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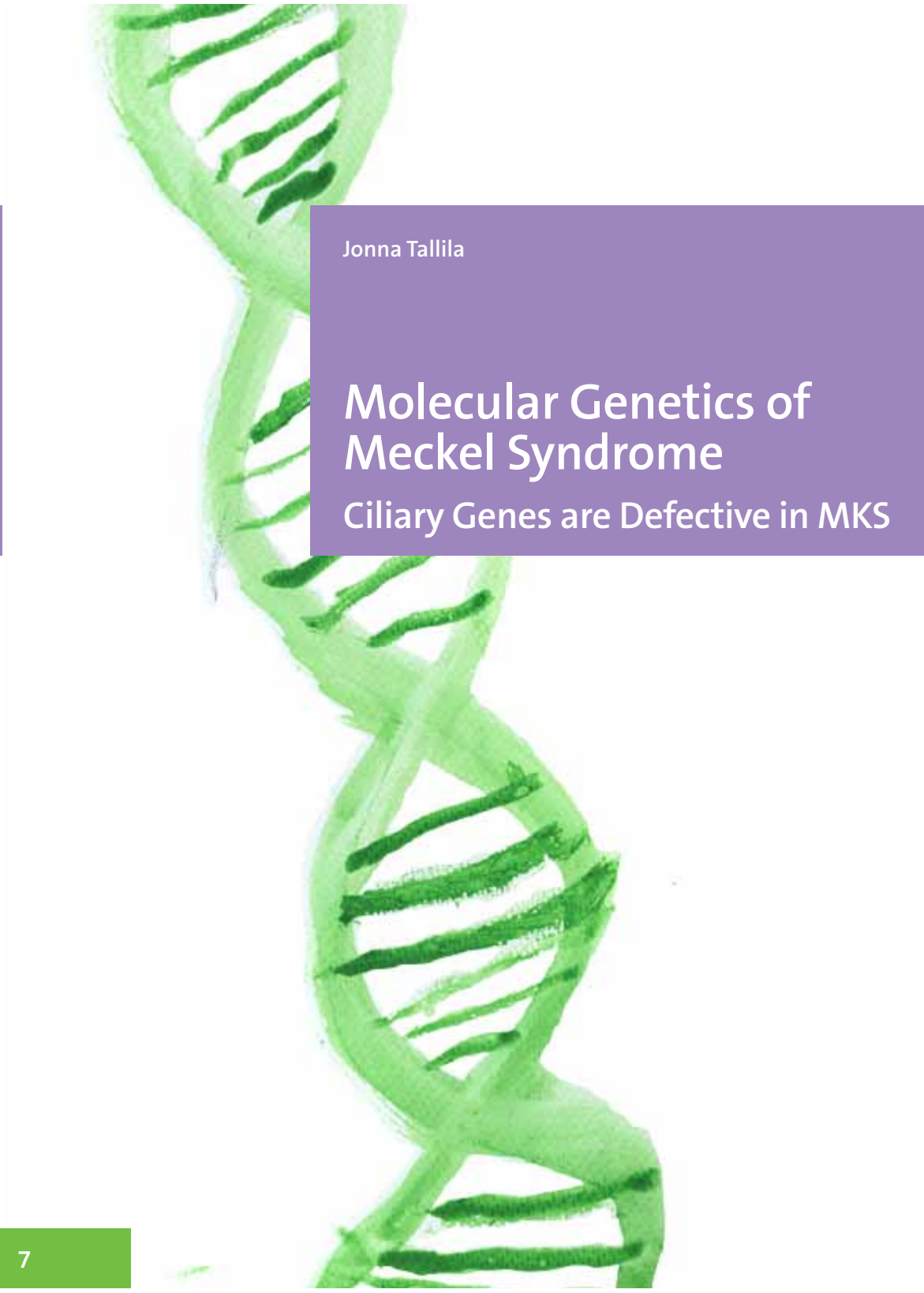

RESEARCH

Jonna Tallila

Molecular Genetics of Meckel Syndrome

Ciliary Genes are Defective in MKS

Jonna Tallila

MOLECULAR GENETICS OF MECKEL SYNDROME

CILIARY GENES ARE DEFECTIVE IN MKS

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Biosciences, University of Helsinki, for public examination in Lecture Hall 2, Biomedicum Helsinki, on 17th of April 2009, at 1 pm.

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”The amazing thing about mammalian development is not that it sometimes goes wrong, but that it ever succeeds.”

Veronica van Heyningen (2000)

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ABSTRACT

Meckel syndrome (MKS) is an autosomal recessive lethal disorder characterized by a combination of malformations. Death occurs already in utero or shortly after birth. Cystic dysplasia of the kidneys with fibrotic changes in the liver and typically occipital encephalocele or some other anomalies in the central nervous system are considered as the minimal diagnostic criteria for Meckel syndrome. In addition, polydactyly is frequently reported in the cases. MKS can be confidently detected and diagnosed by ultrasound already at the 11-14 weeks of gestation.

In this study we identified the first gene implicated in MKS in the Finnish population. The Fin_{major} mutation is in the *MKS1* gene and in 70% of the MKS families the affected fetuses are homozygous and parents heterozygous for the mutation. The carrier frequency is 1% in the Finnish population. The identification of the genetic defect behind MKS also revealed molecular pathways underlying the disease, as the *MKS1* gene was found to encode a protein with a B9 domain, linking its function to cilia.

Since mutations in the *MKS1* gene did not explain the genetic defect in all of the Finnish families, we performed a genomewide SNP scan to locate a new MKS locus. Another major MKS mutation in the Finnish population was identified in the *CC2D2A* gene (*MKS6*). This mutation was found in 20% of the Finnish families and the estimated carrier frequency is 0.5%. The CC2D2A has a Ca^{2+} binding domain and it is also involved in the ciliary functions. Together the *MKS1* and the *CC2D2A* mutations explain over 90% of the MKS cases, making the DNA based diagnostic situation excellent in Finland.

The number of genes behind MKS has increased rapidly in the past years. Identification of the disease genes mutations has also revealed that MKS is an allelic disorder with other syndromes with overlapping phenotypes. Sequence analysis of all the known *MKS* genes (*MKS1, TMEM67/MKS3, CEP290/MKS4, RPGRIP1L/MKS5* and *CC2D2A/MKS6*) in Finnish and non-Finnish families available to us, where the mutation was still unknown, identified mutations in 14 out of the 30 families included in the study. When we collected all the reported

mutations in these genes in different syndromes we could see that there was clearly a genotype-phenotype correlation between the mutations and the syndromes, since the same pair of mutations has never been reported in different syndromes.

Keywords: Meckel syndrome, *MKS1*, *CC2D2A*, *MKS6*, cilia

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TIIVISTELMÄ

Meckelin oireyhtymä (MKS) on peittyvästi periytyvä monioireinen sairaus, joka johtaa kuolemaan jo sikiökaudella tai pian syntymän jälkeen. Taudin keskeisimmät löydökset ovat rakkulaiset munuaiset, fibroottiset muutokset maksassa ja keskushermoston sulkeutumishäiriö, joita pidetäänkin diagnoosin vähimmäisvaatimuksina. Lisäksi polydaktylia, ylimääräiset sormet/varpaat, ovat tyypillinen löydös MKS-tapauksissa. MKS voidaan todeta luotettavasti ultraäänikuvauksessa jo 11. - 14. raskausviikolla.

Tässä tutkimuksessa löydettiin ensimmäinen Meckelin oireyhtymää aiheuttava geeni suomalaisväestössä. Suomalainen valtamutaatio on *MKS1*-geenissä ja 70 %:ssa MKS-perheistä vanhemmat ovat heterotsygootteja (geenivirheen kantajia) ja sairaat sikiöt homotsygootteja mutaatiolle. Mutaation kantajafrekvenssi on 1 %:a suomalaisväestössä. Meckelin oireyhtymän taustalla olevan geenivirheen löytyminen toi tietoa myös aineenvaihduntareiteistä, jotka ovat häiriintyneet taudissa, sillä *MKS1*-geenin koodaamassa proteiinissa on B9-domeeni, joka yhdistää sen toiminnan solun värekarvojen eli cilioiden toimintaan.

Koska mutaatiot *MKS1*-geenissä eivät selittäneet tautia kaikissa suomalaisissa perheissä, teimme koko genomin kattavan SNP-analyysin löytääksemme uuden tautigeenin jäljelle jääneissä perheissä. Toinen valtamutaatio suomalaisväestössä löydettiin *CC2D2A*-geenistä (*MKS6*). Tämä mutaatio selittää taudin esiintymisen 20 %:lla MKS-perheistä. Geenivirheen kantajafrekvenssi on 0,5 %:a. *CC2D2A*-geenissä on kalsiumia (Ca^{2+}) sitova domeeni ja geenin toiminta liittyy myös värekarvoihin. Yhdessä nämä kaksi tunnistettua valtamutaatiota aiheuttavat Meckelin oireyhtymän 90 %:ssa suomalaisista MKS-tapauksista, mikä mahdollistaa loistavan DNA-pohjaisen diagnostiikan taudille.

Viime vuosina on tunnistettu useita geenivirheitä Meckelin oireyhtymän taustalla. Tautigeenien tunnistamisen myötä on paljastunut myös, että MKS on alleelinen tauti muiden oireyhtymien kanssa, joilla on yhteisiä kliinisiä piirteitä Meckelin kanssa. Kaikkien tunnettujen *MKS*-geenien (*MKS1*, *TMEM67/MKS3*, *CEP290/MKS4*,

RPGRIP1L/MKS5 ja *CC2D2A/MKS6*) sekvenssianalyysi suomalaisissa ja ulkomaalaisissa MKS-perheissä, joissa geenivirhe oli vielä tuntematon, paljasti mutaatiot 14 perheessä 30 tutkitusta perheestä. Kerättyämme kaikki *MKS*-geenien raportoidut mutaatiot, saatoimme todeta, että geenivirheiden ja oireyhtymien välillä on selvä genotyyppi-fenotyyppi korrelaatio, sillä samaa mutaatioparia ei ole aiemmin raportoitu eri taudeissa.

Avainsanat: Meckelin oireyhtymä, *MKS1*, *CC2D2A*, *MKS6*, cilia

CONTENTS

ABBREVIATIONS

aa	amino acid
BBS	Bardet-Biedl syndrome
bp	base pair
CC2D2A	coiled-coil and C2 domain containing 2A
cDNA	complementary DNA
CEP290	Homo sapiens centrosomal protein 290 kDa
chr	chromosome
CNS	central nervous system
CNV	copy number variation
del	deletion
DNA	deoxyribonucleic acid
FABB	flagellar apparatus basal body proteome
FDH	Finnish disease heritage
γ	gamma
HGNC	HUGO Gene Nomenclature
HGP	Human Genome Project
HUGO	Human Genome Organisation
IBD	identical by descent
ins	insertion
IFT	intraflagellar transport
JS	Joubert syndrome
kb	kilobase
LCA	Leber congenital amaurosis
LD	linkage disequilibrium
LR	left-right asymmetry of the body plan

Mb	megabase pair
MKS	Meckel syndrome
mRNA	messenger RNA
MTOC	microtubule organizing center
NPHP	Nephronophthisis
NTDs	neural tube defects
PCK	primary ciliary dyskinesia
PCM	pericentriolar material
PCP	planar cell polarity pathway
PCR	polymerase chain reaction
PKD	Polycystic kidney disease
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
SLS	Senior-Locken syndrome
SNP	single nucleotide polymorphism
RPGRIP1L	Homo sapiens RPGRIP1-like
TMEM67	transmembrane protein 67

In addition, the standard one letter abbreviations of nucleotides and amino acids are used.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Kyttälä M, **Tallila J**, Salonen R, Kopra O, Kohlschmidt N, Paavola-Sakki P, Peltonen L, Kestilä M. 2006. *MKS1*, encoding a component of the flagellar apparatus basal body proteome, is mutated in Meckel syndrome. Nat Genet. 38(2):155-7.

II **Tallila J**, Jakkula E, Salonen R, Peltonen L, Kestilä M. 2008. Identification of *CC2D2A* as a Meckel syndrome gene adds an important piece to the ciliopathy puzzle. Am J Hum Genet. 82(6):1361-7.

III **Tallila J**, Salonen R, Kohlschmidt N, Peltonen L, Kestilä M. Mutation spectrum of Meckel syndrome genes. Submitted.

Publication I has appeared also in the thesis by Mira Kyttälä (2006). Some unpublished data is also included into this thesis.

Author Contributions to Publications

I JT participated in DNA and RNA studies and preparation of the manuscript.

II JT contributed to the study design, performed the sequence and data analyses and the functional studies. JT wrote the manuscript.

III JT carried out the study design, performed all the sequence and data analyses and wrote the manuscript.

1 INTRODUCTION

Finnish Disease Heritage (FDH) consists of a group of rare hereditary diseases that are overrepresented in Finland. Most of the diseases cause severe handicap and a heavy burden to the patient and the family. A half of the diseases are lethal sooner or later. Some disorders, in turn, can be effectively treated, provided that the correct diagnosis has been made.

Meckel syndrome (MKS) is an autosomal recessive lethal malformation syndrome, which is considered as a part of the FDH, even though cases are reported in other populations also. Even if overrepresented, the disorders are nevertheless rare also in Finland. MKS is one of the major contributors to neural tube defects. Minimal diagnostic criteria are cystic dysplasia of the kidneys with fibrotic changes in the liver and occipital encephalocele or some other central nervous system malformation. Polydactyly is also frequently reported in the MKS cases. MKS fetuses die already in utero or shortly after birth. Diagnosis can be established by a careful ultrasound examination already at 11-14 weeks of gestation and today, at least in Finland, very few cases are born after a full-term pregnancy.

The first MKS locus on chromosome 17q21-24 was mapped in the Finnish population as early as 1995. Already then the locus heterogeneity was apparent, since all families could not be linked to the regional markers. A few years later the second locus on chromosome 11q13 was identified in families originating from Northern Africa and Middle East. The third MKS locus on chromosome 8q24 was mapped in MKS families originating from the Indian subcontinent. Although the syndrome has been well characterized and the first disease loci were mapped over ten years ago, the disease causing genes have remained unknown. The aim of this study was to identify disease genes implicated in MKS.

2 REVIEW OF THE LITERATURE

2.1 Finnish disease heritage

In the 1970s, after several Finnish disorders had been studied genetically and geologically, the primary theory for Finnish disease heritage (FDH) took shape and the concept of FDH was presented for the first time by Perheentupa in 1972 (Perheentupa, 1972) and for a English readership by Norio in 1973 (Norio et al., 1973). It described 20 genetic disorders that are more common in Finland than in other countries. To date, the FDH consists of 36 inherited monogenic diseases; 32 being autosomal recessive, two being autosomal dominant, and two being X-chromosomal (Norio, 2003c). Almost one third of the diseases cause mental retardation and as many of them show visual handicap. Even if overpresented, the disorders are nevertheless rare also in Finland. The incidence varies between 1:8 000 and 1:100 000. As about 60 000 babies are born per year in Finland, the number of affected cases with one of the relevant disease is approximately six to eight a year, or not even one (Norio, 2003a) (http://www.findis.org).

The Finnish disease heritage has its origins in the special population history of Finland (Peltonen et al., 1999). During the historical era, the Finnish population has endured numerous bottlenecks, both local and national (Norio, 2003b). The majority of the genes of today's Finnish population are thought to originate from small founder populations. The small number of ancient ancestors did not bring all the possible disease mutations along, but rather a random selection (Norio, 2003a). That is why some diseases are more common or only found in Finland and others, that may be common elsewhere, are rarely seen in Finland e.g. phenylketonuria, galactosemia and cystic fibrosis (Peltonen et al., 1999).

In the 12th century the estimated population of Finland was only ~50 000. The population increased rapidly and reached 250 000 habitants in the 16th century, habitation still following mainly the coastal areas (Peltonen et al., 1999). In the 16th century Gustavus of Wasa endorsed the inhabitation of the central and eastern parts of Finland and this movement established the so called late settlement region in Finland. The villages in the late settlement region originate mainly from a small area of Southeastern Finland (Figure 1). The movement as well as wars and epidemics have created significant population bottlenecks that have shaped the unique gene pool over centuries. Regardless of the industrialization and the population migration from rural areas to urban regions, the subpopulations particularly in the late

settlement areas have remained unmixed (de la Chapelle, 1993; Peltonen et al., 1999; Norio, 2003b)

On the whole the isolation of the population has been relatively complete for the past 2000 years, resulting in a significant decrease in the genetic diversity of Finns compared to other populations (Sajantila et al., 1996; Peltonen, 2000). In Finnish subisolates a small number of founders unavoidably resulted in consanguineous marriages, even though relationships between individuals were typically several generations old and unkown to them (Peltonen, 2000). This random inbreeding increased the local incidence of rare disorders and in some Finnish diseases, like LCCS regional clustering of great grand parents can still be observed (Nousiainen et al., 2008). However, in most of the Finnish diseases, the exact relationship between affected cases can not be confirmed (Peltonen et al., 1999).

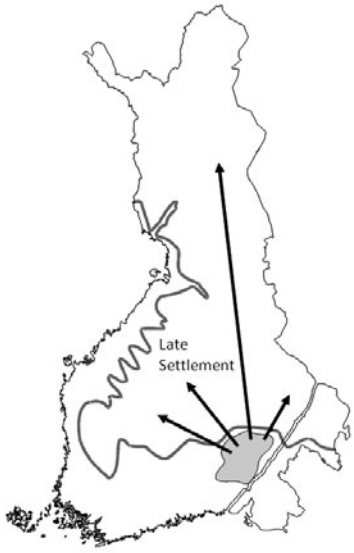

Figure 1. Early habitation of Finland followed the coastal line. In the 16th century the central and eastern parts of Finland were inhabited and this movement established the so called late settlement region in Finland. Picture by courtesy of Teppo Varilo.

Genetic isolates with a history of a small founder population, long lasting isolation and population bottlenecks represent exceptional resources in the identification of disease genes (Peltonen, 2000). In most of the diseases one founder mutation so called Fin_{major} mutation has been found as the main contributor to the disease (Norio, 2003c). However, in some diseases the Fin_{major} mutation accounts only for 70-80%

of the cases e.g. CNF and APECED (Bjorses et al., 1998; Kestila et al., 1998; Peltonen, 2000).

FDH consists of a variety of syndromes ranging from less severe disorders to embryonically lethal disorders (Figure 2). There are five syndromes where the fetuses die already in utero or shortly after birth; Meckel syndrome (MKS), Hydrolethalus syndrome (HLS), Lethal lactic acidosis (GRACILE), Lethal congenital constracture syndrome (LCCS) and Lethal arthrogryposis with anterior horn cell disease (LAAHD) (Table 1). MKS and HLS share many of the clinical manifestations and they were considered as one disorder until 1981, when Salonen et al. described HLS as a new lethal malformation syndrome. The most important difference in the syndromes are the polycystic kidneys seen in MKS cases, where as the kidneys are normal in HLS cases (Salonen et al., 1981). The occurrence of MKS follows the population frequency, where as in most of the diseases regional clustering can be seen (Figure 3).

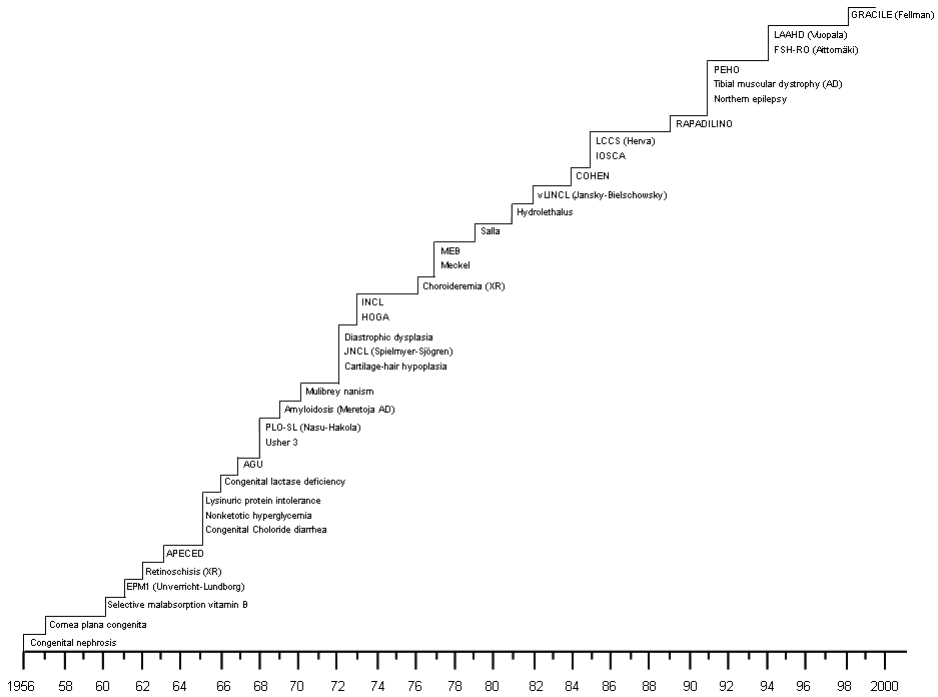

Figure 2. The diseases belonging to the Finnish disease heritage are presented in the Perheentupa's steps. The vertical line of each FDH disease shows the year of its first Finnish publication (modified from Norio et al., 2003).

Table 1. Embryonically lethal diseases belonging to the Finnish disease heritage.

	MIM	Frequency	Main features
Meckel syndrome	249000	1:9 000	Cystic kidney dysplasia, occipital encephalocele and postaxial polydactyly.
Hydrolethalus syndrome	236680	1:20 000	Hydrocephaly with absent upper midline structures of the brain, micrognathia and polydactyly.
Lethal lactic acidosis (GRACILE)	603358	1:47 000	Growth retardation and lactacidosis.
Lethal congenital constracture syndrome	253310	1:25 000	Atrophy of spinal cord motoneurons and fetal immobility accompanied by hydrops, pterygia and multiple joint contractures.
Lethal arthrogryposis with anterior horn cell disease	611890	>1:100 000	Fetal akinesia, arthrogryposis and motor neuron loss.

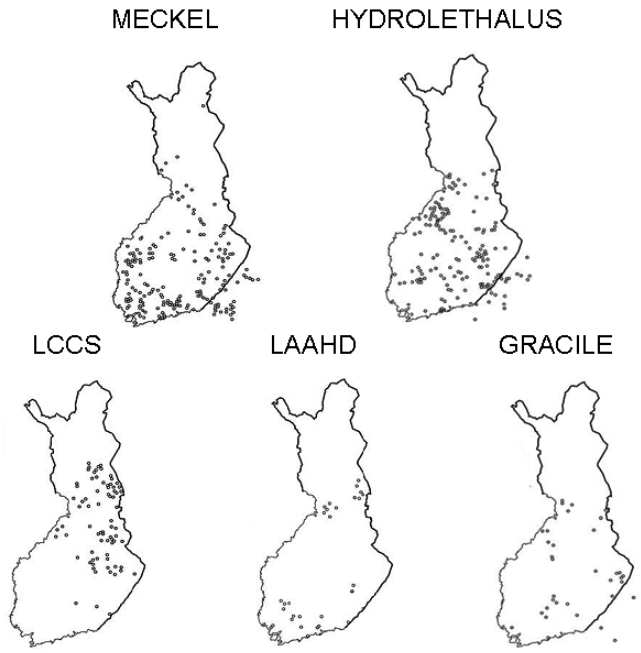

Figure 3. Incidences of the neonathally lethal disorders belonging to the Finnish disease heritage. The prevalence of MKS follows the population density, while in other syndromes regional clustering can be observed (modified from Norio et al., 2003).

2.2 Meckel syndrome

The clinical picture of Meckel syndrome (MKS [MIM 249000]) was first described and illustrated in the literature already in 1684 by Christopher Krahe describing a monsterous child born in Denmark (Kompanje, 2003). In 1934 G. B. Gruber described the syndrome called dysencephalia splanchnocystica and suggested the genetic origin of MKS, since these cases were familial (Gruber, 1934). However, Opitz and Howe suggested in 1696 (Opitz and Howe, 1969) that this syndrome had already been characterized previously by J. F. Meckel the Younger in 1822 (Meckel, 1822) and should be called Meckel syndrome. Currently both Meckel syndrome and Meckel-Gruber syndrome are used.

2.2.1 Main features of MKS

MKS is an autosomal recessive lethal multi-symptomic malformation disorder. There is no treatment for MKS. Mortality is 100% and fetuses die already in utero or shortly after birth. Diagnosis can be established by a careful ultrasound examination already at 11^{th}-14^{th} weeks of gestation when the three major manifestations can be seen: cystic kidney dysplasia, occipital encephalocele and postaxial polydactyly (Figures 4 and 5) (Nyberg et al., 1990; Braithwaite and Economides, 1995; Sepulveda et al., 1997; Gazioglu et al., 1998; Ickowicz et al., 2006). Later during pregnancy the characteristic oligohydramnion can impair visualization. In most cases the parents decide to terminate the pregnancy and the diagnosis is confirmed in postmortem pathological examination. In addition, fibrotic changes in the liver, shortening and bowing of the long tubular bones, ambiguous genital, hypoplastic lungs, and cleft lip palate and club foot can be observed (Opitz and Howe, 1969; Mecke and Passarge, 1971; Fraser and Lytwyn, 1981; Moerman et al., 1984; Salonen, 1984; Paetau et al., 1985; Rapola and Salonen, 1985; Ahdab-Barmada and Claassen, 1990). Also ocular anomalies are frequent in MKS cases (Salonen and Paavola, 1998).

Cystic kidney dysplasia is the most consistent finding of MKS patients. Both kidneys may be enlarged 5-50 times their normal size (with a combined weight of up to 1000 g at full term) (Salonen and Paavola, 1998), but retain their reniform shape and a uniform pattern of histological organization of the renal parenchyma is still present. Size of the cysts varies significantly from minuscule cysts under the capsule to large spherical cysts, which can be even up to 1 cm in deep parts of the

parenchyma. A zone of normal glomeruli and a few undilated tubuli are typically present underneath the capsule (Rapola and Salonen, 1985). There seems to be a failure of the interaction between metanephric duct and metanephric blastema, resulting in the absence of nephrons. The lack of nephrons and a supposed reduction of urine production and fluid flow are reflected in deficient remodeling of early ductal generations into pelves, calyces, medullary pyramids and in almost complete lack of vasa recta and Henle's loops (Blankenberg et al., 1987). Dysfunction of the kidneys in utero often causes oligohydramnion.

The spectrum of the central nervous system malformation ranges from total craniorachischisis at the most severe end to a partial defect of the corpus callosum at the mildest end of the spectrum (Salonen and Paavola, 1998). Occipital meningoencephalocele, the characteristic CNS malformation is seen in 90% of the cases (Paetau et al., 1985). Other major CNS findings are microcephaly, occipital bony defect, absence of olfactory bulbs and corpus callosum, ectopic neuroepithelial rosettes, rhombic roof dysgenesis and prosencephalic dysgenesis (Paetau et al., 1985; Ahdab-Barmada and Claassen, 1990). MKS is considered the most frequent syndromic cause of neural tube defects (NTDs) (Simpson et al., 1991).

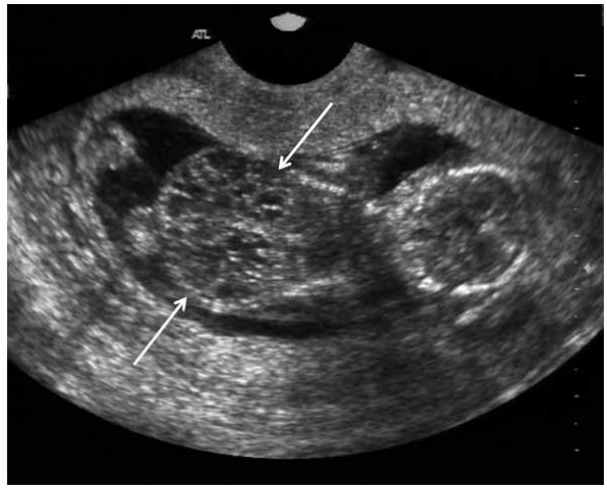

Figure 4. The main features of MKS can be seen already in the end of the first trimester in ultrasound examination. Arrows point to the bilaterally large kidneys with multisystic dysplasia of a fetus at 14th week of gestation. Picture by courtesy of Pirkko Ämmälä.

Fibrocystic changes of the liver are a consistent finding in most MKS cases and have therefore been proposed to be included as one of the diagnostic criteria (Blankenberg et al., 1987). Macroscopically the liver is normal, but histological analyses reveal an increase of bile duct formation and connective tissue of the portal tracts (Rapola and Salonen, 1985). A more detailed analysis of the bile ducts showed that in 77% of the cases a cystic dilation of primitive biliary structures with little portal fibrosis could be detected, while 23% pronounced portal fibrosis (Sergi et al., 2000).

Limb malformations are common, polydactyly being the most consistent finding. Postaxial polydactyly is generally seen in all four extremities, but there is large variation of this feature. Syndactyly, short limbs and club feet even in the absence of oligohydramnion can be observed in MKS fetuses (Salonen, 1984).

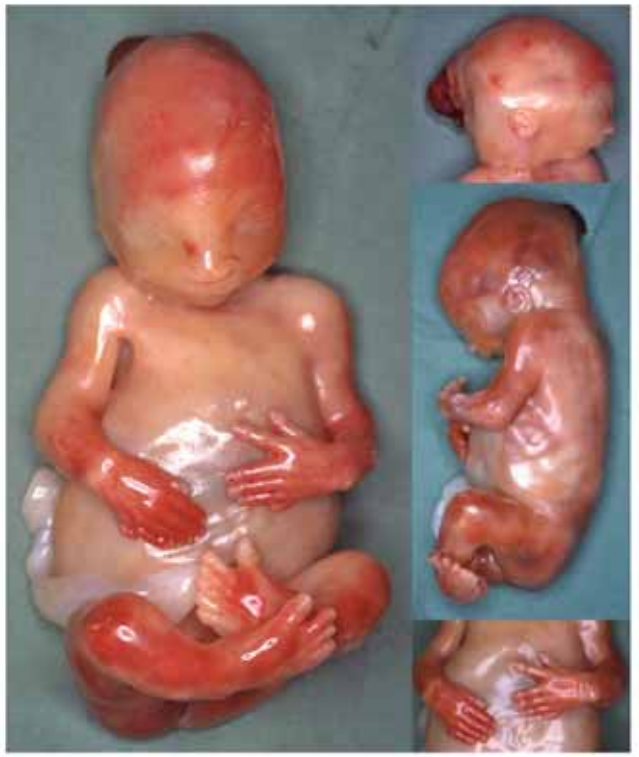

Figure 5. MKS fetus at 21th week of gestation. Main characteristics can be visualized: large stomach due to the polycystic kidneys, encephalocele and polydactyly in hands and feet. Picture by courtesy of Riitta Salonen.

2.2.2 Genetics of MKS

MKS is inherited in an autosomal recessive manner. It is diagnosed worldwide and it affects all racial and ethnic backgrounds; MKS cases have been reported in North America, Europe, Israel and different parts of Asia, e.g. Indonesia, India, Kuwait and Japan (Salonen and Paavola, 1998). Incidences vary greatly between populations, from 1:3 400 to 1:14 000. MKS is enriched in Finland were the carrier frequency is estimated to be 1:9 000, but high frequencies are also reported in some other isolated populations such as Gujarati Indians, Bedouins and Belgians (Figure 6) (Holmes et al., 1976; Seller, 1978; Moerman et al., 1982; Salonen and Norio, 1984; Young et al., 1985; Teebi et al., 1992; Zlotogora, 1997).

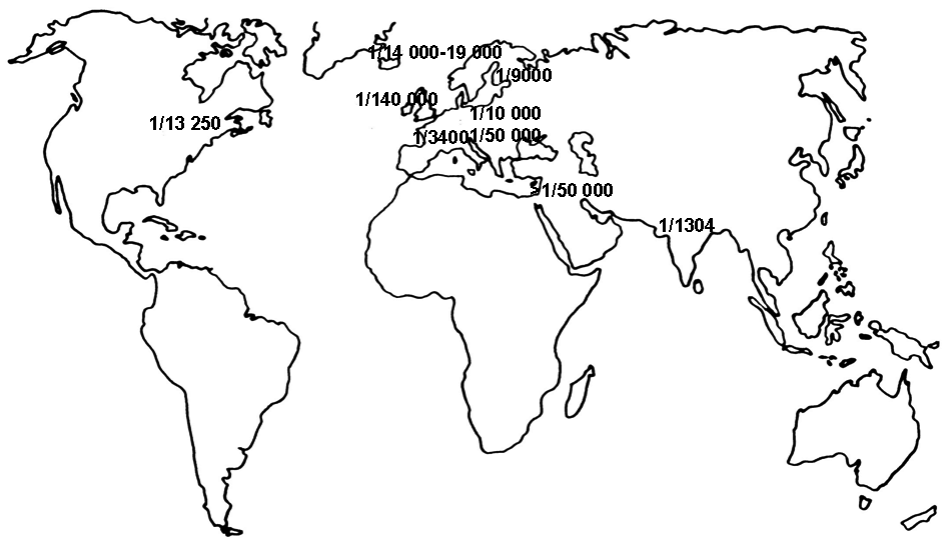

Figure 6. Reported frequencies for MKS world wide. Picture by courtesy of Riitta Salonen.

The heterogeneity of MKS has been well established. The first MKS locus, MKS1 17q21-24 was identified in the Finnish population already in 1995 (Paavola et al., 1995). Already then the locus heterogeneity was apparent, since all families could not be linked to the regional markers. The second locus for MKS on chromosome 11q13 was reported in families that originated from Northern Africa and Middle

East in 1998 (Roume et al., 1998). In 2002 the third MKS locus on chromosome 8q24 was reported in consanguineous kindreds with MKS originating from the Indian subcontinent (Morgan et al., 2002).

2.3 Identification of disease genes

The identification of disease genes is a central step in the understanding of the pathophysiology of diseases. It often provides also new insights into normal human development (Peltonen, 2000). There are many strategies that can be applied in disease gene identification, probably the most important being functional cloning, candidate gene analysis and positional cloning. It is often advantageous to combine the different techniques. Careful clinical evaluation of the cases prior to the candidate gene search is a critical step and fundamental in identifying the disease locus. The choice of approach depends on various things, such as the availability and property of sample and family material and existing knowledge of the biochemical basis of the disease.

2.3.1 Human genome project

Identification of disease genes has changed dramatically during recent years due to the completion of the Human Genome Project (HGP) and the advancement of technological platforms (Figure 7) (Antonarakis and Beckmann, 2006). The project began officially in 1990 and it lasted for 13 years. It was originally planned to last 15 years, but rapid technological advances accelerated the completion date to 2003. In June 2000, the rough draft of the human genome was completed and in February 2001, the working draft was completed, and special issues of *Science* and *Nature* containing the working draft sequence and analysis were published (Lander et al., 2001; Venter et al., 2001). Final HGP papers were published in 2006. The present consensus predicts that the genome consists of about 20,000-25,000 genes (http://www.ornl.gov/sci/techresources/Human_Genome/home.shtml). The sequence of the human DNA is stored in databases (e.g. ncbi.nlm.nih.gov) and is available to anyone on the internet.

Having the sequence has enabled an entirely new approach to biological research. In the past, researchers studied one or a few genes at a time. With whole-genome

sequences and new high-throughput technologies, they can approach questions systematically and on a grand scale. They can study all the genes in a genome, for example, or all the transcripts in a particular tissue or organ or tumor, or how tens of thousands of genes and proteins work together in interconnected networks to orchestrate the chemistry of life. Technology and resources promoted by the HGP have had profound impacts on biomedical research and has revolutionized the wider spectrum of biological research and clinical medicine. Increasingly detailed genome maps have aided researchers seeking genes associated with dozens of genetic conditions.

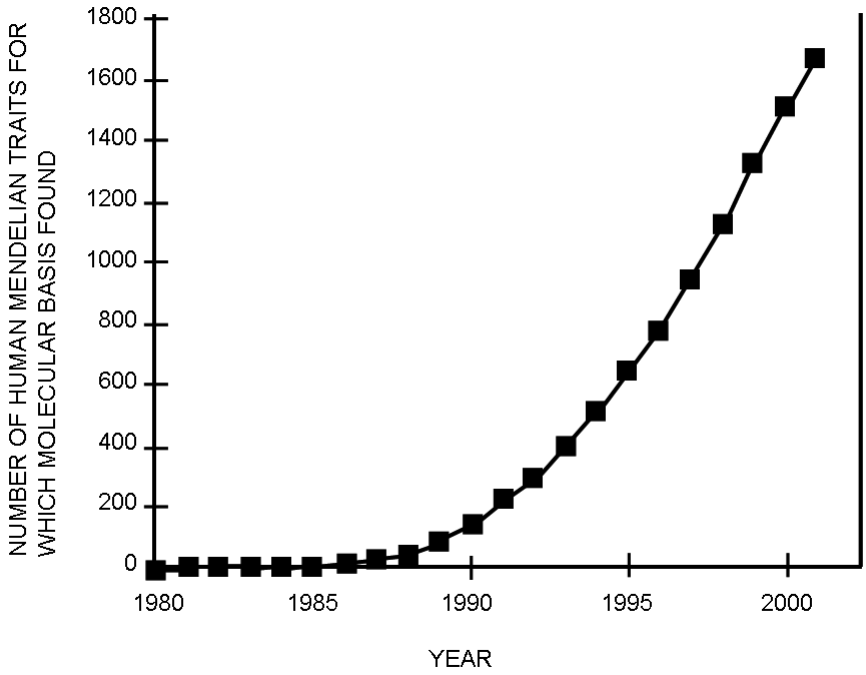

Figure 7. Identification of genes underlying human Mendelian traits (monogenic/single gene diseases) has increased rapidly after the beginning of Human Genome Project. Cumulative data for human Mendelian trait genes (to 2001) include all major genes causing a Mendelian disorder in which causal variants have been identified (modified from (Glazier et al., 2002).

2.3.2 Main approaches

Functional cloning

Functional cloning is the identification of disease genes without information of their chromosomal position, and it has been used in certain instances when the disease has been biochemically well defined (Collins, 1992; Collins, 1995). Examples of gene identification using functional cloning approach include phenylketonuria and sickle cell anemia (Collins, 1995). Prior to the availability of genome sequences this approach was hampered by the difficulty of identifying the disease gene even if the defective protein was known.

Completion of the sequencing of the human genome and other species has brought along a new tool that can be used in disease gene identification and functional analysis of the genes. Comparative genomics is the analysis and comparison of genomes from different species. A basic principle of the comparative genomic approach is that a spesific biological feature is evolutionarily conserved among a group of organisms. A second group of organisms that explicitly lack this same characteristic is then identified. These two groups of organisms serve as positive and negative reference set, respectively, for compiling a list of candidate genes (Nishimura et al., 2005). For example, by comparing the genomes of ciliated versus non-ciliated organisms Li et al. could identify 688 genes that are present in the former but not the latter group. These genes are candidate genes for encoding flagellar and basal body components (Li et al., 2004; Pazour et al., 2005). By comparing the finished reference sequence of the human genome with genomes of other organisms, researchers can identify regions of similarity and difference.

Positional cloning

Disease gene identification based on genomic location, an approach referred to as positional cloning, has been widely used in the research of monogenic diseases (Collins, 1992; Collins, 1995). Positional cloning involves examining DNA markers in families in which an inherited disease is transmitted to search for chromosomal segment that is consistently handed on to the affected family members. This allows the underlying gene to be mapped to a chromosomal region (Wicking and Williamson, 1991). Identifying mutations in one of the regional genes reveals the gene that is responsible for the disease. The advantage of positional cloning is that it allows identifying novel genes for which there is no previous pathogenic hypothesis,

because unlike in functional cloning, the genes are selected for their position, regardless of their function. Positional cloning approach has been used successfully in identification of the disease gene in a number of diseases, including retinoblastoma and cystic fibrosis (Collins, 1992). The so-called positional candidate approach relies on a combination of mapping to the right choromosomal location followed by the survey of the region to see if attractive candidate genes reside there (Collins, 1995). In addition, comparative genomics offers a valuable tool for the candidate gene analysis. The main differences between functional and positional cloning are demonstrated in figure 8.

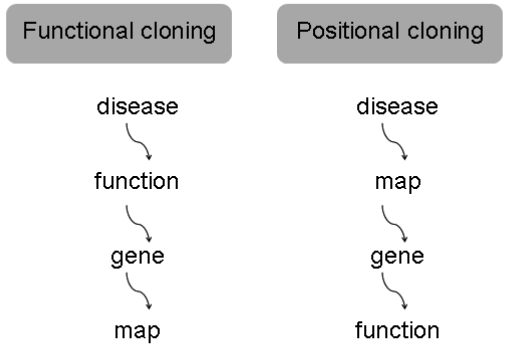

Figure 8. Functional cloning depends upon application of biological information, while positional cloning is initiated by mapping the responsible gene to its correct chromosomal location and requires no prior knowledge of the disease pathogenesis (modified from Collins, 1992).

Polymorphic markers and copy number variation

Individual variation between human genomes is used in human gene mapping. The first generation polymorphic genetic markers were restriction fragment length polymorphisms (RFLPs) (Botstein et al., 1980). There have been considerable advantages since then. The PCR technology made the microsatellite markers a standard tool for linkage analysis (Dearlove, 2002). These are most often di-, tri- or tetra nucleotide repeats that are very polymorphic, showing high levels of allelic variation. The high variability of microsatellites is due to their high mutation rate,

which is explained mainly by slipped strand mispairing during DNA replication (Fan and Chu, 2007).

A single nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide - A, T, C, or G - in the genome differs between individuals and typically has no phenotypic effect. SNPs are less polymorphic compared to microsatellite markers, but they remain more stable along generations due to the lower mutation rate. SNPs are uniformly distributed through out the genome and although the heterozygosity is lower than microsatellites, their global distribution and adaptability to high-throughput genotyping make them very useful to genomewide linkage analyses (Matise et al., 2003).

In the last three years, a new form of genetic variation has been widely reported. Genome structural variation has been known at the cytogenetic and molecular levels for a long time, but its importance at a genome-wide scale was not discovered until recently, with the use of arraybased comparative genomic hybridization and other types of genome-scanning technologies. This variability entails large segments of DNA, typically over one kilobase (kb) and up to several megabases (Mb) and it comprises insertions, deletions, translocations, and inversions of genomic material. So far, the most commonly identified types of variants are gains and loses of DNA, which are called CNVs (copy number variations). CNVs have already been shown to be associated with several complex/common disorders. Interestingly, most of these findings have been obtained by specific analysis of candidate genes or regions. Rare CNVs have been detected in some families of patients affected by Parkinson disease, Alzheimer disease, and chronic pancreatitis (Estivill and Armengol, 2007).

Haplotype, linkage and linkage disequilibrium

Haplotype is a combination of alleles at multiple loci that are transmitted together on the same chromosome. It may refer to as few as two loci or hundreds of loci depending on the number of recombination events that have occurred between a given set of loci. Haplotype block is a set of markers that are statistically associated. Genetic linkage occurs when particular genetic loci or alleles for genes are inherited jointly. Genetic loci on the same chromosome tend to segregate together during meiosis, and are thus genetically linked. Linkage describes the association of two or more loci on a chromosome with limited recombination between them. Linkage disequilibrium (LD) describes a situation in which some combinations of alleles or genetic markers occur more frequently in a population than would be expected. The idea of utilizing linkage disequilibrium and haplotype mapping in disease gene identification is that affected individuals have inherited the gene defect underlying

the disease from the same ancestral relative (founder) who has introduced the mutation to the population (Figure 9). With the genotype data available from the families one can look for shared regions among the affected individuals (disease-containing haplotype). If individuals share a common genomic region, they seem to have same specific markers more often than would be expected. The closer the locus one is to locus two, more unlikely they are separated during meiosis. Non-random associations between polymorphisms at different loci are measured by the degree of LD (Terwilliger, 1995). Current association studies for common disorders and complex traits, aim to detect linkage disequilibrium (LD) between SNPs that genetically mark a given region (tagSNPs) and the functional variants (either at the RNA or protein level) responsible for the phenotypes.

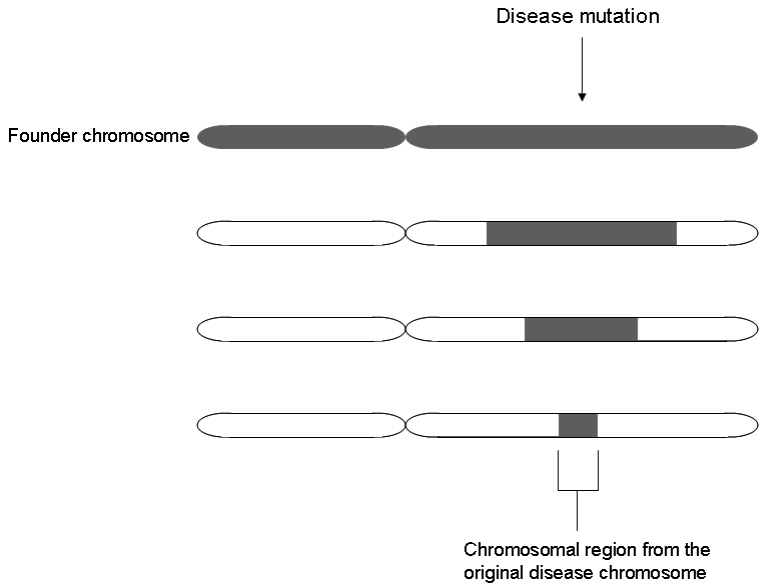

Figure 9. The principle of linkage disequilibrium. Over the course of many generations recombinations have narrowed the closest surrounding area of the mutation to a fairly short chromosomal region.

The HapMap is a catalog of common genetic variants that occur in human beings. It describes what these variants are, where they occur in our DNA, and how they are

distributed among people within populations and among populations in different parts of the world. The project is designed to provide information that researchers can use to link genetic variants to the risk for specific illnesses, which will lead to new methods of preventing, diagnosing, and treating disease. In many parts of our chromosomes, just a handful of haplotypes are found in humans. In a given population, 55 percent of people may have one version of a haplotype, 30 percent may have another, 8 percent may have a third, and the rest may have a variety of less common haplotypes. The HapMap project is identifying these common haplotypes in populations with African, Asian, and European ancestry, since any given haplotype may be more common in one population and less common in another, and newer haplotypes may be found in just a single population. It also is identifying "tag" SNPs that uniquely identify these haplotypes. By testing an individual's tag SNPs (a process known as genotyping), researchers will be able to identify the collection of haplotypes in a person's DNA. The number of tag SNPs that contain most of the information about the patterns of genetic variation is estimated to be about 300,000 to 600,000, which is far fewer than the 10 million common SNPs (http://www.hapmap.org) (Thorisson et al., 2005).

Homozygosity mapping

Homozygosity (or autozygosity) mapping is a useful strategy for disease gene identification in consanguineous families suffering from recessively inherited diseases and in isolated populations. An identical ancestral founder chromosome segment is passed from both parents to the affected child and is said to be homozygous or identical by descent (IBD). A genomic region that is consistently homozygous by descent in all patients is most likely to harbor the disease-causing gene. Segments, homozygous by descent, are expected to be observed also on some other genomic regions in each child of a consanguineous family, with a higher frequency the closer the relationship of the parents, and, in theory, ~6% of the genome of a child of first cousins is expected to be homozygous by descent (Lander and Botstein, 1987). By homozygosity mapping disease gene loci can be successfully identified even in very small families without genotyping all intervening relatives (Carr et al., 2006). Homozygosity can be exploited also in fine mapping the candidate region by searching for a shared, overlapping region of homozygosity between the patients, and thus possibly decreasing the length of the critical region (Lander and Botstein, 1987).

Candidate genes and mutation analysis

In positional cloning approaches, all known or putative genes in a candidate locus are candidate genes. In some cases the regional candidates can be prioritized according to their function, if there is prior knowledge of the disease mechanism. Therefore, functional candidate genes can be positional or non-positional and are chosen based on prior knowledge of the pathology and biochemical basis of the disease.

The sequence of a gene can be altered in a number of ways. Mutations can be classified as point mutations, insertions, deletions, duplications, and inversions (Figure 10). There are three types of point mutations: silent, where the single nucleotide alteration does not change the amino acid, missense, where the amino acid is changed and nonsense, where the nucleotide change leads to an early stop codon, and thus results in a truncated protein. Point mutations that affect the splice sites can also result in aberrant splicing. Insertions add one or more extra nucleotides into the DNA and deletions on the contrary remove them. Insertions or deletions in the coding region of a gene may alter splicing of the mRNA or cause a shift in the reading frame. Mutations can also affect the regulatory elements of the gene.

Much of our information about genome structure, function and evolution depends on sequence data. The chain-termination method published in 1977 (Sanger et al., 1977), also commonly referred to as Sanger or dideoxy sequencing, has remained the most commonly used DNA sequencing technique to date and was used to complete the human genome sequencing. Sequencing projects have traditionally used 400-800 base pair reads. However, there are limitations to the method. The genome contains regions that can not be accessed with traditional sequencing methods, such as long repeats. Very recently, the Sanger method has been partially replaced by several “next-generation” sequencing technologies. The next-generation technologies commercially available today include the 454 GS20 pyrosequencing-based instrument (Roche Applied Science), the Solexa 1G analyzer (Illumina, Inc.), the SOLiD instrument from Applied Biosystems, and the Heliscope from Helicos, Inc. The next-generation technologies have been used for standard sequencing applications, such as genome sequencing and resequencing, and for novel applications previously unexplored by Sanger sequencing (Bentley, 2006; Morozova and Marra, 2008).

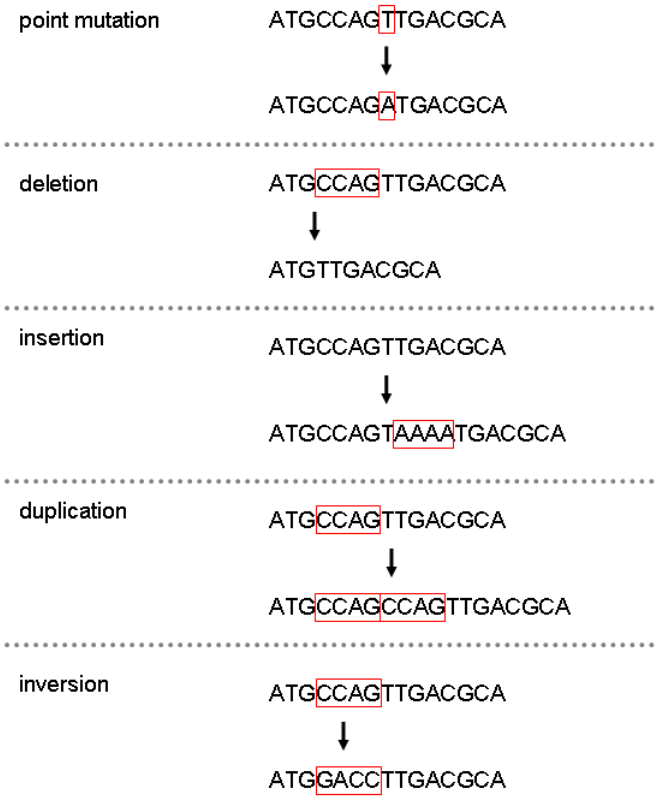

Figure 10. Basic mutation types.

3 AIMS OF THE STUDY

In beginning of this study three MKS loci were mapped, but the disease causing genes remained unknown and the following aims were set for the study:

1. To identify disease gene(s) implicated in Meckel syndrome.

2. To characterize the mutation spectrum of identified MKS gene(s).

3. To characterize the primary function of the gene(s) and protein(s).

4 MATERIALS AND METHODS

4.1 Family material and controls

We have had DNA samples from 56 Finnish MKS fetuses and their parents. During this study the number of families has increased. In some families we have had the samples from healthy siblings in addition. DNA was extracted from blood, tissue, cells and chorionic villus samples and RNA from cells and frozen tissue. All the Finnish MKS cases fulfil the minimal diagnostic criteria (Salonen, 1984). Fibroblast cell lines were available from several Finnish MKS cases. In addition, we had DNA samples from 27 non-Finnish MKS families originating mainly from Europe. In most of these families the DNA sample from the affected fetus and parents were available.

We obtained DNA from 520 Finnish control samples for estimation of the carrier frequency of the *MKS1* Fin_{major} mutation (*MKS1*) and from 575 controls to estimate the carrier frequency of the *CC2D2A* (*MKS6*) Fin_{major} mutation (*MKS6*).

This study has been approved by the ethical committees of the Joint Authtority of the Hospital District of Helsinki and Uusimaa, Finland (28/94, 205/E0/04, 57/E8/04).

4.2 Methods

The disease gene identification process is outlined in Figure 11. The methods used in the original publications are summarized in Table 2. More detailed descriptions of the methods used are in the original publications. The laboratory methods that were used for unpublished data are described briefly below.

Whole mount in situ hybridization

Whole-mount *in situ* hybridization requires the use of non-radioactive probes. We used RNA probes generated by *in vitro* transcription. The probe was first amplified from mouse fibroblast cDNA with *Mks1* spesific primers: F-

AAGGGTTCAGCCAGCAGAGT and R-TGGTTGCCAAACTCCCTTT. The 500 bp fragment was cloned into pGEM-T Easy vector (Promega) and the anti-sense and sense probes labeled with digoxigenin were created with DIG RNA labeling kit (Roche). The bound probe was visualized by incubating the embryos with anti-DIG-antibody coupled to alkaline phosphatase, followed by incubation in substrate for alkaline phosphatase that yields an insoluble colour product.

Phalloidin staining of actin cytoskeleton

The actin cytoskeleton was visualized by phalloidin staining. Fibroblast cells from MKS6 fetus and a control were grown in DMEM solution with 10% serum and antibiotics. Cells were fixed with MES-PFA in PBS and stained with fluorescent labeled phalloidin (Oregon green) to visualize F-actin, following the manufacturers protocol.

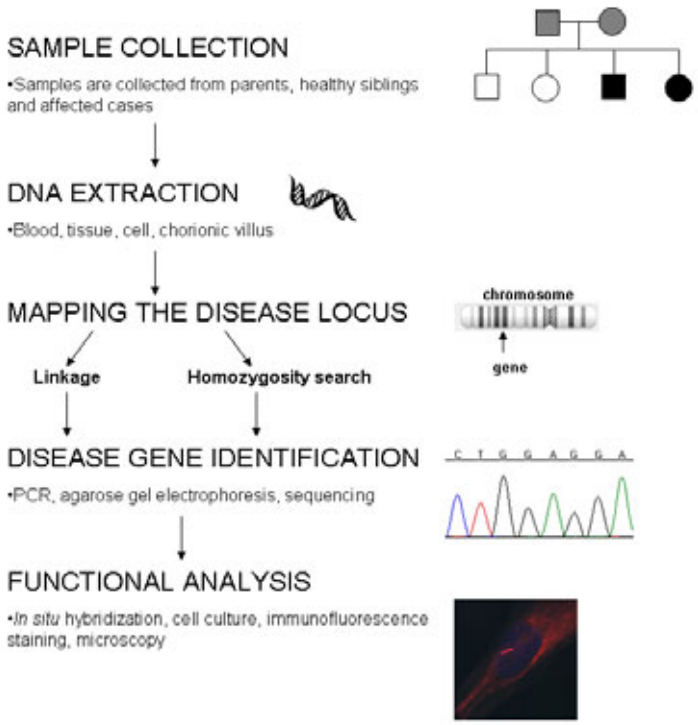

Figure 11. Outline of the disease gene identification process and some examples of methods that can be used in each step of the process.

Table 2. The original publications in which the methods were used are indicated with Roman numerals.

Method	Publication
Agarose gel electrophoresis	I, II, III
Bioinformatic analyses	I, II, III
Cell culture	II
Direct sequencing	I, II, III
DNA extraction	I, II, III
Genomewide SNP scan	II
Genotyping	I
Haplotype analyses	I, II
Immunofluorescence microscopy	II
Immunofluorescence staining	II
Mutation analysis	I, II, III
Polymerase chain reaction (PCR)	I, II, III
Reverse transcriptase (RT-PCR)	I, II
RNA extraction	I, II
RNA *in situ* hybridization	I

5 RESULTS

5.1 Identification of *MKS* genes (I, II)

Originally the *MKS1* locus was mapped in Finnish families to a 13 cM region on chromosome 17q by Paavola and colleagues (1995) (Paavola et al., 1995). Since then, new families participated in the study and helped to define the critical chromosomal region. This newly allocated *MKS1* region contained only five transcripts. Sequencing of four of the regional genes revealed only non-pathogenic polymorphism. The direct sequencing of the *FLJ20345* gene revealed a 29 bp deletion in intron 15, located only six bp from the intron-exon boundary (c.1483-7_35del) (Figure 12). In 26 (70%) of the Finnish families the affected fetuses were homozygous and parents heterozygous for the mutation (Fin_{major}). We could not identify other mutations in the sequence analysis in the remaining 14 Finnish MKS families, meaning that mutations in other, still uncharacterized *MKS* genes or in regulatory elements of the gene would explain the genetic defect in these families.

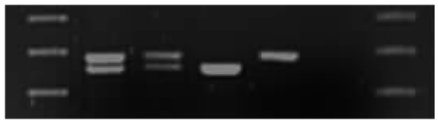

Figure 13. The 29 bp deletion can be visualized on 2% agarose gel. Heterozygous carriers have two bands (+/-), the homozygous MKS fetus has only the lowerband (+) and the healthy control has only the upper band.

Investigation of mRNA from patient fibroblast samples with RT-PCR, demonstrated that the patient transcript misses 83 bp, whole exon 16, compared to the control samples. The presence of this exon in various control tissues was confirmed with cDNAs from the fetal multiple tissue panel (Figure 13). The carrier frequency of the identified mutation was found to be 1% among 1020 Finnish control chromosomes. This carrier frequency correlates well with the prevalence of MKS cases. Since the *FLJ20345* was a novel gene we requested from the HGNC (HUGO Gene Nomenclature) the *MKS1* to be the official gene symbol for the transcript.

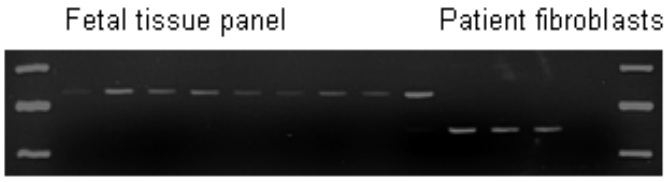

Figure 12. The patients transcripts lack exon 16 compared to the control tissues of the human multiple tissue panel (brain, lung, liver, kidney, heart, spleen, thymus and skeletal muscle).

Since only in 70% of the Finnish cases of MKS was caused by the Fin_{major} mutation (c.1483-7_35del) in the novel *MKS1* gene we started to look for a new MKS locus in the remaining Finnish families. We evaluated the post mortem reports and chose 10 out of 17 available fetuses that met the minimal diagnostic criteria for this study. Given that these families had only one affected fetus and no healthy siblings were available a linkage-based positioning of the underlying locus was not possible. Instead, we assumed that in the isolated Finnish population the cases might share a common mutation and surrounding haplotype and decided to perform a genome wide SNP scan to locate homozygous regions shared by the cases. Illumina genomeviewer was used to exlude copy number variation. Illumina Beadstudio v3.1.0 was used to call genotypes and homozygosity detector option was utilized in the search of extended tracts of homozygosity in each sample using a minimum length of 50 SNPs. The algorithm uses SNP frequencies to calculate the expectation that a single SNP is homozygous in a sample and it can be used to autobookmark samples with extended tracts of homozygosity. As a result of this analysis, six cases were shown to share a common homozygous region (Figure 14). In addition, one case (MKS 40) shared the same region, but when we used PLINK to see the genotypes, we could see that the case was homozygote for a different allelic haplotype than the other five.

Altogether six out of the 10 affected fetuses chosen for the array were found to have overlapping homozygous regions on chromosome 4p15. The size of the homozygous regions varied from 730 kb to 6.8 Mb, all patients sharing a segment of 63 SNPs covering a 565 kb area (see Publication II, Figure 2). One of the regional genes *CC2D2A* was found among the genes of the ciliary proteome making it an excellent candidate for MKS (Gherman et al., 2006).

Sample ID	Bookmark Type	Chr	Start	End	Size [bases]	Author	Comment
MECKEL.206 [13]	LOH	4	8648040	15475912	6827873	Homozygosity Detector	ChiSquare value = 431.083
MECKEL.145 [5]	LOH	4	12683108	16860519	4177412	Homozygosity Detector	ChiSquare value = 307.418
MECKEL.222 [11]	LOH	4	13389323	16652885	3263563	Homozygosity Detector	ChiSquare value = 256.288
MECKEL.122 [2]	LOH	4	13884425	16998305	3113881	Homozygosity Detector	ChiSquare value = 234.907
MECKEL.173 [4]	LOH	4	14807034	16290315	1483282	Homozygosity Detector	ChiSquare value = 115.412
MECKEL.181 [9]	LOH	4	14909996	15640546	730551	Homozygosity Detector	ChiSquare value = 52.242
MECKEL.40 [8]	LOH	4	14985082	15459783	474702	Homozygosity Detector	ChiSquare value = 31.294

Figure 14. Output table from Illumina Beadstudio software. Homozygous region was detected in chromosome 4.

The sequence analysis of the cDNA of *CC2D2A* revealed a four base pair deletion (c.1761-1764delGCAA) in the patient transcript compared to the control. The deletion causes a frame shift after Valine 587 and results in a stop codon at amino acid 616 (p.V587fsX616). Sequence analysis of the genomic DNA revealed that this was due to a C→T substitution (c.1762C>T) in the end of exon 16. This mutation creates a new donor splice site that affects the splicing leading to a defective transcript. Sequence analysis of all the 17 MKS families identified that altogether in 11 families the parents were heterozygous and affected fetuses homozygous for the mutation. All six fetuses that shared the common 63 SNP founder haplotype were homozygous for the *CC2D2A* (*MKS6*) Fin_{major} mutation. When sequencing 575 healthy control samples, three individuals were found to be carriers of the MKS6 mutation; therefore, the estimated carrier frequency is ~0.5% in the Finnish population.

5.1.1 Mutations in Finnish MKS families (I, II and III)

Major mutations in the Finnish population have been identified in two MKS genes (Table 3); The Fin_{major} c.1483-7_35del in the *MKS1* gene, which accounts for ~70% of the MKS cases in Finland and the c.1762C>T in the *CC2D2A* gene, which accounts for ~21% of the cases in Finland. Together these mutations explain the genetic defect behind MKS in over 90% of the cases. Systematic sequence analysis showed that one Finnish MKS case is a compound heterozygote for two likely pathogenic mutations that segregated in autosomal recessive fashion in the *MKS1* gene: an allele resulting in a two base pair deletion that leads to a stop codon (c.392-393delCT, p.S131X) and a Arginine to Tryptophan substitution (c.496C>T,

p.R166W). The amino acid change is predicted to be probably damaging by PolyPhen, but tolerated by SIFT (Publication III, Supplementary Table 2) (Ng and Henikoff, 2002).

Between the identification of the *MKS1* and *CC2D2A* genes other research groups reported the identification of three new genes behind MKS (*TMEM67*, *CEP290* and *RPGRIP1L*). We performed sequence analysis of all the reported genes in the remaining Finnish families, where we could not identify a mutation in the *MKS1* and *CC2D2A* genes. In one of the Finnish MKS families we identified a compound heterozygote mutation in the *CEP290/MKS4*, which segregated in the family. The first mutation was a previously reported AT-deletion in exon 14 (c.1219-1220delAT, p.M407fs421X) and the second a novel deletion in exon 29 (c.3444delAA, p. L1148fs1155X). All the *MKS* mutations found in the Finnish MKS cases, are concluded in Table 3.

Table 3. MKS mutations found in the Finnish population. The two major mutations that account for 90% of the MKS cases in Finland are bolded.

	Allele 1			Allele 2		
	Ex/int	**Mutation 1**	**Effect on protein**	**Ex/int**	**Mutation 2**	**Effect on protein**
MKS1						
	int15	**c.1483-7_35del**	**p.G470fs**	**int15**	**c.1483-7_35del**	**p.G470fs**
	ex4	c.392-393delCT	p.S131X	ex5	c.496 C>T	p.R166W
CEP290/MKS4						
	ex14	c.1219-1220delAT	p.M407fs	ex29	c.3444delAA	p.L1148fs
CC2D2A/MKS6						
	ex16	**c.1762C>T**	**p.V587fs**	**ex16**	**c.1762C>T**	**p.V587fs**

5.1.2 Mutations in non-Finnish MKS families (I, III)

We sequenced all the reported *MKS* genes (five *MKS* genes had been reported in December 2008) in 27 non-Finnish MKS families available to us and identified disease causing mutations in 16 of them. All the *MKS* mutations identified in the non-Finnish MKS cases are presented in Table 4. PolyPhen and SIFT softwares were used in the study to predict the nature of the novel amino acid changes (see Publication III, Supplementary Table 2) (Ng and Henikoff, 2002).

MKS1: In three of the non-Finnish families the affected individuals were homozygous for the Fin_{major} mutation (c.1483-7_35del). One MKS case (F4) of German ancestry was found to be a compound heterozygote for two different mutations in *MKS1*. Both mutations are located at the very beginning of the transcript: a five bp insertion in exon 1 (c.50insCCGGG) causing a frame shift and a T→C substitution in intron 1 (c.80+2T→C), that most likely affects the splicing.

TMEM67 (*MKS3*): Mutations in the *TMEM67 (MKS3)* gene were present in five non-Finnish families. Altogether 9 different mutations were found of which three were novel. In the first family (UM1) the fetus was a compound heterozygote for two novel amino acid changes located in exons 8 and 9 (c.734C>T, p.S245Y and c.888G>T, p.W296C). S245Y is possibly damaging by PolyPhen and not tolerated by SIFT. W296C is probably damaging by PolyPhen, but predicted to be tolerated by SIFT. In the second family (UM2) the fetus was a compound heterozygote for two amino acid changes, a novel Tyrosine to Cysteine substitution (c.161A>G, p.Y54C) and Threonine to Cysteine substitution (c.1588A>G, p.Y513C). The novel Y54C is probably damaging by PolyPhen and not tolerated by SIFT. In the third family (UM3) the affected fetus had a pair of two previously reported amino acid changes (c.672A>T, p.R208X and c.805T>C, p.M252T). In the fourth family (UM4) the father carried the M252T change and the mother carried the C625R change (c.1927A>G, p.C625R) and in the fifth family (UM5) the fetus was a heterozygote for two previously identified amino acid changes (c.1411C>T, p.R454Q and c.2947T>C, p.L966P). Except for family number four, where the DNA sample from the fetus did not work, the segregation was confirmed.

CEP290 (*MKS4*): We detected mutations in *CEP290* in four non-Finnish families. In the first family (UM6) we identified two nonsense mutations, a novel G>T change in exon 5 (c.289G>T, p.E97X) and a C>T substitution in exon 20 (c.1983C>T, p.Q662X) that has been reported previously in a case with CORS (Baala et al., 2007a). In the second family (UM7) both parents were heterozygotes for the AT-deletion observed also in the Finnish family and the father of the third family

(UM8) had this same deletion (c.1219-1220delAT, p.M407fs421X), the mother having c.5850 delT in exon 40 (c.5850delT, p.F1950fs1965X). In the fourth family (UM9) the fetus was homozygous for the same deletion (c.5850delT) in exon 40.

RPGRIP1L (*MKS5*): The analysis of this gene did not reveal any mutations in the families included in the study.

CC2D2A (*MKS6*): We identified three different pairs of compound heterozygote mutations in three non-Finnish families originating from Europe. All the mutations were novel. In the first case the fetus (UM10) had two deletions, one in exon 25 that causes a frameshift and results in an early stop codon (c.3083delG, p.R1028fs) and one in the end of exon 32 that most likely disrupts the splicing (c.4179delG, potential missplicing). In the second family (UM11) we detected a deletion located in the beginning of exon 27 (c.3289delG) that most probably affects the splicing and an amino acid substitution in exon 27 (c.3341C>T, p.T1114M). The change is predicted to be pathogenic by PolyPhen and the fact that it is located in the C2 domain further supports the pathogenic nature of the change. In the third family (UM12) two deletions were found, the one being the same as in the second family and a four base pair deletion in the end of exon 30 (c.3975+1_3delAGTA). This deletion is predicted to destroy a highly conserved splice site residue, since the splice site predictor program NetGene2 (Brunak et al., 1991; Hebsgaard et al., 1996) recognizes the normal splice donor site, but due to the deletion the splice site is lost. Unfortunately, the cDNA was not available in order to confirm the splice defects. In conclusion, five different mutations were discovered of which three are located in the functional calcium binding domain of CC2D2A.

Table 4. All the mutations identified in non-Finnish families.

	Allele 1			Allele 2		
	Ex/int	**Mutation 1**	**Effect on protein**	**Ex/int**	**Mutation 2**	**Effect on protein**
MKS1						
In 3 families	int15	c.1483-7_35del	p.G470fs	int15	c.1483-7_35del	p.G470fs
F4	ex1	c.50insCCGGG	p.P17fs	int1	c.80+2T>C	Missplicing
TMEM67/MKS3						
UM1	ex8	c.734C>T	p.S245F	ex9	c.888G>T	p.W296C
UM2	ex1	c.161A>G	p.Y54C	ex15	c.1588A>G	p.Y513C
UM3	ex6	c.622A>T	p.R208X	ex8	c.755T>C	p.M252T
UM4	ex8	c.755T>C	p.M252T	ex18	c.1927A>G	p.C625R
UM5	ex13	c.1411C>T	p.R454E	ex27	c.2947T>C	p.L966P
CEP290/MKS4						
UM6	ex5	c.289G>T	p.E97X	ex20	c.1983C>T	p.Q662X
UM7	ex14	c.1219-1220delAT	p.M407fs	ex14	c.1219-1220delAT	p.M407fs
UM8	ex14	c.1219-1220delAT	p.M407fs	ex40	c.5850delT	p.F1950fs
UM9	ex40	c.5850delT	p.F1950fs	ex40	c.5850delT	p.F1950fs
CC2D2A/MKS6						
UM10	ex25	c.3083delG	p.R1028fs	ex 32	c.4179delG	Missplicing
UM11	ex27	c.3289delG	Missplicing	ex 27	c.3341C>T	p.T1114M
UM12	ex27	c.3289delG	Missplicing	ex 30	c.3975+3delAGTA	Missplicing

5.2 Characterization of *MKS* genes

5.2.1 Characterization of MKS1 and CC2D2A implicate cilia dysfunction in MKS (I, II)

MKS1 is a novel gene with unknown function, spanning a region approximately 14 kb and including 18 exons. It contains an open reading frame (DQ185029: bp 76-1755, full length cDNA 2.3 kb) for a 559 aa polypeptide containing a conserved B9 domain (pfam07162.1) (Figure 15). There are two other human proteins that contain the conserved B9 domain. One is encoded by the *B9D1* (*EPBB9*) gene on 17p and one by the *B9D2* (*LOC80776*) sequence on 19q. All the three human genes that code for peptides with B9 domains can be found from the list of the human orthologs for genes coding peptides in the flagellar (cilia in human) basal body proteome (Li et al., 2004).

Comparison of the *MKS1* sequence across the species shows high conservation. The MKS1 homolog xbx-7 (R148.1) is expressed in ciliated sensory neurons of *C. elegans* (Efimenko et al., 2005). Genes containing the X–box promoter motif are called *xbx* genes. X-box promoter element is regulated by the transcription factor daf-19, which is a member of the RFX protein family and known to be required for cilia formation in *C. elegans* (Swoboda et al., 2000; Efimenko et al., 2005). A study by Li et al. used comparative genomics of human (having cilia), *Chlamydomonas* (having flagella) and *Arabidopsis* (lacking both) to identify genes specific for ciliary proteins and proposed that the *MKS1* sequence would encode a polypeptide that is conserved in the flagellar (cilia in human) and basal body proteome (flagellar apparatus basal body proteome, FABB proteome (Li et al., 2004). More specifically, the *Chlamydomonas* ortholog of the *MKS1* gene has been suggested to encode a core structural component of the centriole (Keller et al., 2005). Based on the comparative genomics, studies of disorders with similar characteristics to MKS and our preliminary studies we could conclude that MKS1 protein is involved in ciliary functions.

CC2D2A contains 38 exons that encode (bp 73–4758) for a 1620 amino acid polypeptide having a coiled-coil and C2 domain (Figure 15). CC2D2A orthologs are found in all vertebrates and in sea urchin, jellyfish and insects. In order to find out about the functional role of CC2D2A, we reviewed studies that have used different comparative genomics approaches to identify ciliary components and *CC2D2A* is

listed among the flagellar apparatus basal body proteome genes (Avidor-Reiss et al., 2004). Using transgenic worms bearing promoter-GFP reporters it was shown that expression of K07G5.3 (*C. elegans* ortholoque of CC2D2A) is restricted to ciliated cells in transgenic worms further suggesting that the gene has ciliary functions (Blacque et al., 2005).

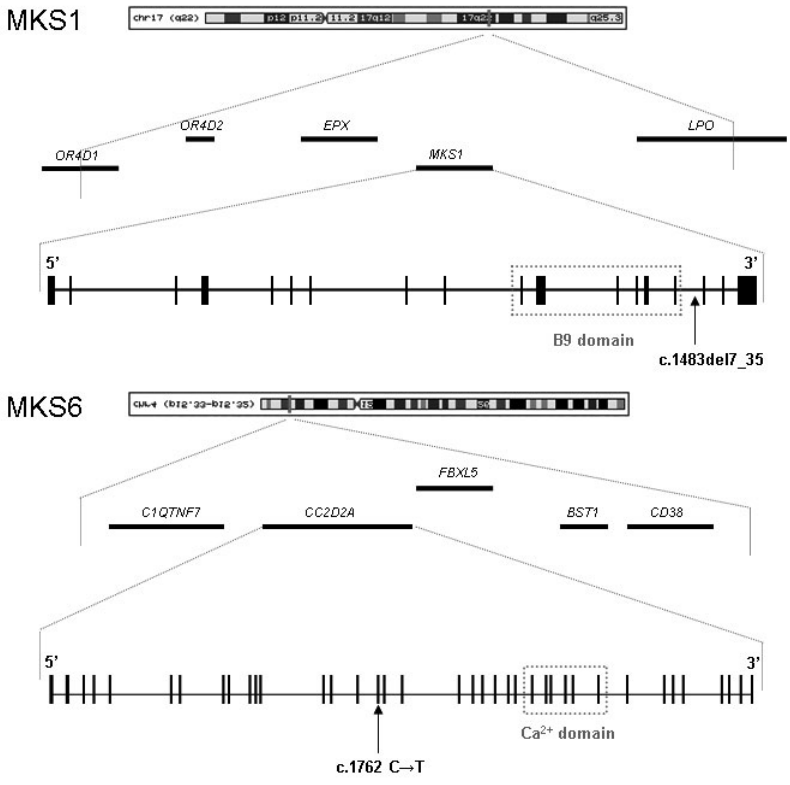

Figure 15. Overview of the critical chromosomal regions of *MKS1* in chr 17 and *MKS6* in chr 4. The major mutations in the genes are pointed by arrows and the functional domains are marked with light grey boxes.

The other clue of the genes function comes from the calcium binding domain, which consists typically of a ~120 amino acid sequence that functions as a Ca^{2+} dependent membrane-targeting module found in many cellular proteins involved in signal

transduction or membrane trafficking. The C2 domain is thought to be involved in calcium-dependent phospholipid binding and in membrane targeting processes such as subcellular localization and it is common in signal transduction proteins (Clapham, 2007). Calmodulin (CaM) is a small, ubiquitos adaptor protein, that amplifies Ca^{2+}'s diminutive size to the scale of proteins and it is the most studied Ca^{2+} sensor in eukaryotic cells (Clapham, 2007; Shen et al., 2008). Shen and collegues used mRNA display technique to find CaM-binding partners in the human proteome and CC2D2A was identified as a potential CaM-binding protein, giving further support for the role of CC2D2A in Ca^{2+}-regulated signaling pathways (Shen et al., 2008).

Calcium is known to have a central role in regulating cilia function as well as the importance of Ca^{2+}-signaling to development is well recognized. It has been proposed that Ca^{2+}-signaling, in the form of pulses, waves and steady gradients, plays a crucial role in the key pattern forming events in embryonic development. Furthermore the role of Ca^{2+} in regulating cilia function and cilia mediated pathways is known.

5.2.2 Functional studies on MKS1 and CC2D2A

In situ hybridization of *Mks1* (I, unpublished data)

In situ hybridization in mouse embryonic sections showed, like the RT-PCR from human fetal multiple tissue panel that Mks1 is expressed in various tissues. The highest transcript level was observed in the bronchiolar epithelium. The expression was abundant in all the tissue types that show malformations characteristic to MKS: brain, liver, kidney, and digits of the upper limbs (Publication I, Figure 1).

Whole mount *in situ* analysis was performed to get a better understanding of the tissues that express *Mks1*. The analysis was done using mouse emryos from day E8 to E10. No expression was seen before embryonic day 9, when the first signals appeared in the developing cochlea. Soon the expression appeared also in all the structures of the central nervous system and the expression only became stronger at embryonic day 10 (Figure 16).

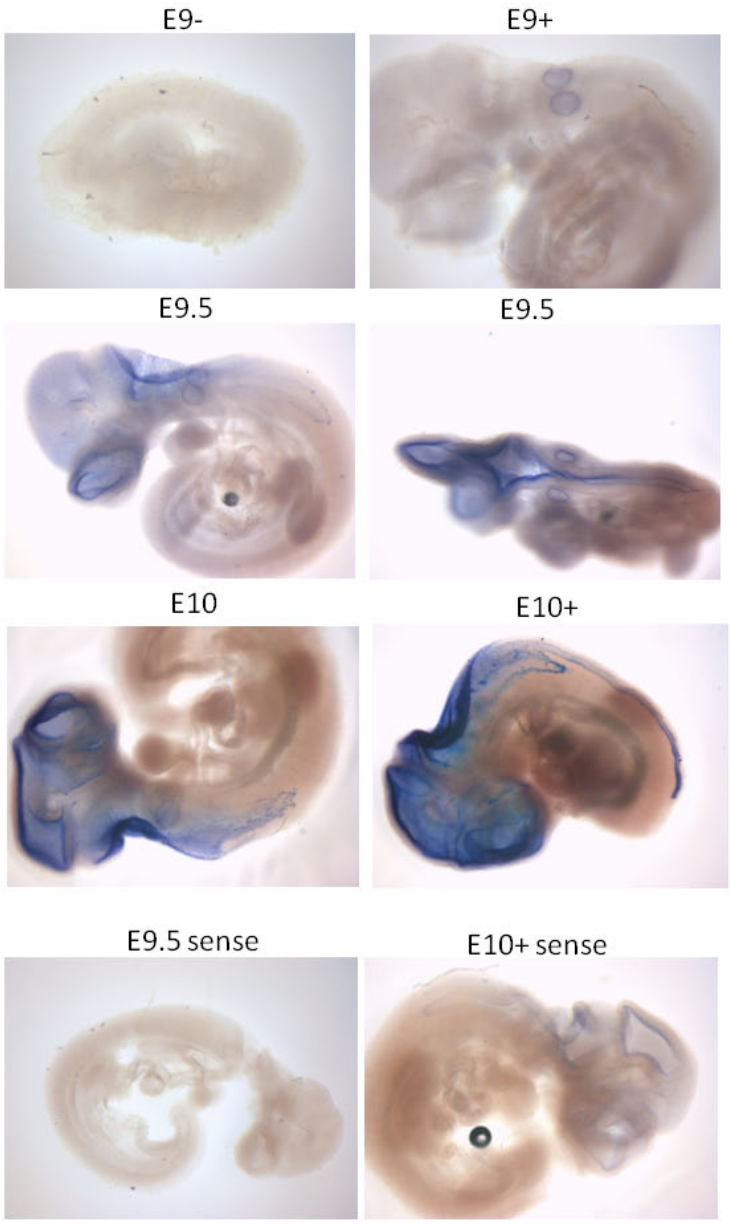

Figure 16. *Mks1* whole mount *in situ* with E9 and E10 embryos and negative sense controls. Expression is especially remarkable in the developing neural tube and brain, and also in the early cochlea.

Functional studies of MKS6 and MKS1 fibroblast cells (II, unpublished data)

To look for any visible defect in the structure of the cilium or centrioles we studied primary fibroblast cells of fetuses, who were homozygotes for the *MKS6* mutation. We also studied the fibroblast cells from a fetus, homozygote for the *MKS1* Fin_{major} mutation. As a result we found out that the centrioles can be seen in fibroblasts of the MKS cases and the healthy control fetus with γ-tubulin antibody, but as a striking contrast, no cilia were detected in patient's cells when using acetylated tubulin antibody while cilia were seen in fibroblasts of a control (Figure 17). Interestingly, in a study by Clotman and collaques they found as well that the biliary cells of some MKS fetuses lacked cilia when liver sections were stained with acetylated tubulin (Clotman et al., 2008), which agrees well with our finding Unfortunately, the genetic background of the studied fetuses was not known.

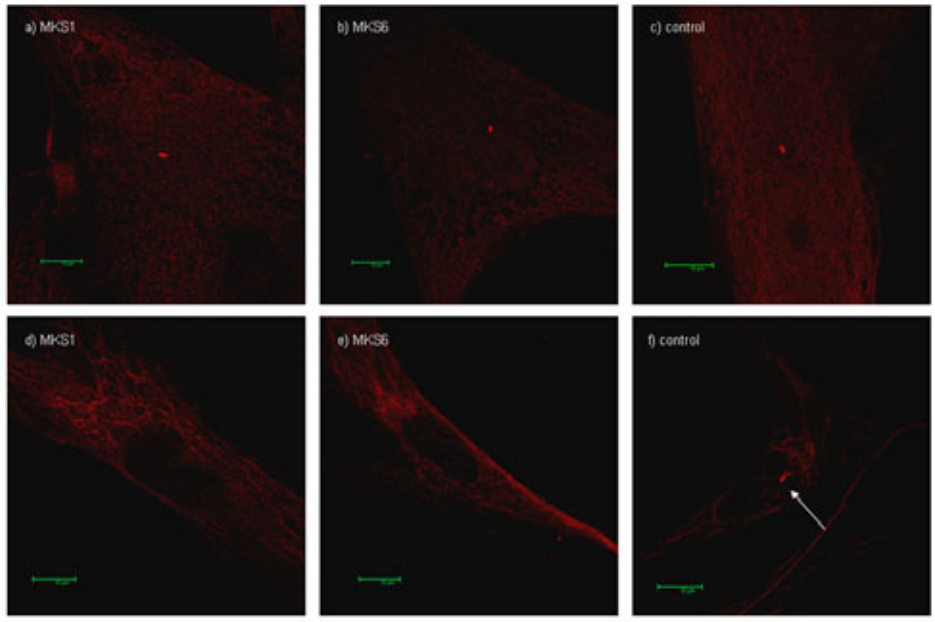

Figure 17. Centrioles were visible with γ-tubulin staining and no differences were detected between MKS cases and controls. Cilia were stained with acetylated tubulin and as a striking contrast to the controls, no cilia were detected in cells from MKS cases. Scalebar is 10μm.

Evidence implicates that the actin cytoskeleton of the cell is involved in the centriole migration during ciliogenesis (Burakov et al., 2003). Pharmacological inhibition of actin prevents basal bodies from moving to the cell surface. Actin may also be involved in anchoring basal bodies once they reach the surface. Basal bodies normally associate apically with a web-like actin network, and experimental treatments that eliminate this network, also prevent basal body docking (Marshall and Kintner, 2008). Since the centrioles were normal and no difference was observed in the centriole migration comparing MKS6 and control cells (see publication II), we wanted to see whether the actin cytoskeleton was impaired in the MKS6 fibroblast cells, and stained the actin cytoskeleton of MKS6 and control fibroblast cells with phalloidin. However, we could not detect any differences between the cell lines (Figure 18).

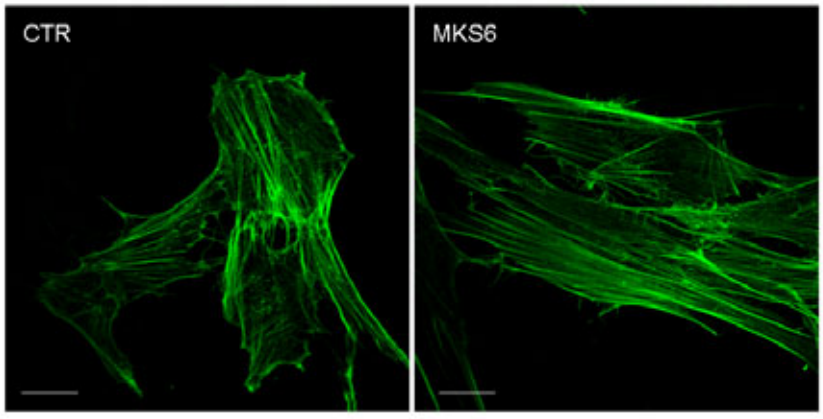

Figure 18. Phalloidin staining of actin cytoskeleton showed no differences in MKS6 fibroblast cells compared to the control cells.

Regional clustering of MKS6 families (unpublished data)

MKS is enriched in the Finnish population, the prevalence being 1:9000 newborn (Salonen and Norio, 1984), but no geographically regional clustering has been observed, unlike in most of the diseases belonging to FDH. The prevalence of MKS follows the population frequency. Even if the families were divided into two groups;

Group1: Families with Fin_{major} mutation (70%) and group2: Families with mutations in other genes (30%), no regional clustering was detected. A recent study showed that genome wide SNP data can be used also in population genetics. The authors showed that in isolated populations like the Finns a clear regional clustering of genotypes can be observed (Jakkula et al., 2008). We applied this strategy with the MKS fetuses chosen for the genome wide SNP array and could see that the cases with the *MKS6* mutation cluster to the west coast of Finland, to the early settlement region. We also collected data from the church records and the information received agrees well with the genotype data. According to the genotype and church record data, the family with mutations in the *CE290/MKS4* gene, originates from the eastern side of Finland (Figure 19).

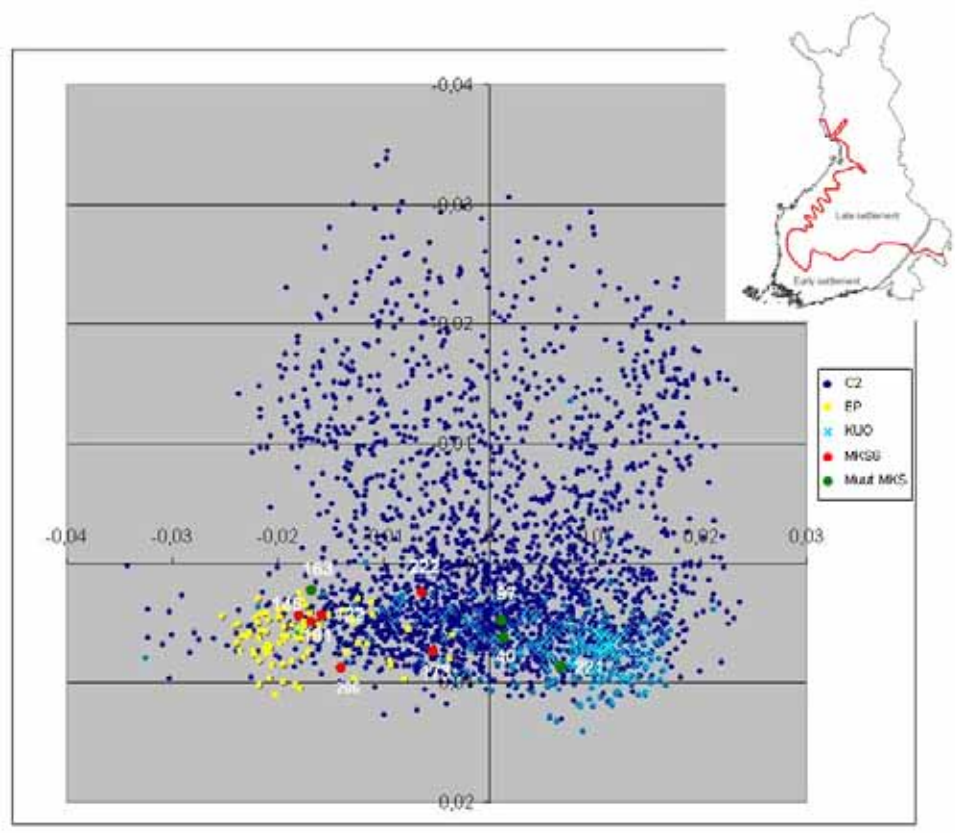

Figure 19. MKS6 cases (122, 145, 173, 181, 206 and 222) cluster with samples from the Southern Ostrobotnia region (EP), while the MKS 221 clusters more closely with the samples from Kuopio (East Finland).

6 DISCUSSION

Prior to this study, very little was known about the molecular genetics of MKS. Three loci had been mapped, but the disease causing genes remained unkonown. The first gene implicated in MKS was identified in the Finnish population and it revealed that MKS is caused by cilia dysfunction. This finding has had a huge impact on studies of MKS as well as on studies of cilia. Until recently most biologists have either ignored cilia or dismissed them as evolutionary remnants of our single-celled days. This view has changed dramatically and it is now clear that cilia perform key tasks in many organs. Cilia and basal bodies have been implicated directly in several developmental processes including left-right asymmetry, heart development, maintenance of the renal epithelium, respiratory function, electrolyte balance in the cerebrospinal fluid, and reproductive fecundity. Some of the most important developmental signals are transmitted, at least in part, through cilia.

The study of monogenic diseases has contributed greatly to our mechanistic understanding of pathogenic mutations and gene regulation, and to the development of effective diagnostic tools. After the identification of the the two major mutations in the *MKS1* and *CC2D2A* (*MKS6*) genes the DNA-based diagnostics of Meckel syndrome in Finland is excellent given that the genetic defect for MKS is now established in over 90% of the Finnish cases, leaving only approximately 10% without a known mutation. In this study we have sequenced only the coding regions of the genes (exons and exon-intron boundaries); consequently it is possible that we missed mutations that are located in the regulatory elements of the genes.

6.1 Genetic heterogeneity of MKS and characterization of the genes

The number of disease genes and loci has increased rapidly in the past few years. To date, five genes, six loci and over 50 mutations have been identified behind MKS. The number will evidently increase, given that these genes explain only a part of known MKS cases world wide. Identification of the disease genes and mutations has also demonstrated that they are not restricted to certain populations and that MKS is an allelic disorder with other syndromes, with overlapping phenotypes.

A common pathogenic pathway provides the molecular basis for overlapping. Disorders that are caused by primary cilia dysfunction are termed as ciliopathies. They range from severe multi-organ phenotypes to mild phenotypes affecting only a

single organ. The identification of the first MKS genes has had a great impact on the studies of the primary cilia, sheding some light into the molecular function and composition of the cilium.

The Finnish population is usually considered relatively homogenous, but the finding that one of the Finnish families established compound heterozygote mutations in the *MKS1* gene, unlike the other 70% of the families, who have the Fin_{major} mutation, was quite surprising. In *CC2D2A/MKS6* the only finding in the Finnish families has been the major mutation that accounts for 20% of the cases. It was also interesting that another family had coumpound heterozygote mutations in the *CEP290/MKS4* gene. Together these findings show that there is more genetic heterogeneity in the Finnish MKS cases than was expected prior to this study.

MKS1 (*BBS13*)

The Fin_{major} mutation in the *MKS1* gene is also common in non-Finnish MKS cases. The fact that the deletion is found also in families with French and English origin made Khaddour and collagues propose that the mutation occurred almost 4000 years ago in a European ancestor, and was introduced in Finland some 1300 years ago (Khaddour et al., 2007). Mutations in *MKS1* can cause Bardet-Biedl syndrome (BBS) or may have a potential epistatic effect on mutations in known BBS-associated loci. Five of six families with both MKS1 and BBS mutations manifested seizures, a feature that is not a typical component of either syndrome (Leitch et al., 2008).

Dawe and colleagues studied the MKS1 and MKS3 proteins and conducted a number of experiments done with MKS1 and meckelin. SiRNA-mediated reduction of *Mks1* in ciliated epithelial cell-line blocked centriole migration to the apical membrane and formation of the primary cilium. Also the cells had less microvilli compared to controls. Co-immunoprecipitation experiments showed that wild type MKS1 and meckelin interact, and in three dimensional tissue culture assays, epithelial branching was severely impaired. Staining of liver and kidney blocks showed that moderate to high levels of MKS1 localized at the proximal renal tubule epithelia, but not glomeruli, and at the biliary epithelium of large bile ducts (Dawe et al., 2007). Functional studies in zebrafish showed that mks1 is necessary for gastrulation movements and that it interacts genetically with known BBS genes (Leitch et al. 2008).

Williams and collaques studied the *C. elegans* orthologs of the three human B9 domain containing proteins. They found that XBX-7 (MKS1), TZA-1 (B9D2) and TZA-2 (B9D1) form a complex and localize to the base of cilia, the transition zone.

Mammalian MKS1 and *C. elegans* xbx-7 are the most diverged of the B9 homologs and show the strongest similarities in the C-terminal regions of the proteins and within the B9 domains. Yeast two-hybrid assay showed a positive interaction between TZA-1 and TZA-2, but not with XBX-7 and the other proteins, suggesting that there is an unknown factor that is responsible for anchoring XBX-7 to the complex. Mutations in the B9 genes in *C. elegans* do not have a great affect on cilia formation unless they are in a combination with a mutation in nphp-1 or nphp-4, that are mutated in NPHP patients. However, no differences were observed in the B9 gene mutants as regard to the localization of NPH-1 and NPH-4, thus there does not appear to be a direct link between B9 proteins and the NPH proteins (Williams et al., 2008).

Two other *Chlamydomonas* orthologs in human are suggested to encode centriole components (POC, proteome of centriole). These genes are mutated in OFD1 (oral-facial-digital syndrome [MIM 258860]) and NPHP-4 (nephronophthisis-4 [MIM 606966]). The authors of the proteomic analysis of *Chlamydomonas* centrioles suggest that OFD1 and NPH-4 are basal body or centriole defects which lead to ciliary dysfunction (Keller et al., 2005). Both of these diseases share similar phenotypic features with MKS.

MKS2

The MKS2 locus on chromosome 11q13 was identified already in 1998 with homozygosity mapping using seven consanguineous families from Northern Africa and Middle East (Roume et al., 1998). The region spans approximately 8 cM; however, the disease gene still remains unknown.

TMEM67 (*MKS3, MGC26979, JBTS6*)

Genomewide linkage search using autozygosity mapping in 8 consanguineous kindreds with MKS originating from the Indian subcontinent identified the third MKS locus on chromosome 8q24 (Morgan et al., 2002). Later, *MKS3* mapping was refined to a 13 Mb interval that is syntenic to the Wistar polycystic kidneys locus in rat. A recessive spontaneous mutation in *wpk* rat was thought to represent a good model for recessive polycystic kidney disease. Later studies indicated that the *wpk* phenotype extended to the central nervous system, where a range of malformations was discovered, including hypoplasia or agenesis of the corpus callosum and hydrocephalus. The MKS and wpk phenotypes (with the exception of the lack of biliary abnormalities in the wpk rat), in addition to the location of the *Wpk* gene, suggested that it was a candidate for MKS. Smith et al. sequenced the human

LOC313067 orthologue, *TMEM67*, in their MKS families and found five different homozygous mutations in them (Smith et al., 2006).

TMEM67 encodes a 995–amino acid seven-transmembrane receptor protein of unknown function. The domain structure of the protein named meckelin provides modest hints as to the pathogenesis of the disease. *TMEM67* is thought to function as a receptor; because it shares some topological (but not sequence) similarities to G protein– coupled receptors and Frizzled receptor. The authors hypothetized that the meckelin might have a role in PCP pathway, since signaling by frizzled in *D. melanogaster* has been linked to PCP. Further evidence of ciliary expression comes from the *C elegans* homolog of MKS3 (F35D2.4) which has a X-box motif, characterictic of proteins found in cilia (Smith et al., 2006).

TMEM67 is a conserved gene that is expressed at moderate levels in fetal brain, liver and kidney, but has widespread, low levels of expression in other tissues. Meckelin localizes to the primary cilium and to the plasma membrane in ciliated cell-lines and primary cells. Dawe et al. also conducted a number of experiments and showed that siRNA-mediated reduction of *Mks3* expression in ciliated epithelial cell-line blocked centriole migration to the apical membrane and formation of the primary cilium. Also the cells had less microvilli compared to controls. In three dimensional tissue culture assays, epithelial branching was severely impaired. Staining of liver and kidney blocks showed that moderate to high levels of meckelin was localized at the proximal renal tubule epithelia, but not glomeruli, and at the biliary epithelium of large bile ducts. Dawe et al. also speculated that meckelin acts as a non-canonical Wnt receptor that links PCP with with actin or tubulin cytoskeleton rearrangements (Dawe et al., 2007).

Baala and colleagues found mutations in the *TMEM67* gene in patients with Joubert syndrome and thus reported that MKS and JS are allelic disorders (Baala et al., 2007b). This was the first report of true allelism between MKS and another disorder, even though there had been suggestions that for example BBS and MKS could be allelic, because of the phenotypic overlap (Karmous-Benailly et al., 2005).

CEP290 (*MKS4, KIAA0373, NPHP6, JBTS5, SLSN6, LCA10, MKS4, BBS14*)

To identify new MKS loci, Baala and colleagues performed a genomewide linkage scan in 8 families unlinked to MKS1, MKS2, or MKS3 and found linkage to chromosome 12. The interval was narrowed to an 8 Mb region containing the *CEP290* gene, which in view of the phenotypic overlap between JS and MKS (Baala et al., 2007a). The finding of Baala et al. of the allelism of these two phenotypes at the MKS3 locus (Baala et al., 2007b), the *CEP290* gene was considered as an

excellent candidate gene, since it was already reported to be mutated in JS (Valente et al., 2006). Sequencing of the 53 coding exons revealed homozygous truncating mutations in three families and compound heterozygous mutations in a fourth family, thus identifying the third *MKS* gene. Sequencing of 20 additional MKS cases identified two additional MKS-affected families with affected individuals carrying compound heterozygous mutations of *CEP290*.

In the mouse model (*rd16* mouse) an in-frame deletion of exons 35-39 in the *Cep290* gene causes early onset retinal degeneration without associated cerebellar or kidney abnormalities (McEwen et al., 2007). CEP290 is localized to the connecting cilium of retinal photoreceptors, the basal body of an inner medullary collecting duct line (IMCD-3), and the centrosome of dividing cells (Wolf et al., 2005; Chang et al., 2006; Sayer et al., 2006). CEP290 interacts with PCM-1, a pericentriolar satellite protein, which is implicated in BBS4 function, and are involved in the ciliary targeting of Rab8, a small GTPase shown to collaborate with BBS protein complex to promote ciliogenesis. This may provide a link between CEP290 and BBS proteins (Kim et al., 2008).

RPGRIP1L (*MKS5 KIAA1005, CORS3, JBTS7, NPHP8*)

To locate a new MKS and CORS (cerebello-oculo-renal syndrome) loci, Delous and colleagues performed genome-wide linkage scans using SNP arrays in seven MKS and six CORS consanguineous families (known MKS and JBTS and CORS loci were excluded)(Delous et al., 2007). They observed homozygosity on chromosome 16q in two MKS families and two CORS families. The minimal critical region overlapped with the syntenic region deleted in the mouse line *Fused toes* (*Ft*), created previously by transgenic insertional mutagenesis. Homozygous *Ft/Ft* embryos die at midgestation, showing exencephaly, polydactyly and laterality defects. Of the four genes (*FTS*, *KIAA1005*, *FTO* and *IRX3*) contained within this overlapping interval, *KIAA1005* was considered as an excellent candidate, as its product has been shown to interact with nephrocystin-4, a ciliary protein defective in NPHP and its *C. elegans* ortholog (C09G5.8) has been found to be enriched in ciliated neuronal cells (Delous et al., 2007).

Delous and colleagues also determined that the RPGRIP1L protein shares 31% identity with RPGRIP1, a protein present at the photoreceptor connecting cilium and mutated in LCA type VI (Delous et al., 2007). RPGRIP1L contains an N-terminal region with five coiled-coil domains, a C-terminal region homologous to the RPGR-interacting domain of RPGRIP1, and a central region with two protein kinase C conserved region 2 (C2) motifs. They also found ubiquitous RPGRIP1L expression in human embryonic and fetal tissues, including brain, forelimbs, and kidney,

confirming its importance in early development (Delous et al., 2007). Staining of RPGRIP1L showed co-localization with CEP290 (Arts et al., 2007).

CC2D2A (*KIAA1345, JBTS9*)

A splice-donor-site mutation was found in a consanguineous Pakistani family with autosomal-recessive mental retardation, in the *CC2D2A* gene (ARMR). RT-PCR amplification of cDNA from affected individuals showed that this mutation results in non-frame deletion of exon 19 (Noor et al., 2008). Noor et al. studied the cellular localization of CC2D2A with overexpression in Cos7 cells and found mainly cytoplasmic localization (Noor et al. 2008), but as Gorden et al. found mutation in *CC2D2A* in patients with JS, they showed that the protein localizes also to the basal bodies as predicted (Gorden et al., 2008). Co-staining with CEP290 antibody demonstrates that recombinant CC2D2A and endogenous CEP290 colocalize at the basal body. Yeast two-hybrid and GST pull-down assays showed interaction of CC2D2A and CEP290. Intrestingly, expression of CC2D2A is substantially higher in fetal brain than in adult brain, which also indicates that it is required during development (Gorden et al., 2008). Owens et al. identified a nonsense mutation in the zebrafish ortholog of CC2D2A (*cc2d2a*), the sentinel (snl) fish (Owens et al., 2008). The cc2d2a transcript is reduced markedly in *snl/snl* fish and 33% of them exhibited pronephric cysts by 6 days postfertilization (Gorden et al., 2008).

6.1.1 Meckel syndrome and allelic disorders

Identification of the disease genes and mutations has demonstrated that MKS shares substantial allelism with other less severe syndromes, including Joubert syndrome, Leber congenital amaurosis, Senior-Loken syndrome, nephronophthisis and Bardet-Biedl syndrome, since the MKS genes are known to be mutated also in these syndromes with overlapping clinical features (Table 5). Embryonically lethal MKS represents the most severe end of the syndromes. Disease gene identification has shown that disease genes and mutations are not restricted to certain populations.

Joubert syndrome (JS [MIM 610688, 610188, 611560]) is a clinically and genetically heterogeneous group of disorders characterized by hypoplasia of the cerebellar vermis with the characteristic neuroradiologic 'molar tooth sign,' and accompanying neurologic symptoms, including dysregulation of breathing pattern and developmental delay. Additional features sometimes associated with Joubert syndrome include retinal anomalies, polydactyly, hepatic fibrosis, and renal disease.

Other clinical features define subtypes of JS, termed Joubert syndrome and related disorders (JSRD), or these related disorders can be referred to as 'cerebello-oculo-renal syndromes' (CORSs). A slightly modified version has proposed that takes into account the most recent genotype–phenotype correlates In this classification, the main neurological signs of JS (hypotonia, ataxia, developmental delay and oculomotor apraxia), along with a neuroradiologically proven MTS, represent the unique mandatory features to diagnose JSRD (primary criteria). Six subgroups of JSRDs are then defined on the basis of additional secondary criteria, as follows: (1) JS subgroup, fulfilling primary criteria only; (2) JS associated with retinopathy; (3) JS associated with renal involvement (either NPH or cystic dysplastic kidneys); (4) CORS subgroup, including JS with both retinal and renal involvement (also termed JS + SLS); (5) COACH subgroup [MIM 216360], including JS with both ocular colobomas and liver abnormalities; and (6) OFDVI subgroup [MIM277170], including JS with both orofacial and digital signs (Valente et al., 2008). Several features overlap with the classic MKS features including polydactyly, fibrotic changes of the liver and CNS malformation (encephalocele). Nine loci behind JS have been identified so far and the disease gene is known in seven of them (*NPHP1*, *AHI1*, *CEP290*, *RPGRIP1L*, *TMEM67*, *ARL13B* and *CC2D2A*) (Gorden et al., 2008; Valente et al., 2008). The second JS locus JBTS2 overlaps with the MKS2 locus on chromosome 11 suggesting allelism between the two diseases also in this locus (Valente et al., 2008).

Bardet-Biedl syndrome (BBS [MIM 209900]) is an important genetic cause of chronic and end-stage renal failure. It is a multi-system disorder, which consists of obesity, retinal degeneration, cognitive impairment, genitor-urinary tract malformations and polydactyly. Polycystic kidneys are the most likely cause of premature death from the syndrome, combined with complications caused by overweight, including type II diabetes, hypertension and hypercholesterolaemia. Although BBS is considered to be a developmental disorder, the only clues in utero may be hexadactyly and hyperechoic kidneys. The diagnosis is often only established once the vision begins to degrade (Tobin and Beales, 2007). To date, fourteen genes behind BBS have been identified (*BBS1*, *BBS2*, *ARL6*, *BBS4*, *BBS5*, *MKKS*, *BBS7*, *TTC8*, *PTHB1*, *FLJ23560*, *TRIM32*, *FLJ35630*, *MKS1* and *CEP290*) (Tobin and Beales, 2007; Leitch et al., 2008). There is currently a debate on the heritance model of BBS, some studies have reported digenic triallelic inheritance (a requirement for three disease alleles, two at one locus and a single in a second BBS gene), while some conclude that homozygous or compound heterozygous mutations at one locus are sufficient for complete disease inheritance (Beales, 2005). In most cases the inheritance of BBS is autosomal recessive. Although BBS is rare (<1:100,000), there is considerable interest in identifying the disease causing genes,

because components of the phenotype, such as obesity and diabetes are common problems in modern society (Mykytyn et al., 2001).

Nephronophthisis (NPHP [MIM 256100]) is a heterogenous group of autosomal-recessive cystic kidney disorders that represents the most frequent genetic cause of chronic and end-stage renal failure in children and young adults (Hildebrandt and Otto, 2005). It is characterized by chronic tubulointerstitial nephritis that progress to terminal renal failure during the second decade (juvenile form) or before the age of five years (infantile form). Nine genes underlying NPHP have been reported (*NPHP1*, *INVS*, *NPHP3*, *NPHP4*, *IQCB1*, *CEP290*, *GLIS2*, *RPGRIP1L*, *NEK8*). The NPHP2 is responsible for the infantile form and the other genes underlie the juvenile form (Salomon et al., 2008). NPHP may be associated with other clinical features, like in Joubert syndrome; NPHP is associated with cerebellar vermis aplasia/hypoplasia, retinal degeneration and mental retardation. Senior-Loken (SLSN [MIM 266900]) is also known as juvenile nephronophthisis with Leber amaurosis. It is an autosomal recessive renal-retinal disorder, characterized by progressive wasting of the filtering unit of the kidney, with or without medullary cystic renal disease, and progressive eye disease (O'Toole et al., 2006).

Leber congenital amaurosis (LCA [MIM 204000]) is generally inherited in an autosomal recessive manner and is characterized by severe retinal dystrophy, causing blindness or severe visual impairment at birth or during the first months of life. LCA represents the most common genetic cause of congenital visual impairment in infants and children. Most vision researchers currently consider LCA the most severe retinal dystrophy without major systemic features. Identification of 14 genes mutated in patients with LCA and juvenile retinal degeneration explain approximately 70% of the cases (*AIPL1*, *CEP290*, *CRB1*, *CRX*, *GUCY2D*, *IMPDH1*, *LCA5*, *LRAT*, *MERTK*, *RD3*, *RDH12*, *RPE65*, *RPGRIP1*, *TULP1*). Several of these genes have also been implicated in other non-syndromic or syndromic retinal diseases, such as retinitis pigmentosa and JS, respectively. *CEP290* is one of the most frequently mutated LCA genes; one intronic *CEP290* mutation (p.Cys998X) is found in approximately 20% of all LCA patients from north-western Europe. LCA patients with *CEP290* and *GUCY2D* mutations seem to have very significant loss of vision, but then remain stable, whereas LCA patients who harbor AIPL1 and RPGRIP1 mutations have progressive loss of vision. Associated phenotypic features include for example keratoconus and cataracts, mental retardation, autism and olfactory dysfunction (den Hollander et al., 2008).

Table 5. Genes mutated in MKS and allelic disorders. In addition, MKS2 locus has been mapped to chr 11q13, JBTS1 locus to chr 9q34 and JBTS2 locus to chr 11q12-13.3, but the genes have remained unknown. MKS genes are bolded, showing which genes are common in diffrent syndromes.

Syndrome	**Number of known genes**					
MKS	5	**MKS1**	**TMEM67**	**CEP290**	**RPGRIP1L**	**CC2D2A**
JS	7	NPHP1	AHI1	**CEP290**	**RPGRIP1L**	**TMEM67**
		ARL13B	**CC2D2A**			
BBS	14	BBS1	BB2	ARL6	BB4	BBS5
		MKKS	BBS7	TTC8	PTHB1	FLJ23560
		TRIM32	FLJ35630	**MKS1**	CEP290	
NPHP	9	NPHP1	INVS	NPHP3	NPHP4	IQCB1
		CEP290	GLIS2	**RPGRIP1L**	NEK8	
LCA	14	AIPL1	**CEP290**	CRB1	CRX	GUCY2D
		IMPDH1	LCA5	LRAT	MERTK	RD3
		RDH12	RPE65	RPGRIP1	TULP1	

6.1.2 MKS and allelic disorders are dictated by the mutations

After analyzing our collection of Finnish and non-Finnish families we were interested in the previously reported mutations, since mutations in the *MKS* genes are also found in other syndromes, and it seems reasonable to assume that there is a genotype-phenotype correlation between the syndromes and the mutations. To test whether the phenotypes of different syndromes are dictated by the nature of the mutations, we collected all the previously published mutations to see whether there really is an apparent contrast between the syndromes and mutations.

In some reports there have been cases where the researches have only identified one mutation that is the cause for MKS. Based on our findings, this is not very recommendable. As we have found out while conducting this study, that one mutation in one gene might not be enough for reporting the right causative gene. In one of the Finnish MKS6 families the mother is a carrier also for the Fin_{major} mutation in the *MKS1* gene. Both of the parents are heterozygous and the fetus homozygous for the c.1762C>T (p.V587fsX616). The Finnish family case demonstrates well the fact that MKS is a monogenic disorder and single pathogenic mutations in two different MKS genes are not sufficient enough to cause the disease. If the other mutation is not detected in the same gene, the disease causing mutation can be another gene altogether and we therefore state that both of the disease causing mutations have to be in the same gene in order to report the gene in question as the disease gene for a MKS case.

However, mutations in other genes may modify the phenotype, even if they are not the primary disease mutations. For example, Tory and colleagues found out that in patients with NPHP and JS-related neurological symptoms have the homozygous *NPHP1* deletion in combination with a heterozygous truncation mutation in *CEP290* and a heterozygous missense mutation in *AHI1* (Tory et al., 2007). Mutations in the MKS genes are found to modify the BBS phenotype (Leitch et al., 2008). Five out of six BBS patients, who manifested seizures, had also a heterozygote mutation in the *MKS1* gene. The modifying effect is possible also in Meckel syndrome and that might explain the differences in the clinical picture. The variable expression of for example *CEP290* null mutations, ranging from nonsyndromic LCA, JS to MKS, suggests an important role of other genetic factors (den Hollander et al., 2008).

MKS mutations represent every type of mutations, there are missense, nonsense, frameshift, or splice-site mutations, all most likely resulting in null alleles. A genotype-phenotype correlation has been hypothesized, but Frank et al. noted that it is currently impossible to predict the patient's phenotype by the mutations' type, location, and/or affected protein (Frank et al., 2008). Our findings additionally support their assumption. Interestingly, but not surprisingly there is a clear distinction between the mutations and syndromes, since none of the reported MKS mutations have been reported in other syndromes as the same combination pair. This indicates that there indeed is a functional role for the type and the location of the mutation. Further understanding of the character of the mutations is needed in order to understand their role in pathogenesis of the syndromes. The reported mutations might help to define the diagnosis in cases where the clinical picture is not apparent.

Yet, a strong genotype-phenotype correlation has been observed, depending on the mutated gene by Khaddour et al, when they compared the clinical findings in MKS1

and MKS3 cases. Postaxial polydactyly is rare in *MKS3* mutated cases in contrast to *MKS1* mutated cases. There is also difference between the type of CNS malformation between *MKS1* and *MKS3* mutated cases, since occipital encephalocele is typically seen in the *MKS1* affected fetuses, but not in the *MKS3* fetuses (Khaddour et al., 2007). In contrast, no clear genotype-phenotype correlation can be seen between *MKS1* mutated cases and *MKS6* mutated cases. Neither there is a strong difference between the Finnish cases were the mutation has not yet been identified compared to the *MKS1* and *MKS6* mutated cases. The only difference observed in the *MKS4* mutated Finnish case compared to the *MKS1* and *MKS6* mutated cases is lack of polydactyly, which is a common feature of *MKS3* mutated cases, but it should be noticed that polydactyly is not found in all *MKS1* or *MKS6* cases either.

In a paper by Paavola and colleagues the authors discussed that not all patients with encephalocele, cystic kidneys and polydactyly have MKS, but if strict diagnostic criteria are applied the diagnosis should be quite unambiguous (Paavola et al., 1997). They further hypothesized that, do atypical cases still really represent MKS, or another cerebro-reno-digital syndrome? Or, could MKS itself be considered as a heterogeneous syndrome with a wide spectrum of phenotypes (Paavola et al., 1997). The identification of the disease genes and the realization that these syndromes are allelic makes this question again relevant. Since all the syndromes have same molecular background, should they be called collectively CORSs? Conversely, one cannot exclude the fact that even though the diseases are caused by mutations in the same genes there can still be seen a clear difference between the mutations and the syndromes. In addition, most of the syndromes caused by *MKS* genes also have their own specific disease causing genes. We therefore think that each syndrome still can and should have own specific criteria and should not be considered as a whole group.

6.2 Cilia

To understand the molecular basis of cilia related disorders it is important to take a closer look to the organelle itself. Currently very little is known about the maintenance of cilia and cilia mediated signaling that is important for normal development, but the amount of information is increasing rapidly. Over the next few years new research will undoubtly provide answers to many of the questions that have arisen.

Cilia and flagella are flexible membrane extensions of the cells and their size varies in length from a few microns to more than 2 millimeters. Although cilia and their

longer cousin's flagella are in fact the same structures, they were given different names before their structures were studied. Typically, cells have one or two long flagella, whereas ciliated cells have many short cilia. The name 'cilia' derives from the Latin word meaning eyelashes. Zimmermann is reported as the first person to observe cilia and was the first to describe the primary cilium already in the 19th century (Zimmerman, 1898). Cilia were forgotten for a long time, but the first link of the dominance of cilia came in 1976, when Swedish biologist Björn Afzelius reported the link between defective cilia and disease. He realized that the symptoms of Kartageners triad, a human disease involving bronchiestasis, male infertility and situs inversus, could be explained as a disease of cilia (Afzelius, 1976). Nevertheless, the prevailing view was that these organelles were cell biological oddities, not worth mentioning, as was the case in some textbooks as late as five years ago.

Cilia can be reviewed as specialized cellular compartments or organelles. They fall in to two classes: motile and immotile. The molecular makeup of motile and non-motile cilia is likely to largely overlap, although each has also components required for their specific functions (Inglis et al., 2006). They also have tissue specific functions during development, tissue morphogenesis and homeostasis (Fliegauf et al., 2007). Ciliar motility is important for moving substances around the cell, such as fluid and mucous, as well as the cell itself. Immotile cilia have a sensory role. Defects in the immotile cilia are frequently associated with kidney cysts, retinal degeneration, polydactyly, obesity, and neural tube defects (Santos and Reiter, 2008). Ciliated cells are commonly a highly specialized, post-mitotic cell type, and are thus non-proliferating, differentiated cells. However, cilia can also form in quiescent cells (G_1 phase), which may undergo later rounds of cell division, and on proliferating cells (Adams et al., 2008). Although cilia are almost ubiquitously present in vertebrate cells, they are restricted to sensory neurons in invertebrates (Wheatley et al., 1996). Even though many differentiated cells have cilia, there are a few cell types where a primary cilium is not formed, e.g. nucleated blood cells, adipocytes and hepatocytes (Alieva and Vorobjev, 2004). A comprehensive list of cells and tissues containing cilia is available at
http://www.bowserlab.org/primarycilia/cilialist.html.

6.2.1 Structure and function

Cilia are assembled as extracellular membrane enclosed compartments (Inglis et al., 2006). The assembly of the cilia is tightly coupled to the cell cycle and occurs from

a plasma membrane-associated foundation called the basal body as cells enter growth arrest/G1, whereas the cilia are discarded shortly before cells enter mitosis (Archer and Wheatley, 1971; Quarmby and Parker, 2005; Pan and Snell, 2007; Pedersen et al., 2008). At the heart of the basal body is a centriole, an important component of the mitotic spindle apparatus in dividing cells. During interphase, however, the centriole moves to the apical plasma membrane (Figure 20).

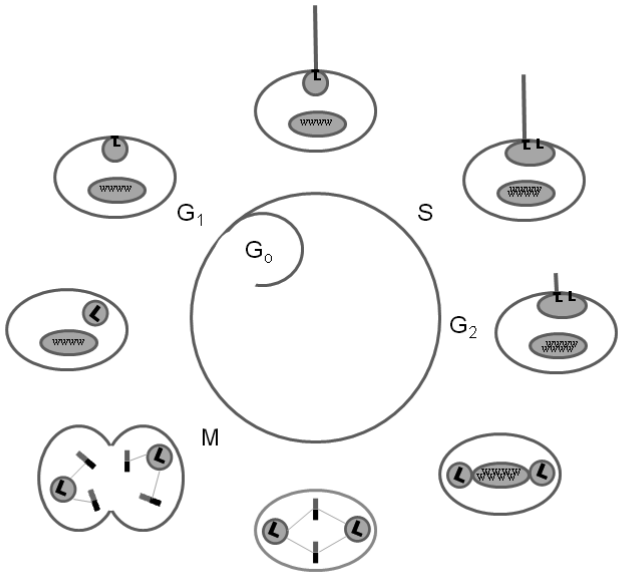

Figure 20. The primary cilium occurs first during G_1 following centrosomal docking to the cell membrane. During this stage as well as the G_0, the cilium functions as a cellular antenna. Upon entering the S phase, the cell's centrioles and DNA begin to replicate. At late G_2 the cilium is disassembled and the centrioles are liberated for mitotic spindle formation. After completion of the cell division, the cells can proceed to ciliary re-assembly in G1 (modified from Santos and Reiter, 2008).

After studying the assembly of the primary cilia under the electron microscope, Sorokin set out three distinct early stages. First, a Golgi-derived vesicle attaches to the distal end of the mother centriole, from which the growing axoneme begins to emerge; the vesicle becomes invaginated as the centriole extends and accumulates accessory structures to become the distal basal body. Second, nearby vesicles fuse with new membrane forming at the ciliary base to create a cover surrounding the

elongating axonemal tube in which microtubule pairs are quickly assembled. In the third stage of the ciliogenesis, the membrane-surrounded axoneme reaches the cell surface and the ciliary membrane fuses with the plasma membrane (Sorokin, 1962; Pedersen et al., 2008). In the fourth stage the axoneme is elongated to form a primary cilium (Figure 21).

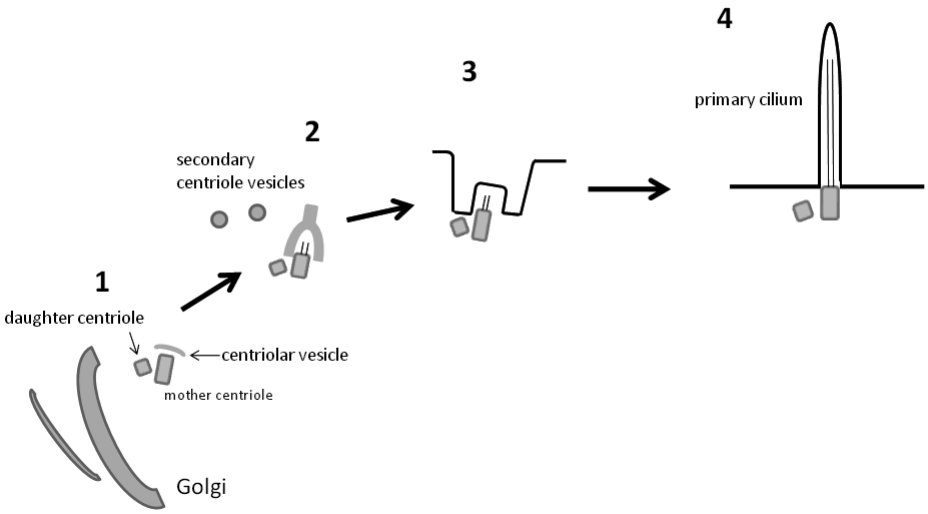

Figure 21. Stages in primary cilium formation. 1) Golgi derived centriolar vesicle localices to the distal end of the mother centriole. 2) Secondary centriolar vesicles fuse with the new membrane to form a sheat that surrounds the elongating axonemal shaft. 3) Membrane bound axoneme fuses with the plasma membrane. 4) After docking the mother centriole to the cell surface and fusion of the sheath with the plasma membrane, the axoneme is elongated to form a primary cilium (modified from Pedersen et al., 2008).

The two centrioles are linked by interconnecting fibers and are surrounded by a proteinaceous matrix known as the pericentrioral material (PCM). Although the composition of the PCM is largely unknown, several proteins such as ninein, polo kinases, and γ-tubulin are required for the nucleation and anchoring of microtubules, making this structure the major microtubule organizing center (MTOC) (Badano et al., 2006).

Generally cilia have been classified into two different groups depending on their structure. There are 9+2 motile eucaryotic cilia and 9+0 immotile cilia also known as the primary cilia. In 9+2 cilia, nine doublet microtubules surround a pair of single microtubules, but in 9+0 cilia there is no central pair. Microtubules are built from tubulin heterodimers composed of α and β tubulin (Nogales et al., 1999). Radial spokes link the outer ring doublets to the inner doublet. Like cilia, centrioles and basal bodies are also made of microtubules. The difference is that they contain 9 sets of triplets and no doublet in the center (Figure 22). The process, how the triplets in the basal body turn into the cilium doublet is not known, but it seems to be cell type-specific and developmentally regulated (Kim et al., 2004). However, owing to numerous exceptions to the two groups of cilia, the distinction of cilia into four subtypes might be more appropriate: 1) motile 9+2 cilia (e.g. cilia in the respiratory epithelial ceels of the airways and ependymal cells lining the brain ventricles), 2) motile 9+0 cilia (e.g. nodal cilia), 3) immotile 9+2 cilia (e.g. vestibular cilia) and 4) immotile 9+0 cilia (e.g. renal monocilia and photoreceptor connecting cilia) (Fliegauf and Omran, 2006).

The ciliary tip harbors the microtubule plus ends, from which axonemes grow. It contains signaling molecules and can undergo morphological changes in response to signaling processes (Fliegauf et al., 2007). Construction of the axoneme (elongation of the axoneme at the distal tip) and maintenance of the cilium requires intraflagellar transport (IFT) (Rosenbaum and Witman, 2002; Santos and Reiter, 2008). IFT was first described in *Chlamydomonas* and has subsequently been found to be essential for the assembly of motile and sensory cilia in many organisms (Pazour and Rosenbaum, 2002). Because no proteinsynthesis occurs within cilia, IFT is needed to move the organelle's structural components from the cell body to the ciliary tip (the anterograde direction) where axoneme synthesis takes place. IFT returns proteins from the cilium to the cell body by means of a retrograde movement (Pazour et al., 1998). The anterograde transport occurs via kinesin motors and the retrograde direction involves a cytoplasmic dynein motor (Cole et al., 1998; Porter et al., 1999). In addition to the role of basal bodies in initializing ciliogenesis, they also serve as the docking and assembly site for proteins involved in the IFT machinery (Deane et al., 2001). IFT does not only deliver ciliary components required for the assembly, maintenance, and length control of motile and sensory cilia, it also carries cilium-based signals that control cell function, gene expression, cell division, animal development, and the onset of human disease (Scholey, 2008). Mutations in genes encoding proteins that participate in IFT cause ciliogenesis defects of both motile and immotile cilia (Fliegauf, et al. 2007).

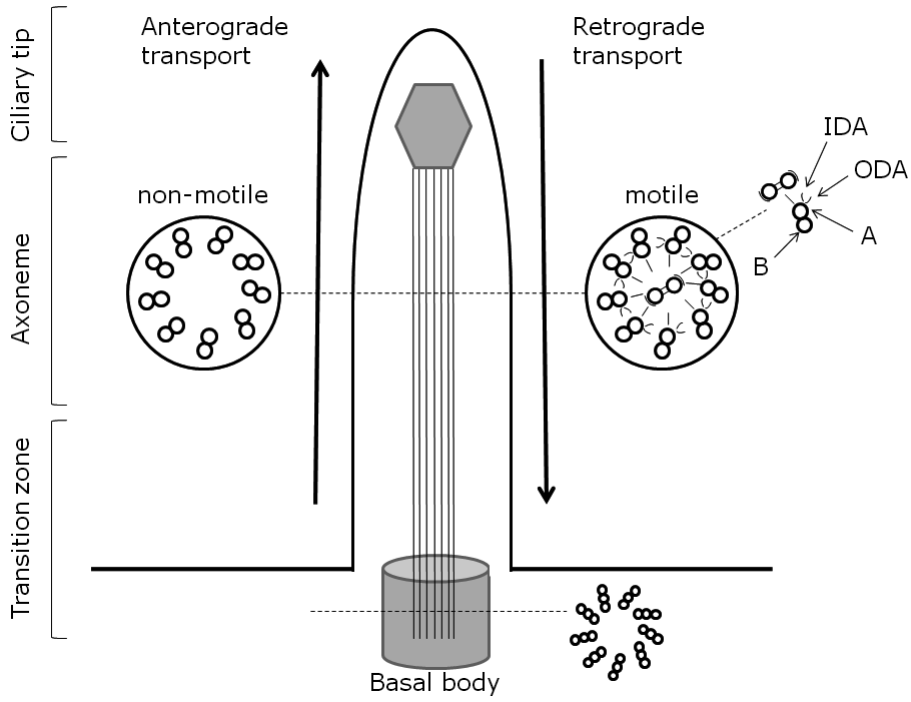

Figure 22. Schematic illustration of the structure of the cilium. Microtubules are built from tubulin heterodimers composed of α and β tubulin (A and B). Cross sections show that in motile 9+2 cilia, nine doublet microtubules surround a pair of single microtubules, but in immotile 9+0 cilia the central pair is missing, as well as the inner and outer dynein arms (IDA and ODA, respectively) and radial spokes that link the outer ring doublets to the inner doublet. Intraflagellar transport (IFT) moves ciliary components required for the assembly, maintenance, and length control of motile and sensory cilia and cilium-based signals that control cell function. The switch between anterograde and retrograde transport takes place in the ciliary tip. The triplet microtubular structure of the basal body turns into the axonemal doublet structure in the transition zone, which might control access to the ciliary compartment.

Cilia act as antennas to sense environmental signaling molecules (Eggenschwiler and Anderson, 2007) and physiological ligands (Christensen and Ott, 2007). Cilia can also generate flows of mucus and cerebrospinal fluid in a beating motion and can act as mechanosensors and flow meters (Praetorius and Spring, 2003). The beating motion also propels spermatozoa (Tobin and Beales, 2007). A dramatic difference in the mechanism of ciliogenesis is seen in the multiciliated epithelia. In

contrast to the primary cilia in most cells, cilia in the airway and ependymal cells are nucleated by basal bodies that form *de novo* in large spherical arrays called deuterosomes (Dirksen, 1991; Marshall, 2008). Defects in deuterosome-spesific cells might result in cilia defects specifically in multiciliated epithelia without having any effect on sensory primary cilia (Marshall, 2008).

The role of cilia in sensing the extracellular environment is best understood in the context of olfaction and photoreception, which occur through a cilium-based signaling pathway. Olfactory neurons that lack either cilia or odorant receptors on their cilia cannot respond to odorants (Singla and Reiter, 2006). The IFT process appears to be especially important in the connecting cilium of the retina, which connects the outer segment to the inner segment of the retina, and is thought to be the exclusive route for newly synthesized protein transport from the inner to outer segment (Mykytyn and Sheffield, 2004). In addition to sensoring odorants and light, cilia can sense movement (Singla and Reiter, 2006).

6.2.2 Cilia and signaling

Primary cilia are found in embryonic tissues and during early postnatal development (Alieva and Vorobjev, 2004). It is emerging that the cilium is indispensable for certain key developmental signaling cascades and it is becoming apparent that many discrete pathways converge at, or are regulated by, the cilium. The ciliary membrane contains various cilia-specific receptors, ion channels and signaling molecules. Flow induced passive cilia bending is required for the mechanosensation of extracellular fluid flow (Fliegauf et al., 2007). A number of cilia-related diseases have been described that are associated with developmental defects affecting the central nervous system, the skeleton or other organ systems. Moreover, it is now clear that cells also use the primary cilia to communicate with each other. For instance, cilia play roles in establishing proper left-right patterning, regulating intracellular calcium levels, and interpreting several intracellular signals (Santos and Reiter, 2008).

The list of cilia-dependent molecular pathways include: PDGFRα growth factor signaling, hedgehog signaling and epidermal growth factor signaling (Tobin and Beales, 2007). These molecular pathways are implicated in almost every major developmental process, including the correct establishment of body plan, left-right axis determination, development and closure of the neural tube and limb formation (Vogel, 2005; Singla and Reiter, 2006). Certain receptors are concentrated in, if not exclusively targeted to, the membrane of the primary cilium, and that the defects of disease arise even if the primary cilia are present, if receptor targeting is defective.

During embryogenesis, the primary cilium signaling pathways are especially important for neurogenesis, for epithelial morphogenesis and differentiation in liver and pancreas as well as kidney development and for epithelial-mesenchymal interactions (Satir, 2008).

Cilia and Wnt

Wnt family members are secreted lipoproteins that regulate both cell proliferation and differentiation (Nusse, 2003). Wnt proteins activate several distinct signaling pathways, classified as either β-catenin dependent (the so-called canonical pathway) or β-catenin independent (the noncanonical pathways, also known as the PCP pathway) (Singla and Reiter, 2006). After Wnt proteins bind to receptors of the Frizzled (Fz) family, Disheveled (Dvl) is recruited to Fz, and glycogen synthase kinase-3β (GSK3β) is inactivated and β-catenin is translocated to the nucleus (Figure 23) (Fliegauf et al., 2007). Upon binding to its receptor Fz the Wnt signal is transmitted to the primarily cytoplasmic protein Dvl, which determinates which Wnt signaling pathway will be activated and acts as a switch between canonical and non-canonical Wnt signaling (Veeman et al., 2003). The PCP pathway depends on many components known to be critical for Wnt signal transduction, including Fz and Dvl. Other components, such as Inturned and Fuzzy, that act downstream of Dvl, are not shared with the Wnt pathway (Santos and Reiter, 2008).

Planar cell polarity (PCP) is a process in which epithelial cells become uniformly polarized to establish an area of aligned structures such as hair orientation. The mechanism requires the asymmetric localization of planar polarity proteins. The disruption of the PCP pathway in mice causes severe neural tube defects (Wallingford, 2006). Studies in vertebrates have indicated that orthologs of genes encoding PCP proteins have roles in the regulation of polarized cell movements, including defects in convergent extension during gastrulation and mediolateral narrowing of the embryo (Keller, 2002). Morpholino knockdown of Inturned and Fuzzy in Xenopus embryos disrupts convergent extension (Park et al., 2006). It seems most likely that a similar mechanism is at work in humans as well.

Among our most recent understanding of neural tube closure is the emerging link between cilia and PCP signaling. The first connection came from the studies of inversin, which was a known ciliary protein (Wallingford, 2006). Mutations in *Inversin* gene cause renal cystic diseases in humans (Otto et al., 2003). Inversin has also been found to both inhibit the canonical Wnt signaling and promote non-

canonical Wnt signaling (Figure 23); however, no developmental defects due to abnormal Wnt signaling have been reported in mouse *inversin* mutants (Scholey and Anderson, 2006).

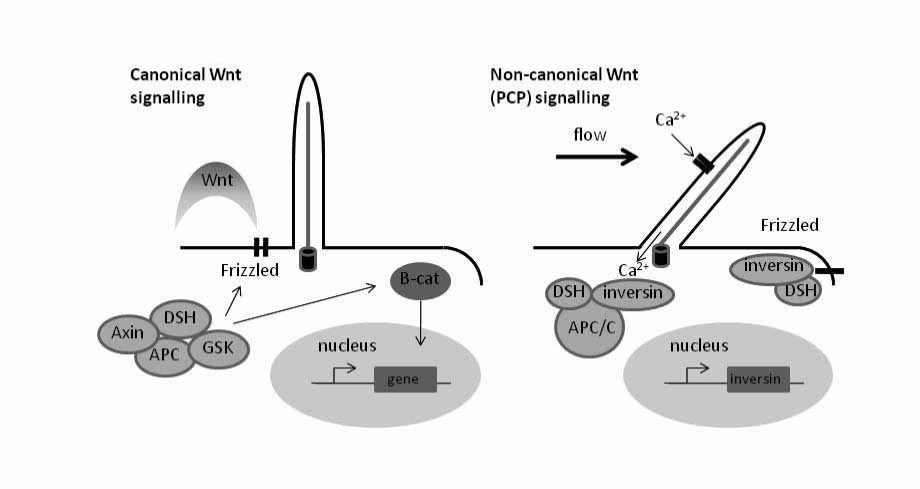

Figure 23. Cilia mediated signalling is thought to act as a switch between canonical and non-canonical Wnt pathways. In the absence of fluid flow, canonical Wnt signalling predominates. On mechanosensation of fluid flow, intracellular Ca^{2+} release causes increased inversin expression, which targets cytoplasmic Disheveled making it unavailable for canonical Wnt signalling (modified from Fliegauf et al., 2007).

There are two models of the possible connections between PCP and cilia: (1) PCP is required for ciliogenesis, in this model PCP signals govern actin dynamics at the base of cilia and are thus required for normal ciliogenesis and in turn for normal Hedgehog signaling, (2) cilia are required for PCP signaling, in this model, cilia act as mechanosensors and act via PCP signals to establish cell polarity and direct cell movement (Wallingford, 2006; Eggenschwiler and Anderson, 2007).

During early kidney development, canonical Wnt signaling is required for metanephric mesenchyme induction and cell proliferation during branching morpohogenesis. Later in development, signaling through the non-canonical Wnt (or PCP) pathway is required to align the mitotic orientation of of proliferating cells of the renal tubules to allow the tubules to lengthen without substantially increasing their diameter (Bisgrove and Yost, 2006). A leading example of the biological

importance of primary cilia is the Oak Ridge polycystic kidney (*orpk*) mouse model of ARPKD (autosomal recessive polycystic kidney disease), the *Tg737*^{orpk}, that has an insertional mutation that disupts the gene coding for the protein Polaris, which localizes to both basala bodies and cilia (Moyer et al., 1994; Taulman et al., 2001). The *Chlamydomonas* and *C.elegans* orthologs of *Tg737*, *IFT88* and *osm-5*, respectively, encode IFT proteins whose disruption leads to defective flagella in both species (Pazour et al., 2000; Haycraft et al., 2001). Abnormal cilia are also observed in the renal epithelium of *Tg737* mice (Pazour et al., 2000; Pazour and Witman, 2003; Badano et al., 2006). The link between cilia and the Wnt pathways, has provided understanding to one of the main features of MKS is cystic kidney dysplasia. The normal Wnt pathways are defective in MKS, since the MKS cases lack cilia

Cilia and Hh

Sonic Hedgehog (Shh) is one of three paralogous vertebrate proteins (related to invertebrate Hedgehog (Hh) protein) that binds to the transmembrane protein Patched (Ptc) at the cell surface. Upon binding, Shh abolishes the inhibitory effect of Ptc on another transmembrane protein, Smoothened (Smo). This relief of inhibition allows Smo to transduce a signal signal to the nucleus via glioma (Gli) transcription factors (Gli1, Gli2 and Gli3), triggering the expression of specific genes (Christensen and Ott, 2007). Supressor of fused (Sufu) is an essential negative regulator of the pathway, which is enriched in the ciliary tip in the absence of Hh ligands (Eggenschwiler and Anderson, 2007). Shh signaling in cilia involves the IFT system, which moves components of the signaling pathway to their functional sites (Figure 24) (Huangfu et al., 2003). Mouse IFT mutants demonstrate that Hh signaling is coupled to cilia. Analysis of neural patterning in double mutant embryos showed that the IFT mutations block Hh signal transduction downstream of Pacthed1 and Smoothened (Eggenschwiler and Anderson, 2007).

Loss of activity of the Shh pathway can cause various birth defects, including holoprosencephaly, polydactyly, craniofacial defects and skeletal malformations (McMahon et al., 2003). Shh is required for the normal patterning of the limb, where it regulates growth, digit number and anterior-posterior polarity (Eggenschwiler and Anderson, 2007). Some of the phenotypes associated with IFT protein dysfunction likely result from their involvement in hedgehog signaling in vertebrates. Hedgehog signal transduction is necessary for the proper development and differentation of many tissue types, and at least two diseases linked to deregulated hedgehog

signaling, Palister-Hall syndrome and Smith-Lemli-Opitz syndrome, share polydactyly as well as renal and heart anomalies with Bardet-Biedl syndrome, that is caused by mutations in genes that encode for IFT proteins (for BBS see 6.1.1) (Blacque et al., 2004). The Shh pathway is disrupted in MKS1 and MKS6 fetuses, since they have no cilia. This finding agrees well with the clinical picture of MKS cases.

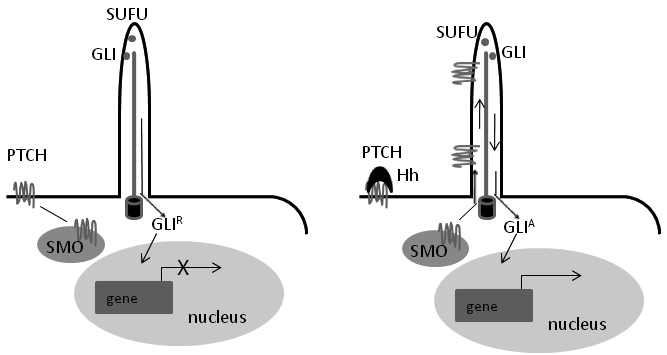

Figure 24. Shh signalling in cilia involves the IFT machinery, which moves components of to their functional sites. The transcription factors GLI and SUFU are transported to the ciliary tip, where GLI is prosessed to create a transcriptional repressor and transported back to the cell body. When Hh ligand binds to its receptor patched-1 (Ptc), SMO is released and transported to the ciliary tip, where it turns off GLI processing (modified from Fliegauf et al., 2007).

An interesting facet of neural tube closure is that the mechanism governing this process in the brain differs significantly from those operating in the spinal cord. The distinction is striking in the classification of neural tube defects (NTDs) upon rostral-caudal location (Wallingford, 2006). It is believed that the PCP pathway is responsible for caudal neural tube defects, whereas Hh pathway accounts for most of the rostral defects (Simons and Mlodzik, 2008). The phenotype displayed by mice lacking PCP function is manifested by a failure of the spinal cord and hindbrain to close (craniorachischisis), but the forebrain closes successfully. Around the time that PCP signaling emerged as a regulator of spinal neural tube closure, a forward genetic screen in mice began uncovering a series of mutants that displayed

exenphaly (defects in the closure of the rostral neural tube). Many of these mutants were found to encode proteins required for the IFT. Further examinations of these mutant mice revealed that the open neural tube defects resulted from the failure in transduction along the Hh signaling pathway, which is critical for patterning of cell fates during embryogenesis (Wallingford, 2006). Patched1 null mice have both rostral and caudal defects (Simons and Mlodzik, 2008). The link between cilia and the Shh pathway might explain one of the main features of MKS, which is the failure of the neural tube closure. The normal Shh pathway is defective in MKS, since the MKS cases lack cilia.

Cilia and left-right asymmetry

Review a series of 67 Finnish MKS cases showed *situs inversus totalis* in three of them, which implies an increased risk for this rare condition in MKS (Salonen, 1984). A link between ciliary motility and the regulation of left-right (LR) asymmetry was proposd when it was observed that some individuals with primary ciliary dyskinesia (PCD) have situs inversus. Earliest asymmetrically expressed genes are active at the node. Nodal cilia rotate in a consistent clockwise direction generating a unidirectional leftward flow of extracellular fluid. Mice that are unable to assemble cilia or with immotile cilia lack the flow and have randomized organ situs (Tabin, 2006). Mouse *iv* (inversus viscerum) mutant results from a mutation in ciliary dynein gene, left-right dynein (*lrd; Dnach11*). In lrd mutant embryos, nodal cilia are immotile (Bisgrove and Yost, 2006). Moreover, artificial generation of directional flow was shown to be sufficient to specify downstream left-right asymmetry and morphogenesis (Tabin, 2006). In other vertebrates, rotational ciliary beating causes also leftward flow across structures that are analogous to the mouse node, including the posterior notochordal plate in rabbits and the Kupffer's vesicle (KV) in zebrafish and medaka. Two models have proposed to explain how fluid flow confers LR asymmetry. The 'morphogen flow' model suggests that signaling proteins are swept to the left side of the node where they initiate downstream signaling pathways. The mechanosensory or 'two cilia' model proposes that two populations of primary cilia exist in the mouse node: central Lrd-expressing motile cilia and peripheral immotile cilia (Bisgrove and Yost, 2006). The nodal flow generated by the motile cilia is thought to be sensed by the immotile, mechanosensory cilia. Both models report an asymmetric Ca^{2+} release that is probably involved in the subsequent events of LR determination (Fliegauf et al., 2007).

6.2.3 Ciliopathies

Given the multiple roles of cilia in development and physiology, it is not surprising that mutations in the ciliary genes can give rise to a number of human monogenic diseases. They are collectively known as ciliopathies and have overlapping clinical features (Figure 25) (Badano et al., 2006; Gibson, 2006; Marshall, 2008). Ciliopathies have a broad range of phenotypes encompassing a number of different autosomal recessive and dominant syndromes (Adams et al., 2008). A non-exhaustive list of 'classical' ciliopathies includes polycystic kidney disease (PKD), retinal degeneration, laterality defects, chronic respiratory problems, situs inversus, hydrocephalus and infertility. More recently cilia-associated disorders, such as Bardet-Biedl syndrome and Alström syndrome, have extended the list to include obesity, diabetes, hypertension, heart defects, and sensory deficits, skeletal, neurological and developmental anomalies (Inglis et al., 2006). Disorders that are caused by dysfunction of the motile cilia include primary ciliary dyskinesia (PCD) and ciliary disorders associated with a situs defect, Kartagener syndrome being an example of the latter, which arises from the dysfunction of the nodal cilia (Zariwala et al., 2007; Adams et al., 2008). Typical ciliopathies and their main features are listed in Table 6 (Afzelius, 2004; Badano et al., 2006; Adams et al., 2008; Marshall, 2008; Sharma et al., 2008).

Ciliopathies can be classified according to whether the there is aberrant function in an intact cilium or complete absence/loss of the mature cilium. The latter is the case with severe multi-organ phenotypes. A specific defect in otherwise intact cilia can affect only a specific tissue/organ (Adams et al., 2008). An overwiew of the molecular aberrations seen in ciliary syndromes are listed in Table 7 (Afzelius, 2004). The most puzzling aspect of ciliopathies is perhaps that different ciliary diseases involve different, often partially overlapping set of symptoms. The clinical complexity of ciliopathies can be understood in the light of basic cell biology of the cilia themselves. Cilia are not just a list of genes, but complex organelles with variable ultrastructures that must assemble and function in different tissue context and in addition some ciliary genes may have roles also in cilia unrelated gene functions (Marshall, 2008). Another important consideration is the relative timing of gene loss in different tissues. Adult-onset ciliopathy can result from the spontaneous loss of heterozygosity of a ciliary gene in patients carrying on mutant allele. This second hit would occur long after embryogenesis and therefore developmental defects, such as polydactyly would not be seen. Timing of ciliopathy onset can be studied using inducible Cre-mediated knockout alleles (Garcia-Gonzalez et al., 2007; Marshall, 2008).

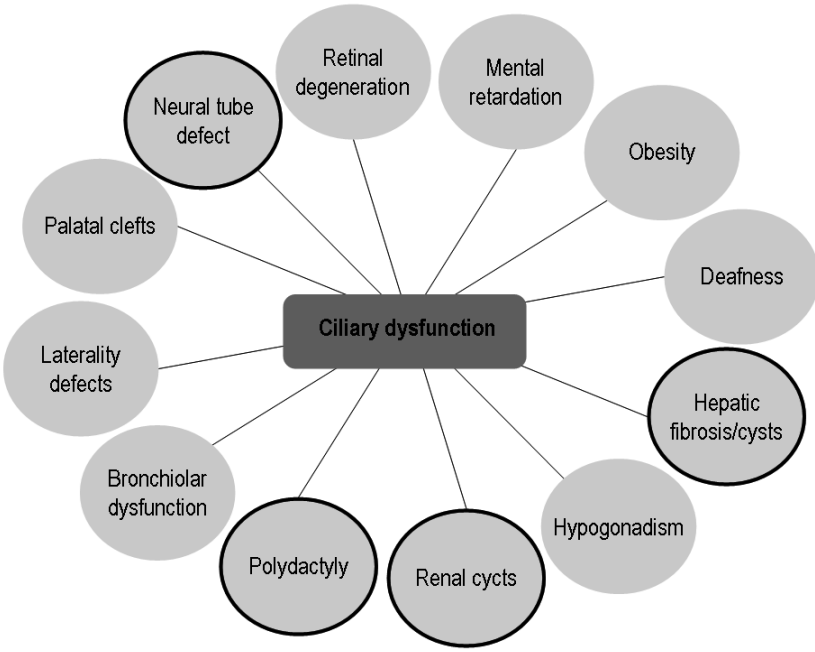

Figure 25. Dysfunction of ciliary proteins gives rise to phenotypes that range from organ specific to broadly pleiotropic. The figure summarizes the pleiotropic effetcs of primary cilia dycfunction in humans. Main features of MKS are highlighted (modified from Gibson et al., 2006).

Table 6. Known human ciliary diseases and their main defects.

Disease	**Main defects**
Primary ciliary dyskinesia (immotile cilia syndrome)	Mucus clearance, hydrocephalus, situs inversus
Kartagener syndrome	Situs inversus, infertility
Polycystic kidney disease -autosomal recessive and dominant	Renal cysts
Bardet-Biedl syndrome	Obesity, polydactyly, mental retardation, kidney abnormalities, hypogenitalism, diabetes
McKusick-Kaufman syndrome	Mesoaxial or postaxial polydactyly, congenital heart disease and hydrometrocolpos
Alström syndome	Cone-rod retinal dystrophy, hearing defects, renal failure, cardiomyopathy, hepatic dysfunction, morbid obesity
Meckel syndrome	Cysts in kidney and liver, CNS malfomation, polydactyly
Joubert syndrome	Renal cysts, ataxia, mental retardation, obesity, oculomotor apraxia, polydactyly, retinal degeneration, hypotonia, neonathal breathing dysfunction
Cerebro-Oculo-Renal syndrome	A form of JS that includes retinal dysplasia and cystic dysplastic kidneys
Oro-Facial-Digital syndrome	Craniofacial abnormalities, postaxial polydactyly, CNS malfomations
Nephronophthitis	Renal cysts, pancreatic and hepatic fibrosis, situs inversus, cerebral vermis aplasia, retinal degeneration, mental retardation
Leber congenital amaurosis	Syndromic and non-syndromic forms of blindness
Senior-Loken syndrome	Main clinical features of both NPHP and LCA
Features associated with many ciliopathies	
Retinis pigmentosa	Progressive retinal degeneration -night blindness, the development of tunnel vision, and an accumulation of retinal pigment-like deposits
Situs inversus	An inversion of the normal left-right asymmetry of internal organs
Infertility	Male or female sterility

Table 7. Molecular aberrations seen in different manifestations of the immotile-cilia syndrome (modified from Afzelius, 2004). See also the illustration of cilia structure (Figure 22)

- No outer and inner dynein arms
- Few outer and inner dynein arms
- No outer dynein arms
- No inner dynein arms
- Short outer dynein arms
- No inner dynein arms and no spokes
- Short or no central microtubules
- No nexin links
- No or short spokes and eccentric central microtubules
- Basal bodies are present but have not grown cilia
- No basal bodies and no cilia
- Cilia with a normal ultrastructure but unable to perform their work

7 CONCLUSIONS AND FUTURE PROSPECTS

In the beginning of this thesis project there were no genes identified and there was no clue of the molecular pathways defective in MKS. Discovery of the first *MKS* gene provided the connection between MKS and cilia and added information to growing body of evidence linking cilia to the organization of cell polarity and tissue patterning during development of many organs. In this this study we were able to identify two genes implicated in MKS and to chareacterize the mutation spectrum of all the reported genes in Finnish and non-Finnish families (Figure 26).

The amount of information concerning MKS and cilia has exploded in the past few years and will continue to increase rapidly. The knowledge of proteins involved in ciliary functions is building up, piece by piece. Even though there are ~1200 number of proteins listed in the ciliaproteome database, there might still be unidentified proteins, since some important ciliogenic proteins may be difficult to identify, because they have close homologs or posses evolutionay conserved domains also present in non-ciliated cells (Leroux, 2007). Also the list most likely includes false positives.

Cilia are not just a list of genes, but complex organelles with variable ultrastructures that must assemble and function in different tissue context. To understand the full spectrum of ciliary disease, we must learn about much more about the cell type-specific differences in cilia. One question is that why might defects in two cilia-related genes lead to distinct diseases? There are several ways this can happen: (1) some ciliary genes may have additional cilia-unrelated gene functions, (2) some mutations may affect the ciliogenesis of only a subset of cilia in the body and (3) genetic defects may affect different ultrastructural modules of cilia and thus influence only a subset of ciliary functions (Marshall, 2008). Another question still remains: how the different mutations in the same genes cause such a variety of disorders as the *MKS* genes? There seems to be a really tight connection between the genotype and phenotype of different mutations and syndromes, since none of the identified mutations are reported as same combinations for different syndromes. What is the underlying molecular mechanism? This key question remains unanswered, but future studies and better understanding of the molecular composition of each ciliary substructure will undoubtly bring us closer in understanding the overall disease mechanisms underlying cilia-related disorders (Fliegauf and Omran, 2006).

An interesting study would be to look at the *MKS* mutation carriers more closely, whether they really are ‘healthy’ in terms of cilia associated features. They might

suffer more e.g. from obesity and hypertension (features seen in BBS patients) or some other cilia dysfunction related phenotype, compared to the rest of the population. No epidemiological studies of *MKS* mutation carriers have been conducted to date. Also the function of the MKS proteins after development, later in life, would be fascinating to study. This could be done for example with conditional knockout mice where the gene is switched of after the critical developmental processes have taken place. We are just beginning to understand the structure and function of the cilia and there are numerous fascinating studies to be conducted. There are only a few animal models of the *MKS* genes to date and there is currently much effort put in generating them. These animal models are an important tool in future studies that will hopefully enlighten our knowledge about the molecular mechanisms behind MKS.

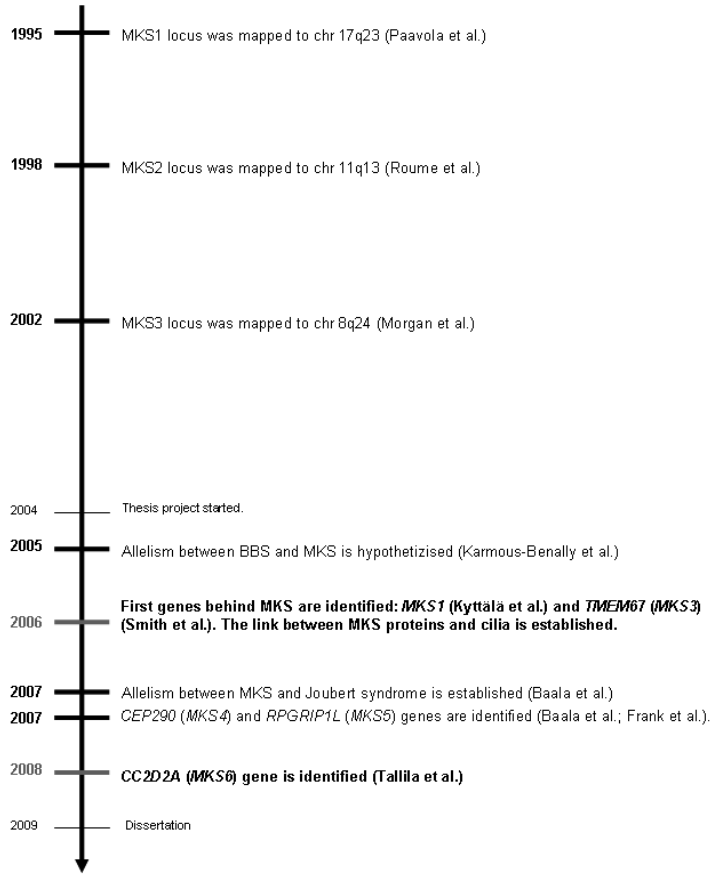

Figure 26. A timeline of MKS research.

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