staining of age-matched Ccdc66⁺⁺ controls (G – L, counterstaining with neutral red) reveals no blue colour marking. OSN - olfactory sensory neurons, SC - supporting cell, P - postnatal day, mo – month/s. 25 µm cryosections, scale bar 20 µm.

In the olfactory bulb of the P4 Ccdc66⁻⁻ mouse, X-gal staining labels the granular-, mitral, inner and outer plexiform as well as the here immature glomerular layer consisting of small glomerular precursors, the protoglomeruli (figure 26 A and D). Moreover, as previously reported for the olfactory bulb of the P17 mouse, the olfactory nerve layer showed highest intensity of reporter gene expression when compared to the other layers. In this section of the P4 Ccdc66⁻⁻ a comparable strong signal is observed in the area of the subependymal zone (figure 26 A, black arrowhead). The X-gal staining of the age-matched Ccdc66⁺⁺ mouse olfactory bulb serves as negative control and shows no staining (figure 26 M) as for all Ccdc66⁺⁺
controls of all investigated stages (figure 26 M - R). In the olfactory bulb of the P10 \textit{Ccdc66}^-/- mouse the distribution and intensity of the X-gal staining is unchanged when compared to the positive staining of the P4 olfactory bulb layers with the exception that the subependymal zone is not intensely labeled, or not truncated. Morphologically, single glomeruli are apparent (figure 26 B and E). In the P17 \textit{Ccdc66}^-/- olfactory bulb, the reporter gene expression pattern is also comparable to stages P4 and P10 with the respective layers marked (figure 26 C and F), again including the subependymal zone (figure 26 C). The glomeruli gain in number and size (figure 26 C and F). At 1 month, the staining in the plexiform layers faints, whereby in turn the glomerular layer is partly labeled by X-gal staining (figure 26 G and J). At 1.5 months the color-filling of the glomeruli as well as the intensity of staining in the olfactory nerve layer increases (figure 26 H and K). At 10 months of age the olfactory bulb of the \textit{Ccdc66}^-/- mouse exposes a comparable \textit{Ccdc66} reporter gene expression in the glomeruli like in the 1.5 months old mouse (figure 26 I and L). In summary, \textit{Ccdc66} reporter gene expression is present in both, olfactory epithelium and olfactory bulb. In addition, both tissues reveal dynamical \textit{Ccdc66} reporter gene expression in both tissues during the postnatal development of the olfactory bulb.
Figure 26: Ccdc66 reporter gene expression in the olfactory bulb during postnatal development of the Ccdc66<sup>−/−</sup> mouse brain. Reporter gene expression is present in the olfactory bulb at all investigated stages of the mouse postnatal development (P4 (A and D), P10 (B and E), P17 (C and F), 1 mo (G and J), 1.5 mo (H and K) and >10 mo (I and L)) with increasing signal intensities in the more mature stages (J - L). The glomeruli are more and more colour filled by X-gal staining precipitates the older the animal. Some slices exhibit accentuation of the SEZ (A and C, less in G and
H). *Ccdc66<sup>+/+</sup>* sections do not show X-gal labelling at any stage (M - R). *Ccdc66<sup>-/-</sup>* and *Ccdc66<sup>+/+</sup>* sections are counterstained with neutral red. GL - glomerular layer, ML - mitral layer, IPL – inner, EPL - external plexiform layer, OIINL - olfactory nerve layer, SEZ – subependymal zone, P – postnatal day, mo – month/s. 30 µm frontal cryosections, scale bar in A-C, G-I, M-R 200 µm, D-F, J-L 100 µm.

**Rostral migratory stream**

During postnatal development of the *Ccdc66<sup>-/-</sup>* olfactory bulb, *Ccdc66* reporter gene expression was visible in the area of the subependymal zone at P4, P17, 1 month and 1.5 months (figure 26 A, C, G, H). Additional coronal sections (olfactory bulb, figure 27 A, B) and sagittal sections (figure 27 C and D) of the 1.5 months-old *Ccdc66<sup>-/-</sup>* mouse brain showed portions of β-galactosidase activity along the location of the rostral migratory stream (RMS; figure 27 C and D, black arrows) that reaches from the subventricular zone to the olfactory bulb subependymal zone.
Figure 27: β-galactosidase activity along the location of the rostral migratory stream in the Ccdc66<sup>−/−</sup> mouse aged 1.5 months. Ccdc66 reporter gene expression was detected in the area of the SVZ and in portions along the RMS (black arrows) that reaches from the SVZ to the olfactory bulb OB (C and D) at the SEZ (A and B). Presentation of Ccdc66<sup>+/−</sup> is not included, because X-gal staining in the Ccdc66<sup>+/−</sup> is lacking in the subependymal zone (figure 26). SVZ - sub ventricular zone, RMS - rostral migratory stream, OB – olfactory bulb, CTX – cortex, HC – hippocampus. 30 µm frontal (A and B) and sagittal (C and D) cryosections (slightly counterstained with neutral red). Scale bar in A - 200 µm, in B and D – 100 µm, in C - 500 µm.

Hippocampus

Ccdc66 labeling in the hippocampus by X-gal staining reveals a change in staining pattern during development (figure 28). At P4 strong labeling is presented in the lateral ventricle (figure 28 A). A moderate similar staining intensity is present in the pyramidal layer of CA1 to CA3 and the granule cell layer of the dentate gyrus and adjacent molecular layer (figure 28 A and B). The intense staining of the ventricle is
the case for the other investigated developmental stages too and is separately addressed in the next section. At P10 Ccdc66<sup>−/−</sup> mice exhibit stained layers in the hippocampus similar to the P4 stage but with increased staining intensities (figure 28 C and D). This is the case especially in CA3 (figure 28 C), and the dentate gyrus outer molecular layer that is now clearly distinguishable from the inner molecular layer (figure 28 D). Furthermore, the apical portion of the stratum radiatum is now strongly labeled (figure 28 D). In addition a diffuse staining in the polymorph layer of the dentate gyrus is detected (figure 28 D). At developmental stage P17, massive Ccdc66 reporter gene expression in the inner molecular layer in the dentate gyrus appears (figure 28 E and F). The X-gal staining of the dentate gyrus polymorph layer and the stratum radiatum diminishes, when compared to P10 (figure 28 D and F). In the 1 month-old hippocampus of the Ccdc66<sup>−/−</sup> mouse the Ccdc66 reporter gene expression is mainly restricted to the pyramidal layers with gradually decreased staining intensities from CA1 to CA3), the granule cell layer and still strongest the inner molecular layer of the dentate gyrus (figure 28 G and H). The polymorph layer displays scattered color spots (figure 28 H). In the 1.5 months (figure 28 I and J) and >10 months-old mice (figure 28 K and L), X-gal staining is present in the CA1 pyramidal layer and the granule layer of the dentate gyrus, including the border of the inner molecular layer. The X-gal staining was also performed with age-matched Ccdc66<sup>+/−</sup> sections in parallel as negative controls. The Ccdc66<sup>+/−</sup> sections do not exhibit staining at any stage (figure 28 M - R). These results demonstrate that Ccdc66 reporter gene expression is present in different layers of the hippocampus during the postnatal development of the Ccdc66<sup>−/−</sup> mouse. Changes of expressing structures and intensities during the postnatal development until adulthood reveal a developmental dependency similar to the olfactory bulb.
Figure 28: Ccdc66 reporter gene expression during hippocampal development of the Ccdc66^-/- mouse. In all presented stages of the Ccdc66^-/- hippocampal development (P4 (A and B), P10 (C and D), P17 (E and F), 1 month (G and H), 1.5 months (I and J) and >10 months (K and L) Ccdc66 reporter gene expression visualization by X-gal staining is present in the PyL of CA1 to CA3 and GrCL of the DG (B, D, F, H, J, L). Additional strong labeling is shown at P10 and P17 in the SR (D and F) and in P10 in CA3 (C). The MoL of the DG exposes X-gal staining at P4 (B), at P10 in the OML (D) and in the inner molecular layer (IML) between P17 and >10 months with decreasing intensities in the older ones. Ccdc66^+/+ hippocampal sections do not display staining due to X-gal exposure and serve as negative controls (M - R). Ccdc66^+/+ sections are counterstained with neutral red (Ccdc66^-/- sections slightly). PyL - pyramidal layer, SR - stratum radiatum, GrCl - granular cell layer, DG - dentate gyrus, MoL - molecular layer, OML - outer molecular layer, IML - inner molecular layer, P - postnatal, mo - month/s. 30 µm frontal sections, scale bar in A, C, E, G, I, K, M - R - 500 µm, B, D, F, H, J, L - 200 µm.
Brain ventricular ependyma

*Ccdd66* reporter gene expression in the brain ventricle coating epithelia revealed a very strong staining signal. The staining of ventricle boundaries appears almost constantly intense throughout all of the investigated stages (from P4 to 10 months; not shown) and is thus only presented exemplarily for the lateral and third ventricles of the P17 mouse (figure 29 A - C). This labelling corresponds to the location of ependymal cells that line the ventricle in a single cell row. Parallel screen for β-galactosidase reaction in the *Ccdd66*+/+ reveals no staining by X-gal (figure 29 D and E).

![Figure 29](image-url)
3.1.4 Discussion

Expression of protein CCDC66 in the mouse brain was evidenced by Western blot analyses with subsequent immune detection earlier. In the presented study, initial data about the expression sites of the Ccdc66 gene and its products are provided in the mouse brain. In the P17 Ccdc66+/+ mouse visualization of Ccdc66 expression by ISH to Ccdc66 RNA displays broad distribution in the investigated regions (olfactory bulb, hippocampus and cerebellum). Surprisingly, an almost identical pattern was observed by ISH in the age-matched Ccdc66−/−. Nonetheless, the proof for Ccdc66 reporter gene expression in the P17 Ccdc66−/− mouse supports widespread Ccdc66 expression in a comparable pattern with most intense signals observed in the olfactory nerve layer and the glomeruli of the olfactory bulb, the molecular layer of the dentate gyrus and the brain ventricle lining ependyma. Furthermore, changes in the expression pattern during the postnatal development in the olfactory bulb and the hippocampus were discovered by Ccdc66 reporter gene expression as well as staining along the rostral migratory stream. In summary, these results indicate a potential role of Ccdc66 expression also in the brain in addition to the retina.

3.1.4.1 Ccdc66 RNA, Ccdc66 reporter gene expression and protein CCDC66 in the mouse brain; on the role of potential short Ccdc66 transcripts and CCDC66 isoforms

An initial comparison of the two detection approaches (X-gal staining and ISH) reveals generally widespread signals of Ccdc66 RNA and reporter gene expression. Interestingly, the Ccdc66−/− mouse brain exposes a comparable ISH pattern to the Ccdc66+/+ mouse. The latter finding might either indicate that the specificity of the probe labeling is not appropriate for ISH on the mouse brain resulting in high levels of background staining or that there is a considerable amount of Ccdc66 RNA present also in the Ccdc66−/− mouse. The successful parallel detection of positive control targets demonstrates the generally proper functioning of the method using the chosen conditions. Furthermore, the specificity of the used Ccdc66 probe has been demonstrated by ISH in the mouse retina. In case the positive labeling in the Ccdc66−/− brain might refer to background staining, this could be due to the differing texture or quality of brain and retinal tissue that might give rise to the different performance of the probe. Minor differences might escape detection given high...
background staining. However, reporter gene expression demonstrated the expression of the trapped gene construct in the Ccddc66+/ mouse at the same sites as by Ccddc66 RNA detection. This result shows that in general there is an interruption of the gene in the Ccddc66+/ brain. The question remains if the ISH signal in the Ccddc66+/ mouse reflects also Ccddc66 RNA or is only background.

As mentioned in the discussion of chapter 2 regarding the detection of an RNA signal in the ganglion cell layer, the presence of remaining small transcript variants is principally possible. This could be one explanation, if the signal is not due to background staining. The first hint into this direction was observed by Western blot experiments with pre-incubation of the CCDC66 specific antibody with the immunization peptide that should result in elimination of specific bands in compared detection with the untreated antibody. This antibody blocking experiments revealed a putative small variant of ~37/40 kDa in the Ccddc66+/+ and Ccddc66−/− mouse retinas and brains (Gerding et al. 2011; Schreiber 2010).

Initial experiments assessing transcript variants on RNA/cDNA level (not presented here) hint to the existence of several short Ccddc66 splice variants in the Ccddc66+/+ mouse, that lack intermediate exons and thus might be able to skip the gene trap by avoiding the splicing of exon 3 to the trap upstream to exon 4. These might encode small CCDC66 isoforms that are still present in the Ccddc66−/− retina (and brain, see 2.1.4). Such variants need additional validation. Another potential small variant that lacks exon 3 is reported to use the first ATG of exon 4 and is comprised in the UCSC database (>uc007stx.1). The listed mRNA is 1559 b long and would be able to encode a ~55 kDa isoform (according to in silico translation by EditSeq). The probe chosen here for the ISH would be able to detect both transcripts. In the brain this would be a considerable amount, but could be an indicator of an explanation of the lack of an obvious phenotype compared to the Ccddc66+/+ or the lack of conspicuous behavioral alterations in the Ccddc66−/− at first sight, as you would suggest when comparing the wide spread reporter gene expression in the Ccddc66−/− retina (2.1.3.2 and 2.1.3.3) and brain (3.1.3.2 and 3.1.3.3).

A rescuing function of alternative spliced variants skipping the gene trap could be the effect or maybe even the expression of a portion of the trapped gene. For example, talin2 that is reported to be a focal adhesion protein that interacts with a variety of proteins encompassing integrin (Horwitz et al. 1986; Xing et al. 2001; Hemmings et
al. 1996), vinculin (Bass et al. 2002) actin (Hemmings et al. 1996) and some others (Chen et al. 1995; Borowsky & Hynes 1998). The expression of a trapped fusion of talin2 and β-galactosidase that includes the N-terminal half (1295 aa of 2453 aa) of the talin2 protein is sufficient for development and survival and did neither obviously effect the health of the mouse nor it results in a specific phenotype. This is quite remarkable, since the above mentioned binding sites are located in the deleted C-terminus (Chen & Lo 2005; Xing et al. 2001). For the interrupted CCDC66 all reported coiled coil domains or the leucine zipper would also not be included in the 34 aa encoded by exon 1 to 3. Chen and colleagues further report that the testis specific talin2, that is normally composed of 30 aa exclusive N-terminus (by additional exons and supposed under different promoter) and about 1300 aa of the WT talin2 is shortened to 204 residues, but does not seem to have a crucial impact on fertility. They propose three explanations including the compensating function of talin1 for the overall need of talin protein function, the N-terminus has an unforeseen function for talin2 and there were slight defects that were not identified or discovered without the hint of insult. These conclusions can be adapted to CCDC66. That is why the sites of increased reporter gene expression were assessed for those mild or not obvious morphological changes (3.2).

3.1.4.2 Ccdc66 reporter gene expression in the mouse brain and parallelizing developmental processes – functional association of Ccdc66 expression

Due to the surprisingly broad variety of Ccdc66 expressing cells in the mouse brain, the focus in the following section is restricted to the observed changes in reporter gene expression during postnatal development and their possible association with cells and structures that could uncover processes that Ccdc66 might affect.

Olfactory bulb, olfactory epithelium and rostral migratory stream

In the olfactory bulb, Ccdc66 reporter gene expression is observed in the granule and mitral layer on a weak level relatively constantly during the monitored postnatal development (P4 to over 10 months). From the beginning on, reporter Ccdc66 gene expression is present to an enormous extent in the olfactory nerve fiber layer where the axons of the olfactory sensory neurons deriving from the olfactory epithelium and specialized glia cells, the so called ensheathing cells, are located. The latter migrate
ahead, support axon growth and later enclose the olfactory sensory neurons (Doucette 1984; Chuah & Zheng 1992; Ramón-Cueto & Valverde 1995; Tennent & Chuah 1996; Windus et al. 2011; Ekberg et al. 2012; Miller et al. 2010). In addition, the olfactory epithelium that develops in the embryonic phase (Treloar et al. 2010), is positively labeled by X-gal staining during the entire postnatal development investigated.

In the olfactory bulb, changes in Ccdc66 reporter gene expression over time are observed in the EPL and the glomerular layer. The EPL is highlighted by X-gal staining during the first investigated developmental stages P4, P10 and P17. In the context of the olfactory bulb development at that time point the EPL consists of dendrites of mature and immature mitral cells (Blanchart et al. 2006). Even though mitral cells and olfactory sensory neurons already unite at the so called protoglomeruli that will define dense zones of synapses, at embryonic days immediately prior to birth (E16 (Treloar et al. 1999), some mitral cells still undergo maturation. They have not reached their final shape and display distal branching tufts and more than one dendrite stretched into the forming glomerular layer. Around P15, after pruning and remodeling, the mitral cells have a mature shape with the single primary dendrite in the glomerular layer and secondary sprouting in the EPL (Blanchart et al. 2006; Malun & Brunjes 1996; Lin et al. 2000).

Further cells of the olfactory bulb that expand their dendrites in the glomeruli and plexiform layer predominantly in the early postnatal period are the periglomerular and granule cells as evidenced in studies regarding the rat olfactory bulb development (Hinds 1968; Altman 1969; Rosselli-Austin & Altman 1979; Bayer 1983; Price & Powell 1970). Both cell-types migrate by the RMS to the OB from their origin in the SVZ (Luskin 1993) and are predominantly generated until P5 (Hinds 1968).

Furthermore throughout life, interneurons migrate through the rostral migratory stream to the olfactory bulb (Lois & Alvarez-Buylla 1994). A sub-portion of interneurons is generated prior to those in the lateral ganglionic eminence, becomes post-mitotic in the embryonic phase and differentiates into variety of mature interneurons of the olfactory bulb, among those periglomerular cells, by postnatal day P21, and a large number remains in the olfactory bulb at P60 (Tucker et al. 2006). Synaptogenesis is already initiated and represented in the whole variety at birth, decreases in the glomerular layer after a peak at P10 - P15, but increases in density
during development in the EPL and internal glomerular layer, perhaps due to the integration of the new interneurons (Whitman & Greer 2007b; Whitman & Greer 2007a).

Regarding the rostral migratory stream mentioned above, Ccdc66 reporter gene expression there might either derive from migrating cells, but could also be associated with the glial tube that coat the walls of the stream (Sun et al. 2002).

In conjunction to the above described processes, development-related Ccdc66 reporter gene expression in the olfactory bulb points to several issues that need to be investigated further: on the one hand, the possible role of Ccdc66 in functionally categorized dendritic outgrowth (EPL and glomeruli) and migration (rostral migratory stream), that already attracted attention in the retinal investigation, and on the cellular level the possible link to glia cells or modified glia cells (rostral migratory stream, olfactory sensory neuron ensheathing cells). The differential identification of Ccdc66 expressing cells by co-labeling with a β-galactosidase antibody and glia specific markers or the cultural isolation and enrichment of olfactory neuron ensheathing cells from the Ccdc66−/− mouse with subsequent X-gal staining could demonstrate whether the strong Ccdc66 reporter gene expression in the olfactory nerve fiber is composed of signals from the olfactory sensory nerve axons and/or the ensheathing cells (Kawaja et al. 2009). Since a lot of the structural connections that are highlighted by X-gal staining postnatal is initiated in the embryonic phase, the investigation of respective structures in prenatal developmental stages would be useful. As a systemic aspect one has to mention, the Ccdc66 reporter gene expression in the olfactory epithelium emphasizes, that Ccdc66 might play a further role in other primary sensory systems. With respect to its potential interaction partners identified and the initial suggestion of Ccdc66 expression in the retina to be probably involved in cellular protrusion and structures with special demands on cytoskeleton structures like the olfactory epithelium, this is a conceivable hypothesis. Furthermore, Mpdz is expressed in the olfactory sensory neurons (Dooley et al. 2009) and Eps8 in the cochlear hair cells (Manor et al. 2011). Hence, these results further point to the presence of a multi-systemic function for protein CCDC66.
Hippocampus

In the hippocampus similar X-gal staining patterns are observed in the pyramidal and the granule cell layer whereas the developmental changes in *Ccdc66* reporter gene expression directs the focus to the *stratum radiatum* and the molecular layer of the dentate gyrus. In the *stratum radiatum*, the layer where the Schaffer collaterals of CA3 pyramidal neurons interconnect with the CA1 pyramidal neurons (Kandel et al. 2000), a clear additional staining appears at P10 and remains in the P17 old *Ccdc66*, but is not or only very faintly present in older stages (1 month, 1.5 months and > 10 months). During the early postnatal period, the dendrites of the embryonic born (embryonic day 16 (E16) to E20 (Bayer, 1980)), migrated and differentiated pyramidal neurons develop, still grow and branch and reach their mature size around the first three postnatal weeks (P21; Burt 1980; Polleux and Snider 2010; Sfakianos et al. 2007). Within this time span the majority of spines are generated in the hippocampus starting on the apical dendrite and increase in density up to P20 (Minkwitz 1976; Wu et al. 2015). The completion and connecting phase of the sprouting dendrites could be one process when *Ccdc66* expression is reflected by an X-gal staining pattern in the *stratum radiatum* at P10 to P17 and fading in the older stages where the maturation is completed (3.1.3.3). This would contribute to the potential functional aspects of previous hypothesis of *Ccdc66* playing a role in cellular process formation dynamics as suggested from retina and olfactory bulb development and supported by the identification of the interaction partners involved in these processes. Furthermore, interneurons are located in all hippocampal subfields (reviewed in Kullmann 2011), and in the *stratum radiatum* gamma-aminobutyric acid (GABA)-ergic interneurons are described to express Eps8 (Huang et al. 2014). *Ccdc66* reporter gene expression in these interneurons is not excluded. Nevertheless, the X-gal staining pattern at P17 looks more like a scattered signal through a broader width of the *stratum radiatum* than in a distinct apical portion that might rather reflect a dendritic aborization pattern. Co-detection with a β-galactosidase and Eps8 antibody is probably able to provide clarity, if the hippocampus is another possible intersection point of Eps8 and protein CCDC66.

In the molecular layer of the dentate gyrus incoming fibers from the entorhinal cortex (commissural and association fibers (Loy et al. 1977; Fricke & Cowan 1978)) form synapses with the dendrites of the granule cells that in turn innervate the CA3
pyramidal neurons and laterally branch into the hilus region. In rodents, the first granule cells are born in the late embryonic phase, but the major portion is still formed postnatally with a peak of neurogenesis at around one week postnatally (Rahimi & Claiborne 2007). The wide span of neurogenesis leads to varying differentiation stages of the cells at a given time point. In the rat, at five days of age granule cells have two or more elementary apical dendrites (Lübbers & Frotscher 1988). They form less basal dendrites, except transient hilar basal dendrites formed by immature granule cells that are retracted in the adults (reviewed in (Wu et al. 2015). The maturation of dendrites and dendritic spines of the granule cells takes place during the first three postnatal weeks (Crain et al. 1973; Wenzel et al. 1981; Lübbers & Frotscher 1988). Intense X-gal staining in the inner molecular layer of the \textit{Ccdc66}^- dentate gyrus is present until one month and less in the older stages (3.1.3.3). If one considers the above mentioned time span of neurogenesis of the granule cells and resulting broad time span of the maturation of dendrite arbors, the latter could still be a roughly parallelized with observed \textit{Ccdc66} reporter gene expression in the \textit{Ccdc66}^- mouse.

Another development-related process in the dentate gyrus molecular layer during the first two postnatal weeks is the migration of interneurons from the outer molecular layer to the inner molecular layer towards the granule cells and the hilus (Dupuy-Davies & Houser 1999; Morozov & Freund 2003). This temporal course matches quite perfectly with obtained \textit{Ccdc66} reporter gene expression and emphasizes once more the process of migration, but also synaptic contact formation that these interneurons form during their movement (Morozov et al. 2006). In conclusion, the integration of known developmental processes in the hippocampal CA1 region and the dentate gyrus to the findings in \textit{Ccdc66} reporter gene expression pattern converge on migration, synapse formation and probably dendritogenesis and/or dendritic maturation.

**Brain ventricle ependyma**

In the \textit{Ccdc66} mouse brain ventricle coating ependyma, an intense \textit{Ccdc66} reporter gene expression was reported, with no remarkable changes over time, but one site of highest reporter gene expression. The brain ventricular ependyma is made up by a single row of ciliated and microvilli harboring ependymal cells that resemble most
epithelial membranes (Bruni 1998). The ependyma builds the border between the cerebrospinal fluid and the brain, are supposed to be secretory and involved in exchange of ions, small molecules and water between the cerebrospinal fluid and neural mass (Bruni 1998). Ccdc66 reporter gene activity is present in the ciliated and microvilli carrying epithelial cells. This is another example for the notion that Ccdc66 is expressed in a variety of different cell types (also content of chapter 4 and 5) and structures.

3.2 Degeneration in the Ccdc66⁻/⁻ mouse brain

3.2.1 Introduction

The functional relevance of Ccdc66 expression in the brain and its consequences are still unclear; the Ccdc66⁻/⁻ mouse does neither constitute an obvious impaired phenotype nor is there any evidence conspicuous behavior as compared to the Ccdc66⁺/+ mouse. Additional Ccdc66 expressing structures in the brain (identified in 3.1.3) might hint to morphological and functional consequences of Ccdc66 deficiency at different developmental stages. In order to further evaluate these regions of intense reporter gene expression in the Ccdc66⁻/⁻ mouse, the following structures including olfactory bulb, hippocampus and brain ventricular ependyma were additionally investigated for degeneration in Ccdc66⁻/⁻ mice on the ultra-structural level by electron microscopy.

3.2.2 Material and methods

Electron microscopy was performed in cooperation with Dr. med. E. Petrasch-Parwez (Department of Neuroanatomy and molecular brain research, RUB). The method was conducted as described before (Petrasch-Parwez et al. 2007). Briefly, 11 months-old Ccdc66⁺/+ and Ccdc66⁻/⁻ mice (three mice of each genotype for the olfactory bulb and one of each genotype for initially assessed hippocampus and ependyma) were euthanized by intraperitoneal injection of a maximal dose of pentobarbital (800 mg/kg Narcoren®, Merial GmbH), intracardially injected with 0.3 ml Liquemin N 25000 (Roche) and perfused transcardially with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 20 min. Brains were dissected and embedded in 2% agarose in
PBS. Embedded brains were coronally cut (1 mm), postfixed with 2% osmium tetroxide in PBS for 30 minutes and dehydrated in an ascending EtOH concentration. Specimens were transferred into a propylene oxide/araldite mixture and finally embedded in araldite. Semithin (0.75 µm) and ultra-thin (0.1 µm) alternating sections were cut with a Leica Ultracut UCT microtome and alternating semithin section counterstained with 1% toluidine blue (pH 9.0). Adjacent ultra-thin sections were contrasted with 5% aqueous uranyl acetate for 5 min followed by a 5-min staining with lead citrate (pH 12). Light microscopic pictures were taken with a DP71 camera (Olympus) mounted on an Olympus light microscope. Ultra-thin sections were viewed in a Philips EM 420 electron microscope, documented by the digital system DITABIS (Digital Biomedical Imaging System). Utilized buffers and solutions are listed in the appendix (appendix, chapter 3.2).

3.2.3 Results

Results of the morphological assessment of the investigated Ccdc66+/+ and Ccdc66−/− mouse brain regions are presented from rostral to caudal positions in the mouse brain.

3.2.3.1 Olfactory bulb

Morphological assessment of the olfactory bulbs of 11 months-old Ccdc66−/− and age-matched Ccdc66+/+ mice revealed no obvious changes when compared on the light microscopically level: semithin sections of the olfactory bulb of both genotypes do not show differences in size or arrangement of mitral, external plexiform, glomerular and olfactory nerve fiber layer (figure 30 A and E for overview, semithin B and F). In the Ccdc66−/− glomeruli on the ultra-structural level, as detected by EM, dark appearing olfactory nerve fibers and their synaptic terminals display characteristic degeneration signs within the glomeruli in terms of dark condensed organelles and lysosomal accumulations (figure 30 C and D, red asterisks). These degenerating olfactory nerve fibers partly form contacts with surrounded dendrites (D; figure 30, panel C and D). The Ccdc66+/+ mouse glomeruli in contrast show normal olfactory nerve fibers and dendrites without any sign of degeneration (figure 30 G and H).
3.2.3.2 Brain ventricular ependyma

The Ccdc66^{-/-} mouse brain was analyzed at the level of the optic chiasm and anterior commissure and compared to respective Ccdc66^{+/+} controls (figure 31 A and D). With focus on the brain ventricular ependyma semithin section in the area of the caudoputamen did not uncover structural differences between Ccdc66^{-/-} and Ccdc66^{+/+} sections (figure 31 B and E). Likewise, electron microscopy of ependymal cells in this area of the ventricle shows normally appearing cells without any sign of degeneration (figure 31 C and F).

3.2.3.3 Hippocampus

Frontal sections of the 11 months-old Ccdc66^{-/-} mouse brain at the level of the rostral hippocampus do not show differences in size of depicted structures when compared to the Ccdc66^{+/+} mouse hippocampal sections (figure 32 A and F). This finding is supported by semithin section of the hippocampus (figure 32 B - D and G - I). CA1 pyramidal cells and granule cells of the dentate gyrus do not reveal a difference in the Ccdc66^{-/-} and Ccdc66^{+/+} hippocampus. Moreover, pyramidal cells display no sign of degeneration by EM of hippocampi of both genotypes (figure 32 E and J).

These results show, that the expression of trapped Ccdc66 gene results in morphological changes, particularly degeneration of the olfactory sensory nerve fibres. In turn, not at all structures that expose high levels of Ccdc66 reporter gene expression show degeneration at that time point of investigation (hippocampus regions and brain ventricle ependyma).
Figure 30: Degeneration in the olfactory bulb of the 11 months-old \textit{Ccdd66}^{-/-} mouse by electron microscopy. The comparison of the \textit{Ccdd66}^{-/-} and \textit{Ccdd66}^{+/+} olfactory bulb frontal sections, presented no differences in their morphological appearance (A and E). The olfactory bulb layers of \textit{Ccdd66}^{-/-} and \textit{Ccdd66}^{+/+} (ML, EPL, GL and OlfNL) display no differences in size or structure as evaluated by semithin sections (B and F). On ultra-structural level \textit{Ccdd66}^{-/-} ONF (in C) and synaptic terminals (T in D) show clear degenerations (red asterisks, C and D) by accumulation of condensed organelles in contrast to unaffected fibers in the \textit{Ccdd66}^{+/+} mouse (D and H). ML – mitral layer, EPL - external plexiform layer, GL – glomerular layer/glo meruli, ONL – olfactory nerve layer, AG – astroglia, D – dendrite, T – terminal. 1 mm frontal sections (A and E), 0.75 µm frontal semithin sections (B and F), 0.1 µm frontal ultra-thin sections (C, D, G, H). Scale bar in A and, E - 1.5 mm, in B and F – 50 µm, in C,D,G,H - 1 µm.
Figure 31: Morphology of brain ventricle ependyma in the 11 months-old Ccdc66<sup>−/−</sup> mouse by semithin sections and electron microscopy. The comparison of the Ccdc66<sup>−/−</sup> and Ccdc66<sup>+/+</sup> frontal sections of the ventricle at the level of the OC and AC, presented no obvious differences (A and D). Neither semithin sections (B and E) nor electron microscopy (C and F) reveal alterations in size or structure of depicted ependymal or caudoputamen cells (B and E). CTX – cortex, V – ventricle, E - ependyma, OC - optic chiasm, AC - anterior commissure, Ci – cilia, MV – microvilli, NCL - nucleus. 1 mm frontal sections (A and D), 0.75 µm frontal semithin sections (B and E), 0.1 µm frontal ultra-thin sections (C and F). Scale bars in A and D - 2 mm, in B and E – 10 µm, in C and F - 1µm.
Figure 32: Morphological characterization of the hippocampus in the 11 months-old Cccdc66<sup>−/−</sup> mouse by semithin sections and electron microscopy. Cccdc66<sup>−/−</sup> and Cccdc66<sup>+/+</sup> frontal sections of the rostral hippocampus, do not differ regarding size of the shown structure (A and F). Likewise, Cccdc66<sup>−/−</sup> and Cccdc66<sup>+/+</sup> semithin sections (B – D and G - I) as well as electron microscopy (E and J) did not discover alterations of hippocampal structure or integrity when comparing Cccdc66<sup>−/−</sup> and Cccdc66<sup>+/+</sup> sections. CC – corpus callosum, HC – hippocampus, TH – thalamus, HTH –
hypothalamus, CTX – cortex, CA – cornu ammonis, PyL – pyramidal layer, PyC – pyramidal cell, SR – stratum radiatum, GrCL – granule cell layer. 1 mm frontal sections (A and F), 0.75 µm frontal semithin sections (B - D, G - I), 0.1 µm frontal ultra-thin sections (E and F). Scale bar in A and F - 2 mm, in B and G – 200 µm, in C, D, H, I - 10 µm, E and J - 2 µm.

3.2.4 Discussion

The ultra-structural investigation of cerebral sites with increased Ccdc66 reporter gene expression demonstrated clear signs of degenerations in terms of inflated olfactory sensory nerve axonal terminals with condensed cell organelles. In contrast, preliminary assessment of the hippocampal and ependyma Ccdc66 reporter gene expressing structures revealed no obvious differences between Ccdc66+/+ and Ccdc66-/-.

The potential role of isoforms that might be translational products of splice variants that circumvent the gene trap and thereby act as rescuing isoforms is discussed in 3.1.4.1. Of course there could also be other compensatory or regulatory mechanisms that countervail and compensate the lack of complete Ccdc66 expression. Several animal models established to prove the importance of a protein in a defined process or developmental process frustrated, disappointed or surprised researchers disproving their hypotheses. For example, even though in vitro studies predicted the α4 integrin subunit to be involved in neural crest migration (Dufour et al. 1988; Sheppard et al. 1994; Stepp et al. 1994), the formation of neural crest-derived tissues was not impaired in mice lacking the neural crest-derived tissues; they were still formed in mutant mice lacking the α4 subunit (Yang et al. 1995). Regarding another integrin subunit α1 as example, a developmental phenotype was not observed in mice lacking the collagen and laminin binding subunit (Gardner et al. 1996) that is supposed to mediate neural crest cell migration and neurite outgrowth (Lallier & Bronner-Fraser 1993; Perris et al. 1993; Tomaselli et al. 1993).

Yet in our case, regarding the presented degeneration of the olfactory nerve fibers in the Ccdc66-/- mouse, the question raises if the degeneration is directly linked to the olfactory sensory neurons or if this is a secondary effect for example by an impaired function of the olfactory ensheathing cells, that express as mentioned in 2.1.4 probably also Ccdd66. The dysfunction of those cells is described to result in a reduced number of olfactory sensory neurons and deprived axon growth (Barraud et
al. 2013). A differentiated ultra-structural investigation of the olfactory epithelium, that is also \textit{Ccdc66} reporter gene expression positive as well as the differentiated determination of \textit{Ccdc66} expression in the olfactory ensheathing cells (discussed in 3.1.4) will hopefully clarify this point. Furthermore, the onset of degeneration in the olfactory nerve fibers shall be temporally determined in order to define whether it is an early event, already impaired during the formation or progressing in time. With this morphological phenotype of the degenerating olfactory nerve fibers in the \textit{Ccdc66\textsuperscript{-/-}} it is tempting to speculate about additional consequences besides the retinal with regard to impaired olfaction performance. In BBS for example there is evidence that the olfactory capacity is reduced caused by a deletion in the \textit{BBS4} Gene (Iannaccone et al. 2005) that is, like some other BBS associated genes, expressed in the olfactory epithelium. An olfactory impairment, taking forms like odor identification or discrimination, is furthermore common in several neurodegenerative diseases like Alzheimers and Parkinson’s disease (between 90\% and 96\% among the patients (Doty et al. 1988; Hawkes et al. 1997). If olfactory impairment is a new characteristic of the \textit{Ccdc66\textsuperscript{-/-}} mouse should be addressed by behavioral studies with odor discrimination tests. In order to screen further for late onset degeneration in the regions of \textit{Ccdc66} reporter gene expression that did not show morphological changes in the age of ten months, as well as to assess further unobvious degenerations in regions that were not in the focus of attention by reporter gene expression, a of staining degenerating nerves by fluoro-jade c assay shall give further insights (Chidlow et al. 2009; Schmued et al. 2005).
Chapter 4 - Compartment localization of protein CCDC66 in mouse retina and brain

4.1 Introduction

Neither the neural cell type (neurons, glia, etc.) nor the cellular compartment localization of protein CCDC66 had experimentally approached yet. Freely accessible programs are available online (e.g. Psort, Proteome Analyst) that search for sequence patterns and provide calculated information about common domains, signal peptides and cellular localization based on different algorithms. For protein CCDC66, the in silico approach results in a variety of predicted compartments for the protein to be located (from nucleus, peroxisomes or cytoplasm; see analyses insight the prediction programs e.g. Psort, Proteome Analyst). In this chapter, the following question shall be addressed in order to clarify: In which cellular compartment/s of mouse retina and brain is protein CCDC66 localized and in which neuronal cell types?

Therefore, a fractionation of mouse retinal and brain whole cell lysates in cytoplasmic, membrane, nuclear and cytoskeleton compartment proteins was performed with subsequent Western blot and immune detection of protein CCDC66. Moreover, double immunofluorescence of CCDC66 in a mixed neural cell culture in conjunction with different neural cell markers (for neurons, glia, oligodendrocytes and neural stem cells) shall bring insight into the cell type distribution and sub cellular localization of protein CCDC66 by the investigation of potential co-localization.

4.2 Materials and methods

4.2.1 Animals and tissues

Retinas and brains for the compartment fractionation were dissected and prepared as described for RNA isolation (2.1.2.4) from at least P17 old Ccdc66+/+ or C57BL6/J mice. For the compartment fractionation of whole cell lysate 6 retinas and 3 brain hemispheres were pooled for each of three replications.
4.2.2 Cell compartment fractionation and Western blot

The cellular fractionation was performed with two fractionation kits (brain: Subcellular Protein Fractionation Kit for Cells, #78840 Thermo Scientific™; retina: Cell fractionation kit, #9038 New England Biolabs GmbH) according to the manufacturers’ instructions with some changes. In short, the frozen tissues were directly transferred and homogenized in the first supplied buffer to isolate cytoplasmic proteins from the suspension. Centrifugation, supernatant removing and suspension steps with the supplied buffers followed in order to isolate proteins of the different fractions. The Pierce fractionation kit enables the separation of soluble and chromatin bound nuclear proteins that were pooled (deviating from the protocol) to a common nuclear fraction. The isolation of the combined cytoskeleton/ nuclear fraction in the NEB kit was modified by the addition of 300 units micrococcal nuclease (#M0247S, New England Biolabs GmbH) and 5 µl 100 mM CaCl₂/100 µl buffer. The protein concentration of the samples was determined by the Pierce™ BCA Protein Assay Kit (#23225, Thermo Scientific™) according to the company’s protocol. For subsequent Western blots, 25 µg protein of each protein fraction with 5 x Laemmli-buffer (final concentration 1x) and 1 M DTT (final concentration 100 mM) were adjusted to equal volumes with RIPA buffer. Samples were denatured for 5 min at 95°C and loaded on a 10% PAA gel. Gel and Western blot equipment were obtained from BioRad (Mini-PROTEAN® Tetra Cell with Mini Trans-Blot® Module, and PowerPac™ Basic Power Supply #165-8033FC). Proteins were separated for ~1.5 hours and transferred to a nitrocellulose membrane (Hybond-C Extra, GE Healthcare Life Sciences; transfer: 100 V, 1.5 hours). PonceauS staining confirmed the complete transfer. Membranes were washed in PBS-T, blocked for 1 h at RT (Western Blocking Reagent, Solution, Roche) and incubated with the primary antibody solution overnight (table 7). On the next day, membranes were washed (6 x 10 min in PBS-T), incubated with alkaline phosphatase conjugated secondary antibody (table 7) for 1 h at RT and washed again (6 x 10 min in PBS-T). Specific detection of target proteins was visualized by the Pierce™ ECL Plus Western Blotting Substrate (Pierce) and detected and documented by the aid of the FUSION-SL documentation system and respective FUSION-CAPT software (Peqlab).
### Table 7: Antibodies for the immune detection of proteins using Western blotting

<table>
<thead>
<tr>
<th>Primary antibody (target)/ species/ product specification, company</th>
<th>Concentration in 1:1 PBS/ blocking solution</th>
<th>Secondary antibody/ product specification, company/ concentration in 1:1 PBS/ blocking solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCDC66/ rabbit polyclonal IgG/ #T-20 sc-102420, Santa Cruz Biotechnology Inc.</td>
<td>1:200</td>
<td>HRP-conjugated goat anti-rabbit polyclonal IgG/ #111-035-003, Jackson ImmunoResearch Laboratories, Inc./ 1:7500</td>
</tr>
<tr>
<td>GAPDH/ rabbit polyclonal IgG/ #ab9485, Abcam</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na,K-ATPase/ rabbit polyclonal IgG/ #3010/Cell signaling technology®</td>
<td>1:1000</td>
<td></td>
</tr>
<tr>
<td>MeCP2/ rabbit monoclonal IgG/ MeCP2 (D4F3) XP®#3456, Cell signaling technology®</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vimentin/ rabbit monoclonal IgG/ Vimentin (D21H3) XP® #5741, Cell signaling technology®</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### 4.2.3 Double immunofluorescence on neurospheres derived mixed neural cell culture

The mixed neural cells culture plates (kindly provided by Prof. Dr. A. Faissner, Cell morphology and Molecular Neurobiology Faculty of Biology and Biotechnology, RUB) were derived from neurospheres that were prepared from the cortex of NMRI mice (embryonic day 12.5, Charles River Laboratories) as described before (Hennen et al. 2011). Cells were cultivated as neurospheres for one week in neurosphere medium, dissociated and plated on polyornithin/lamin coated dishes in mixed neural culture medium. Cells were washed 2 times with PBS, fixed for 10 min in 10% PFA, blocked with 10% NGS in PBS-TX 0.5% for 30 min, before incubation with the primary
antibody in the same solution for 30 min at RT (table 8). Subsequently, cells were washed 3 times in PBS, incubated with the secondary antibody 10% NGS in PBS-TX 0.5% together with TO-PRO®-3 for 30 min at RT (table 8). Cells were cover slipped with Fluorescence Mounting Medium (#S3023, Dako) and staining assessed and documented by a confocal microscope and respective software (Axioplan2, Zeiss). Images were composited with ImageJ. For utilized buffers and solutions (media) see appendix (appendix - chapter 4).

Table 8: Antibodies for double immune fluorescence of cultured cells

<table>
<thead>
<tr>
<th>Primary antibody (target)/species/ product specification, company</th>
<th>Concentration in 1:1 PBS/ blocking solution</th>
<th>Secondary antibodies/company/ concentration in 10% NGS in PBS-TX 0.5% (+ TO-PRO®-3 (#T3605, Life technologies, Inc.; 1:500))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCDC66/ rabbit polyclonal IgG/#T-20 sc-102420, Santa Cruz Biotechnology Inc.</td>
<td>1:20</td>
<td>Alexa fluor 488®-conjugated goat anti-rabbit IgG/#111-545-045, Jackson ImmunoResearch Laboratories, Inc./ 1:300</td>
</tr>
<tr>
<td>Nestin, intermediate filament/mouse monoclonal IgG/#MAB353, Chemicon (Merck Millipore)</td>
<td>1:300</td>
<td>Cy™3-conjugated goat anti-mouse IgG/#115-165-071, Jackson ImmunoResearch Laboratories, Inc./ 1:500</td>
</tr>
<tr>
<td>β-III-tubulin/mouse monoclonal IgG/#T8578, Sigma Aldrich</td>
<td>1:50</td>
<td></td>
</tr>
<tr>
<td>GFAP/mouse monoclonal IgG/#G 3893, Sigma Aldrich</td>
<td>1:50</td>
<td></td>
</tr>
<tr>
<td>MBP/mouse monoclonal IgG/#MAB382, Chemicon (Merck Millipore)</td>
<td>1:50</td>
<td></td>
</tr>
</tbody>
</table>
4.3 Results

In order to gain insight into the cellular distribution of protein CCDC66, cell compartments were fractionized from retina and brain whole cell extracts. Protein CCDC66 localized within these fraction by Western blot and immune detection.

4.3.1 Cell compartment fractionation

Fractionation of retinal cell extracts reveals a predominant localization of protein CCDC66 in the combined cytoskeleton/ nucleus and cytoplasm compartment (figure 33). A faint signal of CCDC66 immune reactivity was further observed in the membrane protein fraction. A putative small variant of protein CCDC66 was solely detected in the cytoskeleton/ nuclear lane (figure 33). The detection of housekeeping proteins of known cellular distribution served as positive control: GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is located in the cytoplasm, Na,K,ATPase in the membrane fraction and the transcription factor MeCP2 (Methyl-CpG-binding protein 2) and the intermediate filament Vimentin in the nuclear/ cytoskeleton compartment (figure 33).

Figure 33: Compartment fractionation of mouse retinal whole cell lysate. In the mouse retina the long CCDC66 isoform (~100-140 kDa) is predominantly located in the cytoplasmic and nuclear/cytoskeleton protein fraction. A putative small isoform (~37 kDa) is solely detected in the cytoskeleton/ nuclear fraction. The confirmation of known distribution of housekeeping proteins within
the separated compartments (GAPDH (kDa) - cytoplasm, Na,K,ATPase (~100 kDa) - membrane, MeCP2 (~75 kDa) & Vimentin (~57 kDa) – nuclear/ cytoskeleton) serves as positive control and confirms the proper fractionation. Protein amount per lane: 25 µg.

The fractionation of mouse whole brain extract into different fractions and the detection of CCDC66 within those show that protein CCDC66 is present in the nuclear and cytoskeleton compartment, while it is slightly detectable in the other fractions of cytoplasm and membrane proteins (figure 34). The potential small CCDC66 isoform likewise appeared mainly in the fraction of nuclear proteins (figure 34).

Figure 34: Compartment fractionation of mouse brain whole cell extract. The long isoform of protein CCDC66 (~100-140 kDa) is predominantly detectable in the nuclear and cytoskeleton compartment and to less extent in the cytoplasm and nuclear protein fraction (black arrowheads). A putative small isoform (~37 kDa) is exclusively present in the cytoskeleton/ nuclear fraction. The control of known distribution of housekeeping proteins (GAPDH (kDa) - cytoplasm, Na,K,ATPase (~100 kDa) - membrane, MeCP2 (~75 kDa) – nucleus & Vimentin (~57 kDa) -cytoskeleton)) serves as positive control and confirms the proper fractionation into the different compartments. Protein amount per lane: 25 µg.

These results indicate that the distribution of protein CCDC66 is located in cellular compartments in the mouse retina and brain with the mainly in the cytoskeleton and
nuclear compartment (retina and brain) and in the cytoplasm (retina). Furthermore, the small putative CCDC66 isoform (1.4.1.2) is predominantly located in the same compartment as the long one - in the retina restricted to the nuclear/ cytoskeleton compartment and in the brain, where these fractions are assessed separately the small isoforms is present in the nuclear compartment.

4.3.2 Co-localization of CCDC66 and neural marker proteins by double immune detection in a mixed neural cell culture

Protein CCDC66 was detected by immunofluorescence and analyzed in conjunction with co-localizing markers for different cell types in a mixed neural cell culture (E12.5 neurospheres, 14 days in vitro, 7 days differentiated). Confocal images show that in the majority of all cells, CCDC66 immune reactivity is present in the nuclei, as indicated by the spatial proximity to the TO-PRO® signal, highlighting nucleic acids (CCDC66 (green), TO-PRO® (blue) figure 35 B, C, E, F, H, I, K, L, white asterisks). The double-labeled immunofluorescence reveals the co-localization of CCDC66 and β-III-tubulin as neuronal marker (β-III-tubulin (red) figure 35 A, CCDC66 (green) figure 35 B, merged (orange) figure 35 C, white arrowheads). The fluorescence signal of CCDC66 is quite delicate and pale, but it is clearly present in soma and the neuronal processes (figure 35 C, merged (orange), white arrowheads).

A similar faint CCDC66 signal is obtained co-localizing with the glial marker GFAP (glial fibrillary acidic protein; (red) figure 35 D, CCDC66 (green) E, merged (orange) F, white arrowheads). Here also, CCDC66 is presented to a small extent in the somata and processes as indicated by the GFAP signal being slightly shifted from red to orange when merged with the CCDC66 signal (figure 35 F, merged (orange), white arrowheads).

Co-localization of the stem cell marker Nestin ((red) figure 35 G, white arrowheads) and the CCDC66 immune detection signal ((green) figure 35 H, white arrowheads) reveals faint staining of protein CCDC66 in stem cells soma and processes (figure 35 I, merged (orange) white arrowheads). A clear co-localization is present of CCDC66 in MBP (myelin basic protein)-labeled oligodendrocytes (MBP (red) figure 35 J, CCDC66 (green) figure 35 K, merged (orange) in L, white arrowheads). In oligodendrocytes CCDC66 is represented in soma and processes as well.
Figure 35: Localization of CCDC66 in neural cells. Confocal images of double immunofluorescence with an antibody against CCDC66 and antibodies against the neural markers β-III-tubulin, GFAP, MBP and Nestin. A weak CCDC66 signal (B, C, E, F, H, J, green, white arrowheads) and co-localization is obtained in the somata and processes of β-III-tubulin positive neurons ((red) A, merged with CCDC66 (green) in C (orange), white arrowheads), GFAP immune reactive glia cells ((red) D, merged with CCDC66 (green, E) in F (orange) white arrowheads) and Nestin immune-reactive neural stem cells ((red) G, merged with CCDC66 (green, H) in I (orange) white arrowheads). In the majority of these cells, CCDC66 detection was further observed in the nuclei, as indicated by co-localization with the TO-PRO® signal (blue) that highlights nucleic acids (B, C, E, F, K, L, white asterisks). A clear and more dominant co-localization is presented by merging of the MBP signal in
oligodendrocytes (red, J) and CCDC66 (green, K) (merged in L (yellow/orange), white arrowheads). E12.5 neurospheres, 14 days in vitro, 7 days differentiated, scale bar - 20 µm.

In summary, these results strengthen the assumption that there is more than a single target cell compartment of protein CCDC66. Furthermore its expression might not be restricted to a single neuronal cell type.

4.4 Discussion
Protein CCDC66 is apparently present in different cellular compartments as evidenced by fractionation of retinal and brain whole cell extracts and subsequent immune detection of protein CCDC66 as well as its co-localization in a mixed neural cell culture.

Protein CCDC66 – cytoskeleton and nucleus
Two isoforms were monitored in the fractionation, the full length protein CCDC66 (long isoform) and a putative short isoform. The compartment-separation of retinal and brain tissue reveals a localization of the long CCDC66 isoform in the nuclear and cytoskeleton compartment (fractions fused in retina, separated in the brain). The putative short variant was localized predominantly in the nuclear, respective nuclear/cytoskeleton compartment.

As initially mentioned, the localization of protein CCDC66 is undefined. Coiled coils are abundant structures found in a diverse number of proteins serving in a broad range of biological processes from transcription factors to intermediate filaments (reviewed in Lupas 1996). In conjunction with previous findings, here, a localization of protein in CCDC66 the cytoskeleton compartment that in turn might allow a scaffold or cytoskeleton-related function of CCDC66 is conceivable. The lack of CCDC66 as a structural or cytoskeleton associated protein involved in the maturation of the OS could lead to processes that prohibit the development of the OS of the photoreceptors in the \textit{Ccdc66}⁻⁻ mouse retina, as photoreceptors degenerate in the \textit{Ccdc66}⁻⁻ model (initial already at P13, Gerding et al. 2011). The expression during phases of synaptic pruning, reorganization (2.1.3.3 and 3.1.3.3) as well as the fact that both identified potential interaction partners have functions regarding
cytoskeleton integrity regulation or stabilization (discussion 2.2.4) makes a structural character of CCDC66 very likely. Similarly RP1, a photoreceptor microtubule-associated protein that is required for correct OS disc assembly and is located at the OS of rod and cone photoreceptors (Liu et al. 2002; Liu et al. 2004). RP1 is involved in retinal degeneration if disrupted (for example Song et al. 2014). Besides the localization of CCDC66 in the cytoskeleton compartment it was also found, together with the small putative CCDC66, in the nuclear protein fraction in retina and brain. In conjunction with reporter gene expression in the retina and brain this could reflect the dot like pattern in proximity to cell nuclei that occurs steadily and not in development-dependent processes (retina – apparently GCL and borders of the INL; olfactory bulb - mitral and granule cells; hippocampus - pyramidal cells and dentate gyrus - granule cells).

For example a protein that is also present in more than one cellular compartment and different tissues, is the neural FMRP (Fragile X mental retardation protein 1) protein, where alternative splicing leads to cytoplasmic or nuclear localization (Sittler et al. 1996). FMRP is an mRNA binding protein that translocates the transcripts to ribosomes. Furthermore FMRP is associated with cell migration and polarity and differentiation (Bardoni et al. 2006). A structural, mRNA transporting function of CCDC66 perhaps involved in a pathway regarding migration and polarity is also possible, since a leucine zipper is present on the proteins C-terminal that is, beside other functions often associated with nucleic acid binding capacities. In order to address this question with respect to CCDC66 as a transport associated protein, it would be possible to test for the miss-localization or displaced accumulation of target proteins due to insufficient mRNA transport. But in this case, a precise hypothesis should be at hand that suggests what target mRNA or protein could be failed to be transported. Other examples of such proteins located in several compartments are the microtubule associated tau protein (Sultan et al. 2011; Mandelkow & Mandelkow 2012) and mPER1 protein isoforms being located in different compartments comparing retina and the suprachiasmatic nucleus (García-Fernández et al. 2007).

In general, the cytoskeleton is also attached to the nucleus, but fractionation could be confirmed as correct by localization of the marker proteins. Furthermore, the identified cellular compartments CCDC66 is localized in are supported by the double immunofluorescence detection of CCDC66 and several markers for neuronal proteins.
and a DNA. Even though the antibody against CCDC66 for IHC (Gerding et al. 2011) is not working on tissue slices reliably, is suitable for the staining of cultured cells due to better antigen retrieval. Ccdc66 reporter gene expression patterns (chapter 2.1.3 and 3.1.3) initially suggest that the expression might not be restricted to a single cell type (for example the retina) and could also have different distributions in other neural cells. Cell culture CCDC66 co-staining reveals co-localization in the soma of GFAP-positive glia cells, nestin labeled stem cells, β-III tubulin labeled neurons and to an increased extent in MBP-reactive oligodendrocytes. All of these cell types were most likely labeled by the investigation of Ccdc66 reporter gene expression in the brain. In cell culture the oligodendrocytes displayed the strongest CCDC66 immune reactivity. Oligodendrocytes are distributed throughout the whole brain, building the myelin sheath of neuronal axons (Kandel et al. 2000). Large fiber tracts in the Ccdc66−/− mouse where the myelin is present in high amounts, like the corpus callosum, were not highlighted by intense reporter gene expression, nor do they appear malformed (3.2). Initial staining of brain slices in the area of the corpus callosum with an MBP antibody, have been performed and should be continued. At first sight, the expression of MBP seems to be slightly increased in the Ccdc66−/− compared to the Ccdc66+/+ mouse (not shown), but this finding has to be validated in further animals. In addition the thickness of the corpus callosum could be measured in order to assess potential slight changes. Another possibility, disregarding the cell type, could be an increased CCDC66 immune reactivity compared to the other cells, because of an actual process within the cell, for instance the ensheathing process of an axon by the oligodendrocytes. However, at first the specificity of the cell culture CCDC66 staining should be demonstrated by the parallel generation of Ccdc66+/+ and Ccdc66−/− neurospheres derived cell culture.
Chapter 5 – Extra-retinal and extra-cerebral *Ccdc66* reporter gene expression sites

5.1 Introduction

Initial Western blot experiments have shown the presence of protein CCDC66 in extra retinal and extra cerebral organs, but to a distinctly lower level. In order to gain insights about protein CCDC66's general function, a *Ccdc66* reporter gene expression in a broad variety of tissues was performed during the postnatal development in the *Ccdc66*\(^{-/-}\) mouse. A widespread initial screen by X-gal staining should identify *Ccdc66* reporter gene expression sites beside retina and brain in order to address the following questions:

1) Where are extra-retinal and extra-cerebral *Ccdc66* reporter gene expressing structures?

2) Are there commonalities among detected sites (together with retinal and brain *Ccdc66* expression sites) that allow conclusions about the general function of CCDC66?

5.2 Materials and methods

5.2.1 Animals, tissues and preparation of cryosections

The tissues for the *Ccdc66* reporter gene expression screen were dissected from the animals for the retinal and brain analyses and treated equally (see 2.1.2.1 - 2.1.2.3). They encompass lung, liver, myocardium, skeletal muscle (*Musculus tibialis anterior*), kidney, spleen, thymus, small and large intestine, stomach, ovary and fallopian tube, testis and epididymis. Tissues of three animals of each genotype (*Ccdc66*\(^{+/+}\) and *Ccdc66*\(^{-/-}\) and age (1 month, 1.5 months, >10 months) were investigated.

5.2.2 X-gal staining

X-gal staining was performed as described in 2.1.2.5 and reporter gene positive structures were assessed by the aid of a mouse histology atlas (Treuting et al. 2012).
5.3 Results

Ccdc66 reporter gene expression is present in the majority of investigated tissues and developmental stages of the Ccdc66+/− mouse (figure 36). Tissue sections of either the 1, 1.5 and > 10 months old mouse are presented together with age-matched Ccdc66+/− tissues that in the majority did not show β-galactosidase expression if not mentioned otherwise (figure 35). Ccdc66 reporter gene expressing structures encompass the bronchi and bronchioles coating epithelium of the lung (figure 36 A, Ccdc66+/− A˚), the myocytes of the myocardium of the heart (figure 36 B, Ccdc66+/− B˚) and in the digestive system the nerve plexus of the small (figure 36 C) and large intestine (figure 36 D, Ccdc66+/− D˚) and stomach (figure 36 E). Tissues of the urogenital system display moderate Ccdc66 reporter gene expression in the kidney, in the area of the tubules (figure 36 F, F˚), in the ovary follicles and surrounding cells (figure 36 G, Ccdc66+/− G˚/H˚) and very intense in the fallopian tube tunica mucosa (figure 36 H, Ccdc66+/− G˚/H˚). In the seminiferous tubules of the testes, differentiating and mature sperm cells are strongly positive for Ccdc66 reporter gene expression (figure 36 I, Ccdc66+/− I˚). Endogenous β-galactosidase activity is detected in the epididymis as indicated by X-gal labeling in the Ccdc66+/− (figure 36 J, Ccdc66+/− J˚) which is consistent earlier reports (Björkgren et al. 2012; Langford et al. 1991). Indistinct X-gal staining is observed in sections of the skeletal muscle (Musculus tibialis anterior, figure 37 A, Ccdc66+/− A˚) and tongue (figure 37 B, Ccdc66+/− B˚).
Figure 36: $Ccdc66$ reporter gene expression in extra-retinal and extra-cerebral tissues of the $Ccdc66^{−/−}$ mouse (adult). $Ccdc66$ reporter gene expression by X-gal staining is detectable in a variety of tissue structures of the $Ccdc66^{−/−}$ mouse (A - J), compared to the $Ccdc66^{+/+}$ control (A´ - J´). These encompass the lung (bronchi/bronchioles, A), the myocardium (myocytes, B), the small (nerve plexus, C) and large intestine (nerve plexus, D) as well as stomach (nerve plexus, E), kidney (tubules, F), ovary (ovary follicle, G) and the fallopian tube (tunica mucosa, G) and the testes (spermatic tubules, I). In the epididymis $Ccdc66$ reporter gene expression is present in both, $Ccdc66^{−/−}$ (J) and $Ccdc66^{+/+}$ (J´), in the latter reflecting endogenous β-galactosidase activity. Negative control staining of the other tissues of the age-matched the $Ccdc66^{+/+}$ mouse displays no labeling (A´ - I´, for tissue identification see respective description A - I). As representative negative control for plexus the large intestine of the $Ccdc66^{+/+}$ mouse is shown (D´). 25 μm cryosections. Scale bar in A, A´, C, D, E, B, B´ - 50 μm, in F, F´, D´, J, J´ - 100 μm, G, G´, H, G´/H´ I - 200 μm, enlargement in G - 50 μm.
Figure 37: Indistinct Ccdc66 reporter gene expression in the skeletal muscle and tongue of the Ccdc66⁻/⁻ mouse (adult). Ccdc66 reporter gene expression is detectable in the skeletal muscle (Musculus tibialis anterior, A) and the tongue (B) of the Ccdc66⁻/⁻ mouse, but not localized to defined structures due to a diffuse signal. X-gal staining of the Ccdc66⁺/⁺ muscle (A') and tongue (B') displays no labeling. 25 µm cryosections. Scale bar 100 µm.

Tissues without detectable Ccdc66 reporter gene expression are presented in figure 38 and encompass thymus (figure 38 A (Ccdc66⁻/⁻), A' (Ccdc66⁺/⁺)), spleen (figure 38 B (Ccdc66⁻/⁻), B' (Ccdc66⁺/⁺)) and liver (figure 38, C (Ccdc66⁻/⁻), C' (Ccdc66⁺/⁺)). Ccdc66 reporter gene expression levels of all investigated tissues with respective structures are summarized in table 9.
Figure 38: *Ccdc66* tissues without detectable *Ccdc66* reporter gene expression (adult). *Ccdc66* reporter gene expression is not detectable in thymus (A), spleen (B) and liver (C). X-gal treatment of respective the *Ccdc66* tissues (A' - C') displays no labeling either. 25 µm cryosections. Scale bar 100 µm.

These results confirm and precisely characterize the location of *Ccdc66* reporter gene expression in tissue sections, like in epithelial cells of the lung, fallopian tube and sperm cells, and besides of that in neural cells of the plexus of the digestive system. Furthermore the gap junction connected myocytes show *Ccdc66* reporter gene expression. In the skeletal muscle (and tongue) β-galactosidase activity is present, but could not yet be localized to a certain anatomical structure. Thymus, spleen and liver do not display *Ccdc66* reporter gene expression. These findings suggest that *Ccdc66* expression might play a role beside the retina and brain.
**Table 9:** *Ccdc66* reporter gene expression in extra retinal and extra cerebral tissues of the *Ccdc66*<sup>-/-</sup> mouse (- not detected, + moderate intensity, ++ intense)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Signal intensity of <em>Ccdc66</em> reporter gene expression in the <em>Ccdc66</em>&lt;sup&gt;-/-&lt;/sup&gt;</th>
<th>Region of <em>Ccdc66</em> reporter gene expression</th>
<th>Cells located in the region of X-gal staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>+</td>
<td>bronchi, bronchioles</td>
<td>ciliated cells, secretory cells</td>
</tr>
<tr>
<td>Myocardium</td>
<td>+</td>
<td>myocardium</td>
<td>myocytes</td>
</tr>
<tr>
<td>Kidney</td>
<td>+</td>
<td>tubules</td>
<td>ciliated tubular cells</td>
</tr>
<tr>
<td>Small intestine</td>
<td>+</td>
<td>nerve plexus</td>
<td>neurons, glial cells</td>
</tr>
<tr>
<td>Large intestine</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stomach</td>
<td>+</td>
<td>follicles</td>
<td>oocyte and surrounding cells</td>
</tr>
<tr>
<td>Fallopian tube</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampulla</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infundibulum/ intramural segment</td>
<td>+</td>
<td><em>tunica mucosa</em></td>
<td>ciliated epithelial cells</td>
</tr>
<tr>
<td>Testes</td>
<td>++</td>
<td><em>seminiferous tubules</em></td>
<td>maturing sperm cells</td>
</tr>
<tr>
<td>Epididymis</td>
<td>endogenous β-galactosidase expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle (musculus tibialis anterior)</td>
<td>+</td>
<td></td>
<td>indistinct</td>
</tr>
</tbody>
</table>
5.4 Discussion

The screen for Ccdc66 expression sites outside the retina and brain in a variety of tissues revealed X-gal staining in several organs with some structures that share common features. Moderate to strong Ccdc66 reporter gene expression is present in the regions where ciliated cells are located, among those the lung bronchi and bronchioles coating epithelia that consist of basal cells, ciliated and secretory cells (Kotton & Morrisey 2014) as well as the kidney tubules coated by the ciliated cells that are common targets in cilia associated disorders (reviewed in Pan et al. 2005; Schock and Perrimon 2002). Furthermore, the tunica mucosa of the fallopian tube in mammals is built of secretory and ciliated cells necessary for the ovum transport (Rocca et al. 1989; Halbert et al. 1989; Halbert et al. 1976) and in the testes the sperm cells that have a single cilium are marked by X-gal staining. The paralleled appearance of Ccdc66 reporter gene expression and the presence of cilia in these regions were already demonstrated for retinal photoreceptors, olfactory sensory neurons and the brain ventricle ependyma (3.1.3.3). These findings point into the direction that this could be a retinal, extra-retinal and extra-cerebral cumulatively common characteristic for Ccdc66 expression, although perhaps not the only and obligatory criterion that allows to predict Ccdc66 expression (see general discussion). Furthermore, in the myocytes of the myocardium (probably corresponding to the gap junction) and in the plexus that comprise nerve cells and supporting glia cells (reviewed in Neunlist et al. 2014), Ccdc66 reporter gene expression is present. The technical implementation of staining the skeletal muscle and tongue should be improved in order to identify the structures where reporter gene expression can be detected. In these structures, location in neural innervating structures could be possible, but also, as evidenced by compartment localization Ccdc66 can be suspected to play a role in the cytoskeleton scaffold.
Chapter 6 - General discussion

6.1 Summary of presented results

The functional characterization of \textit{Ccdc66} gene and protein expression contributes to the understanding of the underlying retinal degeneration mechanism in the \textit{Ccdc66}^{-/-} mouse and elucidates the general role of \textit{Ccdc66} gene and products. By the detection of \textit{Ccdc66} RNA and \textit{Ccdc66} reporter gene expression in the \textit{Ccdc66}^{+/+} and \textit{Ccdc66}^{-/-} P17 mouse, \textit{Ccdc66} expression was localized in several retinal layers (INL, GCL; reporter gene expression further in the OPL) in addition to the previously known appearance in the photoreceptor cells (Gerding et al. 2011). During postnatal development \textit{Ccdc66} reporter gene expression was detected in the IS of the photoreceptor cells, presumably the borders of the INL, the plexiform layers and GCL at all investigated stages (P4, P10, P17, 1 month, 1.5 months and > 10 months) with varying degrees of intensity in plexiform layers (stronger up to P17) and IS (stronger from P17 on). A YTH screen with five different CCDC66 constructs against a retinal cDNA library yielded two possible interaction partners of protein CCDC66: Eps8 (Epidermal growth factor receptor kinase substrate 8) and Mpdz (Multiple PDZ domain protein) that are directly and indirectly involved in cytoskeleton remodelling.

Investigation of \textit{Ccdc66} RNA and \textit{Ccdc66} reporter gene expression in the P17 \textit{Ccdc66}^{+/+} and \textit{Ccdc66}^{-/-} mouse brain revealed additional \textit{Ccdc66} expressing regions and respective layers (olfactory bulb, hippocampus and cerebellum). Accordingly, \textit{Ccdc66}^{+/+} and \textit{Ccdc66}^{-/-} showed comparable \textit{Ccdc66} ISH signals mainly in the same regions. Strongest expression signals were revealed in the olfactory nerve layer, the molecular layer of the dentate gyrus and the brain ventricle coating ependyma. A detailed review of \textit{Ccdc66} reporter gene expression in the olfactory bulb, adjacent olfactory epithelium and hippocampus during postnatal brain maturation (stages mentioned above) revealed relatively constant expression in the olfactory sensory neurons of the olfactory epithelium, olfactory nerve layer, granule and mitral layer of the olfactory bulb, pyramidal and granule cell layer of the hippocampus and the brain ventricle ependyma. Furthermore, along the rostral migratory stream intense β-galactosidase staining was displayed. Development-related changes in \textit{Ccdc66} reporter gene expression were detected in the outer plexiform layer of the olfactory bulb, where the signal decreased over time. In contrast, in the glomeruli of the
olfactory bulb, as well as in the hippocampal stratum radiatum and dentate gyrus molecular layer Ccdc66 expression decreased and spatially varied over time. These particular sites of Ccdc66 expression were additionally analyzed at the ultra-structural level by electron microscopy in over 10 months-old Ccdc66+/+ and Ccdc66−/− mice and revealed clear signs of degeneration in the olfactory bulb, while the hippocampal pyramidal layer of CA1 and dentate gyrus granule cells, as well as the ependymal cells did not show a difference between Ccdc66−/− and Ccdc66+/+ mice.

A cellular fractionation of retinal and brain extracts (of > P17 wild type mice) with Western blot and immune detection reveals the presence of CCDC66 in the nuclear and cytoskeleton compartment in retina and brain, to a lesser extent in retinal cytoplasm. This cellular distribution was supported by double immunofluorescence of CCDC66 in conjunction with antibodies selectively for neurons, oligodendrocytes, astrocytes and undifferentiated progenitors in a neurospheres derived mixed neural culture (at embryonic days 12.5 of wild type mouse neurospheres; differentiated for 7 days). CCDC66 signals were detected in soma, processes and in or in close proximity to the nuclei mainly in oligodendrocytes and with fainter staining in the other cell types.

A screen of Ccdc66 reporter gene expression in extra-retinal and extra-cerebral tissues (in 1 month-, 1.5 months- and >10 months-old Ccdc66−/− and Ccdc66+/+) discovered intense β-galactosidase activity in the regions of ciliated epithelia or cells (lung bronchi and bronchioles, tunica mucosa of the fallopian tube, tubules of the kidney and sperm cells), and moderate in peripheral neural cells (intestine, stomach plexus), and in the myocytes of the Ccdc66−/− mouse myocardium. Skeletal muscle and tongue displayed diffuse β-galactosidase activity. No reporter gene expression could be detected in liver, thymus and spleen, but endogenous β-galactosidase activity in the epididymis.

6.2 Concluding discussion and open questions

The summarized results and related specific open questions are discussed in detail in respective chapters. Hence, in the following only initially posed general research questions regarding the role of Ccdc66 are addressed.
What is the role of *Ccdc66* expression in the pathology of slow retinal degeneration?

Does *Ccdc66* expression in extra-retinal organs indicate additional roles in protein function?

The investigation of the retinal *Ccdc66* expression showed that the role of *Ccdc66* is not restricted to the obviously affected photoreceptors, but encompassed several retinal layers (chapter 2.1). The effects of the transgene *Ccdc66* expression, especially in the plexiform layers, where it parallelizes the establishment of synaptic contacts, have to be assessed in the future, which might clarify whether synaptogenesis might also be involved in the degeneration process. The OPL was already monitored by EM at the age of three months where the OPL was already destroyed in *Ccdc66*<sup>-/-</sup> mice (not shown). Earlier stages of the plexiform layers were not been assessed yet, but would be interesting to be investigated, because of the occurrence of reporter gene expression as early as P4 prior to the formation of the outer segments. The identification of the putative CCDC66 interaction partners Eps8 and Mpdz, that are involved in cytoskeleton dynamics like neurite outgrowth as well as stabilization, scaffolding and anchoring mechanisms (chapter 2.2) further supports the hypothesis of a role for CCDC66 as stabilizing protein. The association of *Ccdc66* reporter gene expression with cytoskeleton stabilizing and dynamical properties was further strengthened by the predominant detection of the protein in the cytoskeleton compartment in retina and brain (chapter 4) and the parallelization of *Ccdc66* reporter gene expression along with differentiation as well as migration processes in the brain (chapter 3.1).

*Ccdc66* reporter gene expression was detected during the time period of neurite maturation in the hippocampal *stratum radiatum* and dentate gyrus molecular layer as well as in the olfactory bulb glomeruli (integration of interneurons in the glomeruli synaptic circuit). Furthermore, adult cellular migration like the interneuron migration through the rostral migratory stream to the olfactory bulb and from the outer to the inner molecular layer in the dentate gyrus are possible processes, where *Ccdc66* reporter gene expression is parallelized with. Neurite outgrowth, differentiation and migration are regulated by RhoGTPases pathways through their impact on actin/cytoskeleton dynamics (reviewed in Etienne-Manneville and Hall 2002) that are affected by Eps8 and Mpdz (see 2.2.4). In case there is a dependency of *Ccdc66*
expression and a RhoGTPase pathway, based on these results one cannot decide in which relation to or which part of the process of the cytoskeleton dynamics would be impacted. First the hypothesis of Ccdc66 in neurite outgrowth, maturation or differentiation should be investigated by outgrowth assays (for example Smit et al. 2003) as well as the assessment of neurite arborization and spine morphology for example by a modified Golgi method in hippocampal CA1 pyramidal neurons (Patro et al. 2013).

Investigation of Ccdc66 expression in the brain points to the same category of function like the extensive exploration of Ccdc66 expression in the retina and furthermore opened a new point of view regarding the general characterization of the Ccdc66<sup>-/-</sup> model by the ultra-structural investigation of Ccdc66 expression sites in the brain (chapter 3.2). The degeneration of the olfactory nerve fibers could hint to an additional pathological phenotype that would promote the state that the Ccdc66-deficient mouse is rather a model for a syndromic type of retinal degeneration than for isolated RP, as initially suggested. For example, the emergence of olfactory dysfunction and mental retardation is suggested to appear in some cases of LCA, even though the latter is also discussed as a secondary effect due to sensory deprivation or is suggested to be excluded in the strict LCA definition (McEwen et al. 2007; Nickel & Hoyt 1982). Also in Kallmann syndrome anosmia is one of the main features beside the sterility (Barraud et al. 2013), and there is evidence of reduced olfactory capacities in cases of the BBS (Iannaccone et al. 2005), where mental retardation or learning disabilities are also reported in patients and an animal model (Baker et al. 2011; Keppler-Noreuil et al. 2011; Davis et al. 2007).

The extra-retinal and extra-cerebral Ccdc66 reporter gene expression at sites that are often associated with ciliopathies (reviewed in Fry et al. 2014) further stresses the possibility of a syndromic variant of retinal degeneration in the Ccdc66<sup>-/-</sup> mouse. Affection of Ccdc66 reporter gene expressing sites thereby obviously may vary, since for example the intense expression in the mouse reproductive structures does not critically affect the reproductive capabilities of the Ccdc66<sup>-/-</sup> mice in both sexes. The functional investigation of Ccdc66 reporter gene expression sites with primary focus on the olfactory system should be assessed by behavioral studies. Further tests should survey the learning and memory performance of Ccdc66<sup>-/-</sup> mice due to the
development-related changes in Ccdc66 reporter gene expression in the hippocampus. The extra-retinal and extra-cerebral expression of the trapped Ccdc66 gene does not reveal drastic effects at first sight (chapter 5), but the tissues should be investigated in detail on the morphological level. With regard to the syndromic aspect as well as the link to degeneration in another sensory system, the investigation of Ccdc66 impact on the acoustic system would be also interesting, not least because of Eps8, one of the possible interaction partners, is located at the tip of the cochlear hair cells.

6.3 Conclusions

Based on presented findings, Ccdc66 gene expression might also play a privotal role in retinal structures besides the photoreceptors and moreover in extra-retinal tissues. Ccdc66 expression in part parallels neurite maturation or synaptogenesis in the retina and neuronal structures (hippocampus and olfactory bulb). In addition, expression for example in the brain ventricle ependyma, olfactory epithelium and sperm cells indicate expression in cells with specialized cytoskeleton structures like cilia. The protein is furthermore localized inter alia in the cytoskeleton compartment and its identified potential interaction partners Mpdz and Eps8 are involved in structural stability and dynamics partly associated with RhoGTPase pathways. All together it is tempting to assume that protein CCDC66 might serve as a structural component in highly specialized regions like for example retinal photoreceptors. In the olfactory sensory neurons the lack of Ccdc66 expression apparently leads to degeneration of axonal terminals too. That in turn might indicate an additional pathological phenotype in the brain and allows further insights into gene function and pathology in human disease. In humans no CCDC66 disease causing mutation was yet identified in RP or LCA patients (Gerding et al. 2011). The extension of a potential functional role of Ccdc66 expression to central structures and extra-retinal and extra-cerebral organs proposes to enlarge the group of patients that are advisable to be tested for a CCDC66 mutation, regarding syndrome types including retinal degeneration for example Kallmann syndrome or BBS, or LCA with additional symptoms (see 6.2).
7. References


Berger, W. et al., 2010. The molecular basis of human retinal and vitreoretinal diseases. Progress in retinal and eye research, 29(5), pp.335–75.


Boettner, B. et al., 2000. The junctional multidomain protein AF-6 is a binding partner of the Rap1A GTPase and associates with the actin cytoskeletal regulator profilin. Proceedings of the National Academy of Sciences of the United States of America, 97(16), pp.9064–9.

References


References


Ma, H. et al., 2014. Suppressing thyroid hormone signaling preserves cone photoreceptors in mouse models of retinal degeneration. *Proceedings of the


References


References


References


8. Appendices

8.1 Buffers and solutions

Instruments or material not mentioned or specified in respective chapters correspond to standard laboratory equipment. All additional chemicals for below-mentioned buffer were suitable for molecular biology purposes and purchased from the following companies/manufacturer: AppliChem, Carl Roth GmbH & Co. KG., Sigma-Aldrich®, Pharmacia, Gibco®, Life technologies, Inc., Roche, Merck Millipore, J.T. Baker (for all chapters).

8.1.1 Chapter 2.1

<table>
<thead>
<tr>
<th>Method</th>
<th>Buffer/solution</th>
<th>Chemicals/composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose-gel electrophoresis</td>
<td>10x TBE, stock solution; for use dilute 1:10 with H₂O bidest</td>
<td>1 M Tris base</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 M boric acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.02 M EDTA</td>
</tr>
<tr>
<td>In situ hybridization</td>
<td>DEPC water; for the preparation of all required buffers for RNA analyses</td>
<td>0.02% DEPC in H₂O bidest</td>
</tr>
<tr>
<td></td>
<td>10x PBS, stock solution; for use dilute 1:10 with DEPC water</td>
<td>100 mM Na₂HPO₄</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mM NaH₂PO₄</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,4 M NaCl (pH 7.4)</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS containing 0.1% tween</td>
<td></td>
</tr>
<tr>
<td>PFA</td>
<td>4% PFA in 1x PBS</td>
<td></td>
</tr>
<tr>
<td>Acidic ethanol</td>
<td>95% EtOH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5% acetic acid</td>
<td></td>
</tr>
<tr>
<td>Proteinase K, stock solution;</td>
<td>200 µg /ml H₂O</td>
<td></td>
</tr>
<tr>
<td>for use dilute 1:200 in proteinase K digestion buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteinase K digestion buffer</td>
<td>100 mM Tris-HCl (pH 8.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 mM EDTA</td>
<td></td>
</tr>
<tr>
<td>TEA buffer</td>
<td>0.1 M triethanolamine (TEA, pH 8.0)</td>
<td></td>
</tr>
<tr>
<td>TEA/AAA</td>
<td>dilute 125 µl acetic acid anhydride (AAA) in 50 ml TEA buffer</td>
<td></td>
</tr>
</tbody>
</table>
| **50x denhardt’s** | 10 mg/ml Ficoll 400  
10 mg/ml Polyvinylpyrollidone  
10 mg/ml BSA, RNase free |
|-------------------|---------------------------------------------------------------|
| **hybridization mix** | 50% formamide  
0.3 M NaCl  
20 mM Tris-HCl pH 8.0  
1 mM EDTA  
x denhardt’s  
10% dextran sulfate  
500 µg/ml tRNA |
| **20x SSC** | 3 M NaCl  
0.3 M Na-citrate  
(pH 7.0) |
| **RNaseA stock solution** | 10 mg/ml in water  
1 mM EDTA  
500 mM NaCl |
| **5%, 2% NGS in PBS-T** | 5% (2%) NGS in PBS  
with 0.1% tween |
| **alkaline phosphatase digestion buffer** | 0.1 M Tris-HCl  
0.1 M NaCl  
50 mM MgCl₂  
(pH 9.0) |
| **NBT stock solution** | 75 mg nitro blue tetrazolium/ ml 70% dimethylformamide/ 30% H₂O |
| **bromo-chloro-indolyl-phosphate (BCIP) stock solution** | 50 mg/ml in dimethylformamide |
| **X-gal staining** | **X-gal staining solution**  
500 mM K₃Fe(CN)₆  
Potassium Ferrocyanide, 100 µl  
500 mM K₄Fe(CN)₆  
Potassium Ferricyanide, 100 µl  
100 mM MgCl₂, 200 µl  
20 mg/ml X-β-gal in DMF (dimethylformimid), 500 µl  
1 x PBS, 9.1 ml |
| **Neutral red staining solution** | 0.3% (w/v) neutral red  
0.1 M acetic acid  
in H₂O bidest |
### 8.1.2 Chapter 2.2

<table>
<thead>
<tr>
<th>Method</th>
<th>Buffer/solution</th>
<th>Chemicals/Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transformation of <em>E.coli</em></td>
<td>LB-medium</td>
<td>0,5% (w/v) yeast extract</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,5% (w/v) NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1% (w/v) peptone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,5% (w/v) agar (optional)</td>
</tr>
<tr>
<td></td>
<td>ampicillin stock solution</td>
<td>0,5 g ampicillin</td>
</tr>
<tr>
<td></td>
<td>dilute 500 µl/l LB medium</td>
<td>0,33 g sodium carbonat</td>
</tr>
<tr>
<td></td>
<td>(50 µg/l)</td>
<td>ad 5 ml H$_2$O bidest</td>
</tr>
<tr>
<td>Plasmidisolation from <em>E.coli</em></td>
<td>lysisbuffer</td>
<td>50 mM Tris-HCl (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>62,5 mM EDTA (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0,4% (w/v) Triton-X 100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,5 M LiCl</td>
</tr>
<tr>
<td>Chemical transformation of yeast</td>
<td>10x TE (dilute 1:10 with H$_2$O</td>
<td>1 M Tris (pH 8.0)</td>
</tr>
<tr>
<td></td>
<td>bident for use)</td>
<td>0,5 M EDTA (pH 8.0)</td>
</tr>
<tr>
<td></td>
<td>10x LiAc (dilute 1:10 with H$_2$O</td>
<td>1 M LiAc</td>
</tr>
<tr>
<td></td>
<td>bident for use)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1x TE/1x LiAc/PEG</td>
<td>1x TE/1x LiAc in 50% PEG</td>
</tr>
<tr>
<td>Plasmid isolation from yeast</td>
<td>yeast lysis buffer</td>
<td>10 mM Tris-HCl (pH 8.0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mM NaCl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mM EDTA,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2% Triton X-100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1% SDS</td>
</tr>
</tbody>
</table>

All media required for the YTH screen were obtained from Clontech Laboratories, Inc. available at (https://www.clontech.com/).
### 8.1.3 Chapter 3.2

<table>
<thead>
<tr>
<th>Method</th>
<th>Buffer/solution</th>
<th>Chemicals/Composition</th>
</tr>
</thead>
</table>
| Electron-microscopy     | rinsing solution                     | 48 mM NaOAc<br>154 mM NaCl<br>4 mM KCl<br>1.36 mM CaCl<br>0.25 mM MgCl$_2$
4% Dextran GDRI (Pharmacia)<br>10 mM nitroprussid natrium (pH 7.4) |
|                         | perfusion solution                   | 2% glutaraldehyde in 0.1 M cacodylatebuffer (pH 7.4)                                   |
|                         | fixation solution                    | 2.5% glutaraldehyde<br>1% PFA<br>0.1 M sodium phosphate                              |
|                         | osmium tetroxide                     | 4% OsO$_4$ in H$_2$O bidest                                                        |
|                         | araldite mixture                     | araldite:DDSA (26:24)+2% DMP30 (2,4,6-Tris (dimethylaminomethyl)phenol)             |
|                         | propylene oxide/ araldite mixture    | propylene oxide: araldite mixture + 3% DMP30                                          |
|                         | toluidine blue staining solution     | 1% toluidine blue<br>1% sodium borate<br>30% saccharose (pH 9.4)                      |
### 8.1.4 Chapter 4

<table>
<thead>
<tr>
<th>Method</th>
<th>Buffer/solution</th>
<th>Chemicals/Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS-PAGE</td>
<td>Running buffer</td>
<td>0.025 M Tris, 0.192 M glycine, 0.1% SDS</td>
</tr>
<tr>
<td></td>
<td>10% PAA separating gel</td>
<td>30% PAA, 3.2 ml 1.5 M Tris (pH 8.8), 3.2 ml 10% SDS, 125 µl H₂O, 5.2 ml 11 µl TEMED 110 µl APS</td>
</tr>
<tr>
<td></td>
<td>5% PAA stacking gel</td>
<td>30% PAA, 833 µl 0.5 M Tris (pH 6.8), 1.25 ml 10% SDS, 50 µl H₂O, 2.9 ml 7.5 µl TEMED 50 µl APS</td>
</tr>
<tr>
<td></td>
<td>5x Laemmli buffer</td>
<td>31% (v/v) 0.5 M Tris (pH 6.8) 10% (w/v) SDS 50% (v/v) glycerol</td>
</tr>
<tr>
<td>Western blotting</td>
<td>transfer buffer</td>
<td>0.1% bromphenol blue 48 mM Tris 39 mM glycine 0.04% (w/v) SDS 20% (v/v) methanol</td>
</tr>
<tr>
<td>Cell culture</td>
<td>PBS stock solution (dilute 1:10 for use)</td>
<td>100 mM Na₂HPO₄ 100 mM NaH₂PO₄ 1.4 M NaCl (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td>10% NGS in PBS-TX 0.5%</td>
<td>10% NGS in 1x PBS with 0.5% triton</td>
</tr>
<tr>
<td>neurosphere medium</td>
<td>DMEM/F12 1:1 (#41966 Gibco, #N4888 Sigma)</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>-------------------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2% (v/v) B27 (#17504-044, Gibco)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 units/ml penicillin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µg/ml streptomycin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200 µg/ml L-glutamine</td>
<td></td>
</tr>
<tr>
<td>mixed neural culture medium</td>
<td>DMEM (#41966 Gibco)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 units/ml penicillin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 µg/ml streptomycin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10% (v/v) HS</td>
<td></td>
</tr>
</tbody>
</table>
8.2 Curriculum vitae

Personal information
Name Schreiber, Sabrina
Date & place of birth 17.07.1985, Brilon, Germany
E-Mail Sabrina.Schreiber@rub.de

Education
08/1996 - 06/2005 Gymnasium Petrinum Brilon
06/2005 Higher education entrance qualification
10/2005 - 12/2010 Biology studies at the Ruhr-University Bochum (RUB)
12/2010 Graduation as Diploma-Biologist
Examination subjects: Neurobiology, Zoology, Microbiology, Human genetics
Diploma thesis: completed in the Department of Human Genetics, Medical faculty, RUB „Das mit generalisierter progressiver Retina-Atrophie assoziierte Protein CCDC66 - funktionelle Charakterisierung im Maus-Modell“
10/2011 - 03/2015 PhD- Study in Neuroscience in the IGSN (International Graduate School of Neuroscience), RUB
10/2011 - 09/2014 Scholarship of the IGSN
10/2014 – 01/2015 Scholarship of the Wilhelm and Günter Esser foundation
10/2014 – today Scholarship supported by the funds center (2168210042, LOM; department 7, Administration of the medical facility)
8.3 **List of publications**

**Co-authorships in peer reviewed journals**


**Conference articles**

**Protein CCDC66 in retinal degeneration** (Poster)

Sabrina Schreiber¹, Denis A. Akkad¹, Elisabeth Petrasch-Parwez², Jörg T. Epplen¹, Wanda M. Gerding¹

¹Department of Human Genetics, Faculty of Medicine, Ruhr-University Bochum, Germany, ²Department of Neuroanatomy and Molecular Brain Research, Ruhr-University Bochum, Germany

Presented at the
- SfN conference, Nov 9-13, San Diego, California, USA
- 9th FENS Forum of Neuroscience, Jul 5-9, Milan, Italy 2014
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Warm thanks to my dear friends that were and are always on my side from the day I met them that is partly many years ago. These include especially Gianna and my other lovely friends from study times, as well as from childhood days. They still accompany me on my way and I am deeply thankful for knowing them, even though I cannot name all explicitly here. The PhD time yield new friends as Anja and Cristina whom I want to thank for great support and wonderful time while sharing this experience together.

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