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REGULATION AND FUNCTION OF CILIARY DYSLEXIA CANDIDATE GENES

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Front cover image shows cilia of renal epithelial cells in human kidney tubuli, visualized with anti-acetylated α-tubulin (red) and anti-DCDC2 (green) antibodies. Cellular nuclei are shown in blue.

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Regulation and function of ciliary dyslexia candidate genes

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To my parents

Elisabeth & Markus

“This inability is so remarkable, and so pronounced, that I have no doubt it is due to some congenital defect”

W. Pringle Morgan, 1896, about dyslexia

„Es wäre auch an die Möglichkeit zu denken, dass die zarte Geissel [...] als eine Art Sinnesorgan der Zelle wirkt [...].“

“It would also be possible to think that the tender flagellum [...] acts as a kind of sensory organ of the cell [...].”

K.W. Zimmermann, 1898, about cilia

ABSTRACT

Dyslexia is defined as an unexpected difficulty in reading despite normal intelligence, senses and instruction. It is the most common learning disability with estimated 5-10% of the population affected. Its heredity is estimated to about 40-60%. Despite the established heredity of the condition, it has been very challenging to pinpoint the underlying genes. In the past 15 years, a number of dyslexia candidate genes have been suggested. A handful of them have been replicated in several studies, including *DYX1C1, DCDC2* and *KIAA0319*. More recently, the very same genes have been independently associated to functions of the cilium. Cilia are microtubule-based organelles present on the surface of most eukaryotic cells.

The aim of this thesis was to investigate the molecular functions of ciliary dyslexia candidate genes and their role at the cilium.

In **paper I**, we found X-box motifs in the promoter regions of *DYX1C1*, *DCDC2* and *KIAA0319* and showed that they are functional and able to bind ciliogenic RFX transcription factors. Knockdown of certain RFX transcription factors altered the expression of *DYX1C1* and *DCDC2,* but not *KIAA0319*. Overall, we strengthened the evidence for *DYX1C1* and *DCDC2* as ciliary genes.

In **paper II**, we identified *DCDC2* as a causative gene for nephronophthisis-related ciliopathy (NPHP-RC) with loss-of function mutations present in two affected families. We observed localization of DCDC2 to the ciliary axoneme of affected organs and demonstrated a crucial role of the Wnt pathway in the pathogenesis of NPHP-RC. 3D modeling in spheroids and *in vivo* modeling in zebrafish confirmed these observations.

In **paper III**, we identified CPAP as an interacting partner of both DYX1C1 and DCDC2. In addition, we observed genetic pathway synergy between *DYX1C1* and *DCDC2* using zebrafish and a human ciliated cell model.

In **paper IV**, we performed transcriptomics on differentiating human neuroepithelial stem cells and characterized the expression of dyslexia candidate genes. We found that some dyslexia candidate genes are upregulated during human neuronal differentiation. Remarkably, we identified the group of ciliary genes as the major group of upregulated genes. In addition, we showed that cilia are present on the surface of neuronal cells throughout differentiation.

In **paper V**, we asked whether dyslexia and ciliopathies might have a common genetic origin by investigating the genome of two individuals with *situs inversus* and dyslexia. We identified rare variants in dynein heavy chain genes likely causing their *situs inversus* phenotype. Their involvement in dyslexia remains to be determined.

In conclusion, the work conducted within this thesis strengthened and expanded on the role of *DYX1C1* and *DCDC2* at the cilium and in ciliopathies and identified the group of ciliary genes as a major gene class in human neuronal differentiation. A link between cilia and dyslexia remains elusive.

ORIGINAL ARTICLES INCLUDED IN THIS THESIS

I. Kristiina Tammimies*, **Andrea Bieder***, Gilbert Lauter*, Debora Sugiaman-Trapman, Rachel Torchet, Marie-Estelle Hokkanen, Jan Burghoorn, Eero Castrén, Juha Kere, Isabel Tapia-Páez, Peter Swoboda
Ciliary dyslexia candidate genes *DYX1C1* and *DCDC2* are regulated by Regulatory Factor (RF)X transcription factors through X-box promoter motifs
The FASEB Journal, 2016 Oct;30(10):3578-3587

II. Markus Schueler*, Daniela A. Braun*, Gayathri Chandrasekar*, Heon Yung Gee*,Timothy D. Klasson, Jan Halbritter, **Andrea Bieder**, Jonathan D. Porath, Rannar Airik, Weibin Zhou, Joseph J. LoTurco, Alicia Che, Edgar A. Otto, Detlef Böckenhauer, Neil J. Sebire, Tomas Honzik, Peter C. Harris, Sarah J. Koon, Meral Gunay-Aygun, Sophie Saunier, Klaus Zerres, Nadina Ortiz Bruechle, Joost P.H. Drenth, Laurence Pelletier, Isabel Tapia-Páez, Richard P. Lifton, Rachel H. Giles, Juha Kere, Friedhelm Hildebrandt
***DCDC2* mutations cause a renal-hepatic ciliopathy by disrupting Wnt signaling**
American Journal of Human Genetics, 2015 Jan 8;96(1):81-92

III. **Andrea Bieder**, Gayathri Chandrasekar, Arpit Wason, Jay Gopalakrishnan, Juha Kere, Isabel Tapia-Páez
Genetic and protein interaction studies reveal pathway synergy between the ciliary dyslexia candidate genes *DYX1C1* and *DCDC2*
Manuscript

IV. **Andrea Bieder***, Masahito Yoshihara*, Shintaro Katayama, Kaarel Krjutškov, Anna Falk, Juha Kere, Isabel Tapia-Páez
Expression of dyslexia candidate genes and ciliary genes during human neuronal differentiation
Manuscript

V. **Andrea Bieder**, Elisabet Einarsdottir, Hans Matsson, Harriet E. Nilsson, Jesper Eisfeldt, Anca Dragomir, Martin Paucar, Tobias Granberg, Tie-Qiang Li, Anna Lindstrand, Juha Kere, Isabel Tapia-Páez
Rare variants in dynein heavy chain genes in two individuals with *situs inversus* and developmental dyslexia
Manuscript

*equal contribution

ADDITIONAL ARTICLES

I. Satu Massinen, Jingwen Wang, Krista Laivuori, **Andrea Bieder**, Isabel Tapia-Páez , Hong Jiao, Juha Kere
Genomic sequencing of a dyslexia susceptibility haplotype encompassing *ROBO1*
Journal of Neurodevelopmental Disorders, 2016 Jan 27;8:4

II. Nancy Y. Yu, **Andrea Bieder**, Amitha Raman, Enrichetta Mileti, Shintaro Katayama, Elisabet Einarsdottir, Bertil B. Fredholm, Anna Falk, Isabel Tapia-Páez, Carsten O. Daub, Juha Kere
Acute doses of caffeine shift nervous system cell expression profiles toward promotion of neuronal projection growth
Scientific Reports, 2017 Sep 13;7(1):11458

CONTENTS

LIST OF ABBREVIATIONS

BBS	Bardet-Biedl syndrome
cDNA	complementary DNA
CNV	copy number variation
Co-IP	co-immunoprecipitation
CPAP	centrosomal-P4.1-associated protein
DTI	diffusion tensor imaging
EMSA	electrophoretic mobility shift assay
FANTOM	functional annotation of the mammalian genome
(f)MRI	(functional) magnetic resonance imaging
GO	gene ontology
GWAS	genome wide association study
hESC	human embryonic stem cell
ICC/IHC	immunocytochemistry/immunohistochemistry
IDA	inner dynein arms
iPSC	induced pluripotent stem cell
JBS	Joubert syndrome
NES cell	neuroepithelial stem cell
NGS	next generation sequencing
NPHP/ NPHP-RC	nephronophthisis/ nephronophthisis-related ciliopathy
NSC	neonatal sclerosing cholangitis
ODA	outer dynein arms
PCD	primary ciliary dyskinesia
qRT-PCR	quantitative real time PCR
RFX TF	regulatory factor X transcription factor
RGC	radial glial cell
RNA-seq	RNA sequencing
RPE1	retinal pigment epithelial cells 1
Shh/Hh	(sonic) hedgehog
SH-SY5Y	subline of the neuroblastoma cell line SK-N-SH
siRNA/shRNA	small interfering/small hairpin RNA
SNP	single nucleotide polymorphism
SNV	single nucleotide variant
STED	stimulated emission depletion microscopy
TPR	tetratricopeptide repeat
WES	whole exome sequencing
WGS	whole genome sequencing
Wnt	wingless homologue (signaling pathway)

1 INTRODUCTION

1.1 HUMAN GENETICS

1.1.1 The human genome

The genetic information is stored in the double helical molecule deoxyribonucleic acid (DNA), which in humans is organized into 24 chromosomes. The genetic code is composed of the nucleotide bases adenine (A), cytosine (C), guanine (G) and thymine (T). The sequence of these bases determines the information stored within the genome and ultimately the phenotype of a being. It comprises around 26,000 protein coding and noncoding genes, regulatory and intergenic regions, and pseudogenes. The first draft of the human genome sequence was published in 2001 by the public effort The Human Genome Project (HUGO) and the company Celera, and was completed in 2003 (Lander et al., 2001; Venter et al., 2001). A decade later, the 1000 Genomes project had sequenced more than 1000 genomes of different populations for use as an openly available resource in an effort to map human genetic variation and contribute to the understanding of genetic disorders (The Genomes Project, 2012). Nowadays, next generation sequencing (NGS) is routinely performed for research purposes and starts to be used in the clinics. The 100,000 Genomes Project promises to bring genetic research yet to another level (Turnbull et al., 2018).

While it has become standard to sequence the human genome, the interpretation of its meaning has proved to be much more complex. Public efforts are now directed at understanding its functions: The Encyclopedia of DNA Elements project (ENCODE) is aiming at mapping all the functional elements in the genome such as genes, transcripts, transcriptional regulatory regions (Consortium, 2011). The Functional Annotation of the Mammalian Genome project (FANTOM) has built an expression atlas of a wide range of tissues at the promoter-level (Forrest et al., 2014). These large-scale projects have shed more light on the actual interpretation of the genome, but a lot remains to be discovered.

1.1.2 Genomic variation and genetic disorders

More than 99.7 % of the genome is identical among all human beings. A small portion of the genome, however, varies between individuals. There are different kinds of variation between human genomes. The most common ones are single nucleotide variations (SNVs) – often termed single nucleotide polymorphisms (SNPs). Copy-number variants (CNVs) are longer, defined sequences of DNA of which the number of copies varies between individuals. While most of those variations are likely harmless, a small fraction is pathogenic and contributes to a disease phenotype. A pathogenic SNV present at a very low frequency in the population is often called a mutation.

How do these variations affect the phenotype of individuals? Monogenic disorders and traits are caused by a single gene and often inherited in a Mendelian mode. Examples of such traits are the ABO blood group, which is determined by a single gene, or disorders such as cystic

fibrosis and sickle-cell anemia, which are caused by a mutation in one gene. On the other hand, polygenic or complex traits are caused by interplay of different genes, often along with environmental factors. Traits as for example eye color and height and disorders such as dyslexia are of complex nature. There is a continuous distribution of traits along the axis monogenic-complex (McCarthy et al., 2008). Most recently, a novel theory was proposed that complex traits are caused by every gene expressed in that tissue, the so called omnigenic hypothesis (Boyle et al., 2017).

The human genetics research field aims at identifying genetic variations underlying a particular phenotype. Generally, this question is addressed by either linkage or association studies. Genetic linkage relies on the principle of co-segregation and recombination in a family pedigree. Association studies compare two groups, one affected with a certain disease state and one unaffected. Susceptibility factors to complex disorders have mostly been identified by association studies, including genome-wide association studies (GWAS) (McCarthy et al., 2008). Association studies have proven useful to determine susceptibility genes in complex traits. However, in some instances, there can be Mendelian forms of phenotypes that are usually complex, which allows for candidate gene discovery. This strategy led for example to the identification of *DYX1C1* and *ROBO1* as dyslexia candidate genes (Hannula-Jouppi et al., 2005; Taipale et al., 2003) (see 1.3.2).

1.2 NEURONAL DEVELOPMENT AND MIGRATION

The brain consists of a large number of interconnected nerve cells. This complex network arises from an initial cell layer consisting of neural progenitors, the neuroepithelium. In the developing cerebral cortex, the ventricular zone contains neural progenitor cells, also called radial glial cells (RGCs) (Fig. 1). Cells differentiate into neurons and migrate forming the cortical plate. The cortical plate gives rise to the six-layered structure of the adult cerebral cortex.

There are two main modes of migration of neurons to their final destination in the cortex: the radial migration of cortical projection neurons and the tangential migration of interneurons from the ganglionic eminence to the cortex (Huang, 2009). Neurons migrating via radial migration are guided by the RGCs. The different layers are generated in an inside-first, outside-last manner: The first neurons that are generated settle in the deep cortical layers whereas later neurons migrate past them to populate the more superficial layers of the cortex. When progenitor cells enter the path of neurogenic division to start differentiation, they follow either of two modes of neurogenesis: In direct neurogenesis, the precursor cell divides, giving rise to one neuron and one RGC. In contrast, in indirect neurogenesis precursor cells undergo an additional cell division thereby giving rise to an intermediate progenitor cell (Fig. 1) (Kriegstein and Alvarez-Buylla, 2009).

Disruption of these processes leads to alterations in the brain and may subsequently lead to neurodevelopmental disorders. It has been proposed that dyslexia is a neurodevelopmental disorder with a possible origin in neuronal migration defects (Gabel et al., 2010; Galaburda et al., 2006).

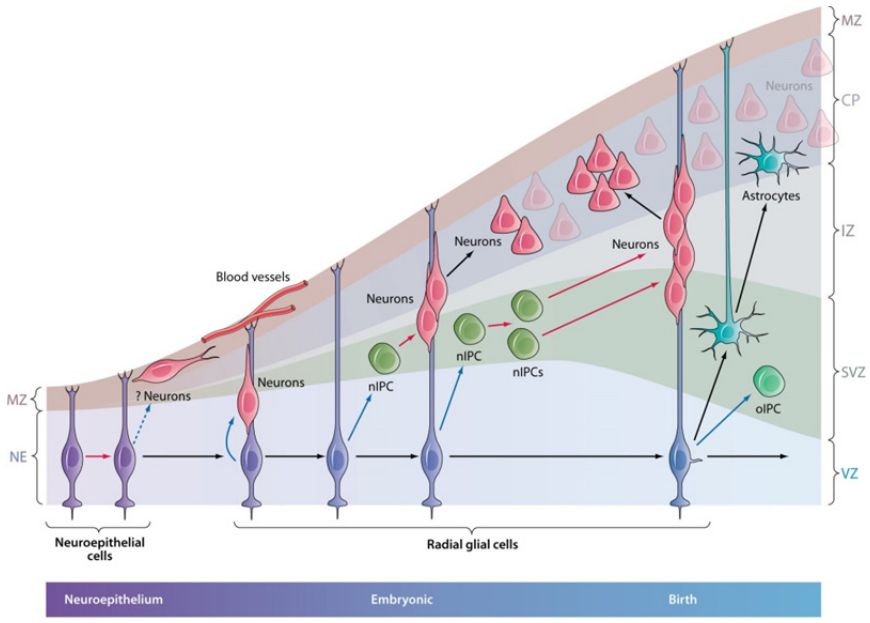


Figure 1: Neurogenesis during cortical development. RGCs divide to give rise to neurons or IPCs, either in a direct or an indirect way and migrate along radial processes to the CP. CP = cortical plate, IZ = intermediate zone, MZ = marginal zone, (n/o)IPC = (neurogenic/oligodendrocytic) intermediate progenitor cell, RG = radial glia, SVZ = subventricular zone, VZ = ventricular zone, arrows: blue = asymmetric division, red = symmetric division, black = direct transformation.

1.3 DYSLEXIA

1.3.1 What is dyslexia?

Dyslexia is defined as a difficulty in reading and writing despite normal intelligence, senses and proper education. One early account in the literature dates from 1896, where a 14-year old boy was reported to be “word blind” (Morgan, 1896). It is nowadays widely accepted as a distinct condition and is listed in the Classification of Mental and Behavioural Disorders by the World Health Organization ICD-10, code F81.0 as “Specific reading disorder” (World Health Organization, 2016). Dyslexia manifests itself from early schooling on and usually persists throughout education and adult life. It can have manifestations such as difficulties reading single words and spelling errors. Interestingly, dyslexia exists in all written languages – which can be as different as English and Chinese (Grigorenko, 2001). The percentage of dyslexia in the population can however vary - it is estimated to be about 5-12% depending on the exact definition of dyslexia (Schumacher et al., 2007). It is thereby the most common learning disability in children. There is also a clear gender bias observed in dyslexia with boys being affected more frequently than girls with a male: female ratio of about 2:1 (Rutter et al., 2004). Dyslexia has a high comorbidity with other learning difficulties, such as

arithmetic disabilities, attention-deficit hyperactivity disorder (ADHD) and specific language impairment (SLI) (Shaywitz and Shaywitz, 2008).

Diagnosis of dyslexia is usually based on standard tests measuring reading skills, taking into account IQ and age for the expected reading ability. Dyslexia is considered the extreme end of a normally distributed range of reading skills in the population so that the border between dyslexic and non-dyslexic might not be sharp (Shaywitz et al., 1992). Dyslexia research has been challenged by difficulties in defining the phenotype (Grigorenko, 2001). In order to increase the understanding of this condition, efforts from the fields of psychology, genetics and molecular biology are combined. There has been a lot of progress during the past decades in understanding dyslexia on a genetic, neurobiological and psychological level (Kere, 2014). Although there are very many different aspects to the condition, it is nowadays generally assumed that there is one underlying complex mechanism. There have been attempts to explain dyslexia with the help of unifying models, such as the phonological theory, the rapid auditory processing theory, the visual theory, the cerebellar theory and the magnocellular theory. However, none of them fully explains all aspects of dyslexia (Paracchini et al., 2016; Ramus et al., 2003).

1.3.2 Genetics of dyslexia

The heritability of dyslexia was suggested already at the end of the 19th century (Pringle Morgan, 1896). Today, there is very strong evidence of dyslexia being a highly heritable condition, which has been supported by twin studies, family studies and molecular genetic studies. Investigations on twins found a concordance rate of dyslexia in monozygotic twins between 68 and 100% and a concordance rate of dizygotic twins ranging from 20% to 55% (Grigorenko, 2001; Scerri and Schulte-Körne, 2010).

Early linkage analysis studies on pedigrees led to the identification of several dyslexia candidate gene loci (DYX), the first ones being DYX1 on chromosome 15, and DYX2 on chromosome 6. Today, several loci have been identified including the regions DYX1-DYX9 (Kere, 2014; Poelmans et al., 2011; Scerri and Schulte-Körne, 2010) (Table 1). For some linkage regions, the causative genes have been pinpointed (Carrion-Castillo et al., 2013). The first dyslexia candidate gene, *Dyslexia susceptibility 1 candidate* 1 (*DYX1C1,* official gene name *DNAAF4,* previously named *EKN1*), cloned in 2003 by Taipale and colleagues (Taipale et al., 2003), is located at the DYX1 locus on chromosome 15q. It was identified in a family in which a translocation breakpoint in that gene co-segregated with dyslexia. In addition, two SNPs -3G/A (rs3743205) and 1249G/T (rs57809907) were significantly associated with dyslexia in a small Finnish cohort. A number of follow-up studies have produced positive and negative replications (Scerri and Schulte-Körne, 2010). At the DYX2 locus, two genes have been identified as the major dyslexia candidates, *Doublecortin domain containing 2 (DCDC2)* and *KIAA0319*, both positively and negatively replicated in a number of studies (Meng et al., 2005; Paracchini et al., 2006; Schumacher et al., 2006). At the DYX5 locus, *Roundabout guidance receptor 1* (*ROBO1)* has been pinpointed as the main candidate (Hannula-Jouppi et al., 2005).

More recently, GWAS for dyslexia have been published. One study combined the data from several European countries to form a cross-linguistic cohort – called NeuroDys – however, the previously described dyslexia variants could not be replicated (Becker et al., 2014). Other GWAS have found association with the genes *ABCC13, DAZAP1, CDC2L1*, *CDC2L2, LOC728661*, *RCAN3, ZNF385D, COL4A2, CCDC136/FLNC* and *RBFOX2* (Eicher et al., 2013; Gialluisi et al., 2014; Luciano et al., 2013). In addition, the role of CNVs in dyslexia has been investigated. Deletions and duplications spanning the genes *DOCK4, PCNT, DIP2A, S100B, PRMT2, GABARAP, NEGR1, ASIC2, DCDC5, CNTNAP2, PCDH11X* and *KANSL1* have also been implicated (Pagnamenta et al., 2010; Poelmans et al., 2009; Veerappa et al., 2013a, b, 2014). These results, however, await replication studies and it thus remains to be determined how important these genes are for dyslexia heritability.

Altogether, despite the strong evidence in favor of dyslexia being heritable, the identification of causative genes remains challenging.

Table 1: Overview of dyslexia candidate genes. Genetic loci for dyslexia that have been recognized as replicated by the Human Gene Nomenclature Committee (http://www.genenames.org/). The loci are named in the order of discovery as DYX1 through DYX9. The genes are listed in chronological order of discovery.

Gene	**Chromosome, locus**	**Degree of reliability**	**Proposed mechanism(s)**
DYX1C1	15q, DYX1	Replicated in many studies	Neuronal migration in embryonal period, regulation of estrogen signaling, ciliary function
ROBO1	3p, DYX5	Replicated	Regulation of axonal and dendritic growth
DCDC2	6p, DYX2	Replicated in many studies	Neuronal migration in embryonal period, ciliary function
KIAA0319	6p, DYX2	Replicated in many studies	Neuronal migration in embryonal period
C2Orf3, MRPL19	2p, DYX3	No replications yet	No known mechanism
PCNT, DIP2A, S100B, PRMT2	21q, no locus named	No replications yet	No known mechanism
MC5R, DYM, NEDD4L	18p, DYX6	No replications yet	No known mechanism
DGK1	7q, no locus named	No replications yet	No known mechanism
CYP19A1	15q, DYX1	No replications yet	Regulation of estrogen signaling
No gene implicated yet	6q, DYX4	Genetic linkage only, no gene	No known mechanism
No gene implicated yet	11p, DYX7	Genetic linkage only, no gene	No known mechanism
No gene implicated yet	1p, DYX8	Genetic linkage only, no gene	No known mechanism
No gene implicated yet	Xq, DYX9	Genetic linkage only, no gene	No known mechanism

1.3.3 Neurobiology of dyslexia

Developmental dyslexia - as opposed to acquired dyslexia, which can result from brain damage after development is completed - is assumed to be a consequence of changes during brain development. Throughout this thesis, the term dyslexia is used as an equivalent for developmental dyslexia. Several studies have aimed at finding structural and functional differences in the brains of dyslexics compared to controls. Early postmortem studies have revealed cortical anomalies in a handful of dyslexic individuals at the microscopic level, mainly ectopias and dysplasias – misplaced neurons that might result from disturbed neuronal migration (Galaburda and Kemper, 1979; Galaburda et al., 1985; Humphreys et al., 1990). The anomalies were present more in the left hemisphere of the brain, which is generally associated with language and reading. Furthermore, in the brains examined in these studies, the planum temporale - a structure in the brain that is asymmetric between the two hemispheres in a majority of the population - was unusually symmetric (Galaburda and Kemper, 1979; Galaburda et al., 1985; Humphreys et al., 1990).

Interestingly, a number of the pinpointed dyslexia candidate genes, namely *DYX1C1, DCDC2, KIAA0319* and *KIAA0319L,* have been shown to affect radial neuronal migration upon knockdown in the rat embryonic neuronal cortex (Fig. 2) (Gabel et al., 2010). The migration defects resulted in heterotopias and ectopias in the adult rats reminiscent of the observations made by Galaburda in postmortem brains of dyslexic individuals (Gabel et al., 2010). Furthermore, Giraud and Ramus proposed a model for how neuronal migration defects might lead to alteration of cortical oscillatory functions, thereby providing a link between dyslexia genetics and phonological processing via cortical anomalies (Giraud and Ramus, 2013). Following up the initial findings, the role of brain asymmetry in dyslexia has been thoroughly investigated. Reduced hemispheric asymmetry in the planum temporale in dyslexics is a finding that is still under dispute and may be gender-specific (Altarelli et al., 2014; Paracchini et al., 2016; Ramus et al., 2017). In summary, language defects seem to correlate with higher or lower degree of symmetry (Leonard and Eckert, 2008).

Functional magnetic resonance imaging (fMRI) studies have shown that certain brain systems are activated differentially in dyslexic compared to normal readers (Shaywitz and Shaywitz, 2008). In normal readers, three major regions are involved in reading: one parietotemporal and one occipitotemporal posterior region and one inferior frontal anterior region (Broca´s area) in the left hemisphere of the brain. In dyslexics, the anterior region is overactivated whereas the posterior regions are underactivated. In addition, hyperactivation of the inferior frontal gyrus has been observed in individuals with dyslexia (Shaywitz and Shaywitz, 2008). Furthermore, a number of studies have shown reduced gray matter in certain areas of the brain in dyslexics measured by MRI (Sun et al., 2010). More recently, there is emerging evidence that there are differences also in white matter between dyslexics and normal readers. For example, SNPs in the dyslexia candidate genes *DYX1C1, DCDC2* and *KIAA0319* have been associated with white-matter volume in the left temporo-parietal region, which in turn correlated with reading ability (Darki et al., 2012). Similarly, in a longitudinal study, a SNP

in *DCDC2* correlated with white matter volume, which predicted reading ability two years after measurement (Darki et al., 2014).

The crossing of the midline of nerve tracts from one brain hemisphere to the other via the corpus callosum is fundamental in sensory processing. Studies of the corpus callosum for a role in dyslexia have produced both positive and negative results (Sun et al., 2010). Another dyslexia candidate gene, *ROBO1*, is known for its role in regulating axonal crossing of the midline. Using binaural suppression as a readout, Lamminmäki and colleagues could demonstrate a weaker interhemispheric suppression of crossing axons that correlated with reduced expression of *ROBO1* in a large family segregating dyslexia (Lamminmaki et al., 2012).

In summary, a number of different brain abnormalities have been reported to associate with dyslexia, however, it has been difficult to establish a unifying theory behind its neurobiology (Ramus et al., 2017).

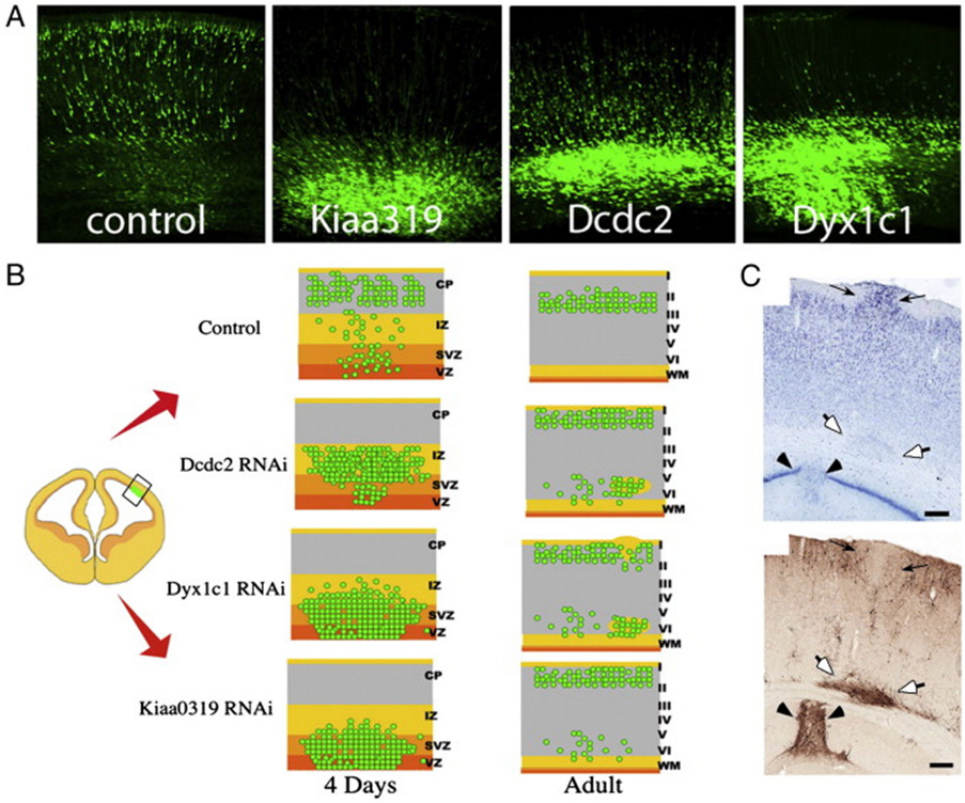


Figure 2: Neuronal migration deficits upon RNAi knockdown of *Dcdc2*, *Dyx1c1*, *Kiaa0319* in rat cortex. A) eGFP transfected cells in the embryonic cortex 4 days after knockdown. Control shows normal dispersion pattern of cells after migration. Cells transfected with RNAi knockdown against the genes show a disturbed pattern. B) Summary of the migration defects 4 days after treatment and in the adult cortex. In the adult, there are heterotopias and ectopias, as well as scattered neuronal displacements. C) Example of adult rat cortex with *Dyx1c1* knockdown: Ectopia (small arrows), white matter heterotopia (open arrows), hippocampal dysplasia (arrowheads). CP = cortical plate, IZ = intermediate zone, SVZ = subventricular zone, VZ = ventricular zone, cortical layers are labelled I-VI, WM = white matter

1.4 CILIA

1.4.1 What are cilia?

The cilium is a microtubule-based organelle present on the surface of most cells (Fig. 3). It was initially discovered by Antoine Philips van Leeuwenhoek at the end of the seventeenth century in protozoans and described as *"incredibly thin feet, or little legs, which were moved very nimbly"* (Satir, 1995). Primary cilia in the tissues of mammals were first described by Zimmermann in 1898 (Zimmermann, 1898). Cilia are highly conserved across species - from the single-cell green algae *Chlamydomonas reinhardtii* to humans. Primary cilia were long considered to be a vestigial organelle and thus ignored by research for a long time. During the last decades, however, the cilium has been rediscovered and associated with a number of important functions.

Cilia come in many different shapes and can roughly be divided into three different subtypes: motile, primary and sensory cilia (Choksi et al., 2014). Motile cilia are responsible for moving fluid in specialized organs, for example the cerebrospinal fluid in the ventricles of brain and spinal cord. They are present in the respiratory epithelium of the lung and in reproductive organs, lining the walls of fallopian tubes and as flagellum of sperms. Cilia are also responsible for establishment of asymmetry in the embryonic node (Shinohara and Hamada, 2017). Motile cilia have a 9+2 microtubule structure; the inner pair of microtubules is required for the movement (Fig. 3 B). The primary cilium is present on almost all cell types of the human body, including neurons. It is important for signaling and cell to cell communication and has also been linked to cell cycle, differentiation and migration (Christensen et al., 2008; Goetz and Anderson, 2010; Pala et al., 2017; Youn and Han, 2018). It has a 9+0 microtubule structure, lacking the inner pair of microtubules (Fig. 3 B). Sensory cilia present in sensory organs, e.g. the kinocilia in the inner ear and photoreceptor cilia in the retina, are responsible for sensing stimuli.

The cilium is attached to the cell by the basal body, which arises from the mother centriole of the centrosome and is surrounded by the pericentriolar material. There is no protein synthesis taking place in the cilium, therefore all proteins have to be transported into the cilium. This is accomplished by the ciliary transport machinery consisting of intraflagellar transport proteins, dynein and kinesin motor proteins (Goetz and Anderson, 2010). Anterograde transport up the ciliary axoneme to the tip is dependent on the kinesin-2 motor (KIF3 motor complex) and the intraflagellar transport proteins IFT B (Fig. 3 A). Retrograde transport down the axoneme to the cell body is dependent on the dynein motor and the intraflagellar transport proteins IFT A. The primary cilium serves as a hub for several signaling pathways, e.g. Hh, Wnt and PDGFRα pathways are signaling via the cilium (Christensen et al., 2008; Pala et al., 2017). The receptors for these pathways are concentrated in the membrane surrounding the primary cilium (Fig. 3 A).

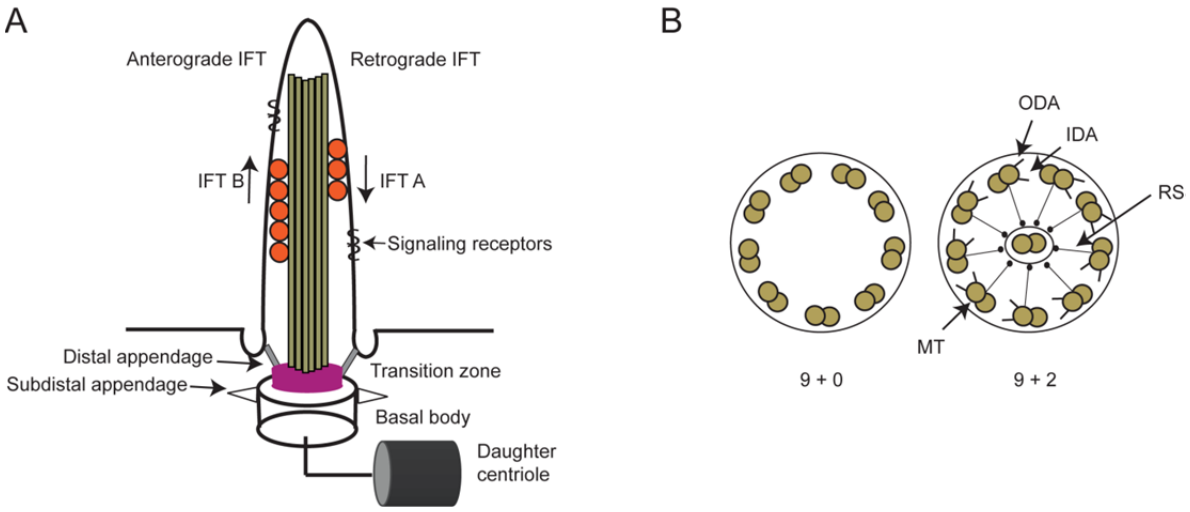


Figure 3: The structure of cilia. A) Schematic drawing of the structure of the primary cilium. B) Cross section of the axoneme of primary and motile cilia. IFT = intraflagellar transport, ODA = outer dynein arms, IDA = inner dynein arms, RS = radial spoke, MT = microtubule doublets.

1.4.2 Ciliopathies

Ciliopathies are a group of rare diseases caused by malfunction of the cilium. The underlying causes are mutations in ciliary genes, typically inherited in an autosomal recessive mode. Ciliopathies display a broad spectrum of symptoms, including kidney cysts, retinal defects, obesity and mental retardation. Examples of ciliopathies are polycystic kidney disease (PKD), Joubert syndrome (JBS) and Bardet-Biedl syndrome (BBS) (Hildebrandt et al., 2011). Some ciliopathies include neurodevelopmental symptoms (Valente et al., 2014) (Table 2).

Primary ciliary dyskinesia (PCD) is a ciliopathy affecting the motile cilia and patients typically display chronic sinusitis and bronchiectasis. In 50% of the cases, it is accompanied by *situs inversus*, a mirror reversal of the internal organs (Kartagener syndrome), caused by the failure of cilia in the embryonic node to establish left-right asymmetry (Knowles et al., 2016). To date, more than 30 genes have been identified as causative (Knowles et al., 2016).

Nephronophthisis (NPHP) and nephronophthisis-related ciliopathies (NPHP-RC) are a group of ciliopathies typically affecting kidney, liver and brain - in some cases, other organs are also involved (Braun and Hildebrandt, 2017). Its underlying genetics is very heterogenous, with more than 90 genes described as causative (Braun and Hildebrandt, 2017).

Table 2: Ciliopathies with brain and cognitive phenotypes. The genes marked in bold are mutated in several of the ciliopathies presented in this table.

Disease/disorder with ciliary phenotype	Brain phenotype/clinical features	Genes involved	References
Meckel-Gruber syndrome (MKS)	Posterior fossa abnormalities, corpus callosum defects, encephalocele, optic nerve hypoplasia, cognitive impairment	***MKS1, NPHP3, TCTN2,*** *B9D1, B9D2,* ***CEP290, TMEM67****,* ***TMEM216****,* ***TMEM231, CSPP1, RPGRIP1L****, CC2D2,* ***BBS2, BBS4, BBS6***	(Szymanska et al., 2014; Waters and Beales, 2011)
Joubert syndrome (JBS)	Cerebellar vermis hypoplasia or aplasia "molar tooth sign", developmental delay or mental retardation, ataxia, retinal dystrophy, oculomotor apraxia, cognitive impairment	*INPP5E, TMEM237, TMEM138,* ***TMEM216, TMEM67, TMEM231,*** *AHIL,* ***NPHP1****, CEP41, ARL13B, OFD1, TTC21B, KIF7, TCTN1,* ***TCTN2,*** *TCTN3,* ***ZNF423,*** *C5orf42,* ***CSPP1,*** *PDE6D,* ***CEP290,*** *CC2D2A,* ***RPGRIP1L,*** *CXORF5, KIAA0556*	(Sanders et al., 2015; Szymanska et al., 2014; Waters and Beales, 2011)
Nephronophthisis and related ciliopathies (NPHP-RC)	Learning disability, cerebellar hypoplasia, hydrocephalus	*NPHP1, NPHP2/INV,* ***NPHP3,*** *NPHP4, NPHP5,* ***CEP290/NPHP6,*** *NPHP7/GLIS2, NPHP8, NEK8/NPHP9, NPHP10,* ***TMEM67/NPHP11,*** *NPHP12, NPHP13/WDR19,* ***ZNF423/NPHP14,*** *CEP164/NPHP15, ANKS6/NPHP16,* ***IFT172/NPHP17****,* ***RPGRIP1L/NPHP18****, NPHP1L/*XPNPEP3*, NPHP2L, IFT20,* ***DCDC2***	(Waters and Beales, 2011; Wolf, 2015)
Bardet-Biedl syndrome (BBS)	Intellectual disability, cognitive impairment, cerebellar hypoplasia, retinal dystrophy	*BBS1,* ***BBS2,*** *BBS3****, BBS4****, BBS5,* ***BBS6,*** *BBS7, BBS8, BBS9, BBS10, BBS11, BBS12, BBS15,* ***IFT172/BBS20****, MGC1203, CCDC28B,* ***TMEM67, MKS1, CEP290***	(Schaefer et al., 2016; Waters and Beales, 2011)
Dyslexia	Unexpected difficulties in learning to read despite normal IQ; small anatomical defects reported in a few dyslexic human individuals; neuronal migration impairment during development in rats	*DYX1C1,* ***DCDC2****, KIAA0319, ROBO1, C2Orf3, MRPL19, CYP19A1, PCNT, DIP2A, S100B, PRMT2, MC5R, DYM, NEDD4L, KIAA0319L, DGKI, CEP63*	(Einarsdottir et al., 2015; Kere, 2014; Scerri and Schulte-Korne, 2010)

1.4.3 Cilia in neuronal development

Primary cilia are present on the surface of mammalian, including human, brain cells (Arellano et al., 2012; Fuchs and Schwark, 2004; Mandl and Megele, 1989; Sipos et al., 2018). They are proposed to have significant roles in development and maturation of neuronal cells (Guo et al., 2015). Patterning and morphogenesis of the telencephalon, a process dependent on Shh signaling, is disrupted in the absence of ciliary or basal body proteins (Lauter et al., 2018; Sarkisian and Guadiana, 2015). For example, animal models with disrupted *Ftm*, *Ift88* or *Ttc21b* display forebrain patterning defects (Sarkisian and Guadiana, 2015; Stottmann et al., 2009; Willaredt et al., 2008; Willaredt et al., 2013).

Cilia are present on the radial progenitor cells extending to the cerebral ventricle (Sarkisian and Guadiana, 2015). Ciliary genes such as *Tctex-1* and *Kif3a* affect the expansion of the

radial progenitor cells via Shh signaling (Li et al., 2011; Wilson et al., 2012). *Arl13b*, a gene mutated in JBS, is required for the proper organization of the radial glial scaffold (Higginbotham et al., 2013). It is still unclear if the cilium has a role in radial neuronal migration, however, it has been reported that cells lose the cilium during migration and regrow it during the postmigratory phase (Sarkisian and Guadiana, 2015). The importance of the centrosome in cell migration and particularly in neuronal migration is more widely recognized (Higginbotham and Gleeson, 2007). On the other hand, radial migration of inhibitory neurons depends on cilia for proper migration as shown in the conditional mouse knockout for *Arl13b* (Baudoin et al., 2012; Higginbotham et al., 2012). Later in neuronal development, cilia play a critical role in the dendritic outgrowth, dendritic branching and synaptic integration and connectivity (Guo et al., 2017a; Kumamoto et al., 2012; Miyoshi et al., 2014).

To date, the role of cilia in human neuronal proliferation, migration and differentiation remains little investigated, as most studies have focused on animal models. An indication that cilia are likely involved in human neuronal development and function is that many ciliopathies include neurological phenotypes. For example, cognitive phenotypes are observed in patients with BBS, JBS and NPHP-RC (Table 2).

1.4.4 RFX transcription factors

The expression of ciliary genes is regulated by different transcription factors. One group of transcription factors known for their role in regulation of ciliary gene expression is the family of regulatory factor X transcription factors (RFX TFs) (Choksi et al., 2014). RFX TFs bind to a conserved motif in the promoter region of a gene, known as the X-box. *RFX* orthologues exist in many species (Choksi et al., 2014). The link between RFX TFs and cilia was initially discovered in the nematode worm *C. elegans*. Absence of *Daf-19*, the only orthologue of RFX in *C. elegans*, caused lack of cilia in sensory neurons (Swoboda et al., 2000). In vertebrates, the family of RFX TFs comprises *RFX1-RFX9* with various degrees of similarity between their domain structures. *RFX1-4* have been linked to ciliary functions (Table 3). The expression of diverse RFX TFs is tissue-specific with many members highly expressed in brain (Aftab et al., 2008; Sugiaman-Trapman et al., 2018).

Table 3: The expression and function of ciliary RFX transcription factors. List of RFX TFs with functional connections to cilia and their phenotypes in vertebrates.

		Ciliary phenotypes	
RFX TFs	**Tissue expression**	**Organism wide**	**Cilia specific**
RFX1	Brain	Homozygous lethal	Not known
RFX2	Organs of laterality, Brain, Kidney, Testis, Epidermis	Left-right asymmetry defects, Defective neural tube closure, Perturbed HH signaling	Truncated dysfunctional motile cilia, Fewer and truncated immotile cilia
RFX3	Organs of laterality, Brain, Pancreas, Epidermis	Left-right asymmetry defects, Hydrocephalus, Malformation of the corpus callosum, Perturbed hormone secretion	Truncated, dysfunctional motile cilia, Aberrant number of immotile cilia, Truncated immotile cilia
RFX4	Brain, Testis	Homozygous lethal, Patterning defects, Reduction/absence of subcommissural organ, Hydrocephalus	Truncated cilia

1.5 MOLECULAR FUNCTIONS OF DYSLEXIA CANDIDATE GENES

1.5.1 *DYX1C1*

Initial studies showed *DYX1C1* expression in several tissues, including brain, and localization to neuronal nuclei and glial cells (Taipale et al., 2003). DYX1C1 shuttles to the cytoplasm and to small cytosolic particles in COS-1 cells upon heat shock (Kaminen-Ahola et al., unpublished). In addition, DYX1C1 localizes to the cytosol of cancer cell lines (Thul et al., 2017) (www.proteinatlas.org). In summary, the compartmental distribution of DYX1C1 in the cell is probably dynamic and cell-type dependent.

Studies on rodent animal models have addressed the role of *Dyx1c1* in neuronal development and in behavior (Table 4). Several reports have shown that knockdown of *Dyx1c1* during embryonic development of the rat brain results in neuronal migration deficits (Fig. 2), (Adler et al., 2013; Currier et al., 2011; Rosen et al., 2007; Wang et al., 2006). Rats with embryonic knockdown of *Dyx1c1* display abnormal auditory processing, spatial learning impairment, working memory deficits and visual attention impairments (Szalkowski et al., 2013; Szalkowski et al., 2011; Threlkeld et al., 2007). In contrast to these results in rat, no disturbances of neuronal migration could be found in a forebrain-specific *Dyx1c1* mouse knockout, but still deficits in learning and memory (Rendall et al., 2015).

More evidence for a role of DYX1C1 in neuronal migration comes from proteomics and transcriptomic studies, supporting the earlier studies in animal models. DYX1C1 interacts with microtubule proteins and regulates genes related to neuronal migration and development. Moreover, a high throughput study revealed interaction with cytoskeletal proteins and further supported a link to chaperone functions and centrosomal proteins

(Tammimies et al., 2013). An *in vitro* random cell migration assay in the human neuroblastoma cell line SH-SY5Y showed that migration speed is increased upon perturbation of *DYX1C1* (Tammimies et al., 2013).

The DYX1C1 protein contains three tetratricopeptide (TPR) repeat domains known to play a role in protein-protein interactions (Fig. 4), (Taipale et al., 2003). DYX1C1 interacts with the heat shock proteins Hsp70 and Hsp90 via the TPR domain and with CHIP, an E3 ubiquitin protein ligase (Chen et al., 2009; Hatakeyama et al., 2004; Tarkar et al., 2013). These interactions indicate a function of DYX1C1 as a chaperone. In addition, an unbiased high-throughput study identified DYX1C1 in the autophagy network (Behrends et al., 2010). DYX1C1 interacts with estrogen receptors (ER) alpha and beta, in the presence of 17b estradiol, thereby suppressing the transcription of ER-dependent targets (Massinen et al., 2009). In an alternative non-genomic pathway, it interacts with ERs to promote their degradation via the proteasome.

Expression of *DYX1C1* is regulated by the transcription factors TFII-I, PARP1 and SFPQ, that bind the dyslexia-associated SNPs -3G/A (rs 37432059) and -10310C/A (rs16787) (Tapia-Paez et al., 2008). Further studies showed that ERα, but not ERβ, binds to a regulatory start site upstream of *DYX1C1* in complex with TFII-I and is enhanced by E2 administration in the presence of the -3G allele, but not the dyslexia associated -3A allele (Tammimies et al., 2012). In addition, methylation at the -4/-3CpG site abolishes the binding of ERβ, thereby repressing transcription of *DYX1C1*. These data provide a link between dyslexia and neuroendocrine pathways and a possible explanation for the sex-specific differences observed in dyslexia. Differential expression of *DYX1C1* by a dyslexia-associated SNP has been reported in another instance (Muller et al., 2018).

Furthermore, DYX1C1 has been proposed as a potential marker in breast cancer and as a biomarker in colorectal cancer (Chen et al., 2009; Kim et al., 2009; Rosin et al., 2012).

1.5.2 *DCDC2*

DCDC2 is structurally related to the family of DCX-containing proteins (Fig. 5), (Coquelle et al., 2006; Reiner et al., 2006). A prominent member of that family is doublecortin (DCX), which is known for its crucial role in neurogenesis and migration. Mutations in *DCX* cause X-linked lissencephaly and its protein product associates with microtubules (Gleeson et al., 1999).

Dcdc2 involvement in brain development and behavior has been examined using rodent models (Table 4). Knockdown of *Dcdc2* during embryonic development of rats leads to disturbed neuronal migration (Fig. 2), (Adler et al., 2013; Burbridge et al., 2008; Meng et al., 2005). In addition, the treated rats display impaired speech sound discrimination (Centanni et al., 2016). In contrast to these results in rats, knockout of *Dcdc2* in the mouse does not lead to neuronal migration defects (Wang et al., 2011). Whereas *Dcdc2* knockout mice display normal neuronal migration, concomitant knockdown of *Dcx* leads to migration defects suggesting compensatory mechanisms among *Dcdc2* and *Dcx* (Wang et al., 2011). Despite

the apparently undisturbed neuronal migration in *Dcdc2* knockout mice, they show defects in rapid auditory processing, working memory and reference memory, long-term memory and visuo-spatial performance (Gabel et al., 2011; Truong et al., 2014). *Dcdc2* knockout mice have increased spike rate and decreased temporal precision of action potential firing in neocortical pyramidal neurons (Che et al., 2014). Whole transcriptome RNA-seq revealed elevated expression of the NMDR receptor subunit Grin2B, providing a link to the observed electrophysiological deficits (Che et al., 2014). In addition, *Dcdc2* knockout mice have enhanced glutamatergic transmission in the cortex (Che et al., 2016).

In vitro, a deletion in intron 2 containing a short tandem repeat termed "READ1", previously reported to be linked to dyslexia, has been shown to alter the expression of *DCDC2* via binding of the ETV6 transcription factor (Meng et al., 2011; Powers et al., 2013).

DCDC2 induces microtubule polymerization and stabilizes microtubules by binding to them (Coquelle et al., 2006; Grati et al., 2015; Jeruschke et al., 2015). It has also been suggested to act on the JNK signaling pathway via interaction with the proteins JIP1 and JIP2 (Coquelle et al., 2006).

DCDC2 has also been attributed roles in cancer, notably prostate cancer, hepatocellular carcinoma, and breast cancer (Cai et al., 2017; Inokawa et al., 2013; Longoni et al., 2013).

1.5.3 *KIAA0319*

Similar to *Dyx1c1* and *Dcdc2*, the role of *Kiaa0319* in neuronal development and behavior has been investigated using rodent models. Embryonic knockdown of *Kiaa0319* impaired neuronal migration in rats (Fig. 2), (Adler et al., 2013; Paracchini et al., 2006; Peschansky et al., 2010). On the behavioral level, the rats displayed impairments in auditory processing and spatial learning (Centanni et al., 2014a; Centanni et al., 2014b; Szalkowski et al., 2012). However, in contrast to the results in rat, knocking out *Kiaa0319* in mice did not result in a neuronal migration phenotype or in behavioral defects in learning and memory (Martinez-Garay et al., 2017).

KIAA0319 has been shown to be present as a transmembrane protein containing several PKD domains, which could function in cell-cell interaction and neuronal migration as well as a transmembrane domain-less protein possibly functioning in signaling (Velayos-Baeza et al., 2008). In addition, Kiaa0319 has been described as a key player in axon growth via Smad2 signaling (Franquinho et al., 2017).

KIAA0319 expression is differentially regulated by transcriptional silencer OCT-1 binding to SNP rs9461045 linked to dyslexia (Dennis et al., 2009).

1.6 DYSLEXIA CANDIDATE GENES AND CILIA

A number of recent studies have shown a connection between dyslexia candidate genes and cilia. The very first study linking a dyslexia candidate gene to cilia showed that *Dcdc2* elongates the ciliary axoneme when overexpressed and alters ciliary signaling via Shh and Wnt in rat neurons (Massinen et al., 2011). In a transcriptomic study comparing ciliated and non-ciliated mouse tracheal epithelial cells, Hoh and colleagues observed that *Dyx1c1*, *Dcdc2* and *Kiaa0319* were upregulated in ciliated cells (Hoh et al., 2012). Based on a set of available transcriptomic data from human ciliated tissues, Ivliev et al. described the upregulation of *DYX1C1*, *DCDC2* and *KIAA0319* in ciliated versus non-ciliated tissues (Ivliev et al., 2012).

For *DYX1C1*, supporting evidence for a ciliary function first came from zebrafish morphants of the orthologue *dyx1c1*. Morphant zebrafish display a full ciliary phenotype including *situs inversus*, hydrocephalus, kidney cysts and defective motile cilia missing inner dynein arms (IDA) and outer dynein arms (ODA) (Chandrasekar et al., 2013). Similarly, mice mutated for *Dyx1c1* have *situs inversus*, ciliary motility defects and respiratory cilia deficiencies (Tarkar et al., 2013). Patients with PCD were shown to have recessive loss-of-function mutations or duplications in *DYX1C1* (Casey et al., 2014; Tarkar et al., 2013). A number of studies have since supported *DYX1C1* as a causative gene for PCD (Guo et al., 2017b; Marshall et al., 2015; Raidt et al., 2014). The *DYX1C1* mutations causing PCD and the functional variants implicated in dyslexia are summarized in Fig. 4. For an overview of ciliary phenotypes associated with *DYX1C1*, see Table 4.

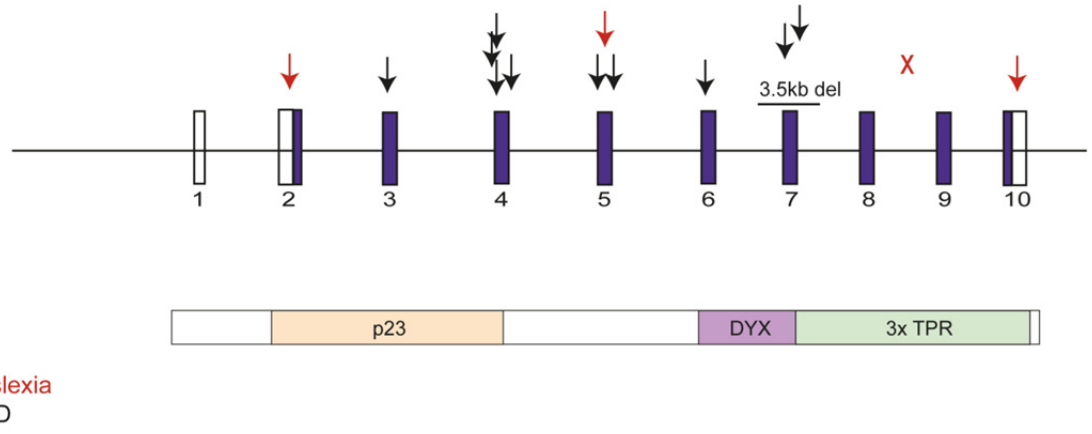
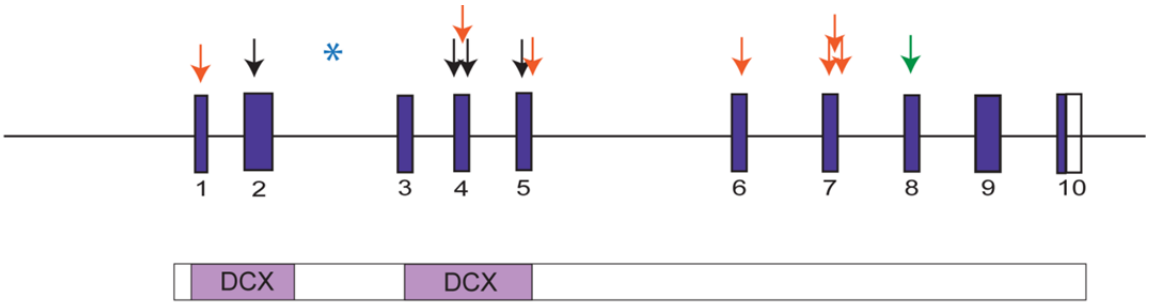


Figure 4: *DYX1C1* gene and protein structure. SNPs associated with dyslexia that have been functionally studied are indicated with red arrows. The translocation breakpoint co-segregating with dyslexia is shown as a red cross. PCD mutations are shown in black. Exons are indicated by numbers 1-10, white boxes = untranscribed exons, blue boxes = transcribed exons. TPR = tetratricopeptide repeat domain.

DCDC2 mutations were discovered in patients with various ciliopathies: Mutations in *DCDC2* were found in patients with NPHP-RC (Paper II), in a family with congenital hearing loss (Grati et al., 2015) and in neonatal sclerosing cholangitis (NSC), a ciliopathy affecting the liver (Girard et al., 2016; Grammatikopoulos et al., 2016). For an overview of mutations in *DCDC2* see Fig. 5. Ciliary phenotypes of *DCDC2* are shown in Table 4.

Robo1, which is important for tangential migration of GABAergic neurons, has been shown to localize to the cilium of interneurons in mice, however, is not classified as a ciliary protein (Higginbotham et al., 2012). Another group of dyslexia candidate genes has been linked to the centrosome or basal body. The gene encoding Centrosomal protein 63, *CEP63,* has been found to co-segregate with dyslexia in a Swedish family (Einarsdottir et al., 2015). Similarly, a deletion encompassing *PCNT* - the gene encoding Pericentrin, a centrosomal protein possibly involved in cilium assembly - was found to co-segregate with dyslexia (Poelmans et al., 2009).



Dyslexia
NPHP-RC
Deafness
Liver-based ciliopathy

Figure 5: *DCDC2* gene and protein structure. The deletion which is associated with dyslexia and functionally studied is shown as a blue asterix. Mutations in ciliopathies are indicated: NPHP-RC (black), deafness (green), liver-based ciliopathy (orange). Exons are indicated by numbers 1-10, white boxes = untranscribed exons, blue boxes = transcribed exons. DCX = doublecortin domain.

Table 4: Summary of the phenotypes upon perturbation of *DYX1C1* and *DCDC2* in different model systems. KD: knockdown, KO: knockout, MO: morpholino, OE: overexpression. "et al." was omitted from all references.

	DYX1C1	***DCDC2***	***References***
Disorder	Dyslexia; PCD	Dyslexia; NPHP-RC; heritable deafness; NSC	Taipale, 2003; Tarkar, 2013; Casey, 2013; Meng et al., 2005; Schumacher, 2006; Schueler, 2015; Grati, 2015; Grammatikopoulos, 2015; Girard, 2016
Cilia length	shortened *(zebrafish MO)*	○ normal (*Rat hippocampal neurons KD, NIH-3T3 cells KD, mouse brain KO, zebrafish MO)* ○ lengthened *(Rat hippocampal neurons OE, NIH-3T3 cells OE, rat ear cells OE)*	Massinen, 2011; Wang, 2011; Chandrasekar, 2013; Schueler, 2015; Grati, 2015
Cilia number	○ reduced *(zebrafish MO)* ○ normal *(mouse KO)*	○ reduced *(human patients, IMCD3 cells KD)* ○ normal *(mouse brain KO)*	Grammatikopoulos, 2015; Girard, 2016; Schueler, 2015; Chandrasekar, 2013; Tarkar, 2013, Wang, 2011
Cilia branching		increased *(rat inner ear cells OE)*	Grati, 2015
Dynein arms	missing IDA/ODA *(mouse KO/zebrafish MO/human)*	normal *(zebrafish MO)*	Tarkar, 2013; Chandrasekar, 2013; Schueler, 2015
Ciliary motility	impaired *(mouse KO/human)*		Chandrasekar, 2013
Ciliary signalling		altered WNT/SHH *(rat hippocampal neurons, NIH-3T3 cells)*	Massinen, 2011; Schueler, 2015
Organ asymmetry	situs inversus totalis *(mouse KO/zebrafish MO/human)*	in some organs *(zebrafish MO)*	Tarkar, 2013; Schueler, 2015
Neuronal migration	○ impaired *(rat KD)* ○ normal *(rat OE)* ○ normal *(mouse KO)*	○ impaired *(rat KD)* ○ normal *(rat OE)* ○ normal *(mouse KO)*	Wang, 2006; Currier, 2011; Rosen, 2007; Meng, 2005; Burbridge, 2008; Adler, 2013; Rendall., 2015; Wang, 2011
Behavioural phenotype	○ abnormal auditory processing; spatial learning impairment; working memory deficits; visual attention impairments *(rat KD)* ○ learning and memory deficits *(mouse KO)*	○ impaired speech sound discrimination *(rat KD)* ○ impairments in long-term memory and visuo-spatial performance, rapid auditory processing, working memory ability *(mouse KO)*	Threlkeld, 2007; Szalkowski, 2011; Szalkowski, 2013; Centanni, 2016; Rendall, 2015; Gabel, 2011; Truong, 2014
Synaptic transmission		glutamatergic transmission enhanced; impaired spike-timing precision *(mouse KO)*	Che, 2016; Che, 2014
Neuronal branching		increased (*C.elegans OE, rat hippocampal neurons OE)*	Massinen, 2011

2 AIMS OF THE THESIS

The overall goal of this thesis was to study the role of ciliary genes linked to dyslexia. This overarching question was approached in different ways and divided into specific questions:

- Study the regulation and molecular functions of the ciliary dyslexia candidate genes *DYX1C1* and *DCDC2* in health and disease (Papers I, II, III)
- Define whether dyslexia candidate genes and ciliary genes have a role in human neuronal development by studying gene expression (Paper IV)
- Determine the underlying genetic cause in patients with a ciliopathy and dyslexia (Paper V)

3 METHODS

3.1 BIOLOGICAL MODEL SYSTEMS

3.1.1 Cell lines (Papers I-IV)

In this thesis, cell lines derived from human and animal tissues were used as model systems. The advantage of using cell lines is that they can be manipulated to address a wide range of biological questions. On the other hand, the *in vitro* systems might not always reflect accurately the biology in living organisms and need to be validated. The following cell lines were used in this thesis:

3.1.1.1 hTERT-RPE1 cells (Papers I, II, III)

Human telomerase-immortalized retinal pigment epithelial cells (hTERT-RPE1, here termed RPE1 cells, ATCC® CRL-4000™) are a widely used model system in cilia research. They are derived from the retinal epithelium. RPE1 cells are used in cilia research thanks to their property of growing a prominent primary cilium by means of serum starvation. In papers I, II and III, RPE1 cells were a tool to investigate regulation of ciliary dyslexia candidate genes and the interactions and localization of their protein products. In paper III, RPE1 cells stably overexpressing CPAP-GFP under the induction of doxycycline were used. The tagging of CPAP with the reporter protein GFP allows for localization of the protein in the cell and for efficient pulldown by GFP-trap.

3.1.1.2 NES cells (Paper IV)

Due to the difficulty in accessing human brain material, previous studies have often used animal models to examine neuronal processes. In order to investigate dyslexia, which is a human-specific phenotype, we here turned to iPSC-derived human neuroepithelial cells in paper IV.

With the advent of reprogramming technology, it became possible to revert the fate of a differentiated cell into a pluripotent cell, so-called induced pluripotent stem cell (iPSC) with a certain set of transcription factors (Takahashi et al., 2007). Both human embryonic stem cells (hESCs) and iPSCs have allowed *in vitro* modeling of neurodevelopmental disorders by using defined neuronal differentiation protocols (Telias and Ben-Yosef, 2014). iPSC-derived long term self-renewing neuroepithelial cells (here termed NES cells) are an intermediate state resembling embryonic neuroepithelial cells (Falk et al., 2012; Tailor et al., 2013). NES cells are self-renewing in the presence of fibroblast growth factor (FGF) and epidermal growth factor (EGF) and spontaneous differentiation can be induced by removal of growth factors (Fig. 6). Their advantage is the relatively short time to reach differentiated neurons compared to pluripotent stem cell-derived neuronal cells.

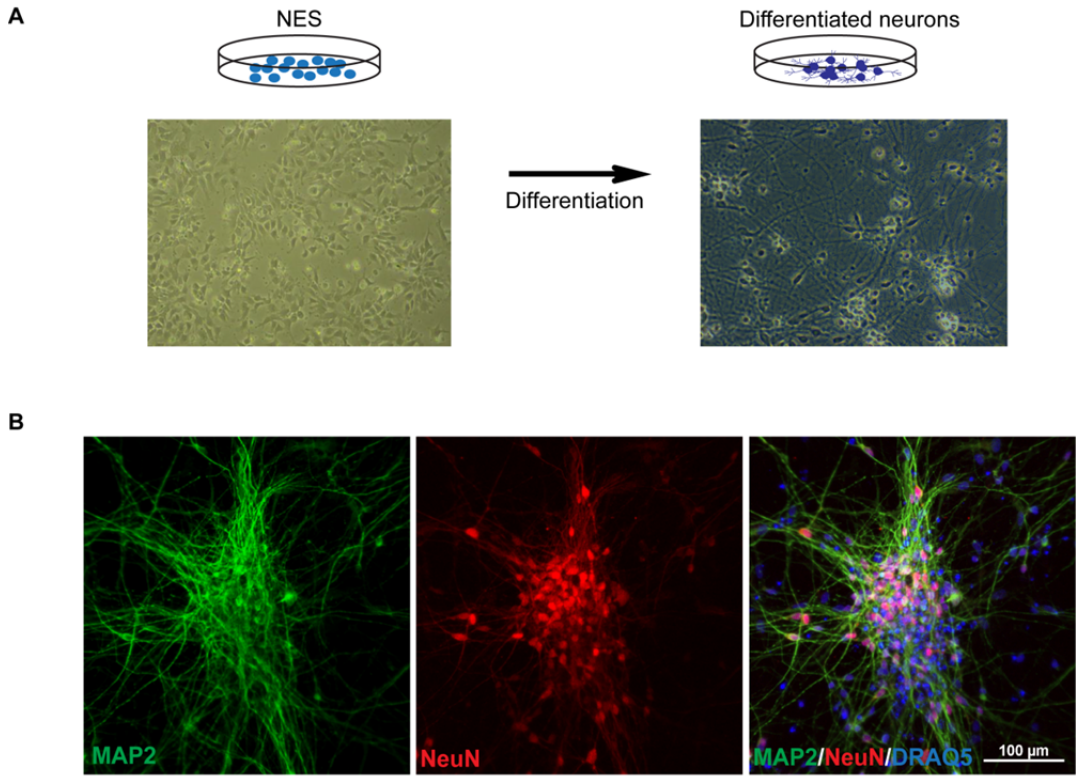


Figure 6: Differentiation of NES cells. A) NES cells differentiating to neurons, visualized in a brightfield microscope. B) NES cells differentiated for 38 days and stained with the neuronal markers MAP2 (microtubule-associated protein 2) and NeuN (neuronal nuclei) and nuclear marker DRAQ5**.**
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3.1.1.3 Other cell lines (Papers I, II, III)

SH-SY5Y (ATCC® CRL-2266™) cells are derived from female human bone marrow and are a subline of the neuroblastoma cell line SK-N-SH (Kovalevich and Langford, 2013). They are often used as a model to study human neuronal processes. In paper I, SH-SY5Y cells were used to study the expression of ciliary dyslexia candidate genes.

The human embryonic kidney cell line HEK293T (ATCC® CRL-3216™) was used in paper II for co-immunoprecipitation assays.

NIH-3T3 cells (ATCC® CRL-1658™) are derived from mouse embryonic tissue and have become a standard fibroblast cell line. They were originally isolated from Swiss mouse embryo tissue and spontaneously immortalized. In paper II, we took advantage of NIH-3T3 cells as a tool for luciferase reporter assays, co-immunoprecipitation assays and assessment of subcellular localization.

mIMCD-3 (ATCC® CRL-2123™) is a transformed cell line derived from the inner medullary collecting duct in the kidney of the mouse. They were used in paper II for a spheroid assay to model kidney development in 3D.

Madin-Darby Canine Kidney (MDCK-II) cells (ATCC® CRL-2936™) are derived from kidney distal tubule from dog. In paper II, we made use of MDCK-II cells to monitor the subcellular localization of proteins.

HeLa cells (ATCC® CCL-2™) are derived from human cervical epithelial adenocarcinoma tissue. In paper III, we took advantage of HeLa cells to investigate the protein-protein interactions among DYX1C1, DCDC2 and CPAP on endogenous levels.

3.1.2 Zebrafish (*D. rerio*) (Papers II, III)

While *in vitro* studies offer a way to test a hypothesis functionally, they might not always accurately reflect the biological conditions. Animal model organisms offer the possibility to directly test hypotheses *in vivo*. In this thesis, zebrafish (*Danio rerio*) served as an *in vivo* model system. The advantages of using zebrafish are its short generation time, the transparence of the embryo and the high conservation of genes compared to humans (Strachan and Read, 2011).

In papers II and III, zebrafish was utilized to investigate the effect of inhibition of the genes *dyx1c1* and *dcdc2b* or a combination thereof. For this purpose, we took advantage of morpholino antisense oligonucleotides. Morpholinos blocking the translation start site of RNA or a splice site in the precursor RNA are microinjected into the single-cell zygote in order to prevent translation of a certain gene (Strachan and Read, 2011). Morpholino-mediated knockdown of a gene in zebrafish is a widely used approach to address early zebrafish development; however it cannot be used for long-term studies because the effect gets diluted during cell divisions.

3.2 METHODS TO STUDY DNA

3.2.1 Next generation sequencing (NGS) (Papers II, V)

Shotgun sequencing of DNA started to be widely used in the beginning of the 21st century as high-throughput sequencing method. In this thesis, we used the sequencing-by-synthesis approach on the Illumina platform (Mardis, 2008). First, the DNA is fragmented into pieces, adapters are ligated and the fragments are bound to the flow cell surface. Fluorescently labelled nucleotides - each nucleotide in a different color - primers and DNA polymerase are added to the flow cell. The incorporated base is imaged by a laser and then removed. This cycle is repeated to produce reads of a few dozens to a few hundreds of bases.

In paper II, we implemented whole exome sequencing (WES) and in paper V whole genome sequencing (WGS) to find disease-causing variants. If assuming that the causative variants are located in the exonic region, WES is a relatively economical strategy with high read depth and direct analysis. WGS is more expensive, but allows a more complete examination of the genome. Its analysis is, however, more complex and the interpretation more difficult even with today´s available tools.

3.2.2 Analysis of NGS data (Papers II, V)

In paper V, we used the GATK tool HaplotypeCaller to call SNVs and small insertions and deletions (McKenna et al., 2010). The variants were then annotated using the tools SNPEff and ANNOVAR (http://wannovar.wglab.org/) (Wang et al., 2010; Yang and Wang, 2015). This resulted in long lists of variants, which had to be filtered according to priorities. Variant prioritization can be complex and a wide range of tools exist to address it (Eilbeck et al., 2017). In the first step, we filtered the detected variants according to their allele frequency in the European population. As reference databases, we used the 1000Genomes project database, the Exome aggregation consortium database and the SweGen database (Ameur et al., 2017; Lek et al., 2016; The Genomes Project, 2012) (www.internationalgenome.org; http://exac.broadinstitute.org; https://swefreq.nbis.se). To assess the consequences of the identified variants, we used the prediction tools SIFT, Polyphen2, MutationTaster, CADD and GERP++ (Adzhubei et al., 2013; Davydov et al., 2010; Kircher et al., 2014; Kumar et al., 2009; Schwarz et al., 2014). For the analysis of structural variants (SVs), we applied the tools CNVnator and TIDDIT. CNVnator relies on the read depth while TIDDIT relies on the detection of split reads (Abyzov et al., 2011; Eisfeldt et al., 2017).

In paper II, the number of SNVs from the WES was narrowed down according to the dbSNP138 (www.ncbi.nlm.nih.gov/projects/SNP) database and according to the homozygosity mapping linkage region.

3.2.3 Sanger sequencing (Papers II, V)

Dideoxy DNA sequencing, more commonly termed Sanger sequencing is a sequencing strategy based on termination by ddNTP incorporation and gel electrophoresis (Sanger et al., 1977). It is a more robust method than the NGS sequencing method, but less high-throughput. Automated Sanger sequencing is used today to validate NGS data. The Sanger sequencing method was applied in papers II and V to validate results obtained via WES and WGS.

3.3 METHODS TO STUDY RNA

3.3.1 RNA-sequencing (Paper IV)

Genome-wide expression profiling by RNA-sequencing is widely used today to identify differentially expressed genes. Mapping and quantification of the transcriptome by RNA-sequencing was first described in 2008 by several groups in parallel. The use, methods and analysis strategies have diversified during the last decade (Conesa et al., 2016; Ozsolak and Milos, 2011). We implemented a modified version of the single-cell tagged reverse transcription (STRT) protocol in paper IV (Islam et al., 2012; Katayama et al., 2013; Krjutskov et al., 2016). The method is optimized for high sensitivity for small amounts of RNA. In addition, it offers the possibility for multiplexing thanks to the use of barcoding. This allows a high number of samples to be run in the same sequencing library, thereby eliminating library bias among samples. poly-T priming is used for reverse transcription. The

method favors the 5´end as opposed to full-length RNA-seq methods. For normalization, we used external synthetic spike-in molecules from External RNA Controls Consortium (ERCC).

3.3.2 Analysis of RNA-sequencing data (Paper IV)

For data processing of the STRT library, we used the STRTprep pipeline (https://github.com/shka/STRTprep/tree/v3dev) (Krjutskov et al., 2016). Differential gene expression was analyzed using the R package SAMstrt (Katayama et al., 2013). The false discovery rate was used as a cut-off for gene discovery. The spike-ins were used to normalize the data and estimate the technical variation.

3.3.3 qRT-PCR (Papers I-IV)

Quantitative real-time polymerase chain reaction (qRT-PCR) is a method to quantify RNA expression. The RNA is copied into cDNA by reverse transcription using poly-T or random hexamer priming. The amount of cDNA present in the sample is then quantified by fluorescent dyes binding to the cDNA during an exponential amplification process using thermal cycling. In studies I-IV, SYBR green dye and Taqman hydrolysis probes were used. SYBR green dye detects double stranded DNA in general while Taqman probes are gene-specific by the use of hybridization probes coupled to fluorophores. An internal control was used to normalize the amount of product of the gene of interest. Specifically, we used *HPRT1* for experiments involving RPE1 cells and *GAPDH* for experiments with NES cells. The qRT-PCR data was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). This relative quantification method compares the expression level of the gene of interest with the expression level of the internal control and with a control sample.

qRT-PCR is a less high throughput method than RNA-seq, but it is fast and specific and used to validate candidate gene findings. In papers I, II and III, we applied qRT-PCR to measure the expression of *DYX1C1, DCDC2* and *KIAA0319* and RFX TFs. In paper IV, qRT-PCR was used to validate the RNA-seq results and confirm upregulation of neuronal differentiation genes, ciliary genes and dyslexia candidate genes.

3.4 METHODS TO STUDY PROTEINS

3.4.1 SDS-PAGE and Western Blot (Papers II, III)

Western blotting allows the detection of proteins according to size using antibodies. The protein samples are denatured using heat and sodium dodecyl sulfate and run on a polyacrylamide gel to separate the products according to their molecular weight by electrophoresis (SDS-PAGE). The protein products are subsequently transferred to a nylon membrane. The protein of interest is then detected by a specific primary antibody, followed by a secondary antibody labelled with an enzyme - in this study horse radish peroxidase (HRP) - and visualized with a light-sensitive substrate.

We used SDS-PAGE and Western Blot in papers II and III to detect protein products pulled-down by co-immunoprecipitations (see 3.4.2).

3.4.2 Co-immunoprecipitation (Papers II, III)

Co-immunoprecipitation (Co-IP) is a method to find interacting partners ("prey") of a protein of interest ("bait"). Antibodies specific to the bait are coupled to beads – usually resin or magnetic beads. The beads are then incubated together with the cellular lysate and the coupled antibodies bind to the bait. Next, the lysate is washed away and only the bait with its prey remains. Those are harvested by elution and are detected by Western Blot with specific antibodies to the interacting proteins (3.4.1).

In paper II, we used co-IPs in order to investigate the interactions of DCDC2 with JNK pathway proteins. In paper III, we used co-IP to capture CPAP and demonstrate its interaction with DCDC2 and DYX1C1.

3.4.3 Immunohistochemistry and immunocytochemistry (Papers I-IV)

Immunocytochemistry (ICC) (in cells) and immunohistochemistry (IHC) (in tissues) is a method to detect a protein of interest by labeling with a specific antibody. Here, we used the indirect labeling method: the primary antibody bound to the target is detected with a secondary antibody, which is labelled with a fluorescent dye. The dye is then visualized by a fluorescent or confocal microscope (see 3.6.1). Several targets can be detected simultaneously by applying a combination of antibodies and dyes.

In this thesis, IHC and ICC were used to study the subcellular localization of DYX1C1 and DCDC2 (Papers I, II, III) and as markers for organelles such as cilia and centrosomes (Papers I-IV).

3.5 OTHER MOLECULAR BIOLOGY METHODS

3.5.1 RNA interference (Papers I, II, III)

RNA interference is a method to deplete RNA of a particular target by using small interfering RNA (siRNA) via the RNA-induced silencing complex (RISC). It allows investigating the effect of a reduced amount of RNA of a certain gene in a direct way without the need to engineer genetic knockouts and has been used widely in many systems including mammalian cells (Martin and Caplen, 2007).

We used siRNA in paper I to knock down RFX factors in RPE1 cells, in paper II to knock down *Ddcd2a* in mouse kidney spheroids and in paper III to knock down *DYX1C1* and *DCDC2* in RPE1 cells.

3.5.2 Luciferase reporter assay (Papers I, II)

Reporter assays offer a way to measure the transcriptional activity of promoters or enhancers. A DNA region of interest containing a sequence suspected to affect gene expression is cloned

in front of a firefly luciferase gene. The luciferase converts a substrate to a luminescent signal, which is detected by spectrophotometer. Renilla luciferase is used as an internal control to normalize the signal.

In paper I, we used a luciferase reporter assay to assess the activity of X-box motives in the promoter region of *DYX1C1*, *DCDC2* and *KIAA0319*. In paper II, we studied by luciferase reporter assay how the Wnt-pathway activation is affected upon *DCDC2* mutation.

3.5.3 Electrophoretic mobility shift assay (EMSA) (Paper I)

Electrophoretic mobility shift assay (EMSA) is a method to investigate protein binding to DNA. The biotin-labelled DNA sequence of interest is incubated together with cellular extracts containing the protein suspected to bind the DNA. The complexes are run on a polyacrylamide gel and separated by molecular weight. The complexes are then transferred to a nylon membrane, crosslinked and detected.

Use of a specific antibody can reveal whether the binding factor is the suspected protein of interest. The complex of the protein of interest plus the antibody will result in a higher molecular weight complex and retarded migration, resulting in a band higher up on the gel, a so-called supershift.

In paper I, we used EMSA to determine whether RFX TFs bind to X-box motives in the promoter regions of *DYX1C1* and *DCDC2*.

3.6 MICROSCOPY

3.6.1 Confocal microscopy (Papers I-IV)

A fluorescent microscope uses light of a specific wavelength to excite a fluorophore. The light is then emitted at a longer wavelength, filtered and collected in a detector. In a confocal microscope, the exciting light is focused via a pinhole on only a small part of the sample, allowing for a higher optical resolution.

In this thesis, we used the confocal microscopes Laser Scanning Microscope (LSM) 510 by Zeiss and A1R Ti confocal by Nikon Instruments for visualization of the samples prepared by ICC and IHC (3.4.3).

3.6.2 Electron microscopy (Papers II, V)

While light microscopy uses photons as a source of illumination, an electron microscope takes advantage of electrons. An electron microscope therefore has a much higher resolution than a light microscope and allows the study of smaller subcellular structures.

In papers II and V, we used transmission electron microscopy (TEM) to visualize the ultrastructure of cilia, including dynein arms.

3.7 BIOINFORMATICS (PAPERS I, IV)

In paper I, we used the EMBOSS fuzznuc tool (http://emboss.bioinformatics.nl/cgi-bin/emboss/fuzznuc) to identify X-box motifs in the promoter regions of dyslexia candidate genes. The motifs were then prioritized according to conservation scores.

For gene ontology (GO) analysis in paper IV, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da et al., 2009). In addition, we applied gene set enrichment analysis (GSEA) (Subramanian et al., 2005), a computational strategy to assess enrichment of a defined list of genes when comparing two conditions.

3.8 BRAIN IMAGING (PAPER V)

Magnetic resonance imaging (MRI) is a brain imaging method applied in neuroscience research, including dyslexia research. Functional magnetic resonance imaging (fMRI) is a method to measure brain activity based on blood flow changes. Blood-oxygen-level dependent (BOLD) contrast measures the change of oxyhemoglobin, the oxygenated form of oxygen in the blood (Mark F. Bear, 2007). Diffusion tensor imaging (DTI) takes advantage of the different diffusion characteristics of water molecules in the brain. Water diffusion is not the same in all directions, for example it is more likely to go along axons than through the cell membrane (O'Donnell and Westin, 2011).

In paper V, we used fMRI to measure which brain regions are activated upon a word generation task in a dyslexic person with *situs inversus* compared to a non-affected control. We also used DTI to study axon bundle trajectories.

4 ETHICAL CONSIDERATIONS

In this thesis, a large part of the experiments were carried out in human immortalized cell lines. These cell lines offer the advantage that they are commercially available and can be easily propagated and manipulated. Thereby, many questions can be addressed without involving animal models or human participants. At the same time, the results gained from these model systems must be considered with care, since the cell lines may not fully represent the physiological conditions in human. Due to the inaccessibility of human brain material, many studies in the field of dyslexia have been carried out in animal models, such as mice or rats, or on post-mortem human tissue. In order to work with a more relevant model, we started working with iPSC-derived NES cells. This system may more accurately model the processes in human brain development. The use of iPSCs also offers the advantage avoiding many of the issues that rose from the use of hESCs.

In paper II and III, zebrafish was used as an animal model. These experiments were important in order to verify that similar processes as the ones observed *in vitro* are acting *in vivo*. The animal suffering was kept to a minimum by limiting the use to early stage animals.

In paper II and V, we used DNA from patients and healthy controls and the probands for brain imaging. All the participants gave their informed consent and participated voluntarily in the study. The use of human research subjects always requires consideration of ethical issues and is monitored by Ethical Review Boards. We estimated that the positive value added by the research carried out in the present studies outweighs the risks for the research participants.

5 RESULTS AND DISCUSSION

5.1 REGULATION AND FUNCTION OF DYX1C1 (PAPERS I, III, IV)

5.1.1 Subcellular localization

We show the localization of DYX1C1 to or around the basal body in RPE1 cells and a co-occurrence with Centrosomal-P4.1-associated protein (CPAP) (Paper I and III). These results are in accordance with previous reports observing localization to the basal body and centrosome and a proteomic study identifying dyx1c1 homologue in Chlamydomonas basal body (Hoh et al., 2012; Li et al., 2004; Tammimies et al., 2013; Wang et al., 2006). Interestingly, the DYX1C1-interacting chaperones Hsp90α, CCT4, CCT5 and CCT8 localize to the centrosome, in a similar pattern as DYX1C1 (Seo et al., 2010; Tarkar et al., 2013; Wang et al., 2015). Others have suggested a localization of Dyx1c1 also in the ciliary axoneme in NIH-3T3 cells, a finding that we did not observe in our model systems (Hoh et al., 2012). In the future, super-resolution microscopy techniques such as stimulated emission-depletion (STED) microscopy might shed more light on the exact subcellular distribution of DYX1C1 (Fig. 7).

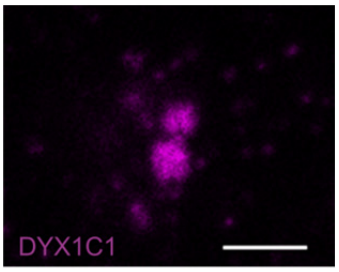

Figure 7: Super-resolution imaging of DYX1C1. Single-channel STED imaging of DYX1C1 in a RPE1 cell. Scale bar = 1 μm.

5.1.2 Tissue-specific expression

In human, *DYX1C1* is expressed mainly in reproductive organs, lung and brain (Forrest et al., 2014; Thul et al., 2017). In zebrafish, *dyx1c1* is expressed during embryonic development and in the adult brain, reproductive organs, liver and kidney (Chandrasekar et al., 2013). *DYX1C1* expression correlates with primary ciliogenesis in RPE1 cells (Paper I). Interestingly, *DYX1C1* RNA is also highly upregulated during NES cell differentiation. This stands in contrast to the expression patterns of the dynein assembly factors *DNAAF1-3*, which are absent or weakly expressed in NES cells (Paper IV). The protein product of DYX1C1 is present in NES-derived cells after 21 days of differentiation (Fig. 8). The expression of *DYX1C1* in human brain is supported by *in vitro* hESC-differentiating neurons, in promoter-specific profiling of gene expression in human tissues in FANTOM5 as well as in protein profiling of human cortex in the human protein atlas (Forrest et al., 2014; Thul et al., 2017; van de Leemput et al., 2014). Overall, these results indicate a role of DYX1C1 beyond dynein preassembly factor of motile cilia and suggest a role during neuronal development and primary cilia, consistent with previous reports (Tammimies et al., 2013).

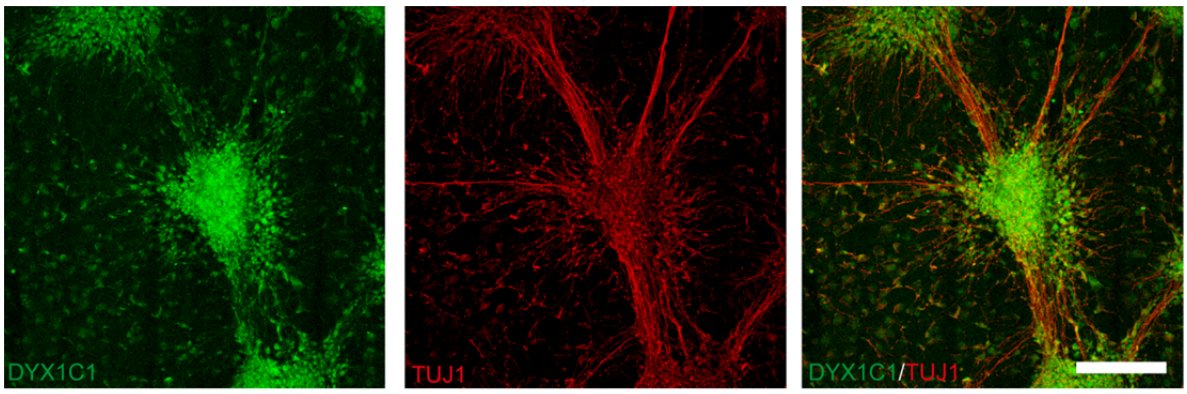

Figure 8: DYX1C1 is expressed in differentiated NES cells. Protein staining of DYX1C1 in NES-derived neuronal cells at 21 days of differentiation. TUJ1 = Tubulin βIII (neuronal marker). Scale bar = 200 μm.

5.1.3 Regulation of expression

The known mechanisms of *DYX1C1* regulation are shown in Fig. 9. In paper I, we add to the knowledge of transcriptional regulation of *DYX1C1* by identifying RFX1 as a repressor. We demonstrated that RFX1 binds to the X-box in the promoter region of *DYX1C1* and that its expression is upregulated upon knockdown of RFX1. This effect was observed in the serum-starved, ciliated cell line RPE1, but not in the non-serum-starved SH-SY5Y cell line. The known role of RFX1 in regulation of ciliary genes (Choksi et al., 2014) supports that this might be a cilia-specific effect. It has been demonstrated previously in non-ciliated cell lines that expression of *DYX1C1* is regulated by the complex of TFII-I, PARP1 and SFPQ (Tapia-Paez et al., 2008). In addition, ERβ transcriptionally regulates *DYX1C1* in an E2-dependent manner (Tammimies et al., 2012). The interplay among different transcriptional regulators may be cell-type specific and depend on the cellular environment, for example the presence or absence of estrogen or presence of cilia. Elucidation of the transcriptional regulation of *DYX1C1* in more detail may provide important insights for the understanding of dyslexia and ciliopathies.

5.1.4 Protein-protein interaction

The protein-protein interaction of DYX1C1 with CPAP and DCDC2 reported here (Paper III) may open up new perspectives on the neuronal migration function of those genes. CPAP is a protein that regulates centrosomal and ciliary length, but more recently has also been implied in neuronal migration (Garcez et al., 2015).

Similar to ERs, CHIP, certain tubulins, LIS and DCDC2, CPAP interacts with DYX1C1 via the p23 domain (Paper III) (Massinen et al., 2009; Tammimies et al., 2013). The p23 domain is known to be important for protein-protein interactions (Garcia-Ranea et al., 2002). Nevertheless, it is somewhat surprising that the TPR domains are dispensable for the interaction, as they have been shown to be crucial for the neuronal migration role of Dyx1c1 and its localization to the centrosome (Tammimies et al., 2013; Wang et al., 2006).

5.1.5 The role of DYX1C1

The functions of DYX1C1 known today are summarized in Fig. 9. Our results provide new evidence for a role of DYX1C1 in primary ciliated cells. While recent studies have confirmed a role of DYX1C1 in motile cilia, likely as a dynein preassembly factor, the role in primary cilia is yet unknown (Chandrasekar et al., 2013; Horani et al., 2018; Tarkar et al., 2013; Yamamoto et al., 2017). An important open question is whether DYX1C1 is involved in ciliogenesis. Chandrasekar et al. reported a reduction of motile and sensory cilia length and number in *dyx1c1* morpholino injected zebrafish (Chandrasekar et al., 2013), (unpublished data). This supports a role for DYX1C1 beyond axonemal dynein assembly and in ciliogenesis. It remains to be determined whether DYX1C1 also affects the growth of primary cilia. Current experiments with perturbation of DYX1C1 in RPE1 and SH-SY5Y cells might answer this question. It is conceivable that DYX1C1 may act as a chaperone for other ciliary proteins than axonemal dynein arms. Most interestingly, recent reports have suggested that regulation of primary cilia is influenced by the ubiquitine-proteasome system, to which DYX1C1 has been linked previously (Shearer and Saunders, 2016). In addition, TPR-domain containing proteins have been assigned a critical role in ciliogenesis (Xu et al., 2015).

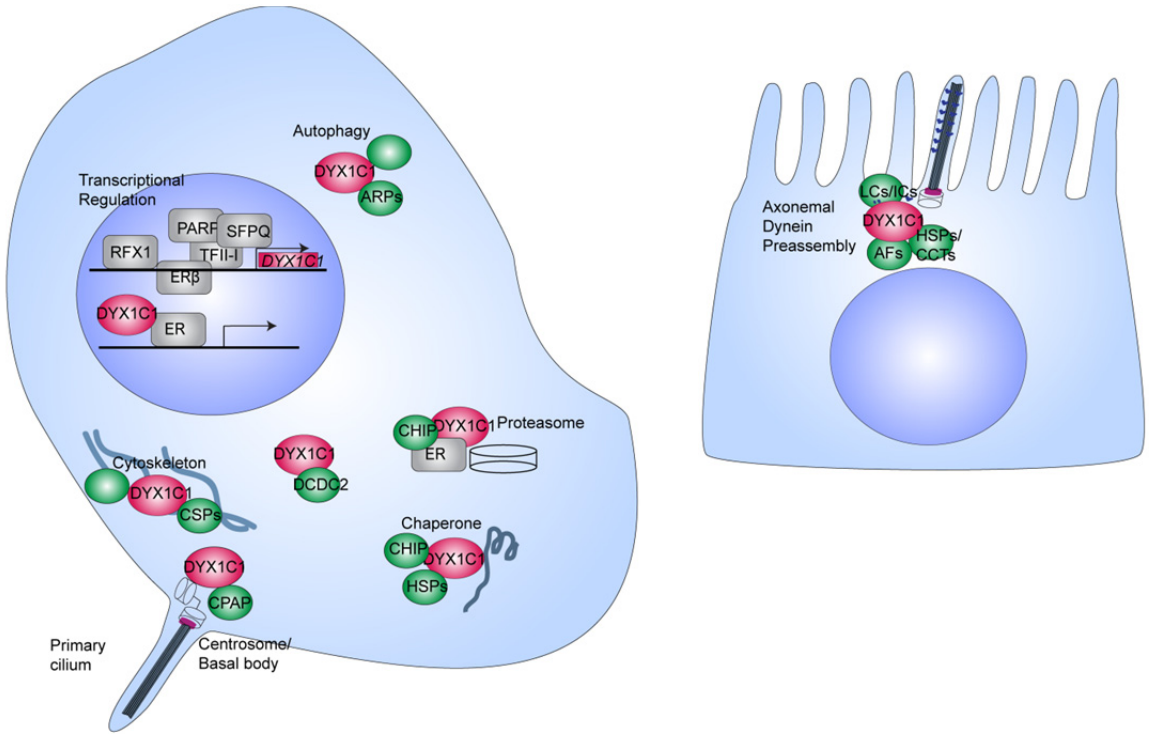

Figure 9: Overview of regulation and function of DYX1C1 placed in the cellular context. Summary of the roles of DYX1C1 in a regular cell (left) and a multiciliated cell (right). ER = estrogen receptor, HSP = heat shock protein, ARP = autophagy-related proteins, CSP = cytoskeletal proteins, LC = light chain, IC = intermediate chain, AF = assembly factor, CCT = T-complex chaperone.

There is no evidence that DYX1C1 acts directly as a transcription factor. However, perturbation studies have shown that it indirectly regulates a number of downstream targets (Tammimies et al., 2013). At the same time, the finding that knockdown of DYX1C1 leads to downregulation of DCDC2 is novel (Paper III). This finding will have to be confirmed by rescue experiments via overexpression of DYX1C1. If the regulation is confirmed, this would open up an exciting new path in dyslexia candidate gene research.

5.2 REGULATION AND FUNCTION OF DCDC2 IN HEALTH AND DISEASE (PAPERS I-IV)

5.2.1 Subcellular localization

We observed localization of DCDC2 at the axoneme of cilia in different tissues and cell lines including primary cilia in human RPE1 cells (Paper I), mouse fibroblast NIH-3T3 cells, MDCK-II cells, human kidney and liver and motile cilia in the mouse brain (Paper II) and human lung (Fig.10). Together with the published results of DCDC2 localization in rat hippocampal neurons and kinocilia in sensory hair cells, it is likely that DCDC2 acts as a ubiquitous component in all mammalian cilia, including primary, sensory and motile cilia (Grati et al., 2015; Massinen et al., 2011). In addition, we frequently observed an accumulation of DCDC2 protein towards the tip of the cilium (Fig. 10). This is in accordance with published observations (Grati et al., 2015).

5.2.2 Tissue-specific expression

Expression of *DCDC2* correlates with ciliogenesis in RPE1 cells (Paper I). No *DCDC2* expression was detected in NES cells by RNA-seq, but an upregulation during differentiation was observed using qRT-PCR (Paper IV). The gene might be expressed at a level below the threshold of detection by the implemented RNA-seq strategy, since it was detected by qRT-PCR, where a larger amount of RNA was used as input. Another possibility is that an alternative transcript might be expressed in brain, which was not detected. *DCDC2* is most highly expressed in kidney, reproductive tissues, and pancreas and expressed at lower levels in liver, lung, brain and thyroid gland (Human Protein Atlas, FANTOM5), (Forrest et al., 2014; Thul et al., 2017). A role for DCDC2 in brain is nevertheless likely. *Dcdc2* is expressed in the mouse brain in a regionally specific way, for example, in layer 4 in the mouse neuronal cortex and in the cerebellum (Li et al., 2013; Reiner et al., 2006).

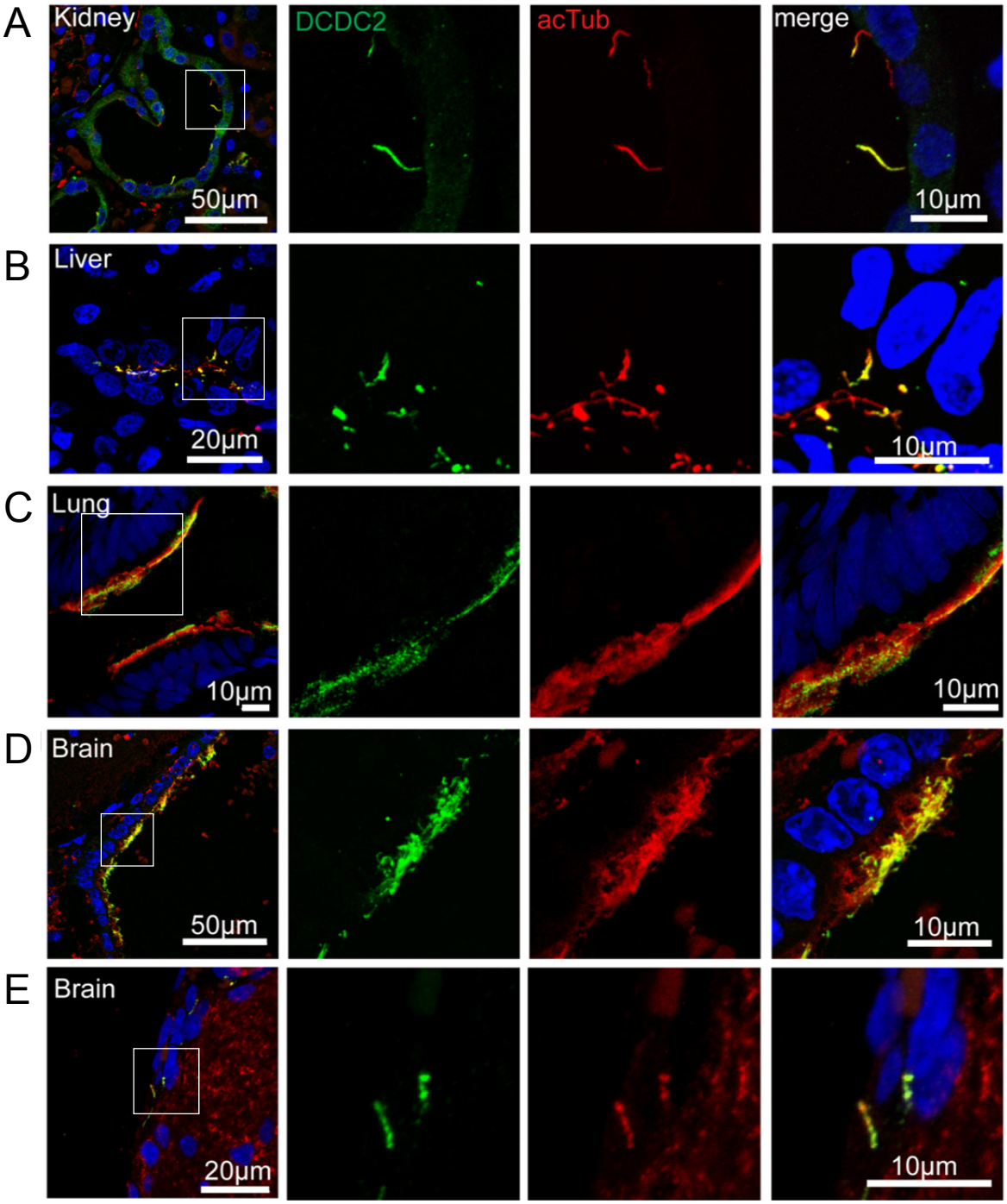

Figure 10: DCDC2 localizes to motile and primary cilia in various organs. A) Human renal epithelial cells B) Human cholangiocytes C) Human respiratory epithelial cells D) Mouse ependymal cells in 3rd brain ventricle E) Mouse brain pia mater cells. Tissue sections were stained for acetylated-α-tubulin (red) and DCDC2 (green). Nuclei are stained with DRAQ5 (blue).

5.2.3 Signaling

In paper II, we identified the Wnt-pathway as the main affected signaling pathway in NPHP-RC caused by *DCDC2* mutations. In addition, we demonstrated the specificity via rescue experiments using specific Wnt-inhibitors. This is in accordance with previous results showing the involvement of DCDC2 in the Wnt-pathway in rat hippocampal neurons (Massinen et al., 2011). However, based on our data, DCDC2 is most likely not involved in the JNK pathway signaling, but further studies are needed to address this. In addition, we observed activation of the Shh pathway by the wildtype but not the mutated constructs of *DCDC2* (Fig. 11). Shh and Wnt pathways are known to act via primary cilia (Goetz and Anderson, 2010). The repression of the Wnt pathway and concomitant activation of the Shh pathway suggests an antagonism between the two pathways. Such an antagonism between the Wnt and Shh pathways has been suggested (Ding and Wang, 2017). The exact molecular mechanism of how DCDC2 affects these pathways is yet unknown.

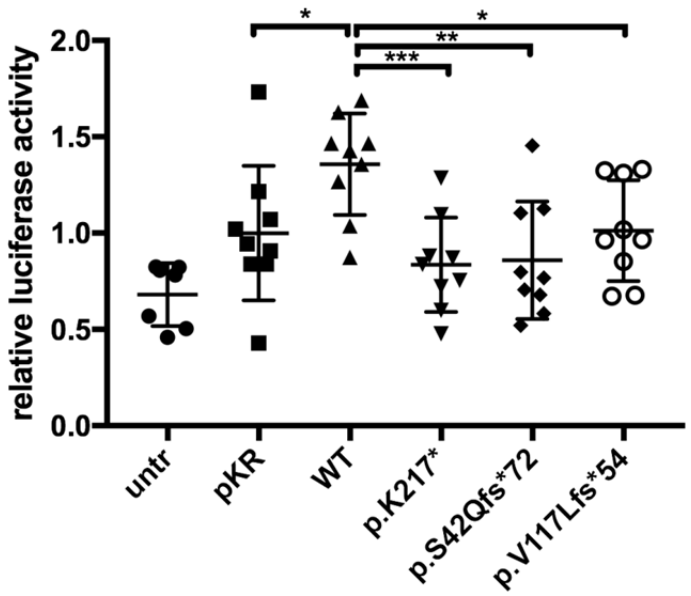

Figure 11: DCDC2 activates Shh signaling. Activation of the Shh pathway in a NIH-3T3 cell line stably transfected with a luciferase reporter containing 8 Gli-binding sites and TK-Renilla and transiently transfected with wildtype and mutated *DCDC2*. Wildtype *DCDC2* induces stronger activation of Shh signaling than mutated *DCDC2*. untr = untransfected, pKR = empty vector, WT = wildtype; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, Student´s t-test.

5.2.4 Ciliopathies

In paper II, we describe *DCDC2* as a causative gene for NPHP-RC in two families by identifying loss-of-function mutations (Fig. 12). Recent studies have added to the evidence of *DCDC2* as a ciliopathy gene: *DCDC2* is mutated in patients with hereditary hearing loss and NSC (Girard et al., 2016; Grammatikopoulos et al., 2016; Grati et al., 2015). These studies support a role of *DCDC2* as a ciliopathy gene and suggest kidney- and liver- specificity as well as implying a role in sensory cilia.

In only 2 out of 800 scanned families, *DCDC2* is implied as the causative gene for NPHP-RC. *DCDC2* is not a causative gene in many patients, in line with the reported high genetic

heterogeneity of NPHP-RC (Hildebrandt et al., 2011). Probably more private, family-specific mutations will be identified in the future.

How can it be that ciliopathies only affect certain organs despite cilia being a ubiquitous organelle? Recently, this complexity of ciliopathies has been emerging (Reiter and Leroux, 2017). Some ciliary genes are expressed in a tissue-specific manner, but that does not explain the whole spectrum of organ-specificity. For example, research in *Drosophila melanogaster* showed that the nano-composition of ciliary components varies according to cell-type and cell-cycle stage, providing one possible explanation for the tissue-specificity of ciliopathies (Jana et al., 2018).

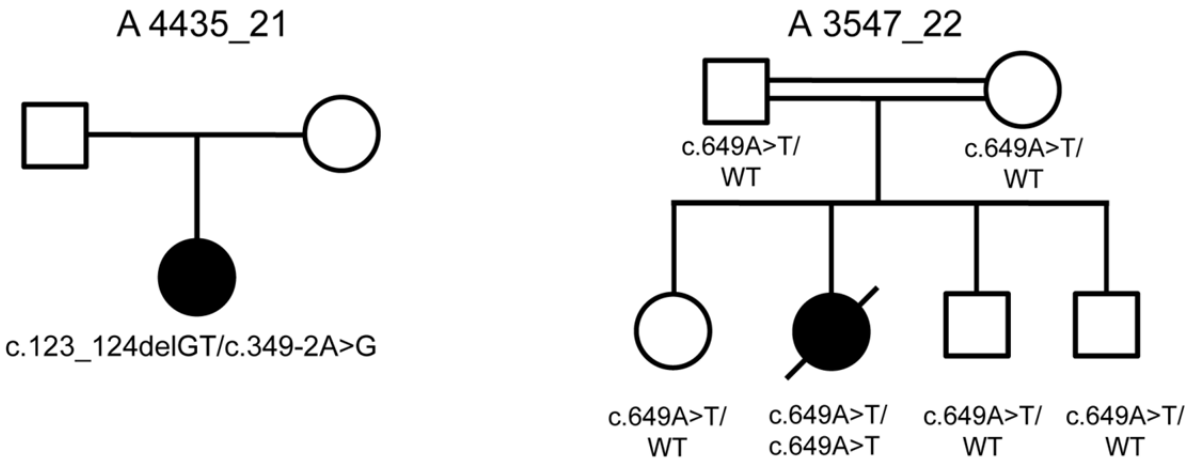

Figure 12: NPHP-RC affected families with mutations in *DCDC2*. The families and mutations are reported in detail in Paper II. c.649A>T = homozygous truncating mutation p.Lys217*; c.123_124delGT = frameshift mutation p.Ser42Glnfs*72; c.349-2A>G = obligatory splice site mutation producing frameshift product p.Val117Leufs*.

5.2.5 Regulation of expression

The transcriptional regulation of *DCDC2* is summarized in Fig. 13. In paper I, we show that RFX2 and RFX3 are activators, possibly in a complex, of *DCDC2*. In contrast, RFX1 acts as a repressor of *DCDC2* transcription. RFX1 and RFX2 bind to the promoter sequences encompassing the X-box motif as shown by EMSA. RFX1-3 are known as ciliogenic transcription factors and have roles in brain development (Choksi et al., 2014). Our data therefore strengthen the role of *DCDC2* in cilia.

In the context of dyslexia, *DCDC2* expression has been suggested to be regulated by the intronic short tandem repeat READ1, associated to dyslexia, and the transcription factor ETV6 (Meng et al., 2011; Powers et al., 2013).

Further studies of the transcriptional regulation of *DCDC2* will be crucial for understanding the role of *DCDC2* in dyslexia and ciliopathies.

5.2.6 The role of DCDC2

The functions of DCDC2 are summarized in Fig. 13. The main emerging molecular function of DCDC2 is microtubule stabilization (Coquelle et al., 2006; Jeruschke et al., 2015; Massinen et al., 2011). DCDC2 likely acts in both motile and primary cilia and probably assumes the same or overlapping roles in both.

DCDC2 may be directly involved in ciliogenesis. Massinen et al. and Grati et al. report longer cilia when DCDC2 is overexpressed in rat hippocampal neurons and inner ear cells (Grati et al., 2015; Massinen et al., 2011) and knockdown of Dcdc2 in IMCD3-derived 3D spheroids leads to reduced ciliation (Paper II). In addition, cilia are lacking in the liver of NSC patients (Girard et al., 2016; Grammatikopoulos et al., 2016). However, we observed no change in cilia number and length upon suppression of *dcdc2b* in zebrafish (Paper II). Similarly, no change of cilia length was observed in *dcdc2* knockout mouse brain (Wang et al., 2011). Overall, DCDC2 might regulate cilia length in a tissue-dependent and possibly species-dependent way.

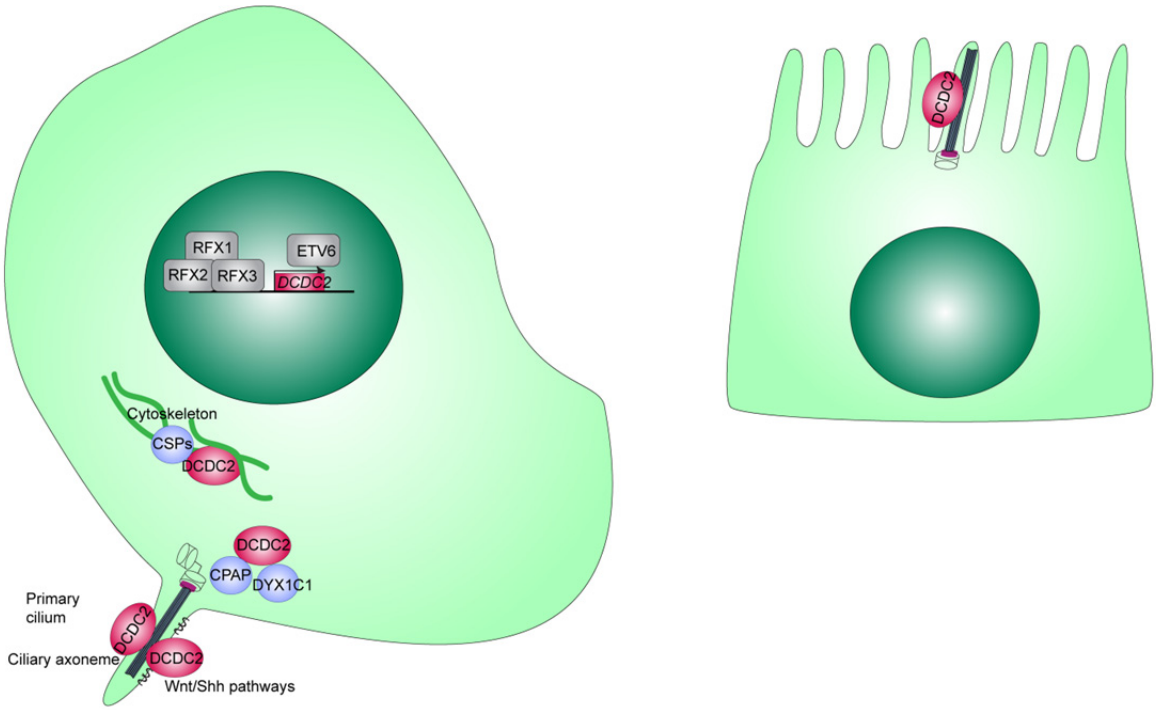

Figure 13: Overview of regulation and function of DCDC2 placed in the cellular context. Summary of the roles of DCDC2 in a regular cell (left) and a multiciliated cell (right). CSP=cytoskeletal proteins

5.3 DYSLEXIA CANDIDATE GENES AND CILIARY GENES IN NEURONAL DIFFERENTIATION (PAPER IV)

The high number of genetic studies in dyslexia stands in contrast to a limited amount of functional studies on the implied genes, especially with regard to human model systems. In addition, there is a limited body of literature on the role of primary cilia in human brain development. We tried to address these issues by characterizing the transcriptome of human NES cells along the differentiation time axis with a focus on dyslexia candidate genes and ciliary genes.

In paper IV, we identified a striking upregulation of ciliary genes during human neuronal differentiation and observed that cilia are present on neuronal cells throughout differentiation (Fig. 14). An involvement of ciliary genes in human neuronal differentiation has not been reported in many studies. An *in vitro* model of differentiating hESCs to neurons has identified GO categories related to cilia as upregulated, consistent with our results (van de Leemput et al., 2014). Regarding dyslexia candidate genes, interestingly, we found that many dyslexia candidate genes are expressed in NES cells. In contrast, we did not observe a significant enrichment of dyslexia candidate genes among upregulated genes. It might be that the dyslexia candidate genes act earlier or later in development or that our implemented model insufficiently reproduces the neurodevelopmental events involved in dyslexia. It is also possible that the list of dyslexia candidate genes includes several false positive observations diluting the statistical test, as the vast majority of the implicated genes have not been replicated.

The strength of this study lies in the use of a human neuronal model. On the other hand, it is limited by the transcriptomics-only approach. Further studies will require a confirmation on protein-level, as the correlation between RNA level and protein level is generally limited (Edfors et al., 2016). In addition, cilia length and number should be quantified over time, in order to allow conclusions about whether ciliary gene expression correlates with ciliary growth. Finally, the functionality of the cilia during differentiation could be addressed by eliminating cilia via disruption of a ciliogenic gene. Such a study would be a valuable addition to the rich body of literature on knockout animal models of ciliary genes in brain.

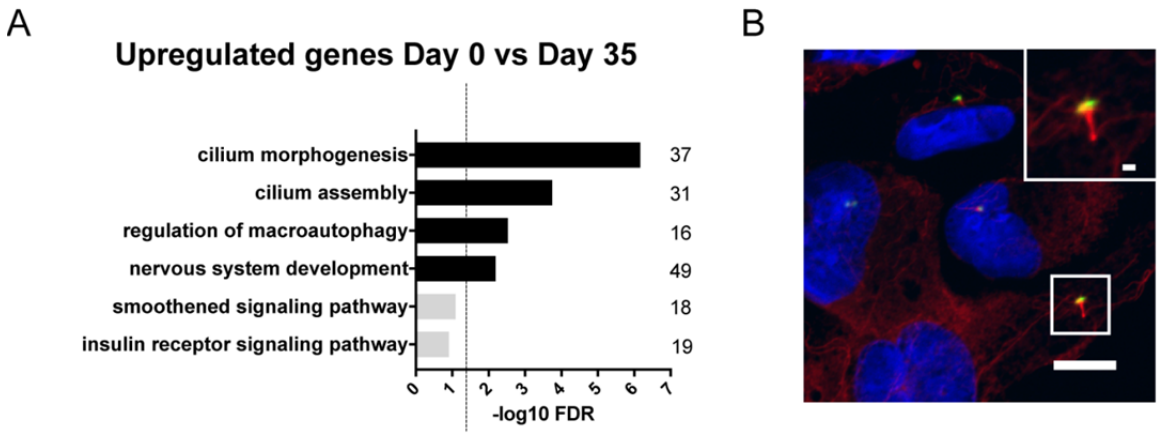

Figure 14: Ciliary genes and cilia in NES cells. A) Gene ontology term enrichment analysis of differentially expressed genes comparing day 0 and day 35 of NES cell differentiation. GO terms of six top ranked biological processes are shown. Terms with FDR<0.05 = black, terms with FDR>0.05 = gray. Dashed line indicates FDR = 0.05. The number of genes in each category is indicated on the right of the bars. B) Cilia in NES cells differentiated for 35 days, visualized with the ciliary marker acetylated-α-Tubulin (red) and the centrosomal marker pericentrin (green). Scale bar = 10 µm, scale bar inset = 1 µm. Nuclei were counterstained with DRAQ5.

5.4 SITUS INVERSUS, THE BRAIN AND DYSLEXIA (PAPER V)

While the relationship between dyslexia candidate genes and cilia has been addressed in several functional studies (paper I), (Massinen et al., 2011), direct examination of individuals with a ciliopathy and dyslexia are so far lacking. Paper V is the first study investigating the underlying genetic causes in individuals with a ciliopathy - *situs inversus* - and dyslexia.

In case 1, the co-occurrence of the phenotypes PCD, dyslexia and scoliosis prompted us to investigate *DYX1C1*, a gene associated with all of these phenotypes (Grimes et al., 2016; Taipale et al., 2003; Tarkar et al., 2013). However, we did not find any interesting variants in *DYX1C1*. The ciliary phenotype - lack of outer dynein arms and intact inner dynein arms - corresponds very well with the proposed causative gene *dynein axonemal heavy chain 5* (*DNAH5)* (Hornef et al., 2006). Also, the prediction tools support a pathogenicity of the identified variants. However, their involvement in dyslexia seems unlikely as there is no co-segregation of the variants with dyslexia in the family.

We studied further the patient from case 1 with MRI, but could not draw any major conclusions about the laterality of the brain. The DTI data revealed that there are more corticospinal tracts in the left hemisphere of the patient, but more in the right hemisphere in the control. This might also be due to handedness, a known factor influencing brain laterality (Knecht et al., 2000). Brain MRI studies of *situs inversus* cases have generally been limited by the rarity of this syndrome. Whether brain laterality is reversed in *situs inversus* mammals is not known yet; it has even been suggested that the brain *situs* is independent of the *situs* of internal organs in humans – opposed to other vertebrates such as zebrafish (McManus et al., 2004). Future research using fMRI to study brain laterality including *situs inversus* subjects

would be valuable and also relevant for the clinic: clinicians performing diagnostic or surgical procedures such as neurosurgery on *situs inversus* individuals should be aware of the brain *situs* (Tubbs et al., 2003).

In case 2, we identified one rare variant in *dynein axonemal heavy chain 11* (*DNAH11)*, but the second variant remains elusive. A reason why we were unable to pinpoint a strong candidate gene with more certainty may be that non-syndromic *situs inversus* has been poorly studied and its genetic causes remain largely unknown (Deng et al., 2015). Moreover, there might be a polygenic or an environmental origin of laterality disorders, which we could not detect with our analysis strategy (Deng et al., 2015).

Overall, the limitation of the small sample size in this study does not allow generalization of the genetic and imaging findings. Systematic inspection of the ciliary gene variants in dyslexia cohorts or of dyslexia candidate genes in ciliopathy cohorts will allow a more comprehensive conclusion about the relationship of cilia and dyslexia.

5.5 IS THERE A LINK BETWEEN DYSLEXIA AND CILIA? (ALL PAPERS)

The finding that some of the most replicated dyslexia candidate genes are ciliary genes prompted us to ask whether there might be a role of cilia in dyslexia. Fig. 15 summarizes the relation between the cilium and dyslexia candidate genes. There are different conceivable scenarios on how cilia deficiency could contribute to the etiology of dyslexia at the molecular level.

Cilia might act as players in neuronal migration: The neuronal migration hypothesis in dyslexia has been proposed based on observations on postmortem brains of dyslexic individuals and on animal studies using *in utero* RNA interference in rats (Gabel et al., 2010; Galaburda et al., 1985). The malformations resulting from the migration defects in turn have been linked to neuronal connection and processing deficits (Galaburda et al., 2006; Giraud and Ramus, 2013). In parallel, primary cilia are involved in directing migration in fibroblasts (Christensen et al., 2008) and cilia are crucial for interneuron migration (Baudoin et al., 2012; Higginbotham et al., 2012). On the other hand, their involvement in radial cortical migration is unclear and it remains undetermined whether radially migrating bipolar or multipolar neurons assemble a transient cilium (Sarkisian and Guadiana, 2015). At the same time, Guo et al. have reported a critical role of ciliary genes in radial neuronal migration (Guo et al., 2015).

Cilia may be responsible for the establishment of brain asymmetry: Brain asymmetry has been associated with several neuropsychiatric disorders including dyslexia, schizophrenia and autism (Trulioff et al., 2017). In addition, cilia have been implied in some of these disorders (Paracchini et al., 2016; Trulioff et al., 2017). It is also known that cilia act in left/right establishment of the body axis at the embryonic node in early development via the nodal pathway (Shinohara and Hamada, 2017). Disturbed cilia might lead to an impaired left-right establishment also in the brain. At the same time, it has been suggested that brain asymmetry raises independently from the main laterality axis (McManus et al., 2004).

Ciliary defects may have a contribution to the etiology of dyslexia in either of the proposed ways described above. However, there are also limitations on these theories. Some evidence seemingly contradict a role of *DYX1C1* and *DCDC2* in dyslexia: patients with ciliopathies and loss-of-function mutations in *DCDC2* and *DYX1C1* do not have dyslexia (Paper II), (Tarkar et al., 2013). An explanation for these observations could be that different types of mutations can have very different outcomes on the phenotypes. There are many cases reported where different kinds of variants in the same gene produce dissimilar phenotypes. For example, *FGFR2* is the causative gene of Apert syndrome, a rare congenital syndrome with craniosynostosis and severe syndactyly (Wilkie et al., 1995). At the same time, a SNP in *FGFR2* is the most significantly associated SNP in a GWAS study with 50,000 breast cancer cases (Easton et al., 2007). Neither do Apert syndrome patients develop breast cancer more frequently, nor do breast cancer patients have any of the symptoms present in Apert syndrome. In dyslexia, it might be that alterations in SNPs in regulatory regions lead to the relatively mild phenotype. SNPs in the regulatory sequences of dyslexia candidate genes have been suggested as risk factors and have been demonstrated to functionally affect transcriptional regulation in some cases (Dennis et al., 2009; Meng et al., 2011; Tammimies et al., 2012).

Another limitation that should be considered is that the earlier results implying *Dyx1c1*, *Dcdc2* and *Kiaa0319* in neuronal migration using knockdown in rats were not replicated in knockout mouse models (Martinez-Garay et al., 2017; Rendall et al., 2015; Wang et al., 2011). An explanation for the discrepancies in phenotype might be the different species examined – rat vs. mouse – or the different technology – shRNA vs. genetic knockout. Recent studies have provided an explanation for the dissimilar phenotypes in knockdown versus knockout animal models (El-Brolosy and Stainier, 2017). The underlying molecular mechanisms explaining such phenomena might be due to genetic compensation (El-Brolosy and Stainier, 2017).

Given the evidence present today, dyslexia will most likely not figure as a ciliopathy *per se*. However, the cilium may have a role in dyslexia to some degree. In a similar way, a role of the cilium is emerging also in other disorders, such as cancer and obesity (Mans et al., 2008; Mariman et al., 2016). In addition, there is a growing body of literature suggesting other neuropsychiatric disorders might also involve cilia (Lauter et al., 2018; Marley and von Zastrow, 2012; Migliavacca et al., 2015; Munoz-Estrada et al., 2018; Paracchini et al., 2016; Trulioff et al., 2017).

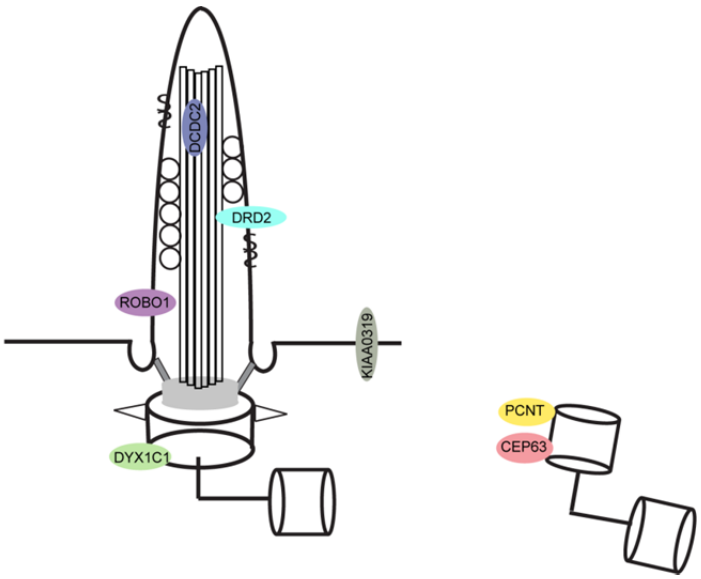

Figure 15: The relation of dyslexia candidate gene products to primary cilium and centrosome. Dyslexia candidate genes of which protein products localize to the cilium or the centrosome or which have been linked to ciliary functions.

6 CONCLUSION AND FUTURE PERSPECTIVES

In the past 15 years, a number of dyslexia candidate genes have been identified by means of genetic studies. More recently, some of the same genes, including *DYX1C1* and *DCDC2,* have been implied in functions at the cilium. In this thesis, we explored the function and regulation of these genes in respect to the cilium and addressed whether ciliary genes might be implied in dyslexia and neuronal differentiation in general.

In paper I, we found that *DYX1C1* and *DCDC2* are regulated by the ciliogenic transcription factors RFX. To date, mutations in ciliogenic RFX TFs have not been identified in patients with ciliopathies nor in dyslexic probands. Based on our results, we propose that future genetic studies in dyslexia and in ciliopathies should thoroughly scrutinize RFX sequences. In addition, further functional studies involving RFX TFs should aim at a systematic identification of RFX-targets using for example chromatin-immunoprecipitation sequencing (CHIP-seq). Such an approach has been undertaken for *Rfx2* by Chung and colleagues (Chung et al., 2014). Furthermore, the mutual regulation among RFX TFs discovered in paper I should be dissected in more detail, as it might reveal additional general roles of RFX TFs.

In paper II, we identified *DCDC2* as a causative gene for NPHP-RC. NPHP-RC displays high genetic heterogeneity and a majority of cases remains unsolved (Schueler et al., 2016). It is therefore likely that many more family-specific mutations will be identified as causative for NPHP-RC in the future. Defining a unifying underlying mechanism remains challenging and so will treatment strategies (Srivastava et al., 2017). Recent studies have also expanded the spectrum of *DCDC2* as a ciliopathy gene (Girard et al., 2016; Grammatikopoulos et al., 2016; Grati et al., 2015). It remains to be determined whether *DCDC2* is a causative gene in more ciliopathies.

In paper III, we proposed a pathway synergy between *DYX1C1* and *DCDC2*. In order to investigate more if DYX1C1 and DCDC2 act in the same pathway upstream or downstream of each other, rescue experiments could be performed to test if knockdown of one gene can be rescued by the other gene. The subcellular localization of the protein complexes involving DYX1C1, DCDC2 and CPAP may be explored more by *in situ* proximity ligation assay (isPLA) – a method allowing detection of protein complexes directly in the tissue. This would allow addressing whether the protein interaction takes place in the cytoplasm or at distinct organelles such as the centrosome. In addition, more precise characterization of the subcellular localization may be achieved by super-resolution microscopy, such as STED.

In paper IV, we identified the class of ciliary genes as a major upregulated gene class in human neuronal differentiation. As ciliopathies often involve neurodevelopmental defects, this finding is interesting also from a clinical point of view, and ought to be investigated further. Specifically, in our system, the number and length of cilia should be quantified in a time-dependent manner and protein expression of ciliary genes assessed to test whether there is a correlation with the transcriptomics results. Next, the functional impact of primary cilia

on human neuronal development should be tested directly by targeted disruption of ciliogenic genes. To this end, iPSCs will be genetically targeted using the gene editing tool CRISPR/Cas9 (Powell et al., 2017) to lack cilia or produce dysfunctional cilia by ablating ciliogenic genes and ciliopathy genes such as *IFT88*, *KIF3A, BBS2* or *DYX1C1*. The edited cell lines can then be phenotypically analyzed during differentiation, for example regarding neuronal markers. The NES model system could also be further expanded to study dyslexia. iPSCs can be derived from dyslexic individuals, established as NES cells and phenotypically characterized. Thereby, the role of neuronal migration in dyslexia may be addressed directly in human by using an *in vitro* cell migration assay. Taking advantage of such an approach, the role of DCX in neuronal migration using NES cells derived from lissencephaly patients was investigated (Shahsavani et al., 2017). In line with this strategy, Tammimies and colleagues examined the role of DYX1C1 in neuronal migration using SH-SY5Y cells (Tammimies et al., 2013). Recently, modeling of human brain development by 3D organoids has opened up new possibilities to study neuronal development and disorders (Lancaster et al., 2013). Modeling of complex polygenic neurodevelopmental disorders using brain organoids combined with more refined analyses such as single-cell RNA-seq has recently also been accomplished (Quadrato et al., 2016). Such a strategy could conceivably also be applied on iPSCs derived from dyslexic individuals and might highlight subtle differences that are expected for a mild phenotype like dyslexia.

Inspection of the genome of two individuals with dyslexia and *situs inversus* identified rare variants in dynein heavy chain genes, but their involvement in dyslexia remains unclear (paper V). Our study was limited by the small sample size available to us. A systematic examination of the dyslexia phenotype in ciliopathy cohorts would allow a more thorough analysis. This approach was hindered by the fact that most often the dyslexia phenotype is not reported in patients with ciliopathies. Alternatively, a systematic examination of ciliary genes, including non-coding sequences, in dyslexia cohorts could be helpful; this path is already being explored by dyslexia geneticists. In addition, functional characterization of the identified variants in cell models will clarify their roles in PCD and dyslexia.

In conclusion, this thesis work has strengthened the evidence for a role of the dyslexia candidate genes *DYX1C1* and *DCDC2* at the cilium in homeostasis and disease. In addition, it has highlighted the group of ciliary genes as a main upregulated class in human neuronal differentiation. However, in individuals with *situs inversus* and dyslexia, no common genetic origin could be pinpointed. Future functional studies using human *in vitro* brain models will shed more light on the general functions of these genes and their involvement in dyslexia. Cilia´s involvement in neuronal development and neuropsychiatric disorders as well as its role in other disorders promises to hold exciting new discoveries for the future.

7 POPULAR SCIENCE SUMMARY

7.1 POPULAR SCIENCE SUMMARY

Dyslexia is defined as a difficulty in reading despite normal intelligence, normal sensory perception and appropriate education. It is a condition affecting about 5-10% of the population, which means that about one in every twenty schoolchildren suffers from dyslexia. It is well known that dyslexia runs in families and that there is a genetic predisposition to it. Studies have identified specific genes that are altered in families with dyslexia. Remarkably, many of these genes are associated with the development of the brain. In other words, in dyslexics, the processes that lead to functional connections in the brain are disturbed. Three of these genes are called *DYX1C1, DCDC2* and *KIAA0319*.
More recently, it has become known that the same genes are not only involved in the development of the brain, but they are also important for the function of hair-like structures on the surface of cells called cilia. A cilium can be considered as the "antenna" of the cell, as a sensor on the cell surface that allows communication with other cells. It can receive signals and send them to other cells. Cilia are found on the surface of almost all human cells, including brain cells. It is known that the function of cilia is impaired in many diseases, including neurodevelopmental disorders. The group of diseases that specifically affects the function of cilia is called ciliopathies. However, little is known about the role of cilia in human brain development and whether there exists a connection between cilia and dyslexia.

In order to investigate the function of the genes *DYX1C1* and *DCDC2*, we used human and animal cell lines. Cell lines can be manipulated in the laboratory to study how certain genes work. We also used DNA directly from humans to find changes in DNA – so-called mutations.
The aim of this work was to investigate the basic functions of genes *DYX1C1* and *DCDC2* in cell and animal models. In addition, it has been investigated how genes associated with dyslexia or cilia are regulated in neuronal development.

In study I, we found that a certain group of so-called transcription factors - factors that control gene expression by binding to DNA - regulate the expression of *DYX1C1* and *DCDC2*. The group of transcription factors is referred to as "regulatory factors X" and is known for the regulation of ciliary genes. We also showed that the protein products of DYX1C1 and DCDC2 are localized in the cell in ciliary structures. We have thus reinforced the evidence that *DYX1C1* and *DCDC2* are ciliary genes.
In study II, we identified mutations in the *DCDC2* gene as the cause of the disorder nephronophthisis-related ciliopathy (NPHP-RC). NPHP-RC is a rare hereditary kidney disease that affects children caused by malfunction of the cilia. We also identified the biochemical signaling pathways underlying the role of *DCDC2* in NPHP-RC.

In study III, we found a genetic interaction between the genes *DYX1C1* and *DCDC2* using cell models and animal models. We concluded that these two genes may act on the same biochemical pathway.
In study IV, we used human neuronal cells made from induced pluripotent stem cells. We found that the group of ciliary genes is activated during human brain development, and conclude that it is likely to be instrumental in human neuronal development.
In study V, we examined the genome of two individuals suffering from dyslexia and a ciliopathy to investigate whether a common genetic cause underlies both conditions. The subjects studied have *situs inversus*, a reversal of the internal organs of the body, which results from a disturbance of the cilia in embryonic development. We found rare mutations in known genes causing *situs inversus*, but we could not conclude the significance of these mutations in dyslexia.

This work has increased the evidence for the importance of *DYX1C1* and *DCDC2* in cilia and ciliopathies and contributed to the understanding of the function and regulation of these genes. In addition, a group of ciliary genes has been described as an important group in human brain development. Future studies will shed more light on the general functions of these genes and their involvement in dyslexia, for example, with human brain models grown in the laboratory. Genetic studies on groups of individuals with ciliopathies and dyslexia could also clarify whether a common genetic basis exists for both disorders. The involvement of cilia in brain development and neuropsychiatric diseases promises exciting new discoveries in the future.

7.2 POPULÄRVETENSKAPLIG SAMMANFATTNING

Dyslexi definieras som oförklarade läsproblem, trots normal intelligens, sensorisk uppfattning och undervisning. Det är en störning som påverkar cirka 5-10% av befolkningen, vilket innebär att ungefär ett av tjugo skolbarn drabbas av dyslexi. Orsaken anses vara avvikelser i hjärnans utveckling. Det är känt att dyslexi förekommer ofta i familjer och man antar en genetisk disposition. Flera studier har identifierat specifika gener som är förändrade i familjer med dyslexi och det är känt att många av dessa gener fungerar i hjärnans utveckling. I dyslektiker störs de processer som leder till funktionella kopplingar i hjärnan. Tre av dessa gener kallas *DYX1C1*, *DCDC2* och *KIAA0319*.
Det har blivit känt mer nyligen att samma gener inte bara har med hjärnans utveckling att göra, utan att de är också viktiga för cilier, hårliknande strukturer på ytan av celler. En cilie kan betraktas som cellens "antenn", en sensor på cellytan som möjliggör kommunikation med andra celler. Den kan ta emot signaler och skicka dem till andra celler. Cilier finns på ytan av nästan alla mänskliga celler, även hjärnceller. Det är känt att ciliernas funktion är rubbad i flera sjukdomar, inklusive psykiska störningar. Den grupp av sjukdomar där funktionen av cilier är specifikt nedsatt kallas ciliopatier. Vi har bara begränsad kunskap om ciliens roll vid utveckling av den mänskliga hjärnan. Det är också okänt om det finns ett samband mellan dyslexi och cilier.

För att undersöka funktionen av gener *DYX1C1* och *DCDC2*, använde vi mänskliga eller animaliska cellinjer. Cellinjer kan manipuleras i laboratoriet för att studera hur vissa gener fungerar. Vi använde också DNA direkt från människor för att hitta förändringar i genomet - så kallade mutationer.
Syftet med detta arbete var att undersöka grundfunktionerna av generna *DYX1C1* och *DCDC2* i cell- och djurmodeller. Dessutom har det undersökts hur gener som är associerade med dyslexi eller cilier regleras i neuronal utveckling.

I studie I fann vi att en viss grupp så kallade transkriptionsfaktorer - faktorer som reglerar genuttryck genom att binda till DNA - påverkar uttrycket av generna *DYX1C1* och *DCDC2*. Gruppen transkriptionsfaktorer benämns "reglerande faktorer X" och är känd för reglering av ciliära gener. Vi visade också att proteinprodukterna från *DYX1C1* och *DCDC2* är lokaliserade i cellen i ciliära strukturer. Vi har därmed förstärkt bevisen att *DYX1C1* och *DCDC2* är ciliära gener.
I studie II identifierade vi mutationer i *DCDC2*-genen som orsak till nephronophthisis-liknande ciliopati (NPHP-RC), en sällsynt ärftlig njursjukdom som drabbar barn. Det är känt att ett fel i cilier leder till denna sjukdom. Vi identifierade också de biokemiska signalvägar som ligger till grund för *DCDC2*s roll i NPHP-RC.
I studie III fann vi en genetisk interaktion mellan generna *DYX1C1* och *DCDC2* med hjälp av cellmodeller och djurmodeller. Vi visade att dessa två gener troligen agerar genom samma biokemiska signalväg.
I studie IV använde vi mänskliga neuronala celler gjorda av inducerade pluripotenta stamceller. Vi fann att ciliagener aktiveras under mänskliga hjärncellers utveckling och dessa gener är sannolikt viktiga för utvecklingen av människans neuroner.
I studie V undersökte vi genomet hos två individer som lider av dyslexi och en ciliopati för att undersöka om en gemensam genetisk orsak kunde bidra till båda tillstånden. Probanderna som undersöktes har *situs inversus*, en inversion av kroppens inre organ, vilket resulterar av en störning av cilier i embryonisk utveckling. Vi hittade sällsynta mutationer i kända gener som orsakar *situs inversus*, men vi kunde inte bevisa om dessa kan kopplas till dyslexi.

Detta arbete har ökat beviset för vikten av *DYX1C1* och *DCDC2* i cilier och ciliopatier och bidragit till förståelsen av funktionen och reglering av dessa gener. Dessutom har gruppen av ciliära gener beskrivits som en viktig grupp vid utveckling av den mänskliga hjärnan. Framtida studier kommer att kasta mer ljus över de generella funktionerna hos dessa gener och deras roll i dyslexi, till exempel med mänskliga hjärnmodeller, som kan odlas i laboratoriet. Genetiska studier på grupper av individer med ciliopatier och dyslexi kan också klargöra om det finns en gemensam genetisk orsak för båda sjukdomarna. Rollen av cilier i hjärnans utveckling och neuropsykiatriska sjukdomar lovar spännande nya upptäckter i framtiden.

7.3 POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG

Legasthenie wird als eine Schwierigkeit beim Lesen trotz normaler Intelligenz, normaler Sinneswahrnehmung und angemessener Bildung definiert. Es ist eine Störung, die etwa 5-10% der Bevölkerung betrifft, was bedeutet, dass etwa eines von zwanzig Schulkindern an Legasthenie leidet. Als Ursache werden leichte Störungen in der Gehirnentwicklung angenommen. Es ist bekannt, dass Legasthenie gehäuft familiär auftritt und dass es eine genetische Veranlagung dazu gibt. Studien haben spezifische Gene identifiziert, die in Familien mit Legasthenie verändert sind. Bemerkenswerterweise hängen viele dieser Gene mit der Entwicklung des Gehirns zusammen. Mit anderen Worten, bei Legasthenikern sind die Prozesse, die zu funktionellen Verbindungen im Gehirn führen, gestört. Drei dieser Gene heissen *DYX1C1*, *DCDC2* und *KIAA0319*.
In jüngerer Zeit ist bekannt geworden, dass dieselben Gene nicht nur in der Entwicklung des Gehirns eine Rolle spielen. Sie sind auch wichtig für die Funktion von haarähnlichen Strukturen auf der Oberfläche von Zellen, die als Zilien bezeichnet werden. Eine Zilie kann als die "Antenne" der Zelle angesehen werden, als ein Sensor auf der Zelloberfläche, der die Kommunikation mit anderen Zellen ermöglicht. Sie kann Signale empfangen und an andere Zellen senden. Zilien befinden sich auf der Oberfläche von fast allen menschlichen Zellen, einschliesslich Gehirnzellen. Es ist bekannt, dass die Funktion von Zilien bei vielen Krankheiten, einschliesslich entwicklungspsychologischer Störungen, beeinträchtigt ist. Die Gruppe von Krankheiten, bei der spezifisch die Funktion von Zilien betroffen ist, wird Ziliopathien genannt. Über die Rolle von Zilien in der menschlichen Gehirnentwicklung ist bisher wenig bekannt. Es ist bisher auch unbekannt, ob ein Zusammenhang zwischen Zilien und Legasthenie besteht.

Um die Funktion der Gene *DYX1C1* und *DCDC2* zu untersuchen, haben wir Zelllinien aus Menschen und Tieren genutzt. Zelllinien können im Labor manipuliert werden, um zu untersuchen, wie bestimmte Gene funktionieren. Wir haben auch DNA direkt von Menschen verwendet, um Veränderungen in der DNA zu finden – sogenannte Mutationen.
Ziel dieser Arbeit war es, die grundlegenden Funktionen der Gene *DYX1C1* und *DCDC2* in Zell- und Tiermodellen zu untersuchen. Ausserdem wurde untersucht, wie Gene, die mit Legasthenie oder Zilien in Zusammenhang stehen, in der neuronalen Entwicklung reguliert werden.

In Studie I fanden wir heraus, dass eine bestimmte Gruppe sogenannter Transkriptionsfaktoren – Faktoren, die die Genexpression durch Bindung an DNA regulieren – die Expression der Gene *DYX1C1* und *DCDC2* regulieren. Die Gruppe der Transkriptionsfaktoren wird als „regulatorische Faktoren X“ bezeichnet und ist für die Regulierung von ziliären Genen bekannt. Auch haben wir gezeigt, dass die Proteinprodukte von DYX1C1 und DCDC2 in der Zelle in Zilienstrukturen lokalisiert sind. Wir haben damit die Hinweise, dass es sich bei *DYX1C1* und *DCDC2* um ziliäre Gene handelt, verstärkt.
In Studie II identifizierten wir Mutationen im *DCDC2*-Gen als Ursache für die Störung Nephronophthise-ähnliche Ziliopathie (NPHP-RC). NPHP-RC ist eine seltene erbliche Nierenerkrankung, die Kinder betrifft. Es ist bekannt, dass eine Fehlfunktion der Zilien zu

dieser Krankheit führt. Wir identifizierten auch die biochemischen Signalwege, die der Rolle von *DCDC2* in NPHP-RC zugrunde liegen.
In Studie III fanden wir unter Verwendung von Zellmodellen und Tiermodellen eine genetische Interaktion zwischen den Genen *DYX1C1* und *DCDC2*. Wir schlossen daraus, dass diese zwei Gene möglicherweise auf demselben biochemischen Signalweg wirken.
In Studie IV verwendeten wir menschliche neuronale Zellen, die aus induzierten pluripotenten Stammzellen hergestellt worden waren. Wir fanden heraus, dass die Gruppe der ziliären Gene während der menschlichen Gehirnentwicklung aktiviert wird, und schliessen daraus, dass sie wahrscheinlich massgeblich an der menschlichen neuronalen Entwicklung beteiligt ist.
In Studie V untersuchten wir das Genom zweier Individuen, die an Legasthenie und einer Ziliopathie leiden, um zu untersuchen, ob eine gemeinsame genetische Ursache beiden Bedingungen zugrunde liegt. Die untersuchten Probanden haben *situs inversus*, d.h. eine Umkehrung der inneren Körperorgane, welche durch eine Störung der Zilien in der embryonalen Entwicklung zustandekommt. Wir fanden seltene Mutationen in bekannten Genen, die *situs inversus* verursachen, jedoch konnten wir keine Schlussfolgerung für die Bedeutung dieser Mutationen in der Legasthenie ziehen.

Diese Arbeit hat die Hinweise auf eine Bedeutung von *DYX1C1* und *DCDC2* in Zilien und Ziliopathien vermehrt und zum Verständnis der Funktion und Regulation dieser Gene beigetragen. Ausserdem wurde die Gruppe von ziliären Genen als wichtige Gruppe in der menschlichen Gehirnentwicklung beschrieben.
Zukünftige Studien werden die allgemeinen Funktionen dieser Gene und ihre Beteiligung an Legasthenie näher beleuchten, beispielsweise mit menschlichen Gehirnmodellen, die im Labor gezüchtet werden können. Genetische Studien an Gruppen von Individuen mit Ziliopathien und Legasthenie könnten ausserdem klären, ob eine gemeinsame genetische Grundlage beider Störungen besteht. Die Beteiligung der Zilien an der Gehirnentwicklung und an neuropsychiatrischen Krankheiten verspricht spannende neue Entdeckungen in der Zukunft.

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